



Down-regulation of the A3 adenosine receptor in human mast cells upregulates mediators of angiogenesis and remodeling



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ABSTRACT

Adenosine activated mast cells have been long implicated in allergic asthma and studies in rodent mast cells have assigned the A3 adenosine receptor (A3R) a primary role in mediating adenosine responses. Here we analyzed the functional impact of A3R activation on genes that are implicated in tissue remodeling in severe asthma in the human mast cell line HMC-1 that shares similarities with lung derived human mast cells. Quantitative real time PCR demonstrated upregulation of IL6, IL8, VEGF, amphiregulin and osteopontin. Moreover, further upregulation of these genes was noted upon the addition of dexamethasone. Unexpectedly, activated A3R down regulated its own expression and knockdown of the receptor replicated the pattern of agonist induced gene upregulation. This study therefore identifies the human mast cell A3R as regulator of tissue remodeling gene expression in human mast cells and demonstrates a heretofore-unrecognized mode of feedback regulation that is exerted by this receptor.

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1. Introduction

Adenosine is an endogenous metabolite, generated under inflammatory conditions. Following its release, adenosine affects multiple cells by binding to four distinct receptors (ARs) classified as: A1R, A2aR, A2bR and A3R, all G protein coupled receptors (GPCRs), which differ in their tissue distribution, pharmacological characteristics, G proteins they couple to and signaling (Brown et al., 2008; St Hilaire et al., 2009). Adenosine has been long implicated in a variety of inflammatory processes including allergic asthma (Spicuzza et al., 2006; Brown et al., 2008; Polosa and Blackburn, 2009). Indeed, adenosine provokes bronchoconstriction in atopic and asthmatic individuals but not in normal subjects (Cushley et al., 1983) and elevated levels of adenosine are found in bronchoalveolar lavage fluid and exhaled breath condensates of allergic inflammation patients (Driver et al., 1993; Huszar et al., 2002; Vass et al., 2006).

The involvement of mast cells in mediating adenosine responses was advocated by the observations of prominent secretion of adenosine following mast cell activation (Marquardt et al., 1984);

potentiation of immunoglobulin E (IgE)-induced mediator release from rodent mast cells by adenosine (Holgate et al., 1980; Peachell et al., 1988); and the inhibition of the bronchoconstrictor response to adenosine by blockers of mast cell derived mediators (Meade et al., 2001; Spicuzza et al., 2006). Animal models have substantiated the central role played by activated mast cells in mediating adenosine hyperresponsiveness (AHR) and have identified the A3R as the major contributor. Specifically, adenosine deaminase (ADA)-deficient mice exhibit extensive lung mast cell degranulation concurrent with elevated adenosine (Zhong et al., 2001); airway responses elicited by adenosine or by the pan-adenosine agonist NECA, are significantly attenuated in A3R- or mast cell-deficient mice, but not in A1R-deficient mice (Tilley et al., 2003; Hua et al., 2008); and finally, AHR develops in mast cell-deficient mice reconstituted with wt, but not with A3R-/- mast cells (Hua et al., 2008). Consistent with this notion, studies employing selective agonists indicated that activation of the A3R stimulates murine lung mast cells degranulation (Reeves et al., 1997; Zhong et al., 2003). However, while the studies in rodent mast cells have provided unequivocal evidence for the involvement of the A3R in mediating the adenosine bronchoconstrictor response, the identity of the corresponding adenosine receptor(s) in humans remains elusive. Selective activation of the A3R potentiates IgE-induced degranulation in primary human lung mast cells (Gomez et al., 2011), but has no effect in human umbilical cord blood-derived mast cells (Hua et al., 2011), or in the LAD2 human mast cell line (Leung et al., 2014), suggesting that this modulatory effect is tissue specific (Gomez et al., 2011). Studies using the HMC-1 human mast

Abbreviations: ARs, adenosine receptors; A3R, adenosine-3-receptor; AREG, amphiregulin; Cl-IB-MECA, 2-chloro-N⁶-(3-iodobenzyl)-adenosine-5'-N-methyluronamide; Dex, dexamethasone; IL1 β , Interleukin 1 β ; IL6, Interleukin 6; IL8, Interleukin 8; IL10, Interleukin 10; IgE, immunoglobulin E; SPP1, osteopontin; VEGF, vascular endothelial growth factor.

