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STIM1 but not STIM2 is an essential regulator of Ca^{2+} influx-mediated NADPH oxidase activity in neutrophil-like HL-60 cells

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ABSTRACT

Extracellular Ca^{2+} entry, primarily mediated through store-operated Ca^{2+} entry (SOCE), is known to be a critical event for NADPH oxidase (NOX2) regulation in neutrophils. While defective NOX2 activity has been linked to various inflammatory diseases, regulatory mechanisms that control Ca^{2+} influx-induced NOX2 activation are poorly understood in SOCE. The role of STIM1, a Ca^{2+} sensor that transduces the store depletion signal to the plasma membrane, seems well established and supported by numerous studies in non-phagocytic cells. Here, in neutrophil-like HL-60 cells we used a siRNA approach to delineate the effect of STIM1 knock-down on NOX2 activity regulated by Ca^{2+} influx. Because the function of the STIM1 homolog, STIM2, is still unclear, we determined the consequence of STIM2 knock-down on Ca^{2+} and NOX2. STIM1 and STIM2 knock-down was effective and isoform specific when assayed by real-time PCR and Western blotting. Consistent with a unique role of STIM1 in the regulation of SOCE, STIM1, but not STIM2, siRNA significantly decreased Ca^{2+} influx induced by fMLF or the SERCA pump inhibitor thapsigargin. A redistribution of STIM1, originally localized intracellularly, near the plasma membrane was observed by confocal microscopy upon stimulation by fMLF. Inhibition of STIM1-induced SOCE led to a marked decrease in NOX2 activity while STIM2 siRNA had no effect. Thus, our results provide evidence for a role of STIM1 protein in the control of Ca^{2+} influx in neutrophils excluding a STIM2 involvement in this process. It also places STIM1 as a key modulator of NOX2 activity with a potential interest for anti-inflammatory pharmacological development.

1. Introduction

Leukocytes are an important part of the host defence against invasion of pathogenic microbes. Superoxide anions generated in neutrophils by NADPH oxidase, referred to as NOX2 [1–3] in the novel terminology, and their derived products are involved in the killing of bacteria. By a sustained production of reactive oxygen species, an inappropriate activation of NOX2 may have pathological consequences. Infiltration and persistent presence of hyper-responsive and easily activated neutrophils may be implicated in the pathogenesis of many diseases (respiratory distress syndromes, rheumatoid arthritis, ischemia-reperfusion injury) [4,5].

Elevation of cytosolic free Ca^{2+} concentration ($[Ca^{2+}]_c$) is crucial for a number of neutrophil responses to a variety of stimuli. This Ca^{2+} signal is often initiated by intracellular stores Ca^{2+} release, which is coupled to the opening of Ca^{2+} channels localized in the plasma membrane, giving rise to a consequent Ca^{2+} influx known as store-operated Ca^{2+} entry (SOCE) [6]. Evidence for the importance of Ca^{2+} influx in NOX2 activation [7,8] is supported by a decrease of superoxide anion secretion when extracellular Ca^{2+} is omitted or chelated by EGTA [7,9–11]. Because $[Ca^{2+}]_c$ elevation is critically involved in the regulation of NOX2 activity, modulation of Ca^{2+} influx might be beneficial under inflammatory conditions.

Inhibition of emerging candidates for store-operated channels (SOCs), such as transient receptor potential canonical (TRPC) and Orai channels [11–14] may be exploited and offered anti-inflammatory therapeutical benefits [15]. However, mechanisms by which channel blockers [16–20] interfere with SOCs are not formally determined and non-specific effects are not excluded. Recently, the stromal-interacting molecules 1 and 2 (STIM1 and STIM2), that are transmembrane proteins located in Ca^{2+} stores, have been identified as potent regulators of Ca^{2+} homeostasis. The function of STIM1 is to act as the Ca^{2+} sensor for endoplasmic reticulum Ca^{2+} content and transduces the Ca^{2+} store depletion signal to SOCs in the plasma membrane [21,22]. In contrast, the physiological function of STIM2 is still unclear. Conflicting results have been reported about a possible role of STIM2 in negatively

Abbreviations: 2-APB, 2-aminoethoxydiphenyl borane; $[Ca^{2+}]_c$, cytosolic free calcium concentration; fMLF, N-formyl-L-methionyl-L-leucyl-L-phenylalanine; siRNA, small interfering RNA; SOCE, store-operated Ca^{2+} entry; SOCs, store-operated Ca^{2+} channels; STIM, stromal-interacting molecule.

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regulating STIM1 [23] or positively regulating SOCE [21,24,25]. So far, no studies have tackled simultaneously the role of both proteins in neutrophils. The mechanism governing SOCE in neutrophils remains controversial and could be partially unrelated to those described in other non-excitable cells.

Given the importance of Ca^{2+} influx in NOX2 activity, we explored whether STIM1 and STIM2 knock-down might negatively affect Ca^{2+} mobilization and consequently whether the regulation of STIM proteins could have a potential impact on the control of inflammatory processes through a decrease of reactive oxygen species production. Our results demonstrated that STIM1 knock-down is associated to a decrease of bacterial chemopeptide fMLF- and thapsigargin-induced Ca^{2+} influx in neutrophil-like HL-60 cells [26–28]. This alteration of SOCE via STIM1 inhibition is correlated to a strong reduction of oxidant production. On the contrary, STIM2 does not participate to Ca^{2+} influx-mediated NOX2 activity. In conclusion, we point out the significance of STIM1 signalling for the correct sequence of NOX2 activation.

2. Materials and methods

2.1. Chemicals

RPMI-1640 medium, fetal bovine serum, L-glutamine, penicillin and streptomycin were obtained from Lonza (Verviers, Belgium). N-formyl-L-methionyl-L-leucyl-L-phenylalanine (fMLF), thapsigargin, EGTA, ionomycin, horseradish peroxidase (HRP) type II were purchased from Sigma Chemicals Co. (Bornem, Belgium). Fura-2 acetoxymethylester (fura-2/AM) and 10-acetyl-3,7-dihydroxyphenoxazine (Amplex Red) were from Invitrogen (Merelbeke, Belgium). SK&F 96365 and 2-aminoethoxydiphenyl borane (2-APB) were purchased from Calbiochem (La Jolla, CA, USA). All other chemicals were of analytical grade and obtained from Merck (Darmstadt, Germany). The physiological salt solution (PSS) used throughout this study has the following composition (mM): NaCl 115, KCl 5, KH_2PO_4 1, glucose 10, $MgSO_4$ 1, $CaCl_2$ 1.25, Hepes-Na 25, supplemented with bovine serum albumin (BSA) 0.1%, pH 7.4. Where indicated, $CaCl_2$ was omitted in PSS.

2.2. Cell culture

The human promyelocytic cell line HL-60 [29] was purchased from ATCC (Manassas, VA, USA) [#CCL-240] and was grown in RPMI-1640 medium supplemented with 10% heat-inactivated fetal bovine serum, L-glutamine (2 mM), streptomycin (100 μg/ml) and penicillin (100 units/ml). The cells were cultured at 37 °C, in a humidified atmosphere of 5% CO_2, 95% air. The culture was passaged twice weekly. The differentiation towards neutrophil-like cells was induced by addition of DMSO (final concentration 1.3%) for 4–5 days [30].

