ERJ Express. Published on January 12, 2012 as doi: 10.1183/09031936.00090511

Caveolin-1 Regulation of Store-operated Ca^{2+} Influx in Human Airway Smooth Muscle

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Running Title: Caveolins and Calcium Influx

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Abstract

Background: Caveolae, plasma membrane invaginations with constitutive caveolin proteins, harbour proteins involved in intracellular calcium ($[Ca^{2+}]_i$) regulation. In human airway smooth muscle (ASM), store-operated Ca^{2+} entry (SOCE) is a key component of $[Ca^{2+}]_i$ regulation, and contributes to increased $[Ca^{2+}]_i$ in inflammation. SOCE involves proteins Orai1 and stromal interaction molecule 1 (STIM1). We investigated the link between caveolae, SOCE and inflammation in ASM.

Methods: $[Ca^{2+}]_i$ was measured in human ASM cells using fura-2. siRNA or overexpression vectors were used to alter expression of caveolin-1 (Cav-1), Orai1 or STIM1. TNFα was used as a representative pro-inflammatory cytokine.

Results: TNFα increased SOCE following sarcoplasmic reticulum Ca^{2+} depletion, and increased whole-cell and caveolar Orai1 (but only intracellular STIM1). Cav-1 siRNA decreased caveolar and whole cell Orai1 (but not STIM1) expression, and blunted SOCE, even in the presence of TNFα. STIM1 overexpression substantially enhanced SOCE: an effect only partially reversed by Cav-1 siRNA. In contrast, Orai1 siRNA substantially blunted SOCE even in the presence of TNFα. Cav-1 overexpression significantly increased Orai1 expression and SOCE, especially in the presence of TNFα.

Conclusions: These results demonstrate that caveolar expression and regulation of proteins such as Orai1 are important for $[Ca^{2+}]_i$ regulation in human ASM cells and its modulation during inflammation.

Keywords: Asthma; Caveolae; Cytokine; Inflammation; Orai1; STIM1

Introduction:

Caveolae are flask-shaped plasma membrane invaginations expressing the scaffolding caveolin proteins-1 though -3 [1, 2], as well as a variety of proteins important in signal transduction [3]. In other tissue types, caveolae and caveolins have been shown to be critical in coordination of signalling mechanisms at both the plasma membrane and internal structures such as the sarco/endoplasmic reticulum [4, 5]. We and others have demonstrated that human airway smooth muscle (ASM) cells express caveolin-1 (Cav-1), and that ASM caveolae contain a number of proteins involved in regulation of intracellular Ca^{2+} ($[Ca^{2+}]_i$) [6-8]. However, the role of caveolae in communication between plasma membrane and intracellular structures has not been explored in ASM.

In ASM, $[Ca^{2+}]_i$ regulation involves both Ca^{2+} influx and sarcoplasmic reticulum (SR) Ca^{2+} release and reuptake [6, 9-11]. We and others have demonstrated that following SR Ca^{2+} depletion (e.g. via agonist stimulation), store-operated Ca^{2+} entry (SOCE) for SR Ca^{2+} refilling occurs in ASM [12-14]. Much previous work (including our own) had focused on the role of transient receptor potential (TRP) channels in regulation of SOCE [12, 15-17]. However, more recent evidence highlights the role of two proteins: Orai1 and stromal interaction molecule 1 (STIM1) [13, 14, 18-20]. Orai1 is thought to be the pore forming subunit of Ca^{2+} release-activated Ca^{2+} channels [21], whereas STIM1 is thought to be localized to the SR (with some plasma membrane expression) and acts as a sensor for SR Ca^{2+} store status [22]. By sensing $[Ca^{2+}]_i$ and intraluminal SR Ca^{2+} levels, STIM1 interacts with plasma membrane Orai proteins in complex and dynamic ways that are still being explored [22-24]. Thus far there are very few studies exploring these novel proteins and their importance for SOCE in ASM [13, 14, 20]. Nonetheless, the plasma membrane localization of Orai1 vs. the SR location of STIM1 raises the

question whether caveolae facilitate interactions between these molecules and thus contribute to SOCE. This has been suggested by a few studies [6, 25, 26] but has not been specifically examined.

The significance of caveolar regulation of SOCE lies in the role of the latter in enhanced airway contractility with inflammation. We have previously shown that the pro-inflammatory cytokine tumour necrosis factor (TNFα) increases SOCE in human ASM [15]. Separately, in a recent study, we showed that TNFα also increases ASM contractility [8]; however the underlying mechanisms that link caveolae, $[Ca^{2+}]_i$ and TNFα are not known. In the present study, we hypothesized that Cav-1 interacts with Orai1 and STIM1 to regulate SOCE, and in the presence of TNFα, caveolar regulation of SOCE contributes to increased $[Ca^{2+}]_i$.

Material and Methods:

Materials: Chemicals and supplies were obtained from Sigma (St. Louis, MO) unless mentioned otherwise. Tissue culture reagents, including Dulbecco's Modified Eagle's Medium F-12 (DMEM F/12) and foetal bovine serum (FBS), were obtained from Invitrogen (Carlsbad, CA). Cav-1 specific caveolin scaffolding domain peptide (CSD) obtained from Enzo Life Sciences Intl, Plymouth Meeting, PA. Negative-CSD obtained from Calbiochem, La Jolla, CA.

Isolation of Human ASM Cells: The techniques for isolation of human ASM cells have been previously published [12]. Briefly, 3rd to 6th generation human bronchi were obtained from surgical lung specimens (exempt and not Human Subjects Research per Mayo Clinic Institutional Review Board). ASM layer was dissected and cells enzymatically dissociated (Worthington Biochemical, Lakewood, NJ), resuspended and seeded into tissue culture flasks or Petri dishes. Cells were maintained for a maximum of 2 subcultures (maintaining ASM phenotype) at 37°C (5% CO_2, 95% air) in DMEM/F12 supplemented with 10% FBS. Experiments were performed in cells serum-deprived for 48 hours.

Subcellular fractionation: ASM cells at confluence were treated (based on the protocol) and homogenized by sonication (5 seconds x 3) in sucrose buffer containing 0.25M sucrose, 20mM Tris-HCl (pH 7.2) and 10μg/ml leupeptin. The resulting homogenate was centrifuged at 2000 x g for 10 minutes to remove cellular debris. The supernatant was centrifuged at 7000 x g for 15 minutes, again at 20,000 x g for 20 minutes and the resultant plasma membrane pellet resuspended in sucrose buffer, while the remaining supernatant was further centrifuged at 100,000 x g for 60 minutes to obtain the microsomal pellet. The final supernatant was saved as the cytoplasmic fraction.

