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Intracellular Chloride Concentration Changes Modulate IL-1β Expression and Secretion in Human Bronchial Epithelial Cultured Cells†

Mariángeles Clauzure[1], Ángel G. Valdivieso[1], María M. Massip-Copiz[1], Consuelo Mori[1], Andrea V. Dugour[2], Juan M. Figueroa[2], and Tomás A. Santa-Coloma[1*]

[1]Institute for Biomedical Research (BIOMED), Laboratory of Cellular and Molecular Biology, National Scientific and Technical Research Council (CONICET) and School of Medical Sciences, Pontifical Catholic University of Argentina (UCA), Buenos Aires, Argentina.
[2]Fundación Pablo Cassará. Buenos Aires, Argentina.
Running Head: Cl^- modulates IL-1β expression and secretion

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* Corresponding author: Dr. Tomás A. Santa-Coloma, Laboratory of Cellular and Molecular Biology (LBCM), Institute for Biomedical Research (BIOMED), Pontifical Catholic University of Argentina (UCA). Alicia Moreau de Justo 1600, 3rd Floor, Bldg. San José, 1107 Buenos Aires, Argentina.
tsantacoloma@gmail.com ; tomas_santacoloma@uca.edu.ar
Tel/fax: 5411-4338-0886

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Abstract

Cystic fibrosis (CF) is caused by mutations in the *CFTR* gene, which encodes a cAMP-regulated chloride channel. Several cellular functions are altered in CF cells. However, it is not clear how the CFTR failure induces those alterations. We have found previously several genes differentially expressed in CF cells, including *c-Src*, *MUC1*, *MTND4* and *CISD1* (CFTR-dependent genes). Recently, we also reported the existence of several chloride-dependent genes, among them *GLRX5* and *RPS27*. Here, varying the intracellular chloride concentration $[Cl^-]_i$ of IB3-1 CF bronchial epithelial cells, we show that IL-1β mRNA expression and secretion are also under Cl^- modulation. The response to Cl^- is biphasic, with maximal effects at 75 mM Cl^-. The regulation of the IL-1β mRNA expression involves an IL-1β autocrine effect, since in the presence of the IL-1β receptor antagonist IL1RN or anti-IL-1β blocking antibody, the mRNA response to Cl^- disappeared. Similar effects were obtained with the JNK inhibitor SP600125, the c-Src inhibitor PP2 and the IKK inhibitor III (BMS-345541). On the other hand, the IL-1β secretion is still modulated by Cl^- in the presence of IL-1RN, IL-1β blocking antibody or cycloheximide, suggesting that Cl^- is affecting the IL-1β maturation/secretion, which in turn starts an autocrine positive feedback loop. In conclusion, the Cl^- anion acts as a second messenger for CFTR, modulating the IL-1β maturation/secretion. The results also imply that, depending on its intracellular concentration, Cl^- could be a pro-inflammatory mediator. This article is protected by copyright. All rights reserved

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Introduction

Little is known regarding the possible role of the chloride anion (Cl^-) acting as a second messenger for Cl^- channels. One channel that modulates the $[Cl^-]_i$ is the Cystic Fibrosis Transmembrane Conductance Regulator (CFTR), the protein responsible for cystic fibrosis [Rich et al., 1990; Riordan et al., 1989]. This channel has the unique characteristic to respond to cAMP signaling, through protein kinase A (PKA), which phosphorylates the CFTR regulatory domain (R), in turn activating its chloride transport activity [Gregory et al., 1990]. The impairment of the CFTR activity in some way produces intracellular signals that regulate apoptosis [Kerbiriou et al., 2009; Kim et al., 1999], cell proliferation [Bellec et al., 2015; Xu et al., 2015], mitochondrial effects [Valdivieso and Santa-Coloma, 2013] and other altered functions that together determine the cystic fibrosis phenotype (not well defined). However, the possible CFTR signaling mechanisms and effectors are largely unknown, except for c-Src, which increases MUC1 expression in cells with impaired CFTR activity [Gonzalez-Guerrico et al., 2002], and IL-1β, which inhibits mitochondrial Complex I and increases oxidative stress upon a CFTR failure [Clauzure et al., 2014]. Since CFTR is a chloride channel, the most obvious candidate for signaling is the chloride anion itself, acting somehow as a second messenger. In this way, the levels of chloride, regulated by the CFTR transport activity (and other chloride channels and transporters), might modulate several cellular functions known to be affected by the Cl^- anion, including specific gene expression [Ohsawa et al., 2010; Roessler and Muller, 2002; Valdivieso et al., 2016]. In this regard, it has been shown that Cl^- can act as a signaling molecule in the bacterium *Halobacillus halophilus*, regulating the expression of specific genes, including the flagellar protein fliC [Roessler and Muller, 2002]. In mammalian cells, Cl^- modulates the G1/S cell-cycle checkpoint through p21 through the stress-activated protein kinases (MAPKs) p38, and JNK [Ohsawa et al., 2010; Shiozaki et al., 2011]. These kinases are also involved in foam cell formation under low intracellular chloride [Wu et al., 2016]. In addition,

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we have recently demonstrated that Cl^- can act as a second messenger modulating the expression of several "chloride-dependent" genes, including *GLRX5* and *RPS27* [Valdivieso et al., 2016], and several studies have shown Cl^- effects in neurons, including the modulation of the expression of $GABA_A$ subunits [Succol et al., 2012]. In fact, a growing number of evidences suggest a role of Cl^- in modulating protein activities and gene expression, acting as a second messenger [Cooper et al., 2001; Grinstein et al., 1992; Menegazzi et al., 2000; Myers et al., 1990; Valdivieso et al., 2016].

Several genes are differentially expressed in cells with impaired CFTR activity [Clauzure et al., 2014; Gonzalez-Guerrico et al., 2002; Massip Copiz and Santa Coloma, 2016; Taminelli et al., 2008; Valdivieso et al., 2012; Valdivieso et al., 2007; Valdivieso and Santa-Coloma, 2013], including *IL-1β* [Clauzure et al., 2014; Grassme et al., 2014]. Thus, by using the IL-1β expression levels of IB3-1 CF epithelial cells as a model system (these cells overexpress basal IL-1β levels [Clauzure et al., 2014]), here we show that changes in the intracellular Cl^- concentrations modulate the steady-state levels of IL-1β mRNA, with similar effects on the secreted protein. The effect of Cl^- on the IL-1β mRNA steady state levels disappear in the presence of the IL-1β receptor antagonist IL1RN or in the presence of IL-1β blocking antibody. However, the secretion of IL-1β is only partially affected in the presence of IL1RN, blocking antibody or cycloheximide, suggesting that Cl^- regulates the IL-1β maturation and secretion. The results also suggest that the secreted IL-1β in turn starts an autocrine positive feedback loop that increases its own mRNA levels.

