Differential Regulation of CFTR by Interferon-γ in Mast Cells and Epithelial Cells

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LIST OF ABBREVIATIONS
C/EBP, CCAAT-enhancer binding protein
ClC, voltage-gated Cl^- channel
CFTR, cystic fibrosis transmembrane conductance regulator
CREB, cAMP response element binding protein
DPC, diphenylamine-2-carboxylate
JAK, Janus kinase
ERK, extracellular signal-regulated kinase
HTB, Hepes Tyrode's buffer
PMC, peritoneal mast cells
JNK, c-Jun NH2-terminal kinase
LAD2, Laboratory of Allergic Diseases mast cell line 2
MAPK, mitogen-activated protein kinase
MQAE, N-(ethoxycarbonylmethyl)-6-methoxyquinolinium bromide
NF-κB, nuclear transcription factor kappaB
NPPB, 5-nitro-2-(3-phenylpropylamino) benzoic acid
PMA, phorbol 12 myristate 13 acetate
PMC, rat peritoneal mast cells
RCMC, rat cultured mast cells
STAT, signal transduction and activator of transcription

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ABSTRACT

Cystic fibrosis transmembrane conductance regulator (CFTR) is a cAMP-dependent chloride channel in epithelial cells; recently we identified it in mast cells. Previous work that we confirmed showed that IFNγ downregulated CFTR expression in epithelial cells (T84), but by contrast, we found that IFNγ upregulated CFTR mRNA and protein expression in rat and human mast cells. IFNγ upregulation of CFTR in mast cells was inhibited by p38 and ERK kinase inhibitors but not a JAK2 inhibitor, whereas in T84 cells IFNγ-mediated downregulation of CFTR was JAK2 dependent, and ERK and p38 independent. Furthermore, IFNγ downregulation of CFTR in T84 epithelial cells was STAT1 dependent, but upregulation of CFTR in mast cells was STAT1 independent. Thus, differential regulatory pathways of CFTR expression in mast cells and epithelial cells exist that are dependent upon either p38/ERK or JAK/STAT pathways, respectively. Surprisingly, IFNγ treatment of mast cells inhibited Cl^- efflux, in contrast to upregulation of CFTR/mRNA and protein expression. However, downregulation of $C1^-$ flux correlated with IFNγ-mediated inhibition of mediator secretion. This and other work suggests that the effect of IFNγ on CFTR expression in mast cells is important for their function.

INTRODUCTION

The cystic fibrosis transmembrane conductance regulator (CFTR) is a cAMP-dependent Cl^- channel that controls transepithelial electrolyte transport, fluid flow and ion concentrations in the intestine, lungs, pancreas and sweat glands (Gibson et al., 2003). Over 1,200 disease-associated mutations in the cystic fibrosis gene have been reported to the Cystic Fibrosis Genetic Analysis Consortium database (www.genet.sickkids.on.ca/cftr/). About 70% of patients with the disease have a deletion of phenylalanine at amino acid position 508 (Δ508) that severely decreases CFTR expression in the plasma membrane and compromises permeability to Cl^-. CFTR expression is temporally and spatially complex and is regulated by many factors including cytokines (Besancon et al, 1994; Baudouin-Legros et al, 2005).

In mast cells, several specific Cl^- conductances have been identified and linked with degranulation. Following antigen stimulation of rat peritoneal mast cells (PMC), there is an increase in Cl^- uptake (Romanin et al, 1991; Friis et al, 1994). Cl^- channel blockers such as 5-nitro-2-(3-phenylpropylamino) benzoic acid (NPPB) inhibit both mast cell Cl^- current and degranulation (Romanin et al, 1991), while diphenylamine-2-carboxylate (DPC), blocks FcεRI-stimulated degranulation and forskolin-induced Cl^- current in PMC (Kulka et al, 2002a). Moreover, mast cell stabilizing compounds, cromolyn and nedocromil inhibit mast cell degranulation, as well as Cl^- ion flux (Alton and Norris, 1996). We have identified CFTR and voltage-gated chloride channel (ClC) family members ClC-2,3,4,5,7 in rat mast cells (Kulka et al, 2002a; Kulka et al, 2002b), and others have identified ClC3, 5 and 7 in human mast cells (Duffy et al, 2001;

Bradding et al, 2003). Thus, given that CFTR in mast cells may be important for their functions, we have studied the regulation of CFTR expression in mast cells.

In epithelial cells, IFNγ downregulates expression of CFTR resulting in a significant decrease in CFTR-mediated Cl^- current (Besancon et al, 1994). IFNγ is a member of a family of inducible secretory proteins produced largely by activated T lymphocytes and natural killer cells (Schroder et al, 2004). IFNγ modulates gene expression by activating Janus Kinase (JAK) resulting in signal transducer and activator of transcription (STAT) 1 binding and phosphorylation. Phosphorylated STAT1 dimerizes and translocates into the nucleus where it binds to γ-activated sequence (GAS) elements and initiates transcription (Schroder et al, 2004). In addition to the JAK/STAT pathway, IFNγ activates other signal-transduction proteins such as p38 mitogen-activated protein kinases (MAPK) and extracellular signal-regulated kinase (ERK) 1/2 MAPK (14). With regard to mast cells, IFNγ can inhibit proliferation, TNF-mediated cytotoxicity, cell differentiation and mediator release (Bissonnette and Befus, 1990; Holliday et al, 1994; Kirshenbaum et al, 1988). We hypothesized that IFNγ may downregulate CFTR expression in mast cells by a JAK/STAT1 dependent pathway, as in epithelial cells. However, in contrast to epithelial cells, we found that IFNγ upregulated CFTR expression in both rat and human mast cells and in a JAK/STAT1 independent manner. Surprisingly, Cl^- flux measurements indicate that IFNγ treatment of mast cells reduces Cl^- flux despite the upregulation of CFTR levels.

Materials and Methods:

Materials

The JAK2 inhibitor, AG-490 (α-Cyano-(3,4-dihydroxy)-N-benzylcinnamide tyrphostin B42), was obtained from Calbiochem (La Jolla, CA), the p38 inhibitor SB202190 ($C_{20}H_{14}FN_3O$) and the ERK inhibitor U0126 (1,4-Diamino-2,3-dicyano-1,4-bis(2-aminophenylthio)-butadiene) were from Cell Signaling (Beverly, MA). Phorbol 12 myristate 13 acetate (PMA) was obtained from Sigma-Aldrich (St. Louis, MO). Stem cell factor, TNF and IFNγ (rat and human) were purchased from PeproTech (Rocky Hill, NJ).