2.3. Fluorescence measurements

Measurement of $[Ca^{2+}]_c$ and H_2O_2 production were concomitantly performed by a double labeling fluorescent technique as described previously [31] with a Quantamaster spectrofluorimeter QM-8/2003 (Photon Technology International Inc., Lawrenceville, NJ, USA). Briefly, differentiated HL-60 cells (1×10^6 cells) were loaded with fura-2/AM (2.5 μM fura-2/AM in PSS) for 30 min at 37 °C, washed three times and resuspended in PSS or Ca^{2+} free-PSS. 30 μM Amplex Red and 1 unit/ml HRP were added during the incubation of cells for 10 min at 37 °C to allow the H_2O_2 production measurement. Cells were placed in the fluorimeter and $[Ca^{2+}]_c$ and H_2O_2 production measurements were obtained as previously described [31]. Results are expressed as $[Ca^{2+}]_c$ augmentation ($\Delta[Ca^{2+}]_c$). Basal and stimulated H_2O_2 productions were determined by calculating the initial and maximal slopes of the tangents (dFluo/dt) of the H_2O_2 production time course curve, before and after stimulation of cells. Production of H_2O_2, expressed in arbitrary units (A.U.), was finally calculated by subtracting basal from maximal fMLF-stimulated amount of H_2O_2.

2.4. Determination of Mn^{2+} influx

Fluorescence measurements were monitored on fura-2 loaded cells at 510 nm at the isobestic point of excitation 360 nm. Mn^{2+} (1 mM) was added to the cell suspension prior to inhibitor (SK&F 96365 or 2-APB) and inducer treatment (fMLF). Finally, ionomycin (10 μM) was added. Ca^{2+} influx was inferred from the rate of quenching (−dFluo/dt) of fura-2 fluorescence.

2.5. Quantitative real-time PCR

Total RNA was isolated from HL-60 cells using the Invisorb spin cell RNA mini kit (Invitek, Berlin, Germany) according to the manufacturer's instructions. First-strand cDNA was prepared from 0.5 μg of total RNA, using the ThermoScript™ RT-PCR System and 50 ng/μl random primers (Invitrogen, Merelbeke, Belgium). Reverse transcription was performed at 25 °C for 10 min, followed by 50 min at 50 °C with subsequent transcriptase inactivation at 85 °C for 5 min. Two units of RNase H were added and incubated at 37 °C for 20 min. PCR primers for STIM1 (forward: 5′-TGGGATCT-CAGAGGGATTTG-3′, reverse: 5′-CATTGGAAGTCATGGCATTG-3′), STIM2 (forward: 5′-CCAGGGCTTTCACTGTGATT-3′, reverse: 5′-CCTCGGCTTAAGGTTGTGAA-3′) and β-actin (forward:5′ TGACC-CAGATCATGTTTGAGA-3′, reverse: 5′-AGTCCATCACGATGCCAGT-3′) genes were designed based on published sequences in GenBank. Real-time PCR was performed on the iQ™5 Real-Time PCR Detection System using iQ™SYBR® Green supermix (Bio-Rad, Nazareth, Belgium) and cDNA synthesized as described above. Real-time PCR was performed following the manufacturer's protocol in a 25 μl reaction volume containing 2 μl of cDNA and 1 μM STIM1, STIM2 or β-actin-specific primer pair. Cycling conditions consisted of enzyme activation for 3 min at 95 °C followed by 40 cycles of denaturation at 95 °C for 15 s and annealing at 60 °C for 30 s. Conditions were optimized to ensure similar amplification efficiencies for all products. Real-time PCRs were done in triplicate and the specificity of the reactions was confirmed by melting-curve analysis. Melting curves for each primer pair showed one specific signal.

To determine the relative expression levels of STIM1 and STIM2, the comparative threshold cycle ($2^{-\Delta\Delta Ct}$) method was used [32]. To control the PCR for variation in the quantity and quality of the RNA added, β-actin (housekeeping gene) was used as an internal control. By subtracting the Ct of the β-actin from the Ct of the gene of interest (STIM RNA), ΔCt was determined. STIM1 in differentiated HL-60 cells was set as the calibrator to determine the relative expression of STIM1 and STIM2 in HL-60 cells. Sequence analysis of PCR-amplified DNA fragments was performed and revealed a perfect match with the sequences of the corresponding STIM genes (data not shown). All experiments were repeated at least three times using three different preparations of mRNA isolated from HL-60 cells.

2.6. RNA interference assays

Double-stranded small interfering RNA (siRNA) of 19 nucleotides targeting human STIM1 and STIM2 (as well as a non-silencing control RNA) were designed and obtained from Eurogentec (Seraing, Belgium). Three synthesized siRNA candidate duplexes *per* gene of interest were tested for silencing efficiency. The most

efficient siRNA (or mixture of siRNA) to mediate knock-down expression of STIM proteins was chosen and used for functional analysis. All experiments were performed with a siRNA duplex for STIM1 (sense: 5′-GGAGGAUAAUGGCUCUAUU-3′, antisense: 5′-AAUAGAGCCAUUAUCCUCC-3′) and a mixture of two siRNA duplexes for STIM2 (sense 1: 5′-AAUUUAGAGCGCAAAAUGA-3′, antisense 1: 5′-UCAUUUUGCGCUCUAAAUU-3′, sense 2: 5′-GUGCACGAACCUUCAUUUA-3′, antisense 2: 5′-UAAAUGAAGGUUCGUGCAC-3′). The scramble siRNA (non-silencing sequence) (sense: 5′-AAUUCUCCGAACGUGUCAC-3′, antisense: 5′-GUGACACGUUCGGAGAAUU-3′) was used as a negative control. All siRNA target sequences chosen in this study were screened by NCBI BLAST searches to predict whether a siRNA would have off-target effects and cause knock-down of a non-targeted gene with which it coincidentally has high homology. No off-target sequences were found confirming the specificity of the duplex. Differentiated HL-60 cells (2×10^6) were transiently transfected with 1–3 μg siRNA specific for STIM1, STIM2 or the non-silencing control sequence using the Nucleofector apparatus (Amaxa Biosystems, Cologne, Germany) according to the manufacturer's protocol. Transfection efficiency was about 80% when using the Nucleofector V kit with program T-019. Approximately 36 h post-transfection, cells were processed for RNA isolation, protein lysates or for H_2O_2 production and Ca^{2+} measurements.

2.7. Western blotting analysis

HL-60 cells were washed twice with PSS and lysed on ice for 30 min in lysis buffer containing Tris 50 mM, pH 7.4, NaCl 150 mM, EDTA 1 mM, Triton X-100 1% and a mix of serine and cysteine protease inhibitors (Roche Molecular Biochemicals, Basel, Switzerland). Lysates were centrifuged at 10,000 × *g* at 4 °C for 10 min. Equal volume of Laemmli 2X buffer was added to supernatants. Total cell lysates were then electrophoresed on 10% SDS-polyacrylamide gel, transferred to nitrocellulose, blocked with 5% non-fat milk, and analyzed by Western blotting using monoclonal anti-STIM1 (BD Biosciences, Erembodegem, Belgium) or polyclonal anti-STIM2 (Abcam, Cambridge, UK) antibodies. Quantification of proteins was performed by densitometric scanning of autoradiograms (Gel-Pro Analyzer, INTAS, Göttingen, Germany).