Materials and Methods

Reagents

Interleukin-1 beta (IL-1β) (Cat. No. I9401), IL-1 receptor antagonist (Cat. No. SRP3327), pepstatin, PMSF, leupeptin, dimethyl sulfoxide (DMSO, culture grade), NADH, dibutyryl-cAMP,

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IBMX, (-)-isoproterenol hydrochloride, p-coumaric acid, tributyltin, nigericin, cycloheximide, H-7 dihydrochloride, and protease inhibitor cocktail (Cat. No. P2714) were purchased from Sigma-Aldrich (St. Louis, MO). IKK inhibitor III (BMS-345541), CFTR(inh)-172 5-[(4-Carboxyphenyl)methylene]-2-thioxo-3-[(3-trifluoromethyl)phenyl-4-thiazolidinone and 4-Amino-5-(4-chlorophenyl)-7-(t-butyl) pyrazolo [3,4-d] pyrimidine (PP2) were from Calbiochem (San Diego, CA). MAPK1/p38 inhibitor (SB203580), MAPKK (MEK1/2) inhibitor (U0126) and JNK inhibitor (SP600125) were from Alomone Labs (Jerusalem, Israel). Trypsin was purchased from Life Technologies (GIBCO BRL, Rockville, MD) and SPQ (6-methoxy-N-[3-sulfopropyl]quinolinium) from Invitrogen (Carlsbad, CA). All other reagents were analytical grade. Antibodies: mouse anti-IκB-α (H-4 mAb, IgG1, Santa Cruz Biotechnology Cat# sc-1643 RRID:AB_627772), mouse anti p-JNK (G-7 mAb, IgG1, Santa Cruz Biotechnology Cat# sc-6254 RRID:AB_628232) were from Santa Cruz Biotechnology Inc. (Santa Cruz, CA); anti-mouse antibody coupled to horseradish peroxidase (polyclonal, W402B) and anti-rabbit antibody coupled to horseradish peroxidase (polyclonal, W401B) was from Promega (Madison, WI); mouse anti-IL-1β (mAb, IgG_1, Sigma-Aldrich Cat# I3642 RRID:AB_477104), rabbit anti-phospho-Tyr- 418-Src antibody (polyclonal, Sigma-Aldrich Cat# S1940 RRID:AB_261460) and rabbit anti-actin antibody (polyclonal, Sigma-Aldrich Cat# A2066 RRID:AB_476693) were from Sigma-Aldrich.

Cultured cells

IB3-1 cells (ATCC Cat# CRL-2777, RRID:CVCL_0338, a bronchial cell line derived from a cystic fibrosis patient with a ΔF508/W1282X CFTR genotype) [Zeitlin et al., 1991] and S9 cells (ATCC Cat# CRL-2778, RRID:CVCL_4461, which are IB3-1 cells transduced with an adeno-associated viral vector to stably express wt-CFTR) [Egan et al., 1992] were purchased from ATCC (www.atcc.org) (these cell lines are no longer provided by ATCC; they are kept now at the John Hopkins University Cell Center). Cells were cultured in DMEM/F12 (Life Technologies,

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GIBCO BRL, Rockville, MD) supplemented with 5 % FBS (Internegocios S.A., Mercedes, Buenos Aires, Argentina), 100 U/ml penicillin and 100 µg/ml streptomycin (Life Technologies, GIBCO BRL, Rockville, MD). Cultures were grown at 37 ºC in a humidified air atmosphere containing 5 % CO_2. All cells were plated at a density of 20,000 cells/cm^2 and adding ~80 µl of medium/cm^2 of cultures dishes/bottles. Before treatments, cells were cultured 24 h in serum-free DMEM/F12 medium.

Inhibition of CFTR chloride transport activity

S9 and IB3-1 cells were incubated as above indicated for 24 h in serum-free medium. Then, the serum-free media was replaced and the cells were cultured for additional 24 h in the presence of a CFTR-stimulation cocktail (200 µM dibutyryl cAMP, 200 µM IBMX and 20 µM isoproterenol), with or without CFTR(inh)-172 (10 µM).

Intracellular Chloride

The SPQ fluorescence was measured as indicative of the relative intracellular Cl^- changes [Maouche et al., 2013; Valdivieso et al., 2011], according to the Stern Volmer equation $Fo/F= 1+Kav.[Cl^-]$ [Krapf et al., 1988]. F is the value of SPQ fluorescence minus Fb (the minimal fluorescence obtained with maximal SPQ quenching in the presence of valinomycin + SCN^- + K^+), and Fo is the fluorescence value in the absence of Cl^- [Krapf et al., 1988]. Since (Kav.Q) >> 1, then $1/F \approx Kav.[Cl^-] /Fo$ and 1/F is proportional (~) to $[Cl^-]_i$. Cells were cultured 48 h as above indicated in 96 well black plates (Greiner Bio-One, Germany; 655090) and loaded the last 24 h with 5 mM SPQ in serum-free DMEM/F12. To determine the SPQ fluorescence levels, the DMEM-F12 medium was changed to Hank's medium (136.9 mM NaCl, 5.4 mM KCl, 1.3 mM $CaCl_2$, 3.7 mM NaH_2PO_4, 0.4 mM KH_2PO_4, 4.2 mM $NaHCO_3$, 0.7 mM $MgSO_4$, 5.5 mM D-glucose and 10 mM HEPES) containing or not 10 µM CFTR(inh)-172, and incubated at 37°C in a 5% CO_2/air incubator for 30 min. The fluorescence was measured in a fluorescence plate

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reader (NOVOstar BMG LABTECH GmbH Ortenberg, Germany) at 37°C. Filters were Ex=340±10 nm, Em=440±10 nm and measurements were performed from the bottom of the plate.

Modulation of intracellular chloride concentration $[Cl^-]_i$

IB3-1 cells were incubated for 1 h in the presence of different Cl^- concentrations (0, 5, 25, 50, 75, 100 and 125 mM). To establish a rapid equilibrium between the $[Cl^-]_i$ and the extracellular chloride concentration ($[Cl^-]_e$), independently of the chloride channels activities, a double-ionophore strategy was used [Valdivieso et al., 2016]. Briefly, IB3-1 cells were washed with Hank´s-gluconate, to remove the remaining extracellular Cl^-, and then incubated for 1 h with different extracellular chloride concentrations $[Cl^-]_e$. The different Cl^- concentrations were obtained combining two high K^+ buffers (High-KCl and High-KNO_3), containing the ionophores nigericin (5 µM) and tributyltin (10 µM), made as previously reported [Valdivieso et al., 2016]. Additional treatments (inhibitors, IL-1RN or blocking antibody) were performed in this 1 h period, incubating the cells in these high K^+ buffers.