Preparation of ASM Caveolar Membranes: Caveolin enriched membrane fractions were prepared from human ASM cells as previously described [6]. Briefly, ASM cells were homogenized in cold buffer A (0.25 M sucrose, 1 mM EDTA, and 20 mM Tricine, pH 7.8) and layered on a 30% Percoll gradient. A series of centrifugations, sonications and resuspensions was used to obtain caveolae-enriched fractions (purity confirmed by Western blot analysis for caveolins, with absence of SR or mitochondrial proteins).

siRNA Transfection: Knockdown of proteins including Cav-1 by siRNA have also been previously described [6, 8]. Transfection was achieved using 20nM siRNA and Lipofectamine 2000 (Invitrogen) in DMEM F/12 lacking FBS and antibiotics. Fresh growth medium was added after 6 h and cells analysed after 48 h. An 18 basepair Cav-1 siRNA duplex (Dharmacon, Lafayette, CO) as previously described [6] a 21 basepair STIM1 siRNA duplex (GCC UAU AUC CAG AAC CGU Utt) and a 21 basepair Orai1 siRNA duplex (Ambion, Austin, TX) targeting human FLJ14466 mRNA (GCA ACG UGC ACA AUC UCA Att) were used. As a negative control, the *Silencer* Negative Control #1 (Ambion) was utilized. Knockdown efficacy and specificity was verified by Western blot analysis.

Cav-1 Overexpression: An mRed-tagged Cav-1 construct (generously provided by Dr. Richard Pagano, Mayo Clinic, Rochester, MN) was transfected into serum-free ASM cells for 24h. Following transfection, DMEM/F12 media with 10% FBS was added for 24 h, and then withdrawn for 48h prior to further experimentation. Expression of Cav-1-mRed was verified by mRed fluorescence within cells, specifically at the plasma membrane.

STIM1-GFP Transfection: Full length STIM1 mRNA was generated using RT-PCR and primers designed from published sequences that include endogenous translation start and stop codons. The PCR product was agar purified to eliminate non-specific PCR product, then cloned

into the TA cloning vector pCR4-TOPO (Invitrogen) and transformed into bacteria. Full length mRNA was cloned into the *Acc I* and *Sac I* restriction sites of the pAcGFP-N1 expression vector. Correct DNA insertion was confirmed by sequencing. ASM cells were transfected using 0.8μg/ml GFP-STIM1 with Lipofectamine.

Western Blot Analysis: Standard techniques based on SDS-PAGE (Criterion Gel System; Bio-Rad, Hercules, CA; either 15% or 4–15% gradient gels) with transfer to PVDF membranes (Bio-Rad) were used. Membranes were blocked for 1 h with 5% milk in TBS containing 0.1% Tween (TBST) and then incubated overnight at 4°C with anti-STIM1 (Abcam,1:1000), Orai1 (Alomone Labs, 1:500) or Cav-1 (Santa Cruz Biotechnology,1:1000) and α-actin (Sigma, St. Louis, MO; 1:5000) antibodies. Following three washes with TBST, primary antibodies were detected using horseradish peroxidase-conjugated secondary antibodies and signals developed by Supersignal West Pico Chemiluminescent Substrate (Pierce Chemical Co., Rockford, IL) and densitometry quantification using a Kodak ImageStation 4000mm (Carestream Health, New Haven, CT).

TNFα exposure: Cells were exposed to 20ng/ml recombinant human TNFα (R&D Systems, Minneapolis, MN) for 48h.

$[Ca^{2+}]_i$ imaging: We have published extensively on $[Ca^{2+}]_i$ imaging of ASM cells [6, 12]. Briefly, human ASM cells were incubated in 5 μM fura-2/AM (Invitrogen) for 60 min and visualized using an epifluorescence imaging system (MetaFluor; Universal Imaging, Downingtown, PA; Nikon Diaphot inverted microscope; 40X/1.3 NA oil-immersion lens; 1 Hz acquisition of 512x512 images via a 12-bit Roper Scientific CCD camera). $[Ca^{2+}]_i$ responses of 20-30 cells per field were obtained using individual software-defined regions of interest. Fura-2 fluorescence was calibrated for $[Ca^{2+}]_i$ using previously described procedures [6].

Store-Operated Ca^{2+} Influx: Previously described techniques were utilized for examining SOCE [6, 12, 15]. Briefly, in the absence of extracellular Ca^{2+} (0 mM Ca^{2+} HBSS containing 1 μM nifedipine and 10 mM KCl), the SR was passively depleted using 10 μM CPA for 5 min after which 2.5 mM extracellular Ca^{2+} was rapidly re-introduced (in the continued presence of CPA, nifedipine and KCl), and the observed $[Ca^{2+}]_i$ response measured.

Confocal Immunofluorescence Microscopy: ASM grown on 4-chambered Lab-Teks (Nalgene Nunc International, Rochester, NY) were fixed in 2% paraformaldehyde for 15 minutes. Cells were then washed in 0.1 M tris-buffered saline (TBS), permeabilized with 0.1% Triton-X in TBS for 15 seconds, washed in TBS and blocked for 60 min in 4% normal donkey serum. Samples were then incubated overnight at 4°C in TBS only (unstained control) or 1 μg/ml of primary anti-Cav-1 (Santa Cruz Biotechnology, Santa Cruz, CA), anti-STIM1 (Abcam, Cambridge, MA) or anti-Orai1 (Santa Cruz Biotechnology) antibodies. Following washes in TBS, samples were incubated for 1 h in Alexa 488 conjugated donkey anti-mouse (1:200; Invitrogen) or Cy3-conjugated donkey anti-rabbit secondary antibodies (1:200 dilution; Millipore, Billerica, MA). Cells incubated with secondary antibodies only served as staining controls. Labeled cells were visualized using a 40X/1.3 or 100X/1.3 oil immersion objective lens on a Nikon Eclipse microscope and a Nikon C2 laser scanning confocal system.

Statistical analysis: Bronchial samples from at least 5 patients were used to obtain ASM cells, with biochemistry and molecular biology protocols being repeated a minimum of 3 times. $[Ca^{2+}]_i$ experiments were performed in at least 20 cells from each bronchial sample. siRNA/mRed/TNFα effects on $[Ca^{2+}]_i$ responses were compared across sets of cells using 2-way ANOVA with Bonferroni correction for repeated comparisons. Statistical significance was established at $p<0.05$. All values are expressed as means $\pm$ SE.

Results:

Relative subcellular localisation of Cav-1, STIM1 and Orai1 in human ASM

Immunostaining of human ASM cells demonstrated substantial co-localization of Cav-1 and Orai1 were primarily expressed within the plasma membrane while STIM1 was expressed within the cytoplasm at the level of the sarcoplasmic reticulum (Figure 1A). There was some level of STIM1 expression also noted near the plasma membrane, albeit to a much smaller extent than intracellular localization. These results were confirmed by Western analysis of cell fractions (Figure 1B). Cav-1 and Orai1 were expressed in plasma membrane fractions, whereas STIM1 was largely expressed in the heavy microsomal fraction.

Overexpression of Cav-1 and TNFα effects

Overexpression of Cav-1 using a Cav-1-mRed construct was confirmed by fluorescence imaging which showed enhanced fluorescence at the plasma membrane within 24h (Figure 2A). Exposure to TNFα not only accelerated the expression of Cav-1- mRed within Golgi but also increased its plasma membrane expression (not shown). Western blot analysis confirmed that transfection with Cav-1-mRed significantly increased Cav-1 expression compared to vehicle (control-mRed transfection). Exposure to TNFα also significantly increased Cav-1 expression. This effect was even further enhanced when cells overexpressing Cav-1 were exposed to TNFα (Figure 2B).