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cell line revealed that the activated A3R induces the expression, though not secretion, of angiopoietin-2, and synergizes with the A2bR in enhancing secretion of this angiogenic factor (Feoktistov et al., 2003). Therefore, the A3R might modulate rather than mediate adenosine responses in human mast cells, presumably at a transcriptional level. Consistent with this notion, we have recently demonstrated transcriptional changes in A3R activated HMC-1 cells by a global microarray approach (Baram et al., 2010). The aim of this study was to investigate the impact of selective activation of the A3R on genes involved in tissue remodeling in severe asthma.

2. Materials and methods

2.1. Cell culture

HMC-1 cells (a kind gift from Dr. J. Butterfield, Mayo Clinic, Rochester, MN), the Jurkat T cell lymphoma and the human prostate cancer cell line PC3 were cultured in RPMI, supplemented with 10% fetal calf serum (FCS), 2 mM glutamine, 100 µg/mL streptomycin, 100 units/mL penicillin and 12.5 units/mL nystatin at 37 °C in a humidified incubator with 5% CO₂. The A549 non-small cell lung carcinoma cell line was grown in high-glucose DMEM supplemented with 10% FCS and 1% of a penicillin–streptomycin–nystatin mixture at 37 °C in a humidified incubator with 5% CO₂.

2.2. Reagents

The A3R agonist 2-chloro-*N*⁶-(3-iodobenzyl)-adenosine-5'-*N*-methyluronamide (CI-IB-MECA), A2aR antagonist 7-(2-phenylethyl)-5-amino-2-(2-furyl)-pyrazolo-[4,3-*e*]-1,2,4-triazolo[1,5-*c*]pyrimidine (SCH58261, Ki 0.7–2.2 nM), A2bR antagonist 8-[4-(((4-Cyanophenyl) carbamoylmethyl) oxy)phenyl]-1,3-di(*n*-propyl)xanthine hydrate (MRS1754, Ki 2 nM), A3R antagonist, 9-Chloro-2-(2-furanyl)-5-((phenylacetyl)amino)-[1,2,4]triazolo[1,5-*c*]quinazoline (MRS1220, Ki 0.65 nM), adenosine, dexamethasone and antibodies directed against phospho-ERK1/2 were purchased from Sigma–Aldrich. A3R targeting siRNA (ADORA3HSS175292) and corresponding control siRNA (negative control medium GC duplex) were purchased from Invitrogen. Antibodies directed against ERK2 were purchased from Santa-Cruz Biotechnology and HRP-conjugated goat anti-mouse and anti-rabbit IgG were purchased from Jackson ImmunoResearch Laboratories.

2.3. RNAi of A3R

HMC-1 cells (5×10^5 cells/sample) were incubated for 48 h with Stealth siRNA Duplex Oligoribonucleotides at a final concentration of 120 nM, prepared according to the manufacturer's instructions in Opti-MEM I Reduced Serum Medium, and in the presence of Lipofectamine 2000. The sequences of siRNA used were: ADORA3 HSS175292(3.RNAI) CACCUUCUAUUUCAUUGUCUCUCUA and UAGAGAGACAAUGAAUAGAAGGUG and medium GC Duplex as a negative control.