Reverse transcription and quantitative real-time PCR (qRT-PCR)

The S9 and IB3-1 cells were cultured as indicated. After incubation, total RNA was isolated by using a guanidinium thiocyanate-phenol-chloroform extraction solution [Chomczynski and Sacchi, 1987]. The quality of RNA was checked by electrophoresis in denaturing formaldehyde agarose gels [Sambrook et al., 1989], and measuring the ratios A260/A230 (greater than 2) and A260/A280 nm (from 1.7 to 2.0). Reverse transcription (RT) was performed by using 4 µg of total RNA, M-MLV reverse transcriptase (100 U, Promega, Madison, WI) and Oligo-dT_{12} in a 25 µl final reaction volume, according to manufacturer´s instructions. The reaction was performed for 90 min at 37 ºC, 5 min at 75 ºC, and then cooled to 4 ºC. The synthesized cDNAs were used immediately for PCR amplifications, or stored at -80 ºC for later use. Quantitative real-time RT-

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PCRs (qRT-PCR) were performed by using an ABI 7500 real-time PCR system (Applied Biosystems Inc., Foster City, CA); the ΔΔCt method was used for comparative quantification. *TBP* (Tata Box Binding Protein) was used as an internal control. Primer sequences for PCR were as follows: *TBP*, 5´-TGCACAGGAGCCAAGAG TGAA-3´ (forward) and 5´-CACATCACAGCTCCCCACCA-3´ (reverse); IL-*1β*, 5´-ACAGATGAAGTGCTCCTTCCA-3´ (forward) and 5´-GTCGGAGATTCGTAGCTGGAT-3´ (reverse); IL-6, 5-GAGAAGATTCCA AAGATGTAGCCG-3 (forward) and 5-AGATGCCGTCGAGGATGTACC-3 (reverse). The cDNA samples (10 µl of a 1:10 of cDNA from reverse-transcribed RNA) were added to 25 µl of PCR reaction mixture containing a final concentration of 2.5 mM $MgCl_2$, 0.4 mM deoxynucleotide triphosphates, 1 U of Go Taq DNA polymerase (Promega), 0.1 X EvaGreen (Biotium, Hayward, CA), 50 nM ROX as reference dye, and 0.2 nM of each primer. The qRT-PCR conditions were as follows: initial denaturation at 95 ºC for 10 min, followed by 40 cycles at 95 ºC for 30 s, 62 ºC for 30 s and 72 ºC for 30 s. Fluorescence signal was acquired at the elongation step, at the end of each cycle. qRT-PCR reactions were carried out in triplicates (intra- and inter-assays by triplicate). The final quantification values were obtained as the mean of the Relative Quantification (RQ) for each biological triplicate (n= 3

Secretion of IL-1β *and* IL-6

Secreted IL-1β and IL-6 were measured in conditioned media from cells. Cells were cultured 72 h as above indicated in p100 dishes. The last 1 h of incubation was done in 5 ml of high K^+ buffer, at different Cl^- concentrations. After incubation, the 5 ml buffer solutions were collected and, only for IL-1β measurements, concentrated to ~250 µl by centrifugation at 3500 g for 30 min at 4ºC by using the Amicon Ultra-15 centrifugal filter units (10.000 kDa cut-off, EMD Millipore, Billerica, MA). IL-1β were measured from frozen concentrate supernatants using the Human IL-1β ELISA set (BD OptEIA™ - Human IL-1β ELISA Set, BD Biosciences). Measurements were performed using a microplate reader (model Benchmark, Bio-Rad,

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Hercules, CA). IL-6 was measured in the same way using the Human IL-6 ELISA set (BD OptEIA™ - Human IL-6 ELISA Set, BD Biosciences), except that the samples were directly measured, without previous concentration.

Protein extraction

Cells were incubated as above indicated, washed twice with cold PBS, scraped with cold extraction buffer (10 mM Tris pH 7.4, 100 mM NaCl, 0.1% SDS, 0.5% sodium deoxycholate, 1% Triton X-100, 10% glycerol) containing the protease inhibitor cocktail (5 ml of cocktail/20 g of cell extract) plus phosphatase inhibitors (2 mM Na_3VO_4, 1 mM NaF and 10 mM Na_2PO_7), and centrifuged at 14000 x g for 20 min at 4°C. The supernatant was stored at - 80°C until use. The protein concentration was measured by using the method of Lowry et al. [Fryer et al., 1986; Lowry et al., 1951].

Western Blot Analysis

Western blots were performed as previously described [Cafferata et al., 2001]. Briefly, total protein extracts (30-50 μg of proteins) were separated on a denaturing SDS-PAGE (15%) and transferred to nitrocellulose membranes. Membranes were blocked with BSA 5% in TBS 1 h and then incubated with primary monoclonal antibodies against IL-1β, IκB-α, p-c-Src, and p-jnk (dilutions 1:1000 in TBS plus Tween-20, 0.05% v/v) for 3 h. The membranes were washed three times with TBS plus Tween-20 (0.05% v/v) for 5 min and incubated for 1 h with goat IgG anti-mouse antibody coupled to horseradish peroxidase (dilution 1:2000 in TBS plus Tween-20, 0.05% v/v), washed three times with TBS plus Tween-20 (0.05% v/v) for 5 min and developed. As internal controls, membranes were reincubated with anti-actin antibody (dilution 1:1000 in TBS plus Tween-20, 0.05% v/v), washed three times as above indicated and then incubated for 1 h with goat IgG anti-rabbit antibody coupled to horseradish peroxidase (dilution 1:1000 in TBS

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plus Tween-20, 0.05% v/v). Results were visualized by using an ImageQuant LAS 4000 system (GE Healthcare Life Sciences, Piscataway, NJ).

Statistics

The assays were performed at least by duplicates and the experiments were repeated at least three times. The results were expressed as the mean obtained from the different independent experiments (interassay comparisons, n=3). One-way ANOVA and the Tukey's test were applied to calculate significant differences among samples ($\alpha = 0.05$). All values are shown as mean ± SEM (n). * indicates significant differences ($p < 0.05$).

Results

The inhibition of the CFTR activity in S9 cells (IB3-1 CFTR-corrected cells) induces upregulation of the IL-1β mRNA

We have previously reported that the expression IL-1β was increased approximately 40 % in IB3-1 CF cells compared to CFTR-corrected S9 cells [Clauzure et al., 2014]. These effects of CFTR over IL-1β expression could be due to the CFTR Cl^- transport activity or to the presence/absence of the CFTR at the cell membrane, independently of its transport activity, as appears to occur with RANTES [Estell et al., 2003]. Thus, to determine whether nor not the CFTR Cl^- transport activity was involved in the IL-1β modulation, we used the CFTR inhibitor CFTR(inh)-172. S9 cells were incubated with 10 µM CFTR(inh)-172 for 24 h in serum-free medium (in the presence of a CFTR-stimulating cocktail composed of 200 µM dibutyryl cAMP, 200 µM IBMX and 20 µM isoproterenol). As shown in Figure 1A, the CFTR inhibitor was able to increase IL-1β expression in S9 cells, reaching the levels of IB3-1 CF cells. The CFTR inhibitor did not show effects on the IL-1β expression in the IB3-1 CF cells, which appear to have already

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a saturated response. These results obtained with the CFTR inhibitor suggest that IL-1β is a CFTR-dependent gene, which can be modulated by the Cl^- transport activity of CFTR and not only by the CFTR absence or reduction from the plasma membrane, as occurs with IB3-1 cells.