Effect of altered Cav-1 expression on TNFα-induced enhancement of SOCE

We have previously reported [6] that transfection of human ASM cells with Cav-1 siRNA results in ~75% reduction in Cav-1 levels. We verified a similar extent of reduction in this set of studies. In cells transfected with Cav-1 siRNA, SOCE was significantly decreased when compared to vehicle control or non-specific siRNA ($p<0.05$; Figure 3). Exposure to 20

ng/ml of TNFα for 48h significantly increased SOCE ($p<0.05$; Figure 3). This enhancing effect of TNFα was significantly blunted in cells transfected with Cav-1 siRNA (Figure 3, $p<0.05$). Cells transfected with Cav-1-mRed showed significantly increased SOCE compared to non-transfected controls (Figure 3). Since only ~40-50% of ASM cells showed high levels of Cav-1-mRed, in separate studies, using software-facilitated regions of interest, we compared SOCE in those cells vs. cells with background fluorescence (which may have overexpressed Cav-1 but not to a large extent). With either method of comparison, cells overexpressing Cav-1 showed significantly higher SOCE. Furthermore, addition of TNFα to these Cav-1 overexpressing cells showed even further increased SOCE (Figure 3, $p<0.05$.)

Effect of TNFα and Cav-1 on STIM1 and Orai1 expression

Considering the role of STIM1 and Orai1 in SOCE, we determined whether the mechanism of TNFα-induced enhancement of SOCE involved increased expression of these proteins. Indeed, exposure to TNFα significantly increased STIM1 and Orai1 in whole cell lysates of human ASM cells ($p<0.05$; Figure 4A). With the understanding that Orai1 is plasma membrane bound, we determined whether caveolae played a role. Indeed, suppression of Cav-1 using siRNA significantly reduced Orai1 expression under control conditions as well as in TNFα exposed cells ($p<0.05$; Figure 4A). In separate experiments, we found that non-specific chelation of plasma membrane cholesterol using 10 mM methyl-β-cyclodextrin (which should reduce caveolar numbers and other lipid rafts) also reduced Orai1 expression (not shown). Overexpression of Cav-1 using Cav-1-mRed significantly increased Orai1 expression which was further enhanced in the presence of TNFα (Figure 4A, $p<0.05$). Interestingly, STIM1 expression with or without the presence of TNFα was not affected by Cav-1 siRNA or Cav-1-mRed (Figure 4A), suggesting the lack of plasma membrane or caveolar STIM1. Indeed, in a separate set of

experiments we analysed caveolar fractions from ASM cells under the same experimental conditions and found that Cav-1 siRNA significantly decreased caveolar Orai1 expression while conversely, Cav-1 overexpression increased Orai1 expression. Exposure to TNFα also increased Orai1 expression, an effect further enhanced when Cav-1 was overexpressed (Figure 4B, $p<0.05$). However, there was negligible expression of STIM1 within caveolar membrane fractions, with no significant changes following TNFα or Cav-1 siRNA or overexpression (Figure 4B).

Relationships between Cav-1, STIM1 and Orai1

Transfection of human ASM cells with Orai1 siRNA significantly lowered the expression of this protein when compared to both vehicle control and negative siRNA ($p<0.05$; Figure 5A), and significantly blunted SOCE ($p<0.05$; Figure 5C). In a separate set of cells, fura 2-loaded human ASM cells exposed to 10 μM histamine demonstrated a typical biphasic $[Ca^{2+}]_i$ response with an initial higher peak followed by a lower plateau level (Figure 5B). In cells transfected with Orai1 siRNA, $[Ca^{2+}]_i$ responses to histamine were significantly decreased when compared to control and negative control cells ($p<0.05$; Figure 5B). Since Orai1 was substantially expressed within caveolae, additional suppression of Cav-1 via siRNA was considered a futile experiment. Accordingly, to demonstrate the relationship between Cav-1 and Orai1, control and Orai1 siRNA transfected ASM cells were exposed to 5 μM Cav-1 inhibitor peptide (CSD; 6 hours) which inhibits the function of Cav-1 without altering its expression [8, 27]. In normal cells, SOCE was significantly reduced by CSD (Figure 5C), functionally linking Cav-1 to SOCE. Combination of Orai1 siRNA with CSD inhibition of Cav-1 further reduced SOCE ($p<0.05$; Figure 5C), further linking Orai1, Cav-1 and SOCE. Additional quality control studies performed using a negative CSD, found no significant difference from vehicle control (data not

shown). Furthermore, we studied SOCE in ASM cells transfected with both Cav-1-mRed and Orai1 siRNA. Overexpression of Cav-1 increased SOCE, while Orai1 siRNA significantly reduced this enhancing effect of Cav-1-mRed on SOCE. To determine the role of caveolar Orai1 in TNFα-induced enhancement of SOCE, we evaluated SOCE in ASM cells transfected with Orai1 siRNA and then exposed to vehicle or TNFα. Orai1 siRNA significantly reduced TNFα-induced increase in SOCE (Figure 5C, $p<0.05$).

Considering the finding that caveolae do not express much STIM1, but the known individual roles of Cav-1 and STIM1 in SOCE, we investigated the functional relationship between Cav-1 and STIM1. In ASM cells transfected with GFP-STIM1, the expression of STIM1 was substantially increased (as expected and confirmed by Western blot; Figure 6B) STIM1 siRNA substantially reduced SOCE when compared to vehicle control, consistent with its known role in regulating SOCE. Conversely, STIM1 overexpression (GFP-STIM1) substantially enhanced SOCE ($p<0.05$ compared to control; Figure 6A). In such cells, Cav-1 siRNA significantly blunted GFP-STIM1 enhanced SOCE, albeit not to control levels ($p<0.05$; Figure 6A).

Discussion:

There is growing evidence that caveolae contain a variety of proteins that play an important role in $[Ca^{2+}]_i$ signalling [1, 2]. By virtue of their shape, caveolae could facilitate interactions between plasma membrane proteins and intracellular organelles. The present study provides evidence that caveolae and specifically Cav-1 facilitate such interactions between the plasma membrane protein Orai1 and the SR protein STIM1 in enhancing Ca^{2+} influx following depletion of SR Ca^{2+} stores (i.e. SOCE). Furthermore, we demonstrate that enhanced $[Ca^{2+}]_i$ by pro-inflammatory cytokines such as TNFα involves SOCE and the proteins Orai1 and STIM1, with a particular role for caveolar Orai1. These findings emphasize the role of caveolae and Cav-1 in regulation of airway contractility and its enhancement in the presence of inflammation. Here, caveolae appear to be important in the communication between intracellular structures such as the sarcoplasmic reticulum and STIM1 vs. plasma membrane $[Ca^{2+}]_i$ regulatory mechanisms such as SOCE, mediated by Orai1.

Caveolae and SOCE

Caveolae are specialized forms of lipid rafts in plasma membrane of most cells. They form dynamic assemblies of sphingolipids and cholesterol containing scaffolding domains with different affinities for proteins resulting in a variety of functions [1]. The constitutive Cav-1 protein is distributed ubiquitously, while caveolin-2 is usually associated with Cav-1 [3, 28]. While caveolin-3 is thought to be "muscle specific" and is expressed in striated muscles [3, 28, 29], we and others have found that caveolin-3 is not expressed within human ASM [6, 30]. Recent studies have established that ASM caveolae contain a number of proteins important to $[Ca^{2+}]_i$ regulation (e.g. agonist receptors, Ca^{2+} influx channels; force regulatory proteins such as RhoA). In canine ASM, it has been established that caveolar-enriched membrane fractions

express Cav-1, L-type Ca^{2+} channels and plasma membrane Ca^{2+} ATPase, but not SR proteins such IP_3R or RyR channels [31].