2.4. RNA purification and quantitative real time PCR

Total cellular RNA was extracted using the PerfectPure™ RNA Purification System (5' prime) according to the manufacturer's protocol. cDNA was generated using 2 µg of total RNA and the high capacity reverse transcriptase (Applied Biosystems) in a total volume of 20 µl. Quantitative real time PCR was performed in an ABI Prism 7900 Sequence Detection System (Applied Biosystems) using the SYBR Green Real-Time PCR Kit (Applied Biosystems) and 20–60 ng of DNA in a total volume of 10 µl, according to the manufacturer's instructions. Relative mRNA levels of the

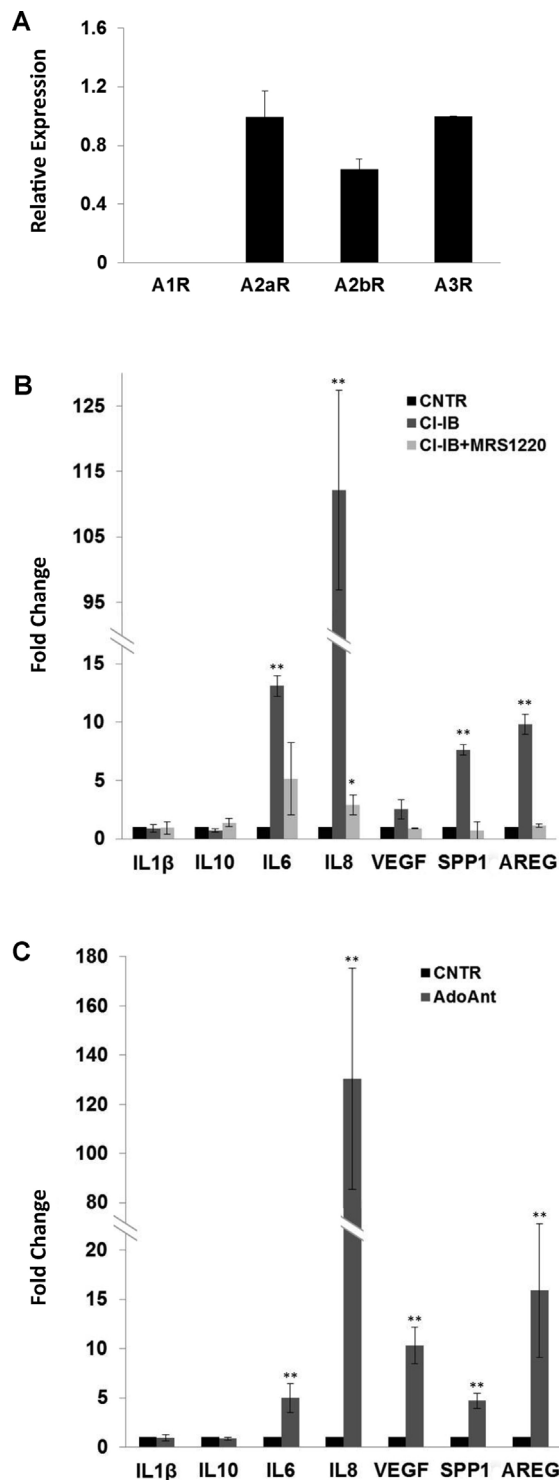


Fig. 1. Effect of CI-IB-MECA induced activation of the A3R on gene expression. (A) The expression levels of the A1R, A2aR, A2bR and A3R in HMC-1 cells (1×10^6 cells/mL) were determined by quantitative real time PCR as described in Section 2. (B) HMC-1 cells (1×10^6 cells/mL) were either left untreated (CNTR), or preincubated for 30 min with 100 nM of MRS1220 as indicated. Cells were then stimulated for 3 h with 100 nM of CI-IB-MECA (CI-IB), as indicated. (C) HMC-1 cells (1×10^6 cells/mL) were either left untreated (CNTR), or preincubated for 30 min with 100 nM of MRS1754 and 100 nM of SCH58261 as indicated. Cells were then stimulated for 3 h with 100 µM of adenosine (AdoAnt), as indicated. The relative expression levels of IL1β, IL10, IL6, IL8, VEGF, SPP1 and AREG were determined by quantitative real time PCR as described in Section 2. The fold changes in expression in treated cells relative to control untreated cells are presented. Data are means $\pm$ S.E.M. from 4 independent experiments. * $P < 0.05$, ** $P < 0.01$.