Rats and PMC Isolation

Male Sprague Dawley rats (300-350 g; Charles River, St. Constant, Quebec, Canada) were housed in a pathogen-free viral antibody-free facility. Rats were sacrificed by cervical dislocation under anesthesia and PMC were isolated as previously described (Kulka et al, 2002a). Briefly, 20 mL of Hepes Tyrode's buffer (HTB, containing (mM): 137 NaCl, 5.5 glucose, 2.7 KCl, 0.5 NaH_2PO_4, 1 $CaCl_2$, 12 HEPES (pH 7.2), and 1% BSA) was injected into the peritoneal cavity and massaged gently for 30 sec; the peritoneal cavity was opened, the buffer collected and kept at 4^{o}C. Following centrifugation at 200 g for 5 min the cell pellet was resuspended in 5 mL of HTB, layered on top of a 30%/80% Percoll gradient, centrifuged at 500 g for 20 min, and the MC were collected from the pellet. PMC were >98% pure and >96% viable as measured by trypan blue exclusion.

Nippostrongylus brasiliensis Sensitization

Sprague Dawley rats were sensitized to *Nippostrongylus brasiliensis* by a single subcutaneous injection of 3000 L3 larvae (Befus et al, 1982). The adult worms were expelled after 10 days, but the mast cells remained sensitized with worm antigen specific IgE for several weeks. The rats were used for experiments 30-40 days post infection.

Cell Culture

The rat cultured MC line (RCMC) 1.11.2 (kindly provided by B. Chan and A. Froese, Winnipeg, Manitoba) was cultured in RPMI 1640 medium containing 5% FBS (Invitrogen, Grand Island, NY), 100 U/mL penicillin, 100 μg/mL streptomycin and 10 mM HEPES (Sigma-Aldrich). The recently established human mast cell line, Laboratory for Allergic Diseases (LAD) 2 (Kirshenbaum et al, 2003) (a generous gift from Drs. Kirshenbaum, Akin and Metcalfe, NIH), was cultured in serum free media (StemPro-34, Invitrogen) supplemented with 2 mM L-glutamine, 100 U/mL penicillin, 50 μg/mL streptomycin and 100 ng/mL stem cell factor. The T84 epithelial cell line was cultured in F-12/DMEM media (Invitrogen) containing 5% FBS, 100 U/mL penicillin and 100 μg/mL streptomycin. All cells were incubated in a humidified atmosphere of 5% CO_2 in air at 37^{o}C.

Western blot

Cells were washed with PBS and 1 x 10^6 cells lysed in buffer containing loading dye solution (Lithium dodecyl sulphate [LDS]) sample buffer (Invitrogen), 10% β-mercaptoethanol (Sigma-Aldrich), 0.1 M dithiothreitol (DTT; Sigma-Aldrich) and

protease inhibitor cocktail (Roche, Indianapolis, IN). Whole cell lysates (30 μg) were separated on 4-12% Bis-Tris SDS-PAGE gels (Invitrogen) and transferred onto nitrocellulose membranes. The membranes were blocked with 3% milk in TBS-0.05% Tween for 1 hr and then probed with primary antibodies against CFTR (clone H-182) and STAT1 (Santa Cruz Biotechnology, Santa Cruz, CA), phosphoSTAT1 (BD Transduction Labs, Chicago, IL), phospho-stress-activated MAPK (SAPK) /c-Jun NH2-terminal kinase (JNK) (Thr183/Tyr185; Cell Signaling Technology), phospho-p38 MAPK (Thr180/Tyr182; Cell Signaling Technology), and phospho ERK1/2 (Thr202/Tyr204; Cell Signaling Technology), or anti-actin (Sigma-Aldrich) in 4% BSA/PBS for 1 hr at room temp. The membranes were washed with TBS-Tween 3X and then incubated with the horseradish peroxidase-linked secondary antibody (sheep anti-rabbit, Jackson ImmunoResearch Laboratories, West Grove, PA, or goat anti-mouse, Santa Cruz Biotechnology) for 1 hr. The membranes were developed with chemiluminescence reagent (Invitrogen) for 1 min and exposed to chemiluminescence film for 1-5 min.

Confocal microscopy

CFTR was localized in sham treated or IFN-γ treated (80 ng/ml, 24 hr) rat PMC and human T84. Following incubation, T84 cells were detached by 10 min incubation with trypsin/EDTA at room temperature and 50,000 PMC or T84 were then cytocentrifuged onto superfrost plus charged slides using a Shandon cytospin 2 (Fisher Scientific, Mississauga ON) at 5 *g* for 6 min in PBS containing 20% FBS. Cells were then air dried overnight, and fixed in 75% acetone: 25% absolute ethanol for 15 min at -

20°C. Non-specific binding sites were blocked by incubation in blocking buffer (PBS containing 3% BSA and 10% normal goat serum) for 2 hr at room temperature. Slides were then incubated with mouse anti human CFTR primary antibody (MA1-935, Affinity Bioreagents Golden, CO) at 1/50 dilution in blocking buffer for 2 hr at room temperature. After three washes in PBS, specific antibody binding was detected with alexa 568 conjugated goat anti-mouse IgM (Molecular Probes, Eugene OR) at 1/2000 dilution in blocking buffer for 1 hr at room temperature. Cell images were obtained using an Olympus FV1000 laser scanning confocal microscope (Carsen Group, Markham ON) with 400X magnification.

Quantitative real-time polymerase chain reaction

Our previous study established that mast cells express CFTR mRNA (Kulka et al, 2002a). In the current study RNA was isolated as described previously (Gilchrist et al, 1997) and quantitative real-time PCR assay was performed using gene-specific fluorescently-labeled primers and a 7700 Sequence Detector (Applied Biosystems, Foster City, CA). All primers and reagents were obtained from Invitrogen. Primers were designed using the LUX® primer design tool, and are listed along with their Invitrogen reference code (Table 1). Each primer set consisted of one labeled (6-carboxy fluorescein (FAM) fluorescent reporter at the 5' end) and one unlabeled primer. Lower case nucleotides in the labeled primer sequence represent hairpin-generating segments of the labeled primer. Data was collected during the annealing/extension phase of PCR and analyzed using the comparative C_t method (Nazarenko et al, 2002).

$^{36}Cl^-$ Flux Measurements

Changes in [Cl^-] were measured by incubating 1 x 10^6 cells/mL with 8.7 mM $Na^{36}Cl$ (ICN, Aurora, OH) in flux buffer (137 mM NaCl, 4 mM KCI, 1 mM $MgSO_4$, 1 mM $CaCl_2$, 20 mM HEPES, 1 mg/mL BSA, 1 mg/mL glucose) at 37°C for 30 min (Friis et al, 1994). The incubation was terminated by transferring 100 µL of the cell suspension onto 120 µL of silicone oil in long, thin Eppendorf tubes. The tubes were centrifuged at 18,000 g for 30 s and then placed into a freezing methanol bath. The bottom of each tube was cut off and placed into a scintillation vial with 48 mM NaOH. Each vial was vortexed for 1 min after which 5 mL of scintillation fluid was added and placed in a Beckman scintillation counter. $^{36}Cl^-$ uptake was calculated based on the specific activity of $^{36}Cl^-$ in the extracellular medium, calculated as the sum of extracellular Cl^- and added $^{36}Cl^-$ (in nmol) divided by the radioactivity of the added $^{36}Cl^-$ (in cpm). All values of $^{36}Cl^-$ uptake were corrected for $^{36}Cl^-$ trapped in the extracellular space, which was determined by measuring cpm immediately after $^{36}Cl^-$ addition (50 ± 10.2 cpm).