In ASM of different species, in addition to Ca^{2+} influx through voltage-gated or receptor-operated channels, influx occurs in response to SR Ca^{2+} depletion. For example, in human ASM, we found that SOCE is triggered by SR Ca^{2+} release via both IP_3R and RyR channels [12, 15]. In this regard, consistent with previous interests in the role of TRPC channels as being key to SOCE, we have also demonstrated a role for TRPC channels in SOCE of ASM [15]. Here, ASM does express several TRPC isoforms including TRPC1, TRPC3, TRPC4 and TRPC6, with siRNA suppression of TRPC3 and TRPC6 (for example) decreasing SOCE [6, 15]. Furthermore, Cav-1 co-localizes with TRPC channels [6] and is important for SOCE regulation in that depletion of lipid rafts with cyclodextrins or suppression of Cav-1 using siRNA decreases SOCE [6, 32].

While this previous work linked Cav-1 to SOCE, the linking mechanism had been thought to be TRPC channels. However, the issue of plasma membrane or caveolar interaction with the SR remained. Here, there has been much recent research on STIM1 and Orai1 as being key to communication between the plasma membrane and SR in triggering SOCE [33]. In regard to the mechanism that senses and triggers SOCE, there have been two models proposed: A) insertional model in which STIM1 translocates to the plasma membrane during SR Ca^{2+} depletion [23] and, B) interactional model where STIM1 is entirely at the level of the SR (although some may be expressed within the plasma membrane) but aggregates and forms clusters with junctional SR following depletion to then activate SOCE channels in the plasma membrane [34, 35]. At this juncture, some studies suggest that Orai1 modulates SOCE (potentially occurring through TRPC channels) [36] while other suggest that Orai1 itself forms

the channel that mediates SOCE [23]. Regardless of which mechanism is involved in SOCE, in an interactional role of STIM1, caveolae and Cav-1 would be ideal candidates to serve as a home for the plasma membrane protein mediating/modulating SOCE, and (via caveolar invaginations) for further facilitating such interactions by increasing physical proximity of plasma membrane and SR.

There is now evidence (including data from our current study) that human ASM expresses STIM1 as well as Orai1, and that suppression of either protein decreases SOCE [13, 14]. What was not known is whether caveolae were involved. Our novel finding is that caveolae are important for STIM1-Orai1 interactions in human ASM SOCE, and that caveolar Orai1 is a key factor to SOCE, as evidenced by our siRNA and over-expression studies. This was further confirmed by the lack of additional inhibitory activity by CSD in the presence of Orai1 siRNA. However, what is not known is whether Orai1 is the actual SOCE channel, or whether Orai1 interacts with TRPC channels to modulate SOCE. Based on previous data on reduced SOCE with TRPC siRNA, and the new data showing Orai1 siRNA suppressing SOCE, we suggest that the latter is the case in human ASM. Accordingly, STIM1 aggregation could lead to interactions with caveolae which contain TRPC as well as Orai1, allowing for multiple levels of regulation. These findings are consistent with a report from human submandibular gland [26] showing that STIM1 clusters with TRPC1 channels and that Cav-1 is required for this interaction.

Caveolae, airway inflammation and SOCE

Inflammation in diseases such as asthma and chronic bronchitis contributes to enhanced bronchoconstriction. Bronchoconstriction results either from an increase in $[Ca^{2+}]_i$ or enhanced Ca^{2+} sensitivity [8, 11]. The fact that overexpression of Cav-1 increases SOCE substantially demonstrates the importance of both in mediating enhancing bronchoconstriction.

In the current study, we used the pro-inflammatory cytokine TNFα which is abundantly produced in the airway and known to be involved in airway inflammation [37]. TNFα is a potent proinflammatory cytokine that is found in bronchoalveolar lavage fluid and sputum from asthmatic patients and has been implicated as a mediator in the pathophysiology of asthma and COPD. We have previously shown that TNFα increases SOCE in human ASM [15], an effect involving upregulated TRPC3 expression. Separately, we and others have demonstrated that lipid rafts and Cav-1 are essential for TNFα–induced activation of signalling pathways associated with ASM contractility and cellular proliferation [8, 38]. Indeed, we recently showed that TNFα increases Cav-1 expression in ASM [8]. Adding to our previous finding of Cav-1 being important for SOCE [6], these studies link TNFα, Cav-1 and SOCE. However, in these studies, the assumption was that TRPC3 mediates this linkage. The results of the present study now demonstrate the importance of caveolar Orai1 in TNFα-induced enhancement of SOCE. Interestingly, although TNFα increased STIM1 expression in whole cell lysates it did not affect its caveolar membrane expression, again highlighting the likely SR localization of this protein in ASM and its role in SOCE regulation. While we have previously found some caveolar expression of STIM1 (confirmed in the present study), its role is less clear. The findings in our present study that altered STIM1 expression has little role in caveolar or TNFα regulation of SOCE suggests a non-SOCE role for caveolar STIM1.

The present data confirm that Cav-1 plays an important role in $[Ca^{2+}]_i$ signalling and its enhancement by TNFα in ASM. The effects of Cav-1 can occur through two scenarios (Figure 7). First, TNFα can increase the number of caveolae and with it the amount of Cav-1 and other proteins important for $[Ca^{2+}]_i$ signalling (e.g. Orai1). With the increased expression of Orai1 and increased SOCE by TNFα, there is now an environment for enhanced caveolae/ Cav-1-mediated

plasma membrane-SR interaction (Figure 7). The finding that Cav-1-mRed overexpression increased SOCE supports this model. Second, significant increases in the expression of the Ca^{2+} sensing protein STIM1 during TNFα exposure, albeit at the level of the SR, could help to sustain refilling of the SR Ca^{2+}. Here, the cytosolic levels of Ca^{2+} may remain high for a longer period (prolonging refilling) due to decreased SERCA expression and activity [11].

In conclusion, these data demonstrate novel data that caveolae and specifically Cav-1 are important regulators of SOCE in human ASM by influencing plasma membrane SR interactions. While both Orai1 and STIM1 are important in TNFα-induced enhancement of SOCE, caveolar Orai1 seems to be particularly important.

Acknowledgements:

This study was supported by NIH grants HL090595 (Pabelick), HL090595-S2 (Pabelick) HL088029 (Prakash) and HL74309 (Sieck), and by grants from the Flight Attendant Medical Research Institute (FAMRI; Sathish, Pabelick, Prakash).

Figure Legends:

Figure 1: Localization of caveolin-1 (Cav-1), STIM1 and Orai1 in human airway smooth muscle (ASM) cells. A. Immunocytochemical staining of human ASM cells and confocal microscopy demonstrated expression of both Cav-1 and Orai1 within the plasma membrane (top row; inset in composite panel magnified 10X). STIM1 was primarily expressed in the intracellular compartments (bottom row; inset in composite panel magnified 10X). Cav-1 secondary stain was Alexa488 (green), while STIM1 or Orai1 were visualized using Alexa568 (red). Composite represents overlap of green and red images from same cell. Scale bar is 10 μm. B. Cav-1, STIM1 and Orai1 expression were also determined in isolated plasma membrane (PM) and microsomal (MS) fractions from ASM cells. Cellular fractions confirmed that Cav-1 and Orai1 were highly expressed in PM fractions, while STIM1 expression was limited to the heavy MS fraction.