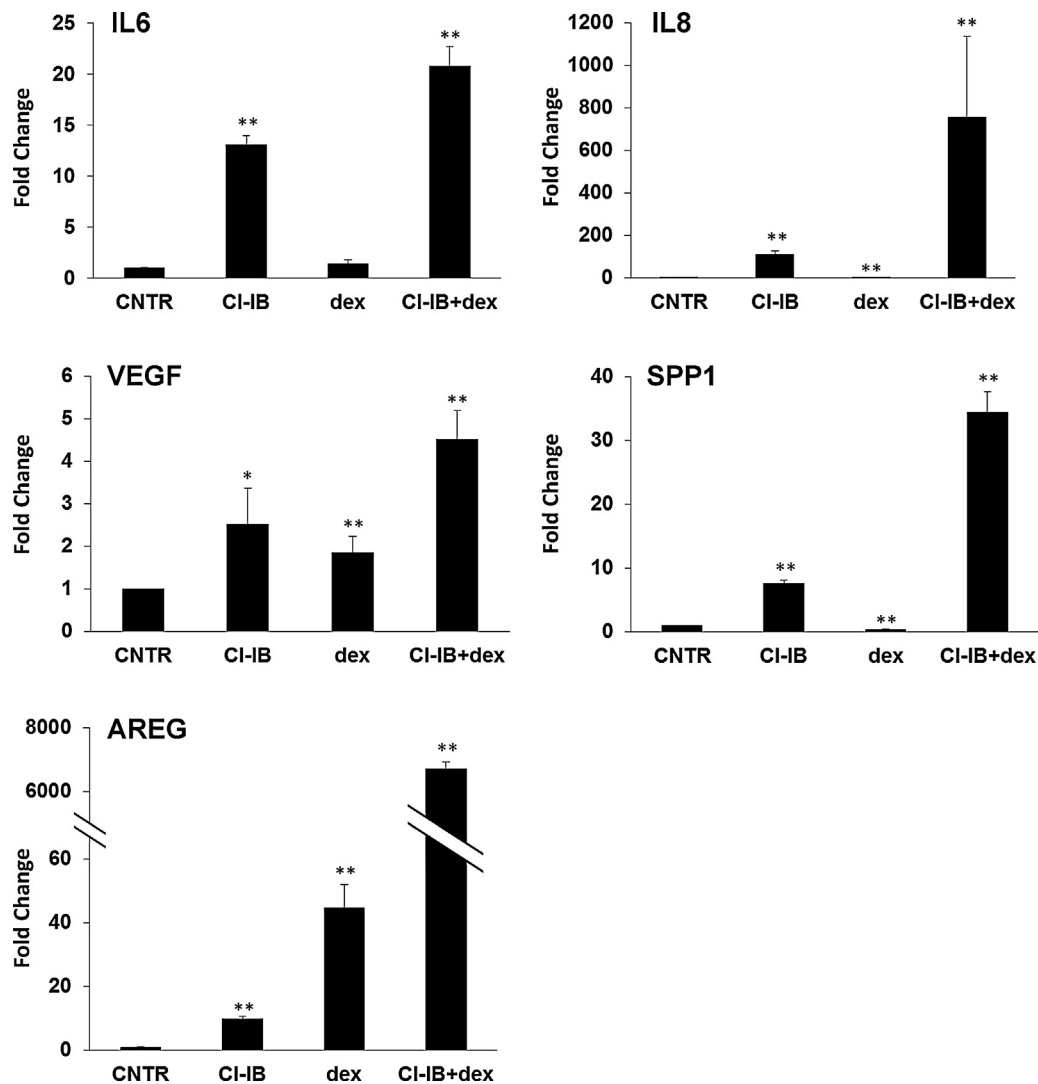


Fig. 2. Effect of dexamethasone on A3R mediated upregulation of tissue remodeling genes. HMC-1 cells (1×10^6 cells/mL) were either left untreated (CNTR), or preincubated for 18 h with $1 \mu\text{M}$ dexamethasone (dex), as indicated. Where indicated, cells were incubated for the last 3 h of incubation with 100 nM of CI-IB-MECA (CI-IB). The relative expression levels of IL6, IL8, VEGF, SPP1 and AREG were determined by quantitative real time PCR as described in Section 2. The fold changes in expression in treated cells relative to control untreated cells are presented. Data are means $\pm$ S.E.M. from 4 independent experiments. * $P < 0.05$, ** $P < 0.01$.

tested genes were calculated by the two standard curves method using HPRT RNA as a reference. The following primers were used: A1R (isoform I): sense: 5'-GTCCGTCCTCATCCTCAC-3'; antisense: 5'-CCACCATCTGTACCGGAGA-3'; A1R (isoform II): sense: 5'-GGGGCACTTGGACAGAAC-3'; antisense: 5'-ACAGGCTGGGCACATCAG-3; A2aR sense: 5'-TGGGAGCCAGAGCAAGAG-3'; antisense: 5'-ACTCTGCATCTCCAAAATCA-3'; A2bR sense: 5'-GAGCTCCATCTTAGCTCTTG-3'; antisense: 5'-TCGTGTCCAGTGACCAAAC-3'; A3R (isoform II): sense: 5'-TTATCTTTACCCACGCTCCAT-3'; antisense: 5'-CTCTGTATCTGACGGTAAGCTTGC-3'; IL8: 5'-AGCTGGCCGTGGCTCTCT-3'; antisense: 5'-CCTTGGCAA-AACTGCACCTT-3'; IL6: sense: 5'-CAGAAAACAACCTGAACCTTCCA-3'; antisense: 5'-AAGTCTCTCATTGAATCCAGATTG-3'; IL18: sense: 5'-TACCTGTCTGCTGTGTA-3'; antisense: 