As a control, to verify that the CFTR inhibitor CFTR(inh)172 actually increased the $[Cl^-]_i$, S9 and IB3-1 cells were loaded with SPQ (the SPQ fluorescence is quenched by Cl^-) and the relative fluorescence was measured [Maouche et al., 2013]. As shown in Figure 1B, the relative 1/F value (the value of S9 control cells was taken as 1; $1/F \sim [Cl^-]$) increased in IB3-1 cells or in cells with treated with CFTR(inh)172. These results suggest, in the first place, that IB3-1 cells accumulates more Cl^- than S9 cells (S9 cells are IB3-1 cells ectopically expressing wild type CFTR), in agreement with their CF genotype/phenotype (CFTR failure). Second, the results suggest that some CFTR activity remains in IB3-1 cells, since the CFTR inhibitor induced a further rise in the $[Cl^-]_i$ of IB3-1 cells. In other words, some CFTR is still operative in IB3-1 cells. More importantly, since the inhibition/mutation of the CFTR activity induces Cl^- accumulation, we hypothesized that Cl^- might be acting as a second messenger for CFTR, upregulating the IL-1β expression, as occurs with other Cl^--dependent genes that we recently described [Valdivieso et al., 2016].

Changes in the $[Cl^-]_i$ modulate IL-1β expression (mRNA and protein)

In order to test whether or not $[Cl^-]_i$ can modulate IL-1β expression, we used a double ionophore strategy (ionophores nigericin and tributyltin) that equilibrates the intracellular and extracellular chloride and H^+ concentrations [Krapf et al., 1988], while keeping constant the pH, cell volume [Nunes et al., 2015] and membrane potential (since these ionophores are non-electrogenic) [Antonenko Yu, 1990; Orlov et al., 1994]. In these conditions, the extracellular and intracellular Cl^- concentrations are equal [Krapf et al., 1988; Valdivieso et al., 2016; Valdivieso et

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al., 2011]. In agreement with our hypothesis, as shown in Figure 2A, the steady-state levels of IL-1β mRNA were modulated by increasing $[Cl^-]_i$. Noteworthy, the modulation was biphasic, with maximal mRNA levels at 75 mM Cl^-. On the other hand, as shown in Figure 2B, the biphasic effect can be observed also in the secreted IL-1β protein, again with a maximal response at 75 mM. Taken together, these results suggest that the $[Cl^-]_i$ is able to regulate IL-1β expression and secretion. Thus, *IL-1β* constitutes an additional Cl^--dependent gene (although the Cl^- effect is indirect, through stimulation of the IL-1β autocrine loop, as shown below). In addition, these results identify the anion Cl^- as the first effector in the CFTR signaling pathway, upstream of IL-1β.

Then, Western blots (WBs) were performed in order to determine the effects of $[Cl^-]_i$, changes on the intracellular levels of IL-1β of IB3-1 cells. As shown in Figure 2C (WB) and 2D (quantification), both the pro-IL-1β and the mature IL-1β levels were modulated by $[Cl^-]_i$, reaching maximal levels of pro- and mature IL-1β at 75 mM Cl^-, in agreement with its mRNA levels. The ratio between mature IL-1β and pro-IL-1β also increased, with maximal ratio at 75 mM Cl^-, suggesting that IL-1β maturation is also increased by Cl^-. These results are in agreement with the increased IL-1β levels in the supernatants of IB3-1 cells in the presence of increased Cl^- concentrations, as it was shown in Figure 2B.

Similar results were obtained with S9 cells (CFTR-corrected IB3-1 cells), as shown in Supplementary Figures S1A-S1D, although with lower levels of IL-1β mRNA and protein compared to IB3-1 cells. Also, the effects of changing the intracellular chloride concentration, $[Cl^-]_i$, were less pronounced in S9 cells compared to IB3-1 cells (Fig. S1A). This is reflected at the protein level, with non-detectable (ND) IL-1β in conditioned media from S9 cells, and more than 3 pg/ml in the conditioned media form IB3-1cells (Fig. S1B). Western blots of intracellular IL-1β also showed a similar pattern, with higher levels of mature IL-1β and a high response to Cl^- changes in IB3-1 cells, compared to S9 cells (Fig. S1C and S1D).

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Effects of different IL-1β pathway inhibitors over IL-1β mRNA expression at different Cl^- concentrations

Since IL-1β produce an autocrine signaling in IB3-1 cells [Clauzure et al., 2014], we then used five inhibitors of the IL-1β signaling pathways to determine if Cl^- was acting through some of them. As shown in Figure 3A, the IKK, JNK and c-Src inhibitors blocked the Cl^- effects on IL-1β mRNA steady-state levels, while the p38 and MEK inhibitors had little effects, which did not reach significance. The IKK and JNK inhibitors also reduced basal IL-1β mRNA levels while the c-Src and p38 inhibitors did not show this effect on basal IL-1β expression.

To confirm the activation of IKK/NF-kB, c-Src and JNK pathways and the effectiveness of each inhibitor when Cl^- concentrations were changed from 5 to 75 mM, the phosphorylation status of c-Src, JNK and IκB-α were tested by Western blots (WBs). As shown in Figure 3B (WB) and 3C (quantification), the phosphorylation of c-Src increased significantly when the Cl^- concentration was changed from 5 to 75 mM. This rise in c-Src activity was abrogated in the presence of the c-Src inhibitor PP2. Similar results were obtained measuring the phosphorylation status of JNK (Figure 3D and 3E): the presence of the JNK inhibitor SP600125 abolished the effects of changing Cl^- from 5 to 75 mM.

Then, to confirm the activation of NF-κB in IB3-1 cells, the relative amounts of IκB-α were measured using WBs of cytoplasmic extracts. As shown in Figure 3F and 3G, the amount of IκB-α was significantly decreased ($p<0.05$) when the concentration was changed from 5 to 75 mM Cl^-, an effect inhibited in the presence of IKK inhibitor III. Altogether, these results suggest that c-Src, NF-κB and JNK are involved in the Cl^- effects over IL-1β mRNA expression. However, as it will be shown below, these Cl- effects are indirect, rather affecting the IL-1β secretion, which in turn, through an autocrine effect, modulates its own mRNA. On the other

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hand, H7, an inhibitor of PKA, PKG and PKC, had no effects on the IL-1β mRNA or protein secretion (Fig. S2A and S2B), so the Cl^- effects are independent of these kinases.

Effects of the interleukin-1 receptor (IL1R) antagonist IL1RN or the anti-IL-1β blocking antibody on IL-1β mRNA expression and IL-1β protein levels

IB3-1 cells were then incubated with the IL1R antagonist IL1RN or with the anti-IL-1β blocking antibody to determine the effects of the IL-1β autocrine loop [Clauzure et al., 2014] on the Cl^- response. Noteworthy, the effects of $[Cl^-]_i$ on IL-1β mRNA expression at 75 mM were completely blocked by the antagonist IL1RN; similar results were obtained by using the IL-1β blocking antibody, although the inhibition was not as good as the one obtained with IL1RN (Figure 4A). On the other hand, as shown in Figure 4B, these effects of IL1RN were not total when the secreted IL-1β protein was tested: when Cl^- was changed from 5 to 75 mMneither the antagonist nor the blocking antibody could avoid a significant rise in the secreted IL-1β protein. These results suggest that the effects of Cl^- over the IL-1β mRNA expression are indirect, through modulation of the secreted IL-1β. In other words, Cl^- does not affect directly the IL-1β transcription but rather its maturation and secretion. Otherwise, even in the presence of IL1RN or IL-1β blocking antibody, changes in the $[Cl^-]_i$ should still affect the IL-1β mRNA expression, which was not the case. Therefore, it appears that $[Cl^-]_i$ affects the IL-1β maturation, and that in turn the secreted IL-1β modulates its own mRNA expression levels.