2.8. Immunofluorescence and confocal microscopy

Differentiated HL-60 cells were collected and rinsed with PSS before plating on sterile round coverslips in a 24-well plate. Cells were allowed to adhere by centrifugation for 5 min at 300 × *g* then stimulated with 1 μM fMLF (or DMSO as vehicle) for 1 min. The stimulation was blocked by addition of ice-cold methanol for 15 min at 4 °C. After washing and permeabilization for 10 min with PBS containing 0.5% BSA and 0.5% Triton X-100 (PBSA-T), non-specific binding sites were blocked for 30 min with 5% human IgG (Sigma, Bornem, Belgium) in PBSA-T. Primary antibodies against STIM1 (BD Biosciences, Erembodegem, Belgium) or control mouse serum (Jackson Immunoresearch, Suffolk, UK) were added for a prolonged incubation of 60 min. After several washes, a cyanine 2-conjugated secondary antibody directed against mouse IgG (Jackson Immunoresearch, Suffolk, UK) was added in addition to wheat germ agglutinin labeled with Alexafluor 633 (WGA-AF633) and DAPI (Invitrogen, Merelbeke, Belgium) for 60 min. Washed samples were mounted in Mowiol/Dabco polymerized overnight at 4 °C. Cell staining was observed on a confocal inverted microscope Zeiss LSM 510 META (Zaventem, Belgium) by using a multitrack configuration, to avoid crosstalk between cyanine 2 and WGA-AF633, and a 63× oil objective.

2.9. Flow cytometry

Antibody labeling was conducted by an indirect immunofluorescence technique and analyzed by flow cytometry. Differentiated HL-60 cells, Jurkat T cells and HeLa cells were harvested and washed twice with incubation buffer (137 mM NaCl, 5 mM KCl, 50 mM Hepes, 1 mg/ml glucose, 10 mg/ml BSA, pH 7.4) and resuspended in PSS. Cells (0.5×10^6) were stimulated with 1 μM thapsigargin for 10 min at room temperature. Stimulation was stopped with an excess of PSS buffer at 4 °C. After centrifugation, cells were first saturated with 5% (v/v) purified human IgG (Sigma, Bornem, Belgium) in incubation buffer then labeled with 10 μg anti-Stim1 polyclonal antibody (Alomone, Jerusalem, Israel) and 10 μg rabbit gamma globulin (Jackson Immunoresearch, Suffolk, UK). Cells were incubated at 4 °C for 30 min with the primary antibodies, then washed once and labeled with the R-PE-conjugated anti-rabbit secondary antibodies (Jackson Immunoresearch, Suffolk, UK) for 30 min at 4 °C. After a final washing step, cells were resuspended in incubation buffer in the presence of 7-AAD (Sigma, Bornem, Belgium) to discriminate dead from living cells. Analyses of cells were performed on a BD FACSCanto™ II analyzer (BD Biosciences, Erembodegem, Belgium) for fluorescence intensity.

2.10. Statistics

Values obtained from different cell batches are expressed as mean ± standard error of the mean (SEM), $n = 3$. Data were analyzed by the ANOVA test. $P < 0.05$ was considered statistically significant.

3. Results

3.1. Endogenous expression of STIM1 and STIM2 in HL-60 cells

Initially, we assessed the expression of the two known human homologues STIM1 and STIM2 in differentiated HL-60 cells. Quantitative real-time PCR was used to determine the relative expression of detected STIM mRNA. The amount of PCR products was normalized to that of β-actin (expression of β-actin mRNA is similar in promyelocytic undifferentiated HL-60 cells and differentiated HL-60 cells, data not shown) and calculated in reference to STIM1 expression in differentiated HL-60 cells (see Section 2). Both STIM1 and STIM2 are expressed in HL-60 cells (Fig. 1A). While STIM1 and STIM2 mRNA are found uniformly expressed in undifferentiated HL-60 cells, STIM1 mRNA is expressed in the greatest abundance in differentiated HL-60 cells. STIM2 mRNA expression pattern was not modified but STIM1 mRNA expression increased moderately during the differentiation process (Fig. 1A).

Western blot analysis was carried out to correlate mRNA level to protein level of STIM and confirm our previous observations. As expected, STIM proteins were found expressed in HL-60 cells. The level of STIM1 protein was higher in differentiated HL-60 cells than in undifferentiated HL-60 cells whereas the level of STIM2 protein remained unchanged during the differentiation process (Fig. 1B).

3.2. STIM1 but not STIM2 activates SOCE in differentiated HL-60 cells

To address the issue whether STIM1 and STIM2 are functionally involved in the SOCE pathway in phagocytic cells, as previously reported in many cell types [21–25], we used specific siRNA sequences to target and selectively suppress both endogenous STIM1 and STIM2 proteins. siRNA individually targeting STIM1 and STIM2 led to an effective inhibition of the relevant target molecule with no discrete suppression of mRNA for non-targeted STIM. Transfection of siRNA specific for STIM1 or STIM2 resulted in a robust decrease of STIM mRNA (Fig. 2A). The ability of each siRNA