5'-TCTTTGGGTAA-TTTTGGGATCT-3'; IL10: sense: 5'-TGCCTAACATGCTTCGAGATCTC-3'; antisense: 5'-CAGCTGATCCTTCAATTGAAAGAA-3'; VEGF: sense: 5'-TGCCCGTGTCTGTAAT-3'; antisense: 5'-TCTCCGCTCTGAGCAAGG-3'; SPP1: sense: 5'-CATGAGAATTGCAGTGATTGCTT-3'; antisense: 5'-CCAGAATCAGCCTGTTAACTGG-3'; AREG: sense: 5'-GATACTCGGCTCAGGCCATTAT-3'; antisense: 5'-CAAATCCATCAGCACTGTGGTC-3'; HPRT: sense: 5'-GACTTTGCTTTCCTTGGTCAGG-3'; antisense: 5'-TCCTTTTACCAGCAAGCTTG-3'.

The data are presented as fold change in mRNA levels in treated vs. control cells.

2.5. Analyses of cellular and released IL8 and amphiregulin (AREG)

For determination of cellular and released levels of IL8 and AREG, cell lysates and cell supernatants of 2×10^6 cells for each sample were assayed by a two-site enzyme linked immunosorbant assay (ELISA) for IL8 and AREG (R&D Systems).

2.6. Determination of protein phosphorylation

ERK1/2 phosphorylation was determined as previously described (Baram et al., 2010). Briefly, HMC-1 cells were serum-starved for 18–20 h, washed and re-suspended to 1×10^6 cells/mL with the same buffer. Following cell stimulation, reactions were terminated by placing the tubes on ice, followed by a brief spin ($14,000 \times g$, 20 s) at 4°C . Cell pellets were lysed by the addition of a lysis buffer [Buffer A comprising: 50 mM Hepes pH 7.5, 150 mM NaCl, 1% Triton X-100, 0.1% SDS, 50 mM NaF, 10 mM sodium pyrophosphate, 2 mM sodium orthovanadate, 10 mM EDTA,