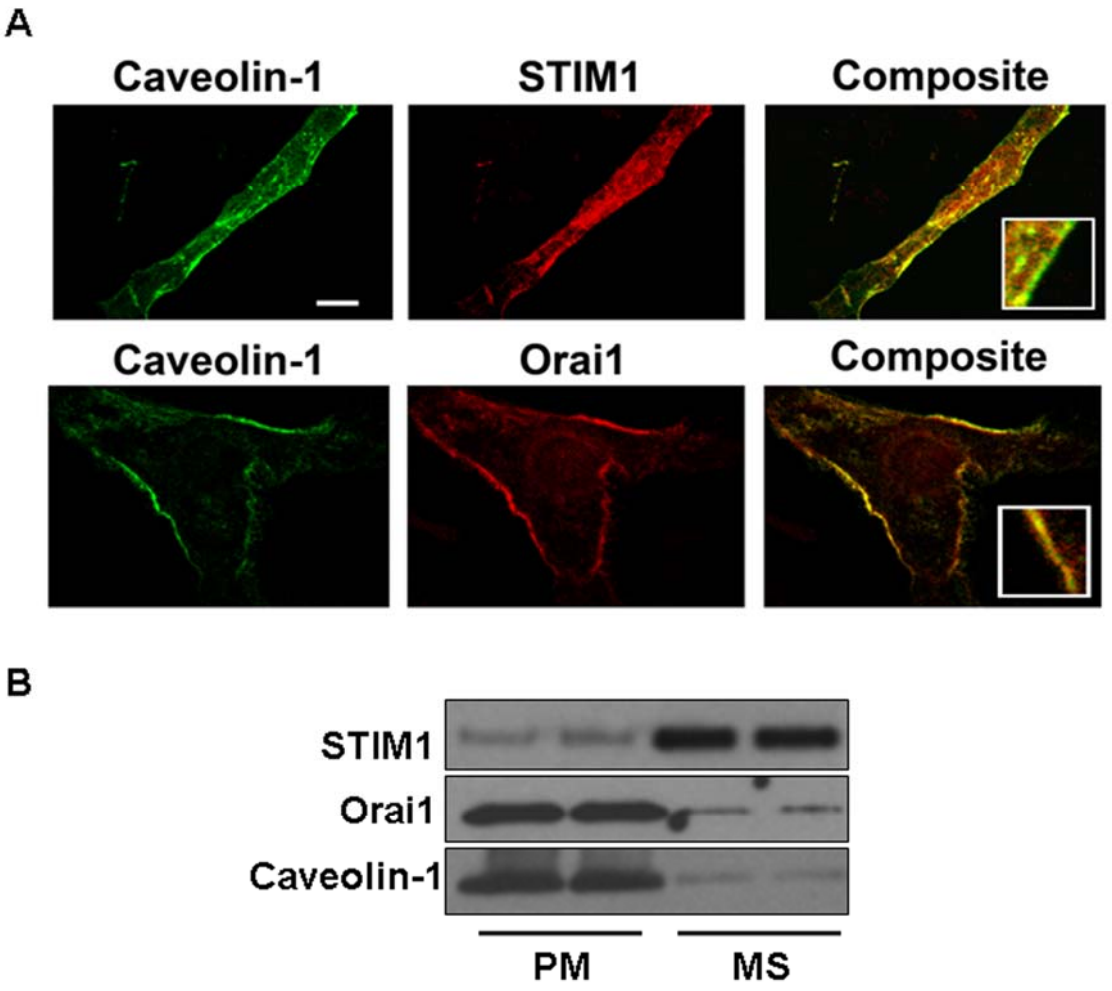

Figure 2: Effect of Cav-1 overexpression and TNFα on human ASM cells. A. Confocal fluorescence imaging demonstrated that overexpression of Cav-1 using Cav-1-mRed significantly increased Cav-1 expression compared to vehicle (control-mRed transfection). B. Western blots analysis showed that exposure to TNFα increased Cav-1 (as shown before). This effect was further enhanced in the presence of Cav-1-mRed. Values are means $\pm$ SE. * indicates significant Cav-1-mRed effect, # indicates significant TNFα effect ($p<0.05$).