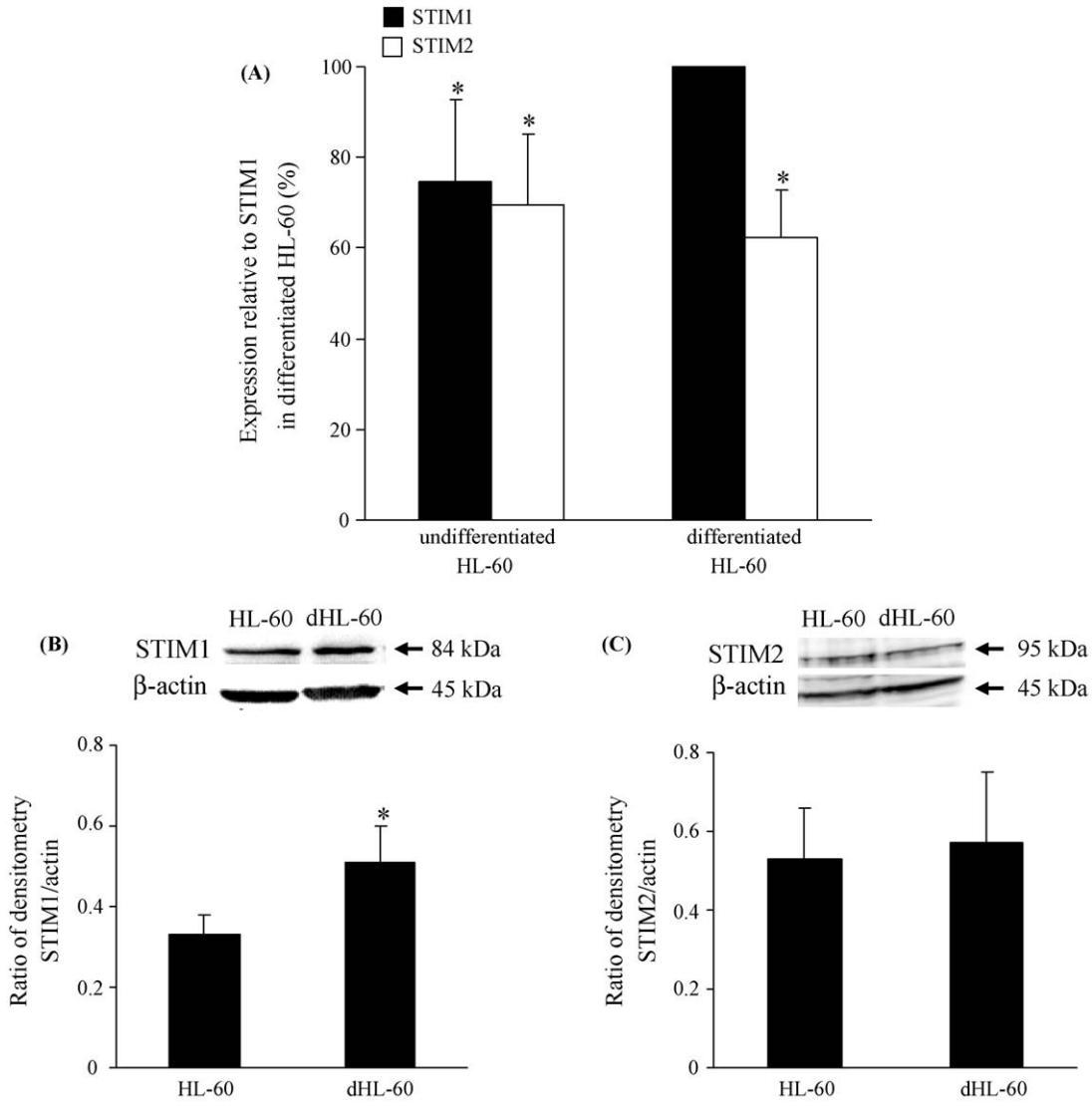

Fig. 1. Expression of STIM in HL-60 cells. (A) STIM1 and STIM2 mRNA expression was determined by real-time PCR in undifferentiated and differentiated HL-60 cells. Data were normalized to β-actin and expressed relative to STIM1 in differentiated HL-60 cells (%). Results are mean ± SEM. *Significantly different from STIM1 expression in differentiated HL-60 cells. (B) STIM1 and (C) STIM2 protein expression in undifferentiated HL-60 cells (HL-60) and differentiated HL-60 (dHL-60) cells was determined by Western blotting. Proteins were detected with specific anti-STIM1 or STIM2 antibodies. A western blot representative of three independent experiments is shown for STIM1 and STIM2. Histograms represent the quantification of (B) STIM1 or (C) STIM2. Protein level was normalized to β-actin. Results are presented as ratio of densitometry and expressed as mean ± SEM. *Significantly different from STIM1 expression in differentiated HL-60 cells.

to inhibit protein expression was evaluated by Western blotting. No evidence of cross-reactivity of the STIM1 antibody with expressed STIM2 and no cross-reactivity of STIM2 antibody with STIM1 were found (Fig. 2B and C). STIM1 protein level was significantly reduced by the specific gene silencing whereas STIM2 protein level was not changed. Similarly, STIM2 siRNA decreased STIM2 protein level without alteration of STIM1 protein levels confirming the specificity of STIM1 and STIM2 knock-down at the protein level (Fig. 2B and C).

Differentiated HL-60 cells transfected with STIM1 siRNA were stimulated with fMLF (0.1 μM), a bacterial peptide known to rapidly increase $[Ca^{2+}]_c$ and activate NOX2 a few seconds later [7–10,28]. As expected, STIM1 siRNA caused suppression of fMLF-mediated $[Ca^{2+}]_c$ elevation by more than 50% (Fig. 3A). To investigate the contribution of STIM1 in SOCE, we used thapsigargin which, by inhibiting Ca^{2+}-ATPase of intracellular Ca^{2+} stores, triggers a relatively slow concentration-dependent release of Ca^{2+} from intracellular stores [33]. Ca^{2+} influx, subsequent to thapsigargin (0.01 μM) stimulation, was approximately abolished by 50% after siRNA treatment, confirming the involvement of STIM1 in SOCE pathway (Fig. 3A). Similarly, the role of STIM2 was also investigated under normal external Ca^{2+} concentrations (1.25 mM). Knock-down of endogenous STIM2 mRNA had no effect, neither on fMLF nor on thapsigargin-initiated Ca^{2+} responses (Fig. 3A). To verify that STIM1 knock-down alters Ca^{2+} influx and not internal Ca^{2+} release in differentiated HL-60 cells, we performed an additional experiment in which STIM1 siRNA-transfected HL-60 cells were stimulated first with fMLF in Ca^{2+}-free medium to deplete intracellular Ca^{2+} stores. Extracellular Ca^{2+} was then added to monitor Ca^{2+} influx (Fig. 3B). We found that addition of fMLF in Ca^{2+}-free medium elicited an initial small rise in cytosolic Ca^{2+} similar in both control and STIM1 knock-down cells. Re-addition of extracellular Ca^{2+} resulted in a rapid Ca^{2+} influx into the cells which was reduced in HL-60 cells transfected with STIM1 siRNA (Fig. 3B). These observations supported the concept that STIM1 is not involved in Ca^{2+} release from stores but is essential for SOCE-mediated Ca^{2+} influx in neutrophil-like HL-60 cells.