MQAE Measurements

Fluorescence measurements were performed in HTB. Gluconate, Br^-, or I^- buffers were identical to HTB except that 137 mM NaCl was replaced by equivalent amounts of sodium gluconate, NaBr, or NaI, respectively. One million cells/mL were incubated with 5 mM of the Cl^--sensitive dye N-(ethoxycarbonylmethyl)-6-methoxyquinolinium bromide (MQAE, Molecular Probes, Eugene, OR) in 1 mL HTB for 30 min at 37°C. Cells were washed twice and resuspended in 50 µl HTB. MQAE is quenched by Cl^-

anions and thus, when Cl^- leaves the cell, it dissociates from MQAE and fluorescence increases. MQAE fluorescence was excited at 350 nm and the emission was measured at 450 nm with a PTI spectrofluorimeter (Photon Technology Int., London, Ontario), using Felix software (version 1.42). All experiments were performed at 37°C. To produce a driving force for Cl^- efflux, the cells were added to 1 mL of gluconate buffer and MQAE fluorescence was monitored for up to 10 min. Cl^- efflux was calculated as the initial rate of change of MQAE fluorescence after addition of cells to the gluconate buffer. For quantitative analysis, the data collected in the first 60 sec were fitted using linear regression, and the slope was used as a measure of Cl^- efflux. All traces were normalized to initial baseline reading (buffer, no cells). In some experiments 10 worm equivalents/mL of *N. brasiliensis* antigen was added to the cell suspension, and changes in fluorescence were monitored for up to 10 min.

Statistics

All data is presented as mean of at least three independent experiments with standard error of the mean (SEM). Where indicated, data was analysed using a paired *t*-test for sample means, ANOVA or the Tukey-Kramer multiple comparisons test.

RESULTS

Interferon-γ upregulates CFTR mRNA and protein expression in rat and human mast cells

PMA, TNF and IFNγ downregulate CFTR expression in epithelial cells (Nakamura et al, 1992; Sen et al, 1993; Besancon et al, 1994). To determine if CFTR in mast cells was similarly regulated, rat RCMC were treated with PMA, TNF or IFNγ for 24 hr and CFTR expression was identified by western blotting as we have done previously (confirmed using isotype controls for flow cytometry, western blot and immunohistochemistry, Kulka et al, 2002a). As expected, TNF and IFNγ decreased CFTR expression in T84 cells (Fig 1A). Surprisingly, however, TNF and IFNγ upregulated CFTR expression in RCMC. PMA had no detectable effect on CFTR expression in RCMC or T84 (Fig 1B). Because IFNγ upregulates STAT1 expression (Hu et al, 2002), membranes were stripped and re-probed with anti-STAT1. As expected, STAT1 protein (visible as a double band, representing STAT1α and STAT1β) was upregulated in both RCMC and T84 by PMA, TNF and IFNγ.

Confocal analysis of CFTR expression in T84 cells showed a largely cytoplasmic distribution and as expected from the results of western blot analysis, the intensity of CFTR staining was decreased following IFNγ treatment (80 ng/mL; 24 hr, Fig. 1C). By contrast, CFTR expression in rat PMC was increased following IFNγ treatment, and appeared to be associated with granules (Fig 1D). Studies of nonpermeabilized cells identified some CFTR in a plasma membrane-like distribution on T84 cells, but there was no obvious CFTR with such a distribution on PMC (not shown).

To further characterize upregulation of CFTR in mast cells, CFTR mRNA expression following IFNγ dose response and time course treatments was analyzed in RCMC, human LAD2 and T84 cells (Fig 2). Quantitative PCR analysis confirmed that IFNγ (10 ng/mL) significantly ($p<0.05$) upregulated CFTR mRNA expression in RCMC by 3 hr following treatment and the magnitude of this upregulation was 39 $\pm$ 13 % at 12 hr. In LAD2 MC the upregulation of CFTR was statistically significant by 8 hr of IFNγ treatment and by 12 hr was 54 $\pm$ 17 % greater than in untreated cells (Fig 2A). Significant upregulation of CFTR was induced in mast cells within 8 hr with as little as 1 ng/mL (RCMC) or 10 ng/mL (LAD2) of IFNγ (Fig 2B).

By contrast, IFNγ significantly decreased CFTR mRNA expression in T84 cells within 3 hr (Fig 2A and B) and by 12 hr the magnitude of decrease was 49 $\pm$ 4 % at 12 hr of treatment. The IFNγ effect was dose dependent such that 1, 10 and 100 ng/mL of IFNγ decreased CFTR mRNA expression in T84 cells by 23, 50 and 83 % after 8 hr (all statistically significant decreases compared to the untreated group).

Western blot analysis showed that by 6 hr, CFTR expression was increased compared to untreated RCMC and by 24 hr, CFTR expression was significantly upregulated (Fig 2C). In T84 cells, decrease in CFTR protein expression was observable at 6 hr and remained low at 24 hr (Fig 2D). In both T84 and RCMC, STAT1 protein was upregulated after 6 hr of treatment and remained elevated up to 24 hr.

IFNγ upregulation of CFTR is inhibited by MAP kinase inhibitors

To determine which IFNγ signalling pathways were activated in mast cells as compared to epithelial cells, RCMC were treated with IFNγ and whole cell lysates were analysed for phosphorylated STAT1, p38, ERK, and JNK (Fig 3). IFNγ activated STAT1, ERK and p38, but not JNK phosphorylation. STAT1 was activated at 5 min and remained activated for up to 30 min. ERK2 (bottom band) was constitutively activated, but phosphorylation of both ERK1 (top band) and ERK2 (bottom band) was induced after 5 min. p38 was activated at 15 min, later than STAT1 or ERK.

To determine if JAK/STAT1, p38 or ERK signalling pathways were involved in IFNγ-mediated upregulation of CFTR, RCMC, LAD2 and T84 cells were treated with IFNγ in the presence of a JAK2 inhibitor (AG-490), a p38 kinase inhibitor (SB202190), and an ERK MAP kinase inhibitor (U0126). In RCMC and LAD2, AG-490 did not affect IFNγ-mediated upregulation of CFTR protein expression, but both SB202190 and U0126 partially inhibited IFNγ-mediated upregulation of CFTR (Fig 4). By contrast, in T84 cells, AG-490 blocked IFNγ-mediated downregulation of CFTR. Membranes were stripped and reblotted with anti-STAT1 to compare regulation of another IFNγ responsive protein. STAT1 upregulation in RCMC was sensitive to AG-490 but in human LAD2 cells, STAT1 upregulation was inhibited by AG-490, SB202190 and U0126. T84 cells were similar to the RCMC in that STAT1 upregulation was only blocked by AG-490 but not SB202190 and U0126. Densitometry analysis of three independent blotting experiments confirmed that AG-490 did not affect IFNγ-mediated upregulation of CFTR protein expression in both LAD2 and RCMC cells (Fig. 4B, $p<0.05$).