As shown in Figure 4C and D, both IL1RN and the IL-1β blocking antibody significantly ($p<0.05$) reduced the amount of pro and mature IL-1β, and the response to Cl^- changes from 5 to 75 mM Interestingly, the ratio between mature and pro-IL-1β still increases in the presence of IL1RN or blocking antibody, suggesting that the $[Cl^-]_i$ also affects this ratio. Thus, it appear that $[Cl^-]_i$ modulates the IL-1β maturation (ratio between pro and mature IL-1β) and secretion, and then, indirectly, its own mRNA expression. IB3-1 cells incubated with cycloheximide did not

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modify the effect of Cl^-, further supporting the idea that Cl^- effects on IL-1β are posttranslational (maturation/secretion) (Supplementary Fig. S2A and S2B).

Finally, we tested if similar effects of Cl^- could be observed for a different cytokine, IL-6. As shown in Supplementary Fig. S3A, changing Cl^- from 5 to 75 mM produce stimulation of the IL-6 mRNA. This effect was blocked by IL1RN (Fig. S3A), suggesting that IL-6 mRNA is stimulated by the IL-1β autocrine loop. These results are in agreement with reported results showing that IL-1β regulates IL-6 expression and secretion [Cahill and Rogers, 2008; Kandere-Grzybowska et al., 2006; Xia et al., 2015]. However, contrary to IL-1β, changes in Cl^- from 5 mM to 75 mM showed no effects on the secretion of IL-6 (Fig. 3B). IL1RN only slightly reduced the secreted IL-6, but no effects were observed by changing the Cl^- concentration in the presence of IL1RN. Taken together, these results imply that Cl^- affects only the expression of the IL-6 mRNA through the secretion of IL-1β induced by Cl^- , without affecting the IL-6 secretion. This is reasonable, since the mechanisms of activation and secretion of IL-6 and IL-1β are different. IL-6 contains a classical signal peptide that target the cytokine to the ER to be transported through the constitutive secretory pathway. On the other hand, IL-1β does not have a signal peptide and possess a complex pathway of activation and secretion, not fully understood [Murray and Stow, 2014]. Thus, in IB3-1 cells, Cl^- is not affecting the IL-6 secretory pathway and does modulate the IL-1β non-classical secretory pathway.

Discussion

The results obtained show that changes in the $[Cl^-]_i$, modulate the expression levels of IL-1β mRNA and protein, in a biphasic way. Therefore, depending of the starting intracellular concentration, a decrease in $[Cl^-]$ can either increase or decrease the secreted IL-1β. These results also indicate that Cl^- acts as a second messenger of CFTR, modulating the expression

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of IL-1β, indirectly, through stimulation of the IL-1β maturation/secretion. The secreted IL-1β in turn up-regulates its own expression, through an autocrine positive feed-back loop. Interestingly, Cl^- is not the only anion that may behave as a second messenger. Another anion, the phosphate (PO_4^{3-}) anion, has been also recognized as a possible second messenger involved in diverse cellular functions [Khoshniat et al., 2011; Naviglio et al., 2006; Spina et al., 2013].

The inhibitors of IKK, c-Src and JNK were able to abrogate the Cl^- effects on the IL-1β mRNA expression. These results are in agreement with previous reports showing that IL-1β stimulates its own expression in a vicious cycle of autocrine signaling through NF-κB [Arlt et al., 2002]. According to the present results, this occurs through IKK, c-Src and JNK signaling. The p38 and MEK inhibitors had only a slight effect that did not reach significance. On the other hand, the PKA, PKG and PKC inhibitor H7 [Hidaka et al., 1984] have no effects on the IL-1β mRNA expression levels. However, since the antagonist of IL1R1 and anti-IL-1β blocking antibody completely blocked the effects of changing the $[Cl^-]_i$ on IL-1β expression, Cl^- appear to regulate the IL-1β maturation or secretion and only indirectly its mRNA. In other words, if Cl^- were modulating the IL-1β mRNA expression, this modulation should be still operative in the presence of IL1RN or IL-1β blocking antibodies. Since this was not the case, the only alternative is that Cl^- is affecting the IL-1β maturation and secretion. This was corroborated by the fact that IL1RN can block the IL-1β priming (Figure 4A) but only partially its secretion (Figure 4B). This is also reflected by the fact that the ratio between mature IL-1β and pro-IL-1β increases when Cl^- is changed from 5 mM to 75 mM, independently of the presence or not of IL1RN or IL-1β blocking antibodies. In addition, this idea is further supported by the fact that cycloheximide (CHX) had no effects on the increased secretion of IL-1β induced by changing Cl^- from 5 to 75 mM, suggesting that translation processes are not involved in the Cl^- signaling that determines increased IL-1β secretion. Interestingly, CHX did not affect the IL-1β mRNA expression,

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implying that the accumulated IL-1β protein was enough to start the IL-1β loop and mRNA modulation in this short incubation time (1 hour).

Thus, as illustrated in Figure 5, the results obtained suggest that Cl^- may act as a second messenger for CFTR, being the first element in its signaling cascade, upstream of IL-1β: CFTR → Cl^- → IL-1β. The response is biphasic for both mRNA and protein, with optimal IL-1β expression at 75 mM. The Cl^- ion appears to be affecting IL-1β maturation/secretion and not its mRNA expression, which is actually modulated by the autocrine effect of the secreted IL-1β. Interestingly, Verhoef et al. [Verhoef et al., 2005] have postulated an inhibitory effect of Cl^- on the activation of caspase-1 and IL-1β secretion by the P2K7 receptor. It should be pointed out however, that in that work the results were obtaining replacing the extracellular Cl^- by gluconate, without measuring the intracellular Cl^- concentration, membrane potential, intracellular and extracellular pH or cell volume (parameters fixed in the present work using tributyltin and nigericin). Perhaps all these variables contribute to the final $[Cl^-]_i$ concentration or have influence over the inflammasome activation in the absence of the ionophores nigericin and tributyltin. However, here this double ionophore strategy, as mentioned above, keeps the pH, volume and membrane potential clamped so these parameters should not have influence on the Cl^- -stimulated secretion. Further studies are required to elucidate the precise mechanism by which $[Cl^-]_i$ modulates IL-1β maturation and secretion.

In conclusion, Cl^- act as a second messenger for CFTR and is able to modulate *IL-1β* gene expression, although indirectly, through stimulation of the IL-1β autocrine loop, which is initiated by the enhanced secretion of mature IL-1β induced by Cl^-. We have previously demonstrated that increased ROS levels in cells with impaired CFTR activity are mainly due to IL-1β autocrine effects [Clauzure et al., 2014]. The same occurred with the mitochondrial Complex I inhibition [Clauzure et al., 2014]. However, IL-1β effects are not only limited to mitochondria and the consequences for the cell physiology of the IL-1β modulation under different $[Cl^-]_i$ can be

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multiple and relevant. The results also imply that Cl^- could be a pro-inflammatory mediator, depending on the intracellular concentration, since this anion can modulate IL-1β secretion and indirectly stimulate its autocrine loop.