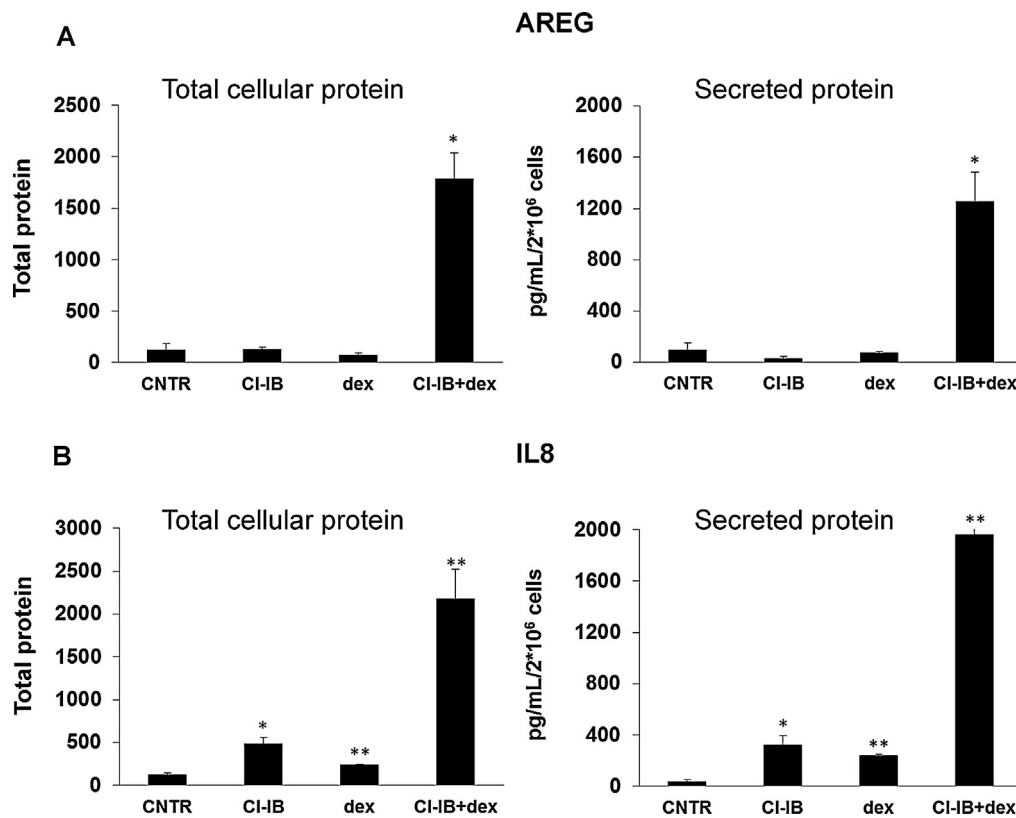


Fig. 3. Effect of dexamethasone on cellular and secreted levels of AREG and IL8 in A3R activated cells. (A) HMC-1 cells (2×10^6 cells/mL) were either left untreated (CNTR) or incubated for 24 h with either 1 μ M dexamethasone (dex), or 100 nM of CI-IB-MECA, or both, as indicated. The cellular and secreted amounts of AREG were then determined by ELISA assay as described in Section 2. (B) HMC-1 cells (2×10^6 cells/mL) were either left untreated (CNTR) or incubated for 18 h with 1 μ M dexamethasone (dex), as indicated. Where indicated, cells were incubated for the last 3 h of incubation with 100 nM of CI-IB-MECA (CI-IB). The cellular and secreted amounts of IL8 were determined by ELISA assay as described in Section 2. Data are means $\pm$ S.E.M. from 3 independent experiments. * $P < 0.05$, ** $P < 0.01$ compared with the control untreated cells.

2 mM EGTA, 1 mM PMSF, protease inhibitor cocktail (Boehringer Mannheim), phosphatase inhibitor cocktail-1 (Sigma–Aldrich)] and centrifuged for 15 min at $12,000 \times g$. Supernatants were mixed with $\times 5$ concentrated Laemmli sample buffer, boiled and subjected to SDS-PAGE and Western blotting.