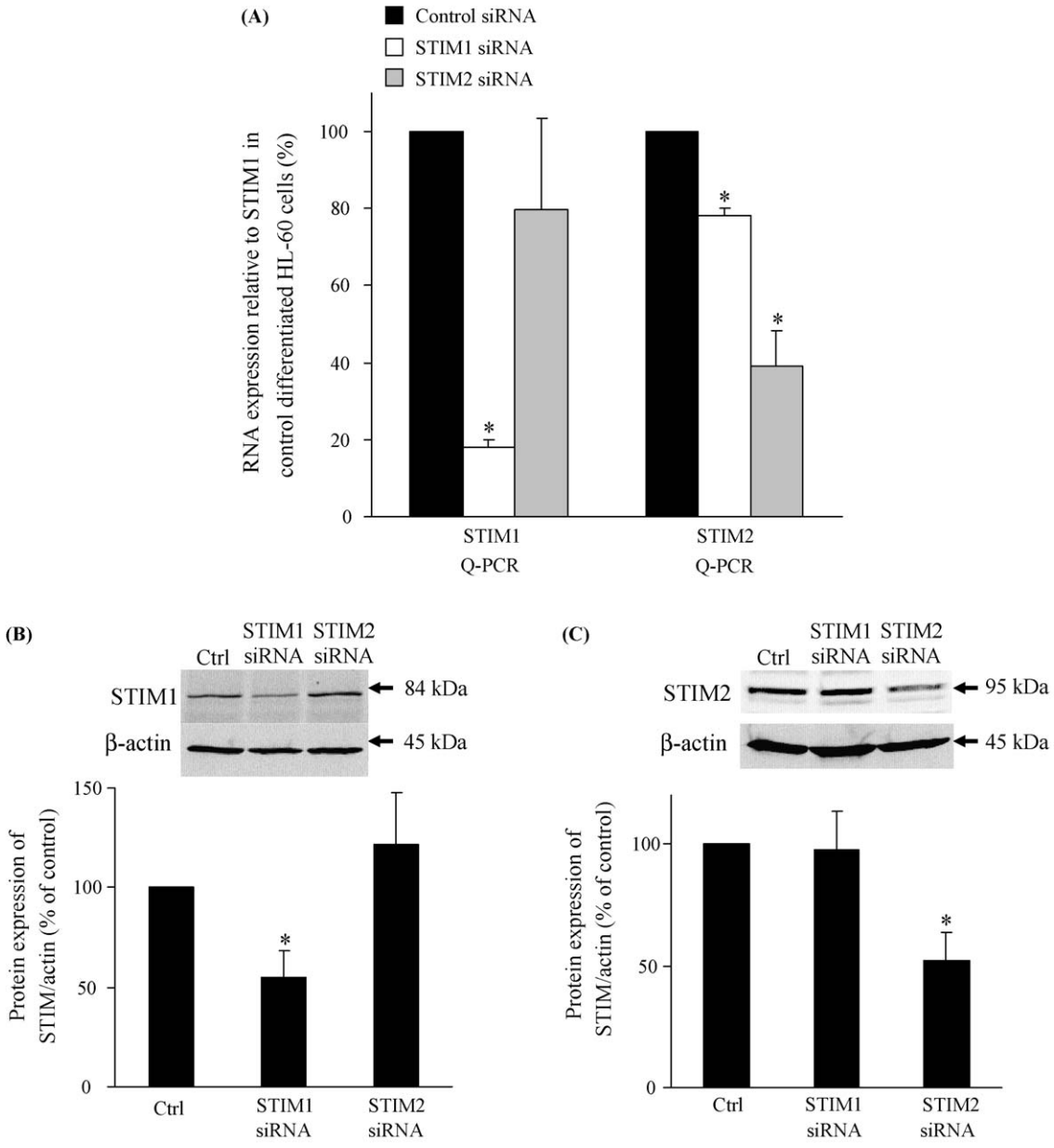

Fig. 2. Decrease of STIM1 and STIM2 expression after siRNA treatment. (A) Efficiency and specificity of STIM1 and STIM2 siRNA were determined by real-time PCR analysis. Differentiated HL-60 cells were transiently transfected with STIM1 or STIM2 siRNA. The amount of STIM1 and STIM2 mRNA in cells was normalized to β-actin and calculated in reference to STIM1 and STIM2 mRNA obtained from cells transfected with a non-silencing sequence used as control. Results are expressed as mean ± SEM from three independent experiments. *Significantly different from control. Specificity of (B) STIM1 and (C) STIM2 antibodies was determined by western blot analysis. A western blot representative of three independent experiments is shown. Histograms show efficiency of (B) STIM1 and (C) STIM2 siRNA on protein expression. Protein level was normalized to β-actin and expressed as % of control (non-silencing siRNA sequence). Results are expressed as mean ± SEM from three independent experiments. *Significantly different from control.

To further confirm the predominant role of STIM1 in extracellular Ca^{2+} entry, Ca^{2+} influx into cells was monitored by fura-2 fluorescence quenching using Mn^{2+} (1 mM). Subsequent to the addition of fMLF (0.1 μM), a pronounced decline of fura-2 isofluorescence was observed in stimulated cells (Fig. 4A). This decrease in fura-2 fluorescence, resulting from a reduced divalent cation influx, is attenuated in the presence of STIM1 siRNA. The rate of Mn^{2+} influx induced by fMLF in cells transfected with STIM1 siRNA is decreased by more than 50% and is identical to the one observed when cells are subjected to the purported SOCE inhibitors 2-aminoethoxydiphenyl borane (2-APB) [16,17] (10 μM) or SK&F 96365 [18,19] (10 μM) (Fig. 4B). Hence, these results argue that SOCE requires functional STIM1 protein in granulocytes.

3.3. Cellular localization and translocation towards the membrane of STIM1 in differentiated HL-60 cells upon fMF stimulation

Since it has been reported that a fraction of STIM1 is present in the plasma membrane of HEK-293 cells [19], we studied STIM1 surface expression in differentiated HL-60 cells by flow cytometry analysis. No surface labeling of STIM1 was observed, when an antibody reacting with the extracellular N-terminal domain of STIM1 was used (Fig. 5A). In contrast, STIM1 was detected on the plasma membrane of Jurkat T cells (Fig. 5C) and HeLa cells (data not shown). Thapsigargin did not trigger an elevation in the amount of plasma membrane STIM1 in all cell types (Fig. 5B and D). Although many studies based on overexpression of STIM1 have shown that