To confirm the actions of AG-490, SB202190 and U0126, RCMC were treated with IFNγ (10 ng/mL) in the presence of these inhibitors and STAT1, p38 and ERK1/2 phosphorylation was assessed by western blotting (Fig 5). As expected, AG-490 but not SB202190 or U0126 inhibited STAT1 constitutive and IFNγ-induced phosphorylation (Fig. 5A). U0126 inhibited ERK1/2 constitutive and IFNγ-induced phosphorylation (Fig. 5B). SB202190 inhibited IFNγ induced p38 phosphorylation (Fig. 5C).

IFNγ inhibits both constitutive and antigen-induced Cl^- flux in mast cells

To determine the effect of IFNγ on Cl^- flux in resting and antigen-IgE activated mast cells, we employed two methods: measurement of $^{36}Cl^-$ uptake and assessment of Cl^- sensitive fluorescence using MQAE. Studies with $^{36}Cl^-$ have shown that IFNγ treatment decreases Cl^- uptake of PMC (Fig 6A, $p<0.01$). A time-course of PMC Cl^- uptake shows that IFNγ did not have an effect at the earlier treatment points (less than 2 hr) but decreased Cl^- uptake at 20 and 24 hr (Fig 6B). Similar results were obtained with $^{36}Cl^-$ uptake measurements in RCMC (data not shown).

Fluorescence measurements were performed with mast cells loaded with MQAE in HTB solution and chloride efflux was measured after placing cells in gluconate buffer. Figure 6C shows that IFNγ treatment significantly reduced Cl^- flux in sensitized PMC not challenged with antigen ($P<0.05$; n=8 and 11, control and IFNγ treated cells, respectively). Following antigen challenge (10 WE/mL) the magnitude of the IFNγ mediated depression in Cl^- efflux was reduced (Fig. 6D). Although antigen challenge in the absence of IFNγ treatment showed a trend towards reduced Cl^- efflux, this was not

statistically significant (P>0.5, n=3). Identical results were obtained in both PMC and RCMC under conditions when the cells were loaded in gluconate buffer and placed in HTB to measure Cl^- influx (data not shown).

Measurements of halide permeabilities indicated that Br^- was more permeable that Cl^- and I^- in PMC cells (Br^-(1.34) > Cl^-(1.00) ≥ I^- (0.68), n=3 in each set). Similar results were obtained with RCMC (Br^-(1.19) ≥ Cl^-(1.00) > I^- (0.61), n=3 in each set). The halide permeability sequence, $Br^- \geq Cl^- > I^-$, is characteristic of CFTR Cl^- channels (Illek et al, 1999), and suggests that CFTR channels are an important component of Cl^- flux in mast cells.

DISCUSSION

This is the first study demonstrating that CFTR expression is regulated differently in epithelial and non-epithelial cells. Moreover, we show that IFNγ-induced upregulation of CFTR in MC involved MAPK signalling pathways, whereas IFNγ-induced downregulation of CFTR in epithelial cells involved JAK/STAT pathways. Paradoxically we also show that despite IFNγ upregulation of CFTR in mast cells, IFNγ treatment depressed mast cell Cl^- flux in multiple assay systems.

It is now well established that CFTR gene expression is regulated in a complex, cell- and stimulus-specific manner that may involve both transcriptional and posttranscriptional mechanisms. For example, TNF decreases CFTR mRNA in human colonic epithelial cells but not in airway epithelial cells, whereas IL-1β increases it only in airway epithelial cells (Baudouin-Legros et al, 2005). While stimulation of CFTR gene expression by IL-1β involves activation of the CFTR promoter (Brouillard et al. 2001), downregulation of CFTR by TNF and IFNγ involves mainly posttranscriptional mechanisms (Baudouin-Legros et al, 2005). The results of this study show that in mast cells both TNF and IFNγ increase CFTR mRNA, but whether this process affects CFTR gene transcription and/or mRNA stability is presently unknown. However, the fact that IFN-γ increased CFTR protein levels to a greater extent than the mRNA, suggests that IFNγ treatment may increase mRNA stability rather than CFTR gene transcription.

IFNγ modulation of gene expression is mediated by both STAT1-dependent and independent pathways (Gil et al, 2001). Our results show that IFNγ activates STAT1, ERK1/2 and p38 but not JNK, suggesting that these pathways are also induced in mast

cells. Using inhibitors to JAK/STAT, ERK and p38, we determined that in both rat and human mast cells, CFTR upregulation is JAK/STAT independent but requires activation of the MAPK pathways mediated by ERK and p38. In T84 cells, IFNγ-mediated downregulation of CFTR is inhibited by AG-490, but is unaffected by the p38 and ERK inhibitors. STAT1 expression, by comparison, is upregulated by IFNγ in both mast cells and T84 epithelial cells and is inhibited by AG-490, suggesting that STAT1 upregulation is a positive feedback mechanism that sensitizes mast cells to IFNγ as previously observed in human macrophages (Duffy et al, 2001). In human LAD2 cells, IFNγ-mediated upregulation of STAT1 is also sensitive to ERK and p38 inhibitors perhaps indicating the importance of MAPK pathways in IFNγ signalling. Therefore, IFNγ activates at least two pathways in mast cells - the JAK/STAT pathway responsible for upregulation of STAT1 and the p38/ERK pathway(s) that is responsible for upregulation of CFTR. Although ERK activation is involved in CFTR upregulation, PMA activates ERK but does not upregulate CFTR (Fig 1). This suggests that ERK activation requires activation of other molecules, perhaps p38, for upregulation of CFTR mRNA.

The exact role of CFTR in mast cell function is unknown and is the subject of other work in our lab. To date we have established that DPC, a drug known to inhibit CFTR, but to also have other activities, blocks FcεRI-stimulated degranulation of PMC (Kulka et al, 2002a). Moreover, knockdown of CFTR expression by antisense oligonucleotides in the human mast cell line HMC-1 reduces Cl^- flux, adhesion to fibronectin and calcium ionophore A23187 induced degranulation and IL-6 production (A. Schwingshackl and R. Dery, unpublished results). Our working hypothesis is that

CFTR in mast cells is an important component of Cl^- flux and perhaps of other activities, as recognized for epithelial cells (Rowe et al, 2005).