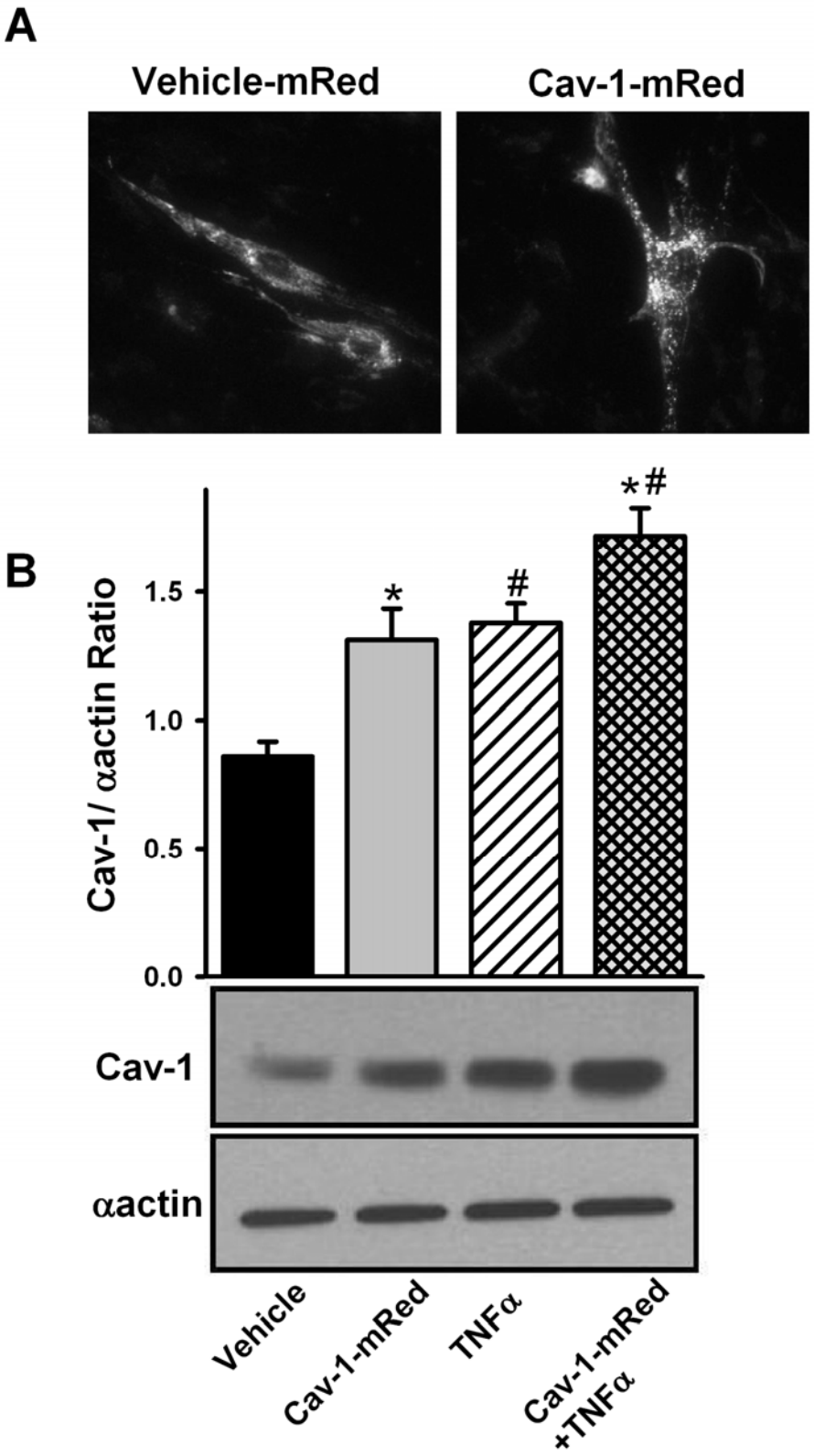
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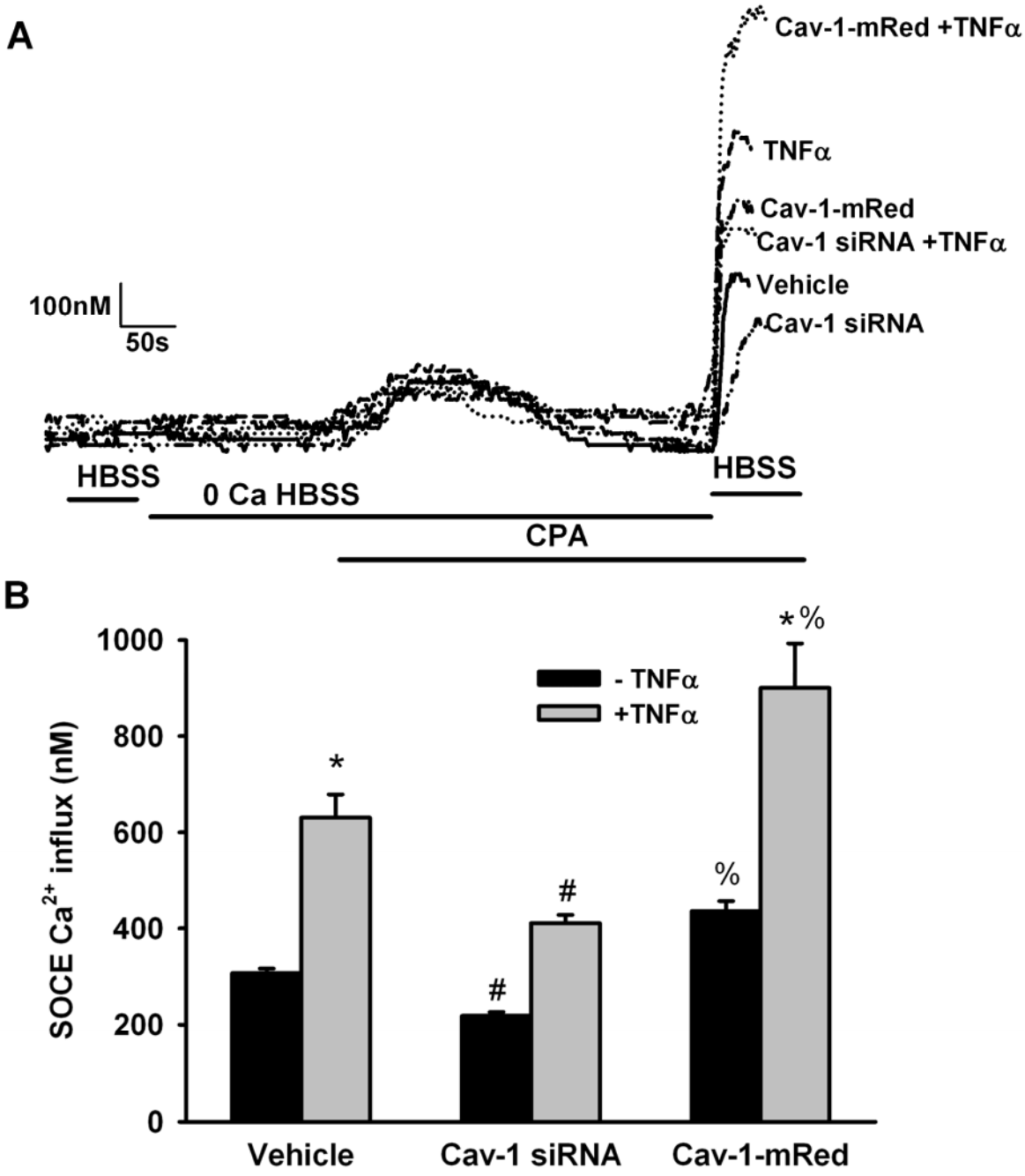
Figure 3: Effect of the pro-inflammatory cytokine TNFα, Cav-1 small interference RNA (siRNA) and Cav-1 overexpression (Cav-1-mRed) on SOCE in human ASM cells. A. Representative $[Ca^{2+}]_i$ tracings demonstrating SOCE in the different experimental groups. After removal of extracellular Ca^{2+}, SR Ca^{2+} stores were depleted with cyclopiazonic acid (CPA). Subsequent rapid introduction of extracellular Ca^{2+} resulted in activation of SOCE (in the continued presence of CPA). B. Overnight exposure to TNFα significantly increased SOCE in comparison to vehicle control. In cells transfected with Cav-1 siRNA, SOCE was significantly decreased compared to vehicle control and TNFα. Overexpression of Cav-1 using Cav-1-mRed significantly increased SOCE compared to vehicle control. Exposure to TNFα further enhanced SOCE in the presence of Cav-1-mRed. Values are means $\pm$ SE. * indicates significant TNFα, # indicates significant siRNA effect, % indicates significant Cav-1-mRed effect ($p<0.05$).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Cav-1-mRed +TNFα
TNFα
Cav-1-mRed
Cav-1 siRNA +TNFα
Vehicle
Cav-1 siRNA
100nM
50s
HBSS
0 Ca HBSS
HBSS
CPA
B
SOCE Ca2+ influx (nM)
1000
800
600
400
200
0
- TNFα
+TNFα
*
#
#
%
*%
Vehicle
Cav-1 siRNA
Cav-1-mRed

Figure 4: Effect of TNFα and Cav-1 on STIM1 and Orai1 expression in human ASM cells. A. Exposure to TNFα increased both STIM1 and Orai1 expression in ASM cell lysates. In Cav-1 siRNA transfected cells Orai1 expression significantly decreased in both in cell lysate and caveolar fractions (A and B). In Cav-1-mRed transfected cells Orai1 expression significantly increased in both in cell lysate and caveolar fractions in the presence of TNFα. Interestingly; STIM1 expression was very low within caveolar membrane fractions in comparison to Orai1 and did not show significant changes (B). Values are means ± SE. * indicates significant TNFα, # indicates significant Cav-1 siRNA effect, % indicates significant Cav-1-mRed effect ($p<0.05$).