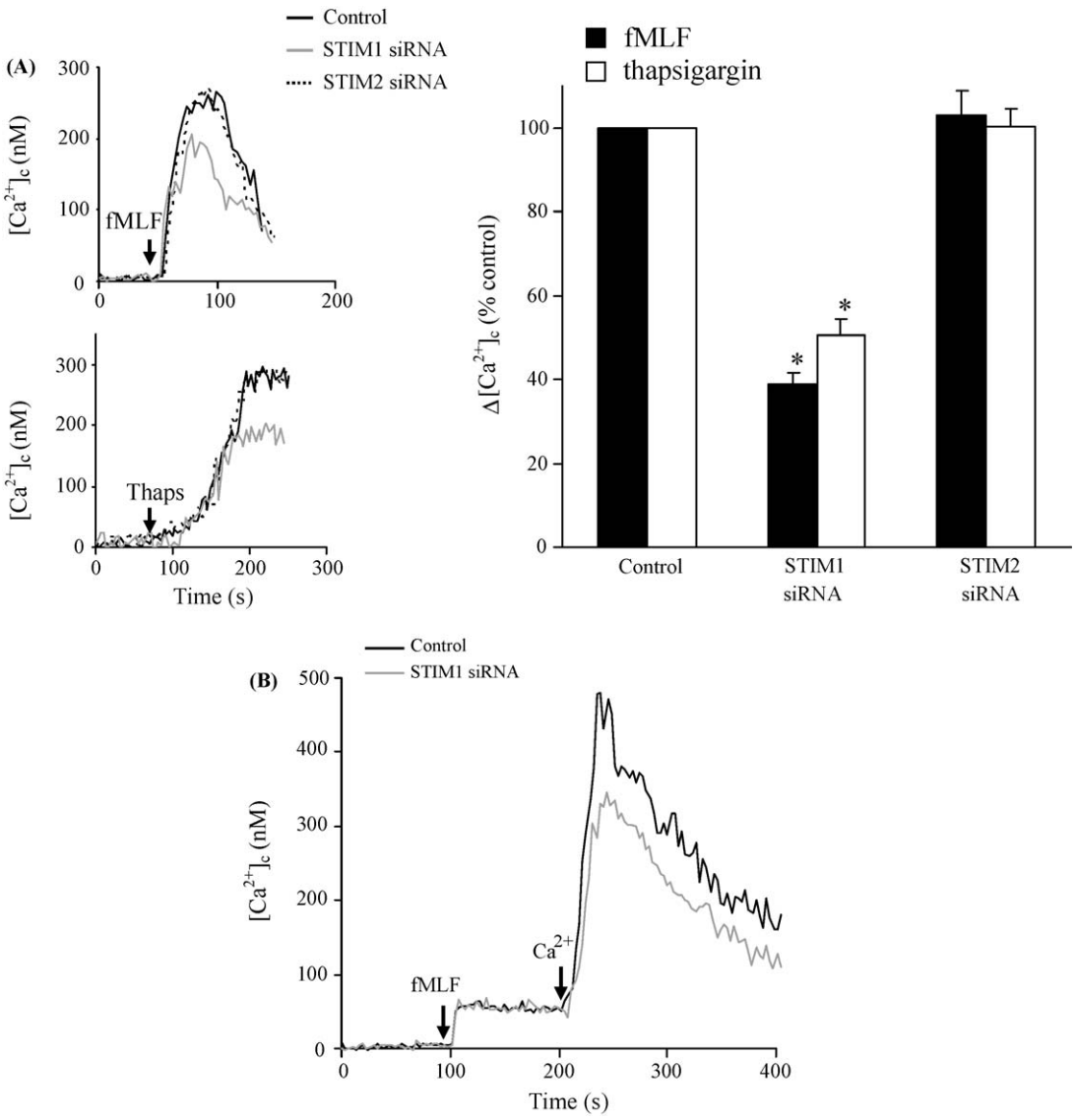

Fig. 3. STIM1 but not STIM2 knock-down inhibits Ca^{2+} influx but not Ca^{2+} store depletion in differentiated HL-60 cells. Differentiated HL-60 cells were transiently transfected with STIM1 or STIM2 siRNA. (A) Transfected cells were assessed for $[Ca^{2+}]_c$ elevation after stimulation by fMLF (0.1 μM) or thapsigargin (0.01 μM). Left panel shows representative traces for variations of fMLF or thapsigargin-induced Ca^{2+} entry and right panel the quantitative data of the traces based on the maximal $[Ca^{2+}]_c$ elevation observed (fMLF-induced $[Ca^{2+}]_c$ peak and thapsigargin-induced sustained phase) expressed as % of control (non-silencing siRNA). (B) Transfected cells, suspended in the Ca^{2+}-free medium, were stimulated with fMLF (0.1 μM) followed by the restoration of 1.25 mM extracellular Ca^{2+} as indicated by the arrows. Cells transfected with a non-silencing sequence were used as control. Time courses representative of three independent experiments are shown to illustrate the changes in $[Ca^{2+}]_c$.

depletion of Ca^{2+} stores caused the translocation of the protein to sites of endoplasmic reticulum-plasma membrane apposition, this event has not been reported in neutrophils. We observed in preliminary studies that the relocalization of overexpressed STIM1 into punctuates near the plasma membrane occurs with a very low frequency in stimulated cells (data not shown). Therefore, we examined the localization of endogenous STIM1 by immunofluorescence in cells fixed after stimulation onto coverslips in order to determine whether redistribution of STIM1 occurred in non-transfected cells. In non-activated cells, STIM1 protein was seen in a spherical distribution around the nucleus (Fig. 6). Activation of differentiated HL-60 cells with fMLF (1 μM) for 1 min resulted in a redistribution of STIM1 into punctuate structures in the plasma membrane region (Fig. 6). This observation corroborates the fact that activation of Ca^{2+} influx is associated with STIM1 redistribution near the plasma membrane in neutrophils.

3.4. STIM1 but not STIM2 is associated to NOX2 activation

Our data clearly established that a functional STIM1 and its redistribution play a major role in SOCE in a model of neutrophils. Because the activation of NOX2 is dependent on Ca^{2+} influx, we hypothesized that STIM1 inhibition might contribute to a reduction in NOX2 activity. To evaluate the effect of STIM1 knock-down in the regulation of NOX2 activity, H_2O_2 production was measured in differentiated HL-60 cells transfected with STIM1 siRNA. Whereas the peak of $[Ca^{2+}]_c$ was approximately reached 15 s after fMLF stimulation (0.1 μM) (Fig. 3A), H_2O_2 production was detected after a 40 s latency following the addition of fMLF (Fig. 7A in insert). Although $[Ca^{2+}]_c$ rapidly decreased, the maximal rate of H_2O_2 production remained sustained for a duration estimated to 1 min (Fig. 7A in insert). Then, the rate of H_2O_2 production decreased reflecting NOX2 deactivation.

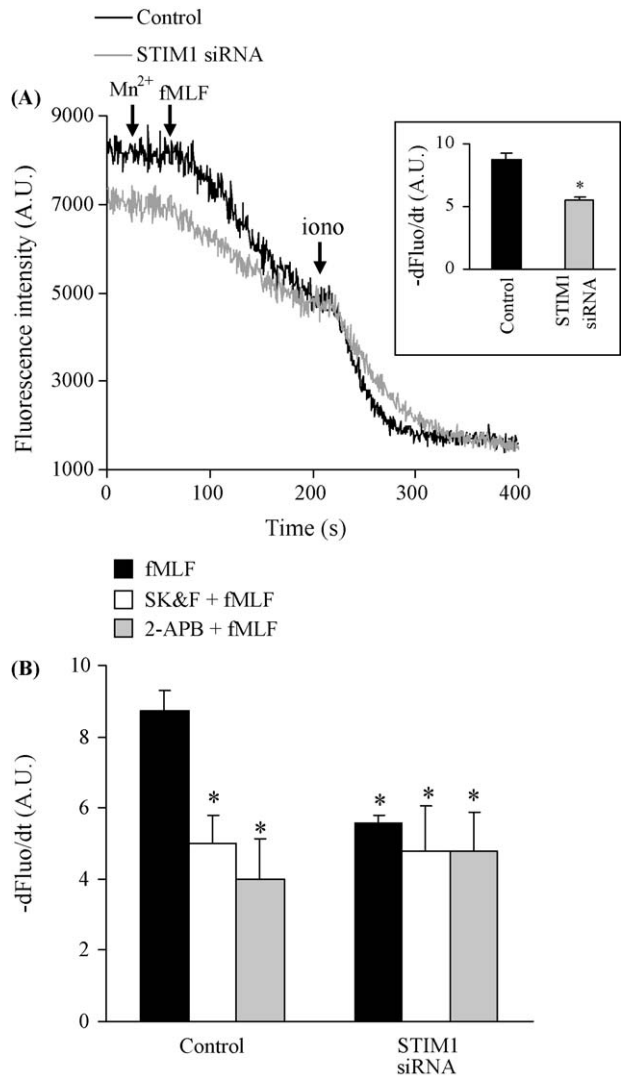

Fig. 4. STIM1 has a predominant role in extracellular Ca^{2+} entry in differentiated HL-60 cells. Differentiated HL-60 cells were transiently transfected with STIM1 siRNA. Transfected cells were assessed for fluorescence quenching by Mn^{2+} (1 mM) entry. Cells suspended in the presence of 1.25 mM extracellular Ca^{2+} were subjected (B) or not (A) to 10 μM SK&F 96365 or 2-APB followed by fMLF (0.1 μM). The histograms show the quantitative data of the traces calculated as mentioned (see Section 2). Cells transfected with a non-silencing sequence were used as control. *Significantly different from control.

Knock-down of STIM1 by siRNA resulted in substantial suppression of the H_2O_2 production induced by fMLF (0.1 μM) in differentiated HL-60 cells (STIM1 siRNA: 43 ± 13% control) (Fig. 7A). STIM1 siRNA reduced the maximal intensity of H_2O_2 production without affecting the duration of production and latency.

As STIM2 siRNA did not influence Ca^{2+} influx, we verified that this treatment is also inefficient in NOX2 activation; STIM2 knock-down did not alter fMLF-induced H_2O_2 production (Fig. 7A).