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Legends to Figures

Figure 1: Effects of CFTR inhibitor CFTR(inh)-172 on IL-1β expression and $[Cl^-]_i$. IB3-1 and S9 cells were preincubated for 24 h in serum-free DMEM/F12 and then treated with CFTR(inh)-172 for additional 24 h. A: Quantitative real-time RT-PCR of IL-1β mRNA expression levels of S9 e IB3-1 cells incubated with 10 μM of CFTR(inh)-172 for 24 h in serum-free medium (in presence of CFTR-stimulating cocktail). B: S9 e IB3-1 cells were loaded overnight with 5 mM SPQ (Cl^- fluorescent probe) in serum-free media. The SPQ fluorescence F was measured 30 min post stimulation with 10 μM of CFTR(inh)-172 in Hank`s buffer (in presence of CFTR-stimulating cocktail). The results were expressed as 1/F, taken the value corresponding to S9 control cells as 1. Measurements were performed in triplicate and data are expressed as mean ± SE of three independent experiments (n=3). * indicates $p<0.05$ compared with basal S9 cells.

Figure 2: Changes in the intracellular chloride concentration modulate IL-1β expression. IB3-1 cells were preincubated for 24 h in serum-free DMEM/F12. After, the cells were incubated at different chloride concentrations (0, 5, 25, 50, 75, 100 and 125 mM) in the presence of the ionophores nigericin (5 μM) and tributyltin (10 μM) for 1 h and the cellular IL-1β mRNA, internal or secreted protein levels were determined. A: Quantitative real-time RT-PCR of IL-1β mRNA expression levels in IB3-1 cells. B: ELISA quantification of the IL-1β present in culture media. C: Representative WB of pro-IL-1β, mature IL-1β and actin of whole cellular lysates from IB3-1 cells. D: Densitometric quantification and statistical analysis of the results shown in panel C. The results were expressed in arbitrary units, taken the value corresponding to 0 mM chloride as 1. Measurements were performed in triplicate and data are expressed as mean ± SE of three independent experiments (n=3). * indicates $p<0.05$ compared with 0 mM Cl^-

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Figure 3: Effects of p38, IKK, JNK, MEK1/2 and p-c-Src inhibitors over IL-1β mRNA levels in response to $[Cl^-]_i$ changes. IB3-1 cells were incubated for 1 h at 5 or 75 mM Cl^-, in the presence of tributyltin (10 μM) and nigericin (5 μM) and treated as indicated; p38 Inh: p38 inhibitor SB203580 (5 μM), IKK Inh: IKK inhibitor III (2 μM), JNK Inh: JNK inhibitor SP600125 (5 μM), MEK1/2 Inh: MEK1/2 inhibitor U0126 (5 μM); c-Src inh: c-Src inhibitor PP2 (5 μM). A: Quantitative real-time RT-PCR of IL-1β mRNA expression levels. B: Representative WB of p-c-Src and actin. C: Densitometric quantification and statistical analysis of the results shown in panel B (p-c-Src/actin ratio). D: Representative WB of p-JNK and actin. E: Densitometric quantification and statistical analysis of the results shown in panel D (p-JNK/actin ratio). F: Representative WB of IkB-α and actin. G: Densitometric quantification and statistical analysis of the results shown in panel F (IκB-a/actin ratio). The results were expressed as arbitrary units, taken the value corresponding to 5 mM Cl^- as 1. Measurements were performed in triplicate and data are expressed as mean ± SE of three independent experiments (n=3). * indicates $p<0.05$ compared with untreated 5 mM Cl^- IB3-1 cells.

Figure 4: IL1RN and anti-IL-1β blocking antibody on chloride effects. IB3-1 cells preincubated for 24 h in serum free media were additionally incubated for 1 h at two different chloride concentrations 5 and 75 mM plus IL-1 receptor antagonist (10 ng/ml) or anti-IL-1β blocking antibody (30 ng/ml) and the cellular IL-1β mRNA, internal or secreted protein levels were determined. A: Quantitative real-time RT-PCR of IL-1β mRNA expression levels in IB3-1 cells incubated with IL1RN and anti-IL-1β blocking antibody. B: ELISA quantification of the IL-1β present in culture media. C: Representative WB of pro-IL-1β, mature IL-1β and actin of whole cellular lysates from IB3-1 cells. D: Densitometric quantification and statistical analysis of the results shown in panel C. The results were expressed in arbitrary units, taken the value corresponding to untreated 5 mM chloride as 1. Measurements were performed in triplicate and

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data are expressed as mean ± SE of three independent experiments (n=3). * indicates $p<0.05$ compared with untreated 5 mM Cl^- IB3-1 cells.

Figure 5: Autocrine IL-1β loop and chloride effects on IL-1β expression in IB3-1 cells. The figure illustrates the interactions among the different proteins, kinases or small molecules involved in this work. Cl^- acts as a second messenger for CFTR, modulating IL-1β expression. We postulate that Cl^- is able to modulate IL-1β expression first through stimulation of the IL-1β maturation/secretion. Then, the secreted IL-1β creates a positive feedback loop (autocrine loop) that, even in the presence of CHX, further stimulates its own mRNA expression. c-Src, NF-κB and JNK are important IL-1β effectors of this loop, although the exact points of regulation and Cl^- dependency remain to be determined. The interactions were drawn by using the software Pathway Studio (v10, Elsevier). Arrows with the + symbol represent stimulations and those with the -| symbol represent inhibition. Green ellipses: small molecules; red sickle-vertex: kinases; purple rectangle: disease (CF).

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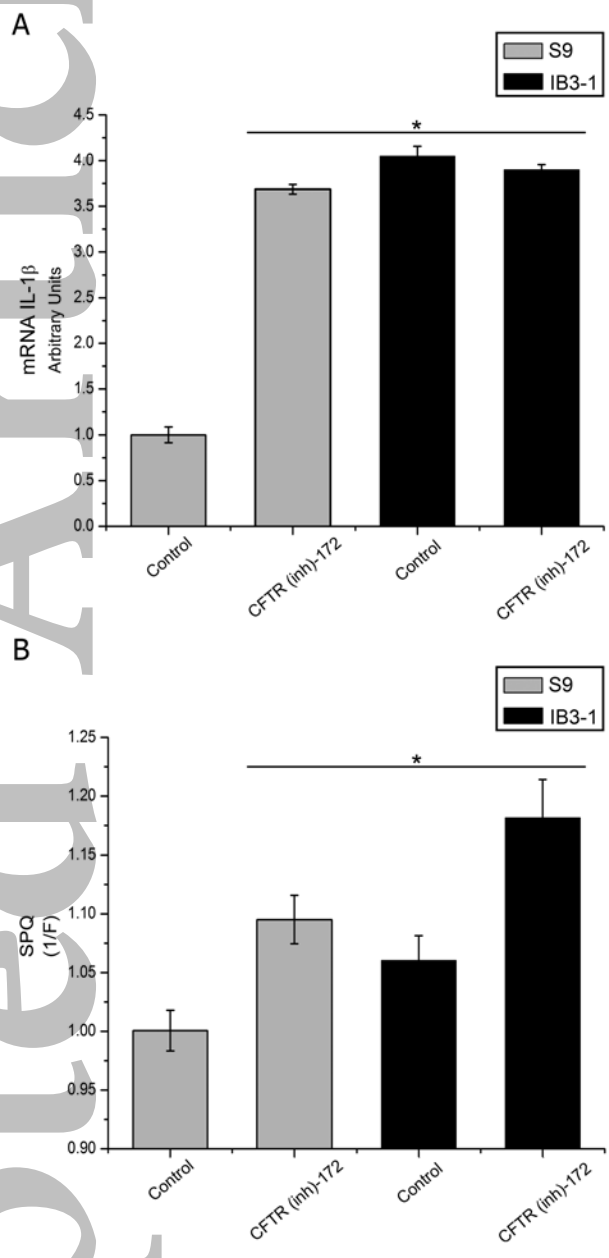