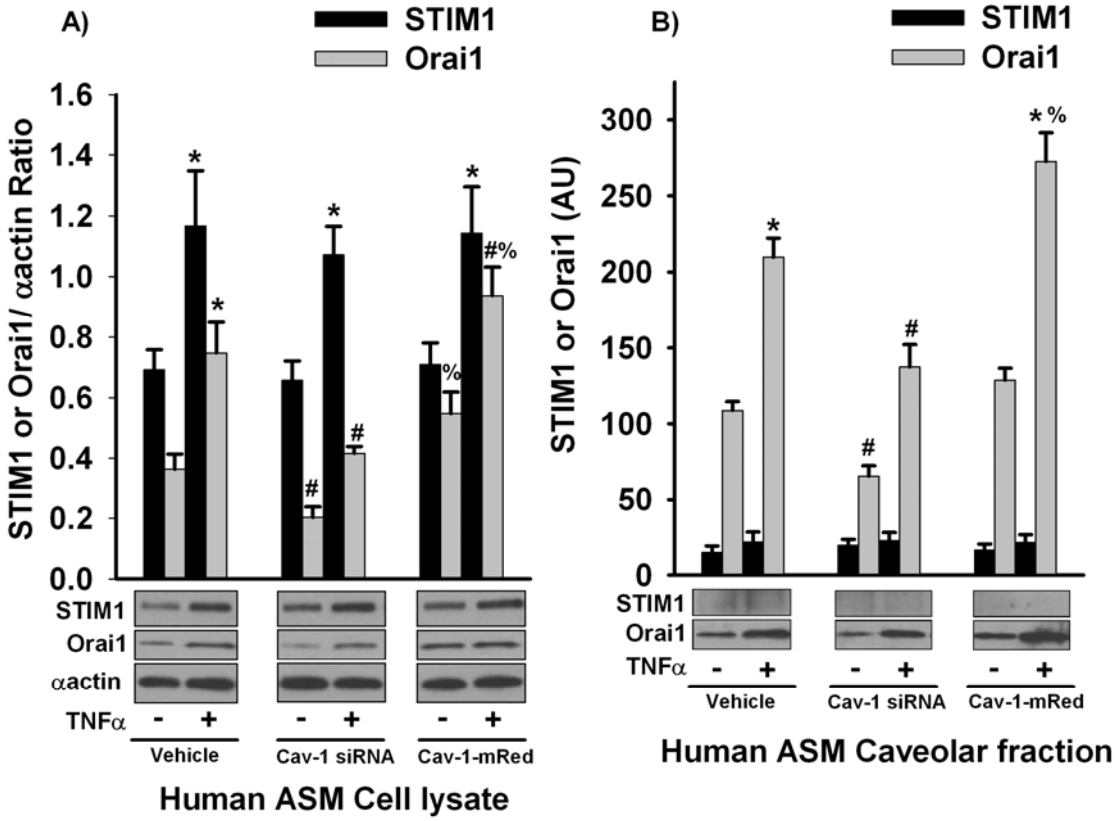


Figure 5: Effect of Orai1 siRNA on $[Ca^{2+}]_i$ and SOCE in human ASM cells. A: Transfection efficiency of Orai1 using siRNA was verified by Western blot analysis. Smooth muscle αactin served as loading control. B: Orai1 siRNA significantly reduced histamine-induced $[Ca^{2+}]_i$ responses compared to vehicle and negative control. C: Orai1 siRNA significantly reduced SOCE compared to vehicle control. Exposure to 5 μM Cav-1 specific scaffolding domain inhibitor peptide (CSD; 6 hours) significantly reduced SOCE (confirming the Cav-1 siRNA effect on SOCE in Figure 3). Combination of Orai1 siRNA and CSD also significantly reduced SOCE compared to vehicle control but not in comparison to Orai1 siRNA or CSD alone. Cav-1-mRed significantly increased SOCE, which was decreased by Orai1 siRNA. Orai1 siRNA significantly reduced SOCE in the presence and absence of TNFα, confirming the importance of Orai1 during airway inflammation. Values are means $\pm$ SE. * indicates significant Orai1 siRNA effect, # indicates significant CSD effect, & indicates significant Cav-1-mRed effect, %indicates significant TNFα, ($p<0.05$).