Following siRNA assays indicating that a reduction of STIM1 expression resulted in strong reduction of NOX2 activity, we abrogated SOCE using SK&F 96365 or 2-APB on cells transfected with STIM1 siRNA. We hypothesized that such a measure should not reduce Ca^{2+} influx in differentiated HL-60 cells treated with STIM1 siRNA. We observed that the reduction of fMLF-induced H_2O_2 production was similar to the one observed in untreated transfected cells (Fig. 7B) confirming that NOX2 activity is tightly associated to SOCE via STIM1 regulation.

4. Discussion

SOCE is the primary mechanism of regulated Ca^{2+} entry into non-excitable cells. Although sustained Ca^{2+} influx has been studied for decades, the proteins involved have only been identified recently. STIM1, a sensor of Ca^{2+} within the endoplasmic reticulum lumen, has been involved in the regulation of SOCs by interactions between proteins in Ca^{2+} stores and protein components of SOCs in the plasma membrane. The sequence of events can be briefly summarized as follows: depletion of internal Ca^{2+} store causes the redistribution of STIM1 into punctuates near the plasma membrane and its co-localization with Orai1 and TRPC channels. STIM1 induces clustering of Orai1 within the plasma membrane resulting in the activation of SOCs. Although the formation of a ternary complex between TRPC, Orai1 and STIM1 has been described (in which TRPC is the pore-forming SOCs and Orai1 is the regulatory subunit that confers store depletion sensitivity to TRPC), the mechanism by which SOCs activation is accomplished is still unknown. Neutrophils are central effectors of the innate immune response to inflammation in humans notably through the generation of reactive oxygen species by NOX2. Dysfunctional NOX2 can be associated to a number of inflammatory diseases such as rheumatoid arthritis, asthma... Because NOX2 is known to be regulated by Ca^{2+} influx, detailed understanding of the mode of regulation of SOCE leading to NOX2 activation is of considerable importance [7,8,11,34]. In this aim, we investigated the specific role of STIM1 in Ca^{2+} influx-regulated NOX2 activity.

A high level of STIM1 protein is readily detected in HL-60 cells consistent with its potential critical role in SOCE. In differentiated HL-60 cells, STIM1 knock-down by specific siRNA caused a marked reduction of extracellular Ca^{2+} entry without affecting intracellular Ca^{2+} store depletion. The use of thapsigargin and purported pharmalogical SOCE inhibitors allows us to conclude that STIM1 is a critical component of SOCE in neutrophils. STIM1 is originally located to intracellular sites in our model of neutrophils. In accordance with the original observation of Liou et al. [21], we found that, upon Ca^{2+} store depletion, STIM1 redistributes into punctuate structures and moves closer to the plasma membrane. Thus, STIM1 may be involved in SOCE by promoting interaction between Ca^{2+} stores and plasma membrane in neutrophils.

STIM2, a STIM1 homolog, has also been found to constitutively activate Ca^{2+} entry [24]. However, this Ca^{2+} mobilization is only observed when STIM2 is expressed at high levels. On the other hand, STIM2 interaction with STIM1 has been shown to have a powerful inhibitory effect on SOCs activation [23]. Although the role of STIM2 is controversial, both proteins may have a coordinated role in regulating SOCE. To resolve the question whether STIM2 has a regulatory effect on Ca^{2+} entry in neutrophils, we investigated the effect of STIM2 knock-down on $[Ca^{2+}]_c$. Suppression of STIM2 had no effect on Ca^{2+} influx. Despite this difference between STIM1 and STIM2, an important role of STIM2 for Ca^{2+} homeostasis in neutrophils should not be excluded since it might function as a feedback regulator that stabilizes basal cytosolic and endoplasmic reticulum Ca^{2+} concentration [25].

Because Ca^{2+} influx regulates NOX2 activation, control of SOCs activation may reduce physiopathological neutrophil activation. By using STIM1 knock-down to directly inhibit extracellular Ca^{2+} entry, NOX2 activation is partially prevented in differentiated HL-60 cells.

Using two purported pharmalogical SOCE inhibitors (2-APB and SK&F 96365) [16–20], we provide evidence that STIM1-induced Ca^{2+} entry is a critical step in the control of the oxidative response, a feature which is not observed when STIM2 expression is decreased. Ca^{2+} influx occurring through STIM1-mediated SOCs activation is a relevant event in the oxidative response activation but a mechanism other than SOCE may be also considered. This

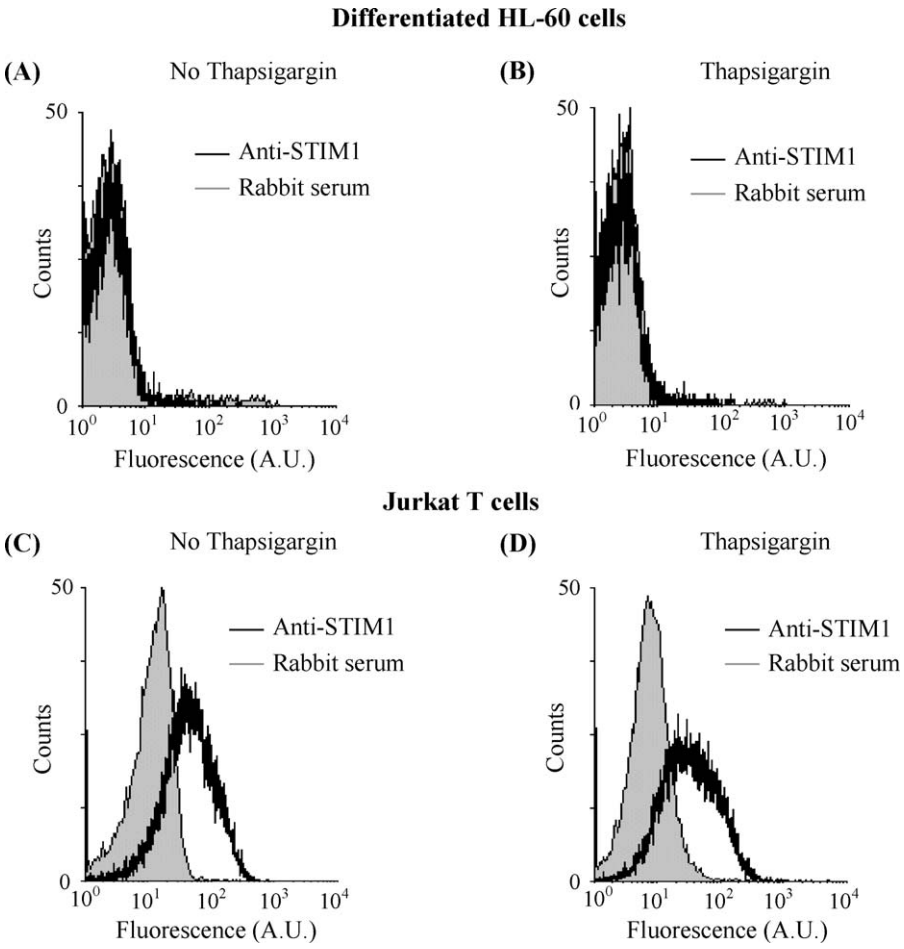

Fig. 5. Membrane localization of STIM1. (A and B) Differentiated HL-60 cells and (C and D) Jurkat T cells were subjected or not to thapsigargin (1 μM) for 10 min then incubated with a polyclonal anti-STIM1 antibody before staining with R-PE-conjugated anti-rabbit and flow cytometry analysis. Results are expressed as fluorescence intensity histograms of STIM1 expressing cell population compared to negative control (rabbit serum).