Figure 1

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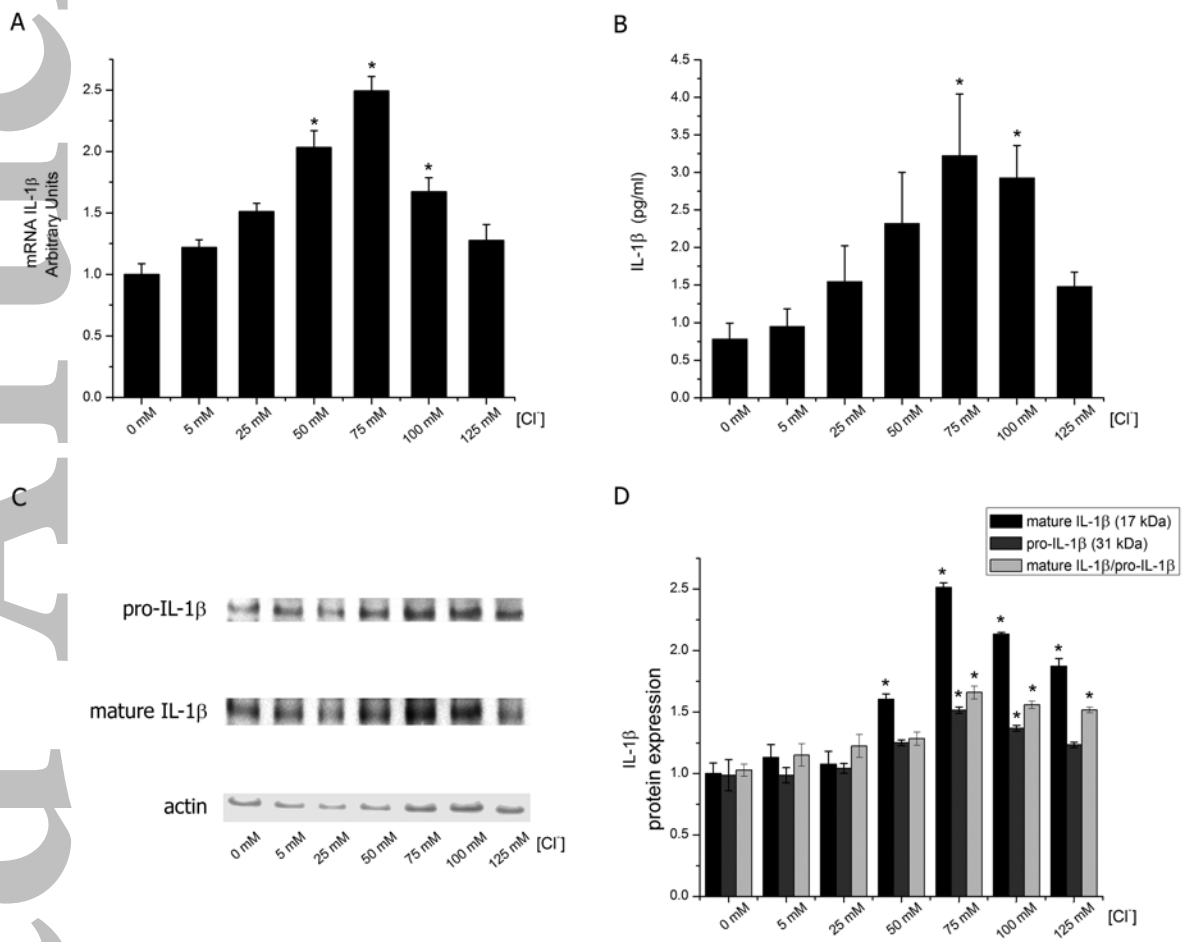

Figure 2

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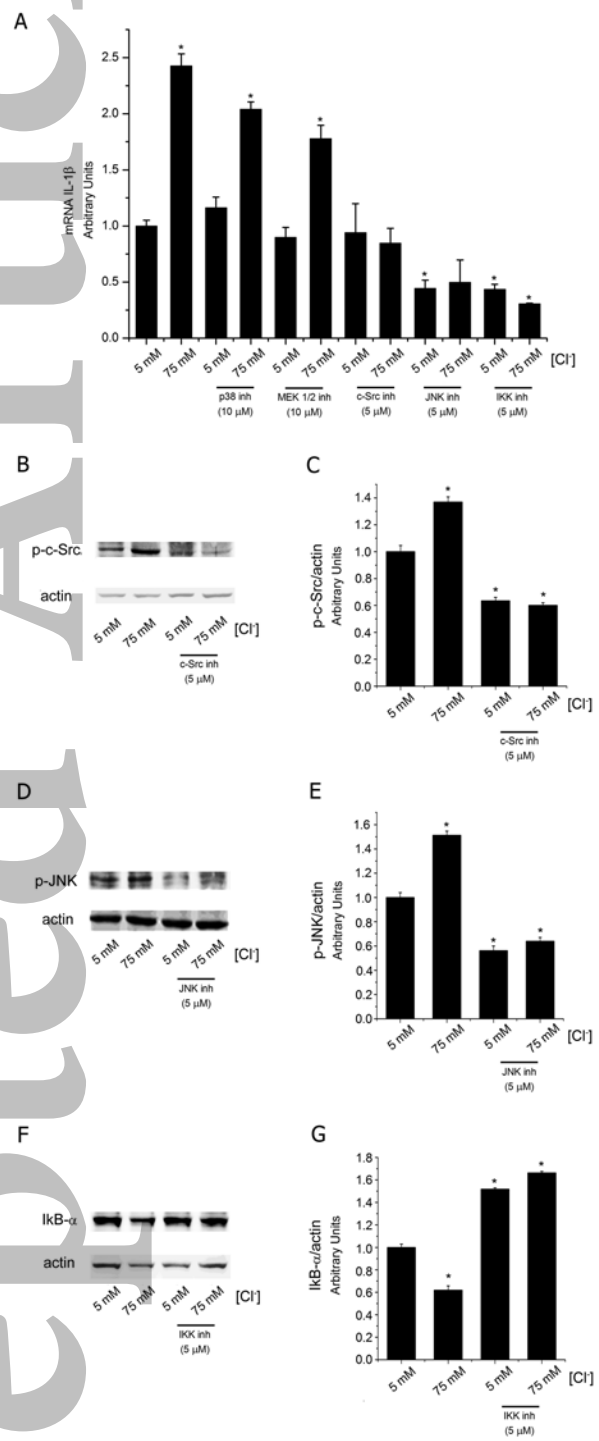