In turn, the role of mast cells in cystic fibrosis is unclear. Recently, mast cells have been recognized as important players in innate and acquired immune responses (Marshall, 2004). Moreover, there are increased numbers of mast cells in nasal polyps from cystic fibrosis patients compared to non-cystic fibrosis patients and many show signs of activation in cystic fibrosis (Henderson and Chi, 1992). Differences have also been found in mast cell numbers in human fetal trachea between cystic fibrosis and non-cystic fibrosis specimens (Hubeau et al, 2001). Interestingly, mast cell numbers and mast cell specific genes and others genes associated with innate immunity are upregulated in the intestine in CFTR null mice that show a severe intestinal phenotype (Norkina et al, 2004). Thus, the role of mast cells in cystic fibrosis warrants further investigation.

The finding of IFN-γ mediated increase in CFTR expression and decrease in Cl^- flux could be explained in several ways. For example, if IFNγ treatment leads to cell depolarization, this would tend to reduce Cl^- flux under our experimental conditions, perhaps by channels other than CFTR. Alternately, IFNγ may modulate expression of other proteins involved in Cl^- flux, e.g. SNARE proteins, which inhibit CFTR activity by decreasing channel open probability (Cormet-Boyaka et al, 2002). It is also possible that although IFNγ increases CFTR expression in mast cells, this may not involve maturation of CFTR and its translocation to the plasma membrane, where it could be fully functional. Indeed, our confocal studies of CFTR expression support the hypothesis that the increase in CFTR expression in MC is mainly observed intracellularly, most likely in

association with granules (Fig 1D). In addition, studies of the biosynthetic processing and intracellular trafficking of CFTR indicate that CFTR undergoes constitutive endocytosis and recycling (Picciano et al. 2003). Thus, IFN-γ treatment could affect the balance between CFTR degradation and recycling back to the plasma membrane, reducing the effective amount of CFTR in the plasma membrane.

The role of IFNγ-mediated upregulation of CFTR in mast cell physiology is difficult to determine. Further studies are required to characterize the functional effects of increased CFTR on mast cell functions such as Cl^- transport, degranulation and mediator release in response to stimuli such as allergens. Furthermore, the transcription factors involved in CFTR upregulation in mast cells must also be examined to provide insight into regulation of the CFTR promoter. The mechanisms that modulate CFTR gene expression through extracellular and intracellular signals may ultimately provide targets for therapy in cystic fibrosis where CFTR expression is abnormal.

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Footnotes:

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Figure Legends

Figure 1. Western blot analysis of CFTR and STAT1 expression in untreated (lane 1), PMA treated (lane 2), TNF treated (lane 3) and IFNγ treated (lane 4) T84 (panel **A**) and RCMC (panel **B**). T84 cells were treated with PMA (10 ng/mL), human recombinant TNF (10 ng/mL) or IFNγ (10 ng/mL) for 24 hr. RCMC were treated with PMA (10 ng/mL) or rat recombinant TNF (10 ng/mL) or IFNγ (10 ng/mL) for 24 hr. Cell lysates were resolved on an 4-12% SDS-PAGE and blotted with anti-CFTR, anti-STAT1 or anti-actin antibody. Western blots shown are representative of three independent experiments. Effect of 24 hr IFN-γ treatment (80 ng/ml) on CFTR expression was characterized in T84 cells (Panel C) and rat peritoneal mast cells (Panel D), using confocal laser scanning microscopy (bar on the figure represents 10 μm).

Figure 2. Quantitative PCR analysis of CFTR expression in RCMC LAD2 and T84 cells following different times and doses of IFNγ treatment (A and B). Asterisks represent statistical significance as determined by student t test, compared to untreated sample (time=0) in each case ($p<0.05$). The dose used in Fig. 2A was 10 ng/mL, and in Fig. 2B treatment was for 8 hr. Western blot analysis of CFTR, STAT and actin expression in RCMC and T84 following a timecourse (hr) of 10 ng/mL IFNγ treatment (C and D). (n=3)

Figure 3. Western blot analysis of STAT1 and MAP kinase activation following IFNγ treatment. RCMC were treated with 10 ng/mL of IFNγ for indicated times and cell lysates were probed with antibodies to phosphorylated STAT1, p38, ERK or JNK. Blots were stripped and probed with anti-actin to show equal loading. Representative of three independent experiments.

Figure 4A. IFNγ-mediated upregulation of mast cell CFTR protein requires activation of JAK2 and/or p38 and ERK. RCMC, LAD2 and T84 cells were treated with IFNγ (10 ng/mL) with or without JAK2 inhibitor (AG-490; 30 mg/mL), p38 inhibitor (SB202190; 30 mg/mL), or ERK MAPK inhibitor (U0126; 30 mg/mL) for 24 hr. Representative of three independent experiments. 4B. Densitometry summary of data in Fig. 4. RCMC were treated with IFNγ (10 ng/mL) with or without JAK2 inhibitor (AG-490; 30 mg/mL), p38 inhibitor (SP202190; 30 mg/mL), or ERK inhibitor (U0126; 30 mg/mL) for 24 hr and expression of CFTR (black bars) and STAT1 (grey bars) was analysed by western blot. (n=3 independent experiments; error bars represent SEM). Asterisks represent statistical significance as determined by student t test ($p<0.05$).

Figure 5. RCMC were treated with IFNγ (10 ng/mL) alone (IFN) or pretreated with AG-490 (IFN+A; 30 mg/mL), SB202190 (IFN+S ; 30 mg/mL) or U0126 (IFN+U ; 30 mg/mL) for 10 min, then stimulated with IFNγ (10 ng/mL) and for 1 to 30 min. Cell lysates were analyzed for phosphorylation of STAT1 (A), ERK1/2 (B) or p38 (C) and β-

actin (D) was assessed as a loading control**.** Representative of three independent experiments.

Figure 6. IFNγ decreases $C1^-$ flux. Rat peritoneal mast cells were purified (>95%) and treated with IFNγ at various doses (A; 24hr) or for various times (B; 80 ng/mL). After treatment, mast cells were washed and incubated with $^{36}C1^-$ for 30 min. Mast cells were spun through oil and radioactivity of cell pellets was measured in a scintillation counter (n=5 independent experiments; $p<0.01$ compared to untreated). Effects of IFNγ on $C1^-$ flux in PMC as measured by MQAE (7C,D). Effects of IFNγ (80 ng/mL, 24 hr pretreatment) on $C1^-$ efflux from sensitized rat peritoneal mast cells. After IFNγ pretreatment cells were loaded with the $C1^-$ sensitive dye N-(ethoxycarbonylmethyl)-6-methoxyquinoliaium bromide (MQAE) and the driving force for $C1^-$ efflux involved placing them in gluconate buffer with or without antigen challenge (10 worm equivalents/mL); (n=8 to 11 independent experiments; $p<0.01$).