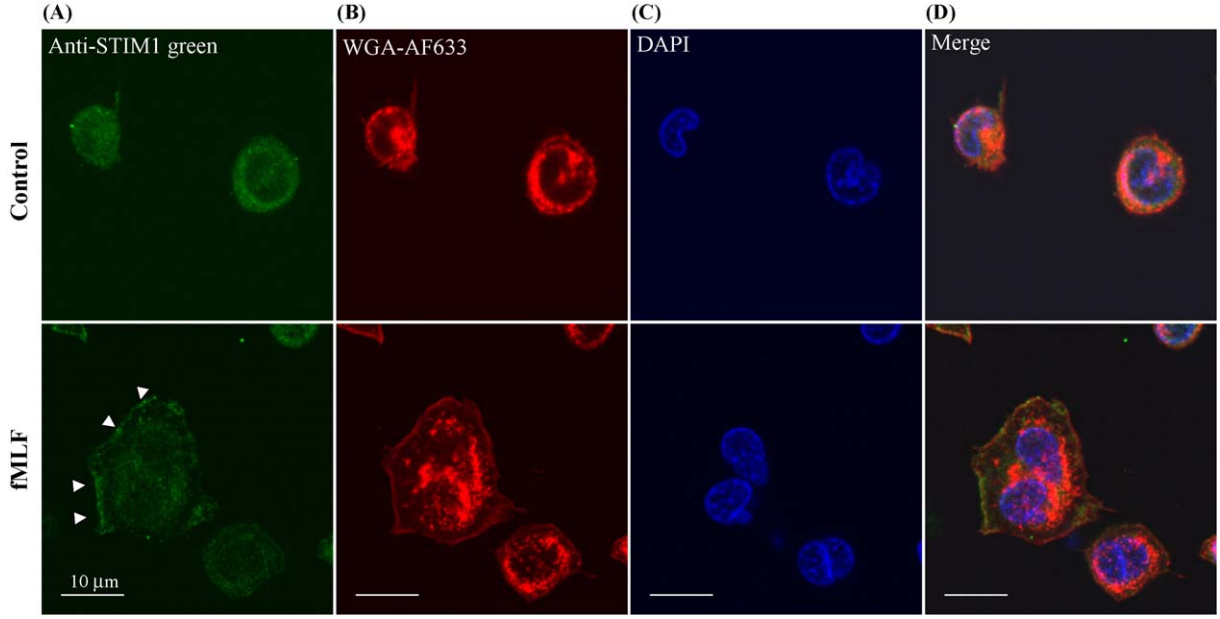

Fig. 6. Endogenous STIM1 protein forms punctuate clusters close to the plasma membrane region following fMLF stimulation in differentiated HL-60 cells. Differentiated HL-60 cells were plated and stimulated or not (control) with fMLF (1 μM) for 1 min in the presence of extracellular Ca^{2+}. (A) Cells were immunostained with anti-STIM1 (green) antibody, (B) plasma membrane was labeled with WGA-AF633 (red), (C) nucleus was labeled with DAPI (blue), (D) merged fluorescence. The white arrows indicate STIM1 punctate clusters.

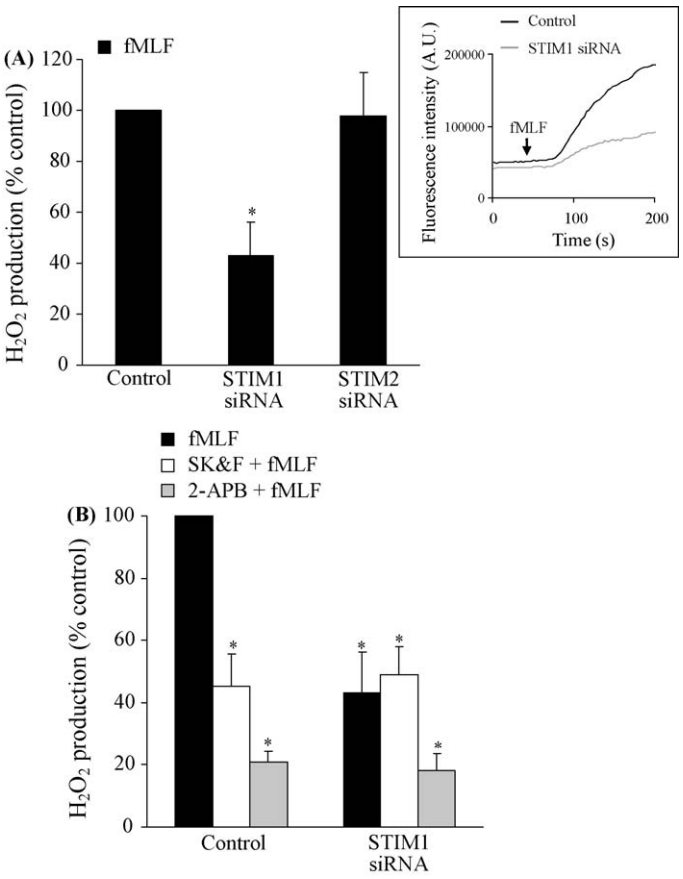

Fig. 7. STIM1 but not STIM2-mediated SOCE inhibition modulates NOX2 activity. Differentiated HL-60 cells were transiently transfected with STIM1 or STIM2 siRNA. Cells suspended in the presence of 1.25 mM extracellular Ca^{2+} were assessed for H_2O_2 production after (A) stimulation by fMLF or (B) treatment with 10 μM SK&F 96365 or 2-APB followed by fMLF (0.1 μM) stimulation. The histograms show the quantitative data of the traces (insert). Cells transfected with a non-silencing sequence were used as control. *Significantly different from control.

suggestion was provided by Itagaki et al. [35] based on multiple observations. For example, when lysophosphatidic acid is applied to HL-60 cells in Ca^{2+}-free medium, no store depletion is observed but Ca^{2+} influx is detected immediately after re-addition of external Ca^{2+}. In addition, lysophosphatidic acid has stimulatory effects on NOX2 in a concentration-dependent manner. Further, recent data obtained with HL-60 cells revealed that NOX2 could be activated by non-SOC channels [12]. Thus, two separate Ca^{2+} signalling pathways can be involved in NOX2 regulation. Our data establish that STIM1 participates in NOX2 activation through SOCE. However, the role of STIM1 in NOX2 activity may not be restricted to the activation of SOC channels, as it has been shown that STIM1 regulates Ca^{2+} channels activated by a pathway independent of the depletion of intracellular Ca^{2+} stores or any consequence of such depletion [36]. Thus, STIM1 is capable of acting on diverse Ca^{2+} channels and consequently emerges as a key regulator of NOX2 activity.

Discovery of the role of STIM1 in SOCE and the evidence provided by our study for its importance in phagocytic cells strongly suggest that signalling events controlling STIM1 activation may be rightfully considered as a possible way to regulate NOX2 activation. Consistent with this, our observations show that STIM1 knock-down results in a down-regulation of NOX2 activity. Thus, strategies, which negatively influence signal transduction mediated by STIM1, may be beneficial in reducing inflammatory damages. Determination of the events dependent on STIM1, including molecular identity of soluble messenger, mechanism of SOCs activation and additional proteins involved in the endoplasmic reticulum-plasma membrane junction, assume a considerable importance for restraining oxidant production by neutrophils during inflammation.

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