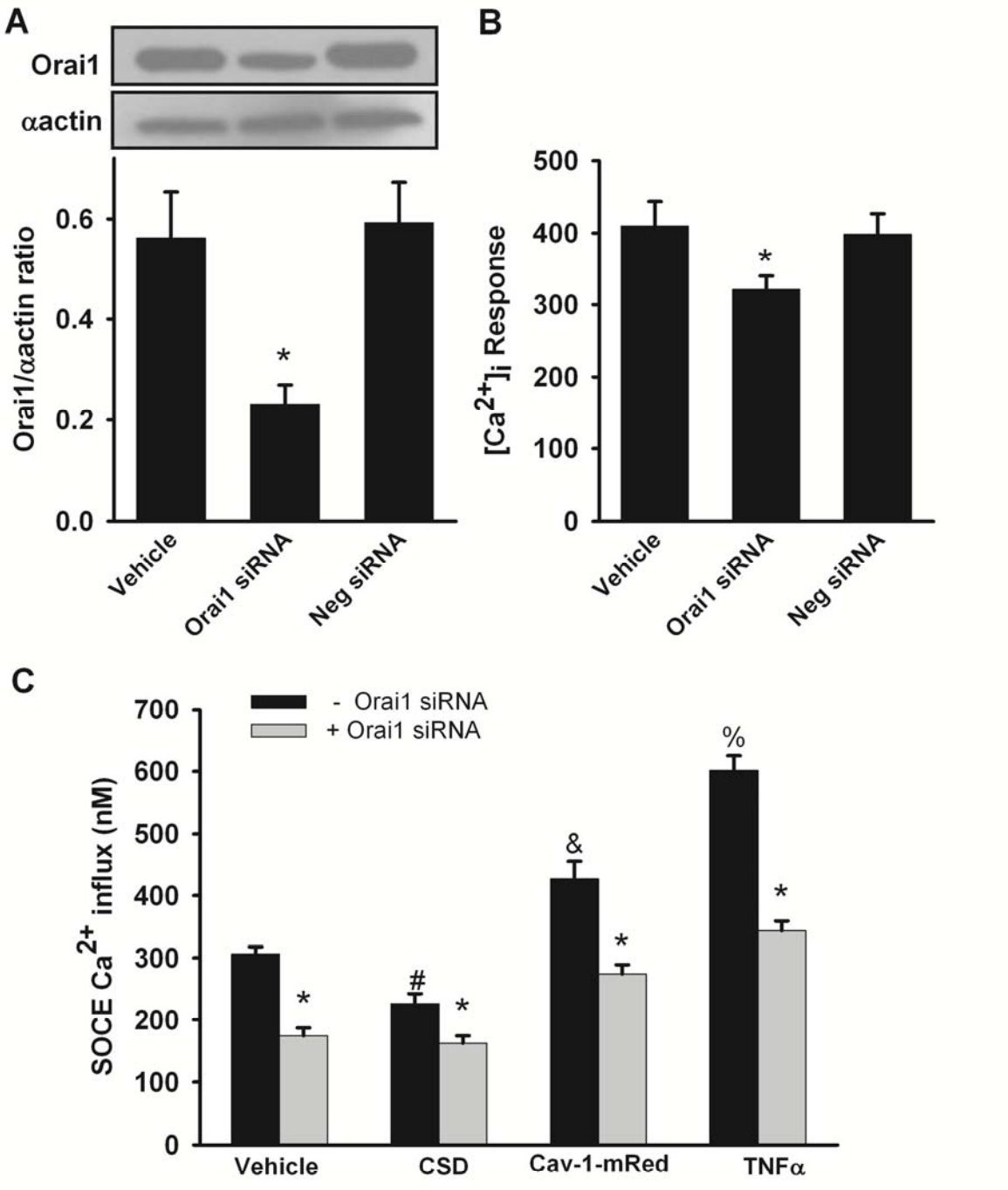

A
Orai1
αactin
Orai1/αactin ratio
0.0
0.2
0.4
0.6
*
Vehicle
Orai1 siRNA
Neg siRNA
B
[Ca2+]i Response
0
100
200
300
400
500
*
Vehicle
Orai1 siRNA
Neg siRNA
C
- Orai1 siRNA
+ Orai1 siRNA
SOCE Ca2+ influx (nM)
0
100
200
300
400
500
600
700
*
#
*
&
*
%
*
Vehicle
CSD
Cav-1-mRed
TNFα

Figure 6: Effect of STIM1 overexpression and Cav-1 siRNA on SOCE in human ASM cells. A: Overexpression of STIM1 using GFP-STIM1 significantly increased SOCE when compared to vehicle control (transfection control). STIM1 siRNA significantly reduced SOCE when compared to vehicle control. Cav-1 siRNA transfection partially reduced the GFP-STIM1-mediated increase in SOCE. B: GFP-STIM1 transfection significantly increased STIM1 expression confirmed by western blot analysis. Values are means $\pm$ SE. * indicates significant GFP-STIM1 effect, # indicates significant Cav-1 siRNA effect, % indicates significant STIM1 siRNA effect, ($p<0.05$).

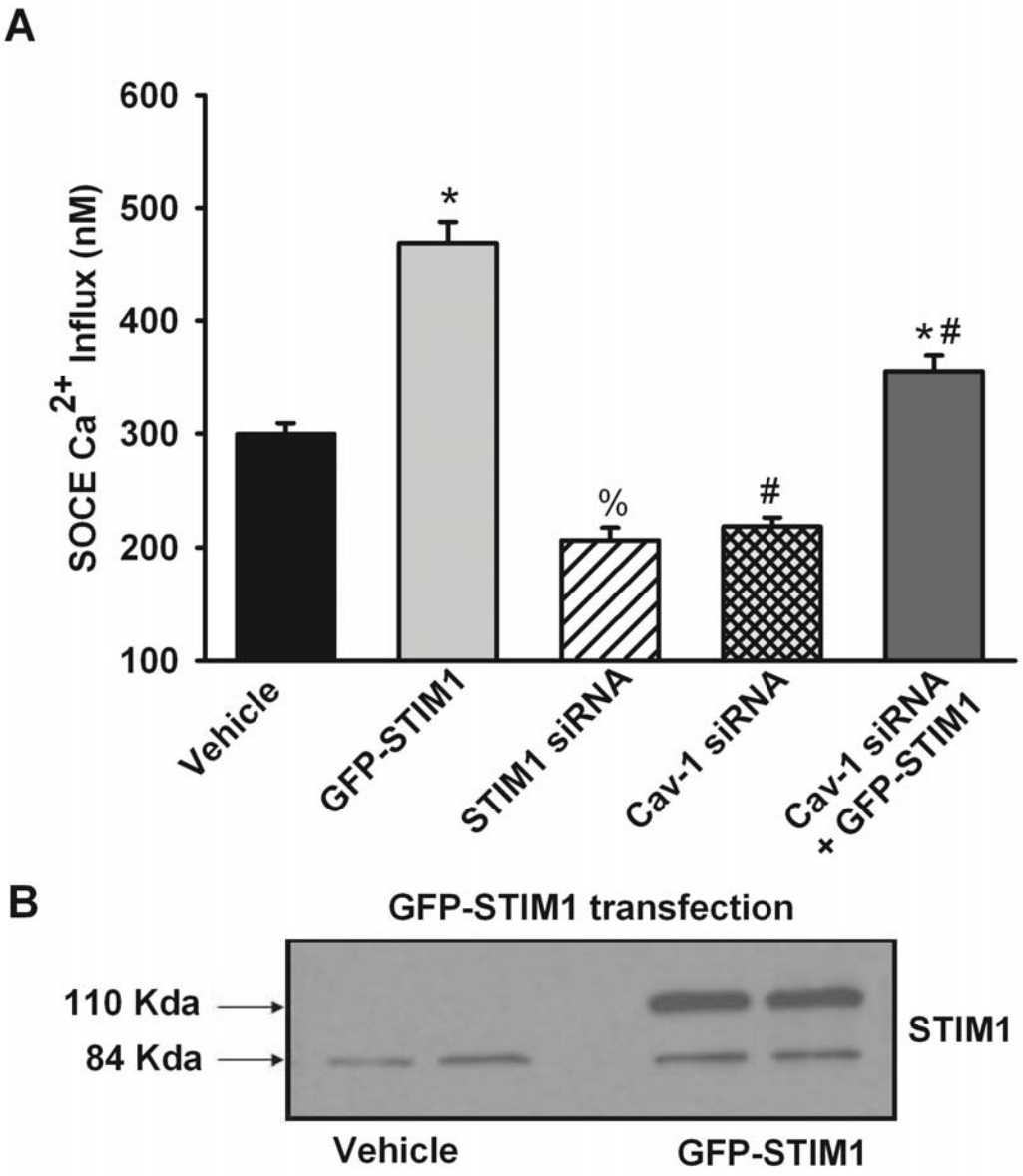
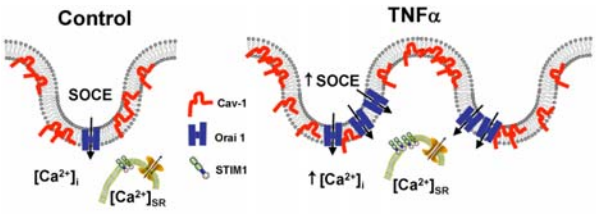

A
600
500
400
300
200
100
SOCE Ca2+ Influx (nM)
*
%
#
*#
Vehicle
GFP-STIM1
STIM1 siRNA
Cav-1 siRNA
Cav-1 siRNA + GFP-STIM1
B
GFP-STIM1 transfection
110 Kda
84 Kda
STIM1
Vehicle
GFP-STIM1

Figure 7: Schematic of TNFα effects on caveolae and SOCE components (STIM1 and Orai1). Caveolae in ASM cell membranes express Cav-1 as well as major SOCE player Orai1. Sarcoplasmic reticulum contains STIM1 and other Ca^{2+} regulatory proteins. Exposure to TNFα increases the expression of Cav-1 as well as Orai1 with more caveolae being formed or with more regulatory proteins within caveolae. These changes contribute to increased $[Ca^{2+}]_i$ in ASM.



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