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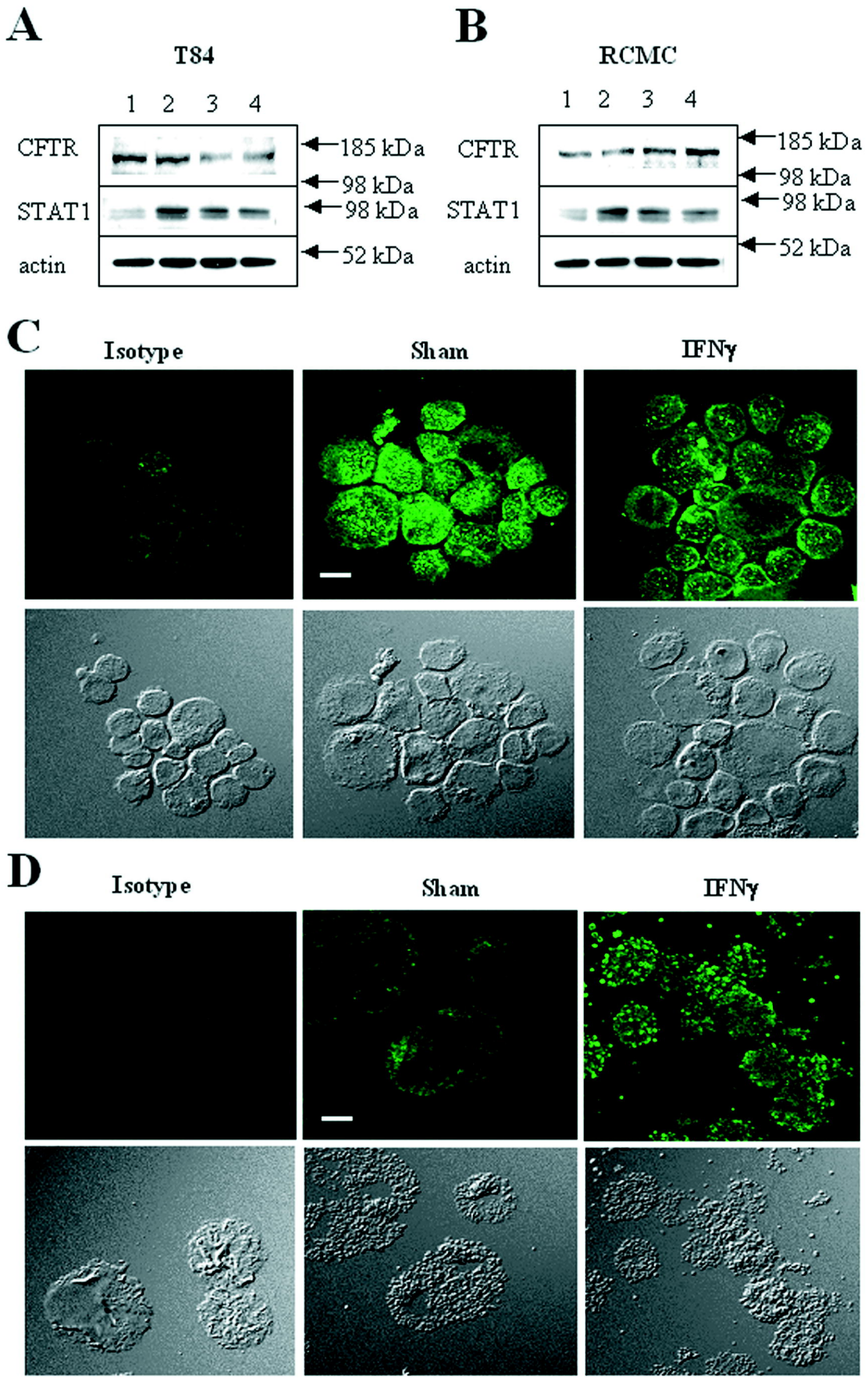
Table 1. List of qRT-PCR primers used in experiments

Product	GenBank accession #	Primer sequence	Invitrogen order code
Human CFTR (forward) (Unlabeled)	NM_000492	5`-GCATACTGCTGGGAAGAAGCAA-3`	hCFTR_1017RL_973FU
Human CFTR (reverse) (FAM labeled)	NM_000492	3`-gactcgACATAGGCTGCCTTCCGAGtC-5`	hCFTR_1017RL
Rat CFTR (forward)	XM_347232	5`-CGCAGGTTCTCAGTGGACGA-3`	rat CFTR_1986RL_1976FU
Rat CFTR (reverse) (FAM labeled)	XM_347232	3`-cacctgACTGTTTGGCTTTGTTCCAGGtG-5`	rat CFTR_1986RL
Human/rat b-actin (forward)	NM_001101	5`-GACGAGGCCCAGAGCAAGA-3`	actin_304RL_257FU
Human/rat b-actin (reverse) (FAM labeled)	NM_001101	3`-caactgTCTCCATGTCGTCCCAGtTG-5`	actin_304RL


A
T84
1 2 3 4
CFTR
STAT1
actin
185 kDa
98 kDa
98 kDa
52 kDa
B
RCMC
1 2 3 4
CFTR
STAT1
actin
185 kDa
98 kDa
98 kDa
52 kDa
C
Isotype
Sham
IFNγ
D
Isotype
Sham
IFNγ