Figure 3

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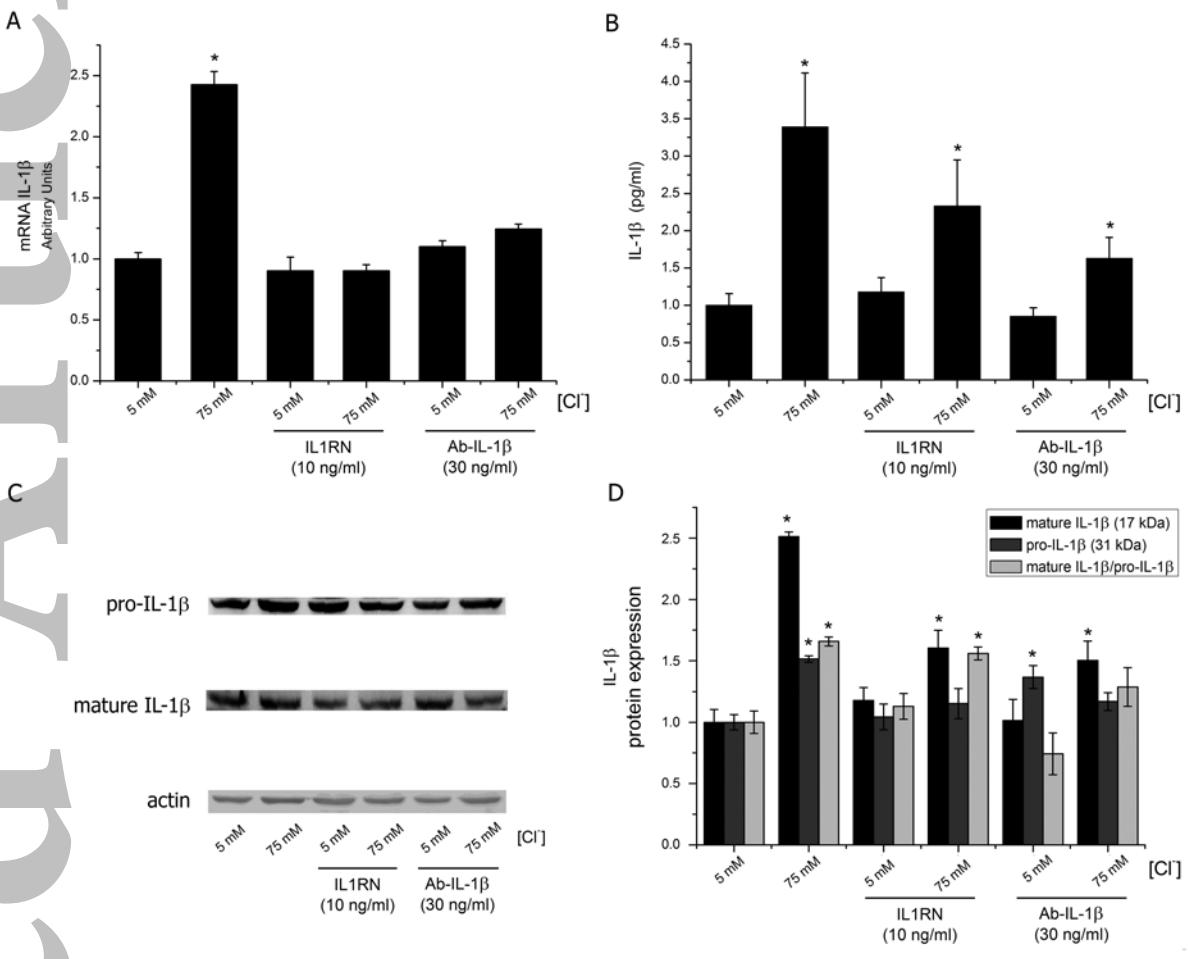

Figure 4

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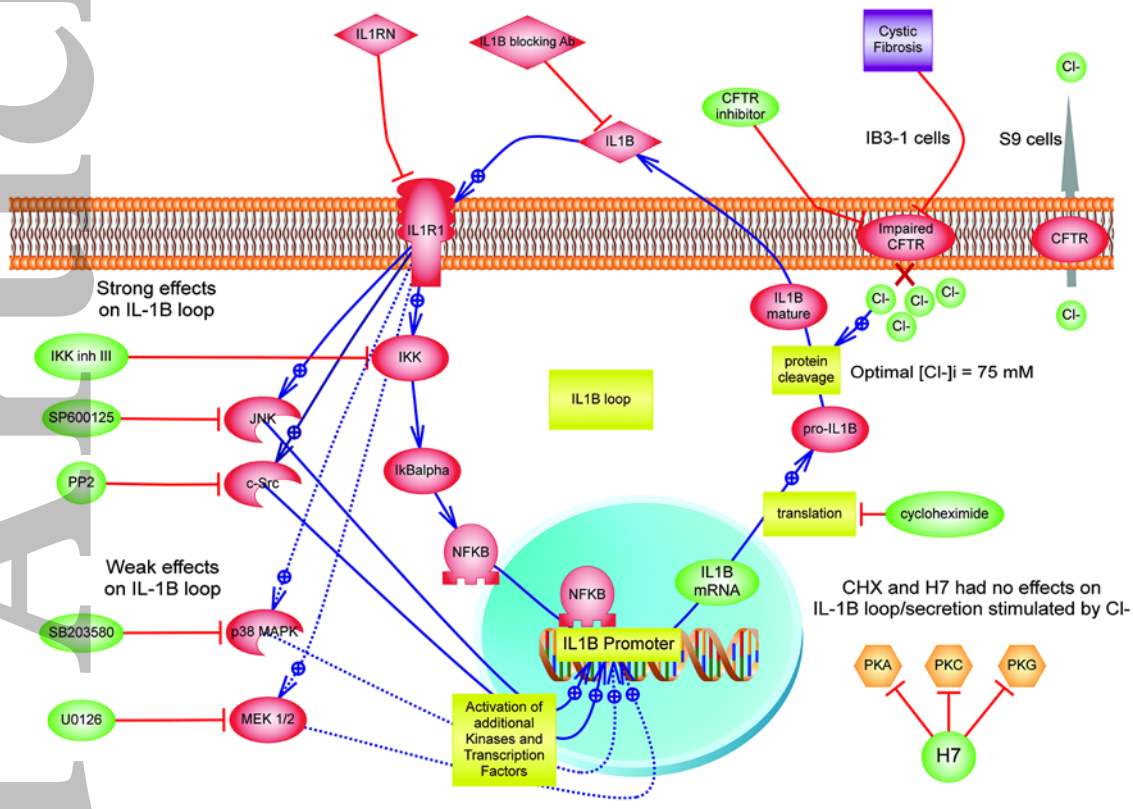

Figure 5

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