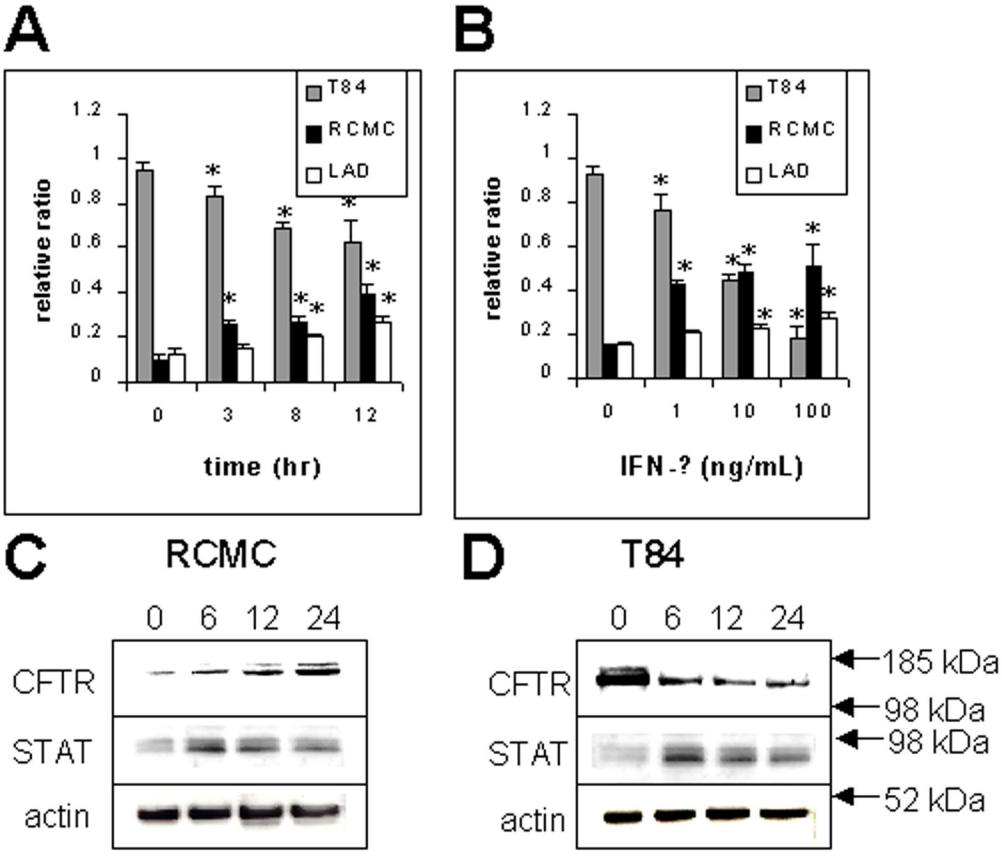

A
B
T84
RCMC
LAD
relative ratio
1.2
1
0.8
0.6
0.4
0.2
0
0
3
8
12
time (hr)
0
1
10
100
IFN-? (ng/mL)
C
RCMC
D
T84
0 6 12 24
CFTR
STAT
actin
185 kDa
98 kDa
98 kDa
52 kDa

Fig. 2

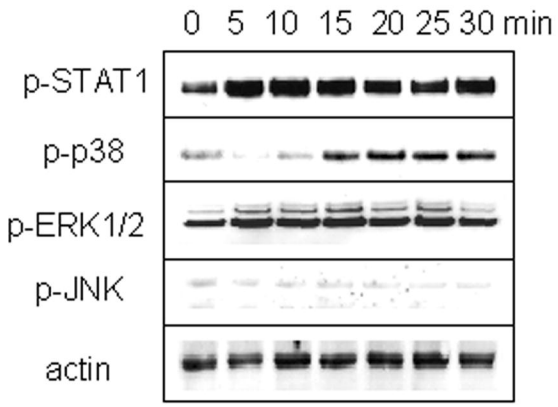


Fig. 3

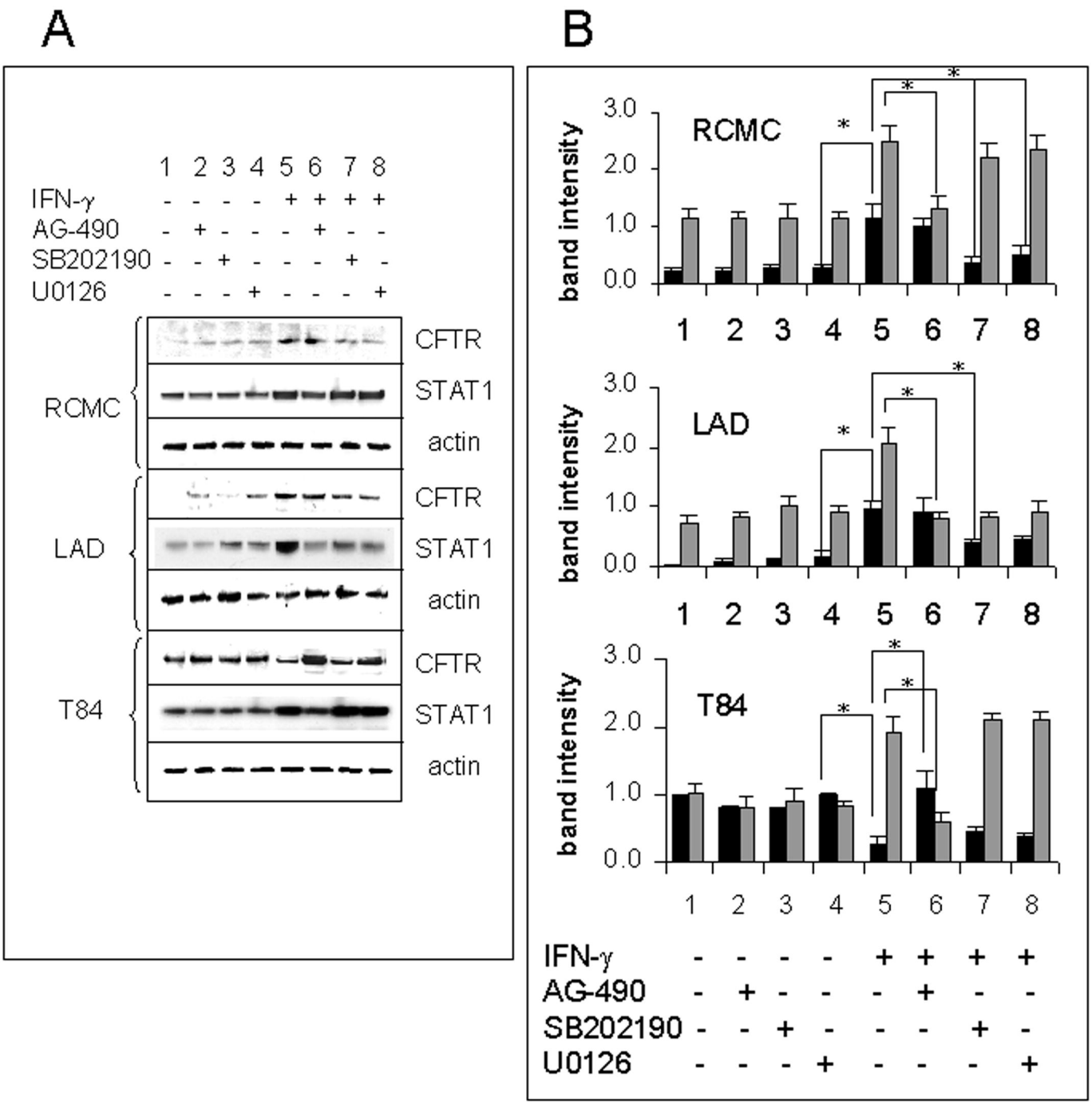

A
B
1 2 3 4 5 6 7 8
IFN-γ - - - - + + + +
AG-490 - + - - - + - -
SB202190 - - + - - - + -
U0126 - - - + - - - +
RCMC
LAD
T84
CFTR
STAT1
actin
band intensity
3.0
2.0
1.0
0.0
*

Fig. 4

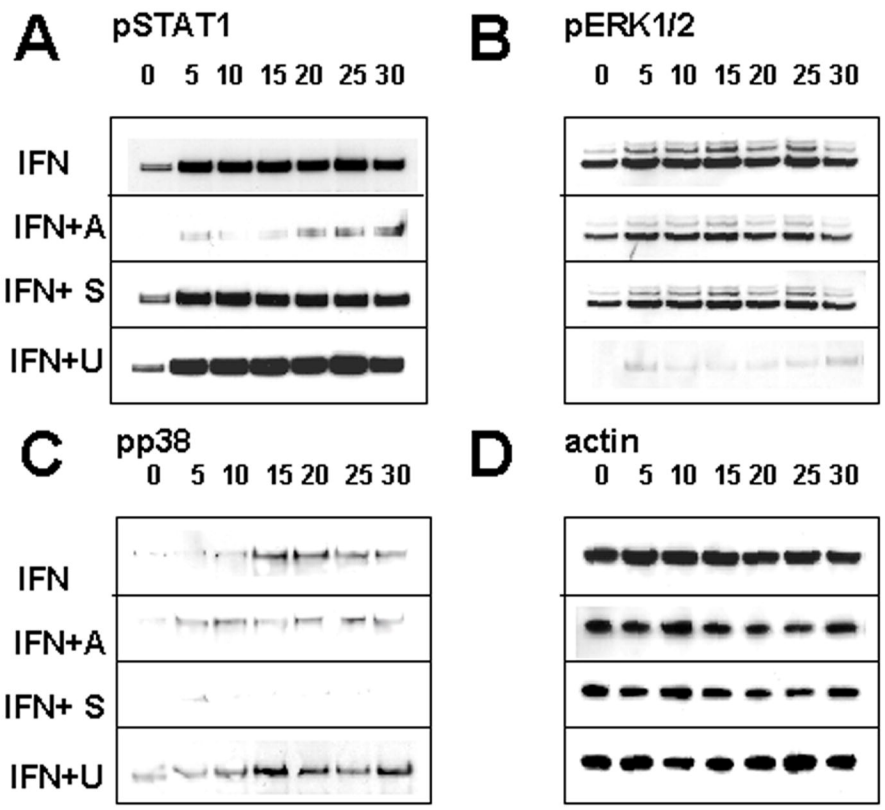

A
pSTAT1
0 5 10 15 20 25 30
IFN
IFN+A
IFN+ S
IFN+U
B
pERK1/2
0 5 10 15 20 25 30
C
pp38
0 5 10 15 20 25 30
IFN
IFN+A
IFN+ S
IFN+U
D
actin
0 5 10 15 20 25 30

Fig. 5

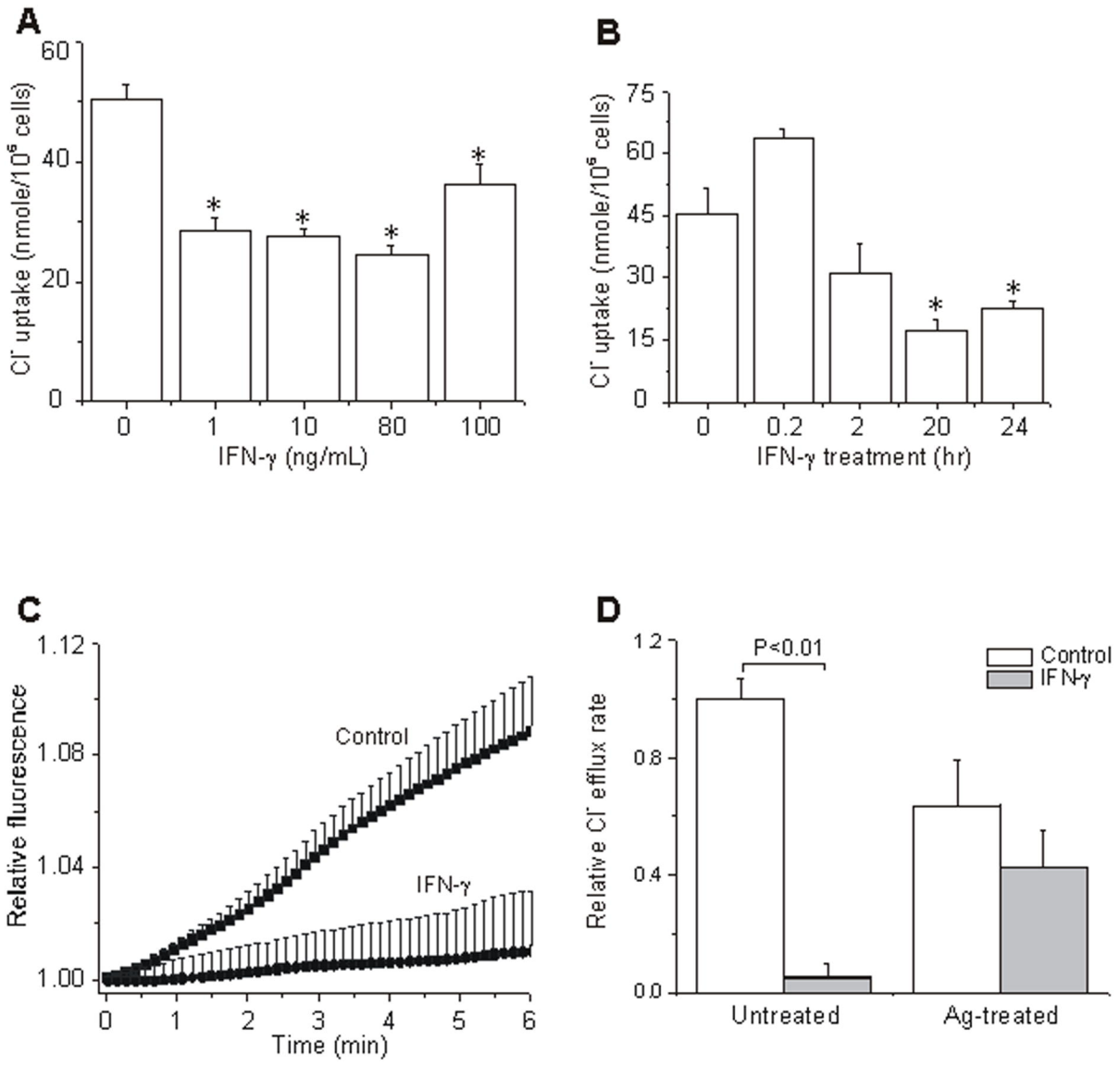

A
Cl⁻ uptake (nmole/10⁶ cells)
60
40
20
0
0
1
10
80
100
IFN-γ (ng/mL)
B
Cl⁻ uptake (nmole/10⁶ cells)
75
60
45
30
15
0
0
0.2
2
20
24
IFN-γ treatment (hr)
C
Relative fluorescence
1.12
1.08
1.04
1.00
Control
IFN-γ
0
1
2
3
4
5
6
Time (min)
D
Relative Cl⁻ efflux rate
1.2
0.8
0.4
0.0
P<0.01
Control
IFN-γ
Untreated
Ag-treated

Fig. 6