2.7. Statistical analysis

Data are expressed as means $\pm$ S.E.M. P -values were determined by two-sample equal variance two-tailed Student t -test.

3. Results

3.1. Agonist binding to the A3R upregulates genes involved in tissue remodeling

To begin characterizing the transcriptional profile of A3R activated human mast cells we chose to use as our model the HMC-1 cells, which respond to selective activation of this receptor and of which large numbers can be obtained. First, we analyzed the expression profile of the ARs and confirmed that in consistence with previous reports (Feoktistov and Biaggioni, 1995; Meade et al., 2002), HMC-1 cells express endogenously the A3R, alongside the A2a and A2b receptors, but not the A1 adenosine receptor (Fig. 1A). These results have therefore indicated that HMC-1 cells share with human lung mast cells a similar profile of AR expression (Gomez et al., 2011). We then analyzed the expression patterns of genes whose functions are linked with vascular remodeling in asthma. Specifically, we tested cytokines that are involved in angiogenesis, including IL6, which enhances transcription and production

of VEGF (Cohen et al., 1996; Wei et al., 2003), IL8, which stimulates endothelial cell proliferation and enhances angiogenesis in asthma (Keglowich et al., 2013), and VEGF, the vascular endothelial growth factor. We also analyzed fibrogenic genes including osteopontin (SPP1) and amphiregulin (AREG) whose upregulation was noted during airway remodeling in severe asthma (Wang et al., 2005; Frenzel and Weiss, 2011) and correlated with asthma severity (Novali, 2001; Enomoto et al., 2009; Kohan et al., 2007, 2009). IL1 β and IL10 were tested as respective representatives of pro and anti-inflammatory cytokines. CI-IB-MECA, a selective agonist of the A3R, increased significantly the mRNA levels of all the tested genes, except for IL1 β and IL10 (Fig. 1B). Therefore, the gene signature of the A3R-activated mast cells was consistent with an activity of inflammatory tissue remodeling. In particular striking was the impact of A3R activation on IL8, whose expression level increased by more than 100 fold (Fig. 1B).

Recently, the A3R agonists IB-MECA and CI-IB-MECA were shown to exert cytotoxic effects independently of the A3R (Mlejnek et al., 2013). Therefore, we next investigated the contribution of the A3R to the upregulation of the tissue remodeling genes by assessing the sensitivity of this process to treatment with an A3R antagonist. Indeed, MRS1220, a selective antagonist of the A3R, markedly inhibited gene upregulation induced by CI-IB-MECA (Fig. 1B), therefore supporting the involvement of the A3R in these responses.

To substantiate our results even further, we also quantified the relative expression of the tested tissue remodeling genes in cells that were activated by adenosine in the combined presence of the A2aR and A2bR antagonists, SCH58261 and MRS1754, which leave only the A3R active. The results of these experiments revealed a gene pattern that was similar to the one displayed by

CI-IB-MECA treated cells. Hence, whereas IL1 β and IL10 remained unaltered, the expression of IL6, IL8, VEGF, SPP1 and AREG has significantly increased, whereby IL8 expression increased to the highest extent (Fig. 1C). Therefore, collectively, these results confirmed the involvement of the A3R in the upregulation of angiogenic and fibrogenic genes that are the hallmark of tissue remodeling during severe asthma.

3.2. Dexamethasone synergizes with the A3R in upregulating tissue remodeling genes

Severe asthma is often associated with resistance to corticosteroids, a commonly prescribed therapy for asthma (Sher et al., 1994). Therefore, we next analyzed the impact of dexamethasone on A3R provoked responses. When applied alone, dexamethasone did not affect the expression level of IL6, dexamethasone reduced the basal expression levels of IL8 and SPP1 and increased significantly the transcripts of both VEGF and AREG (Fig. 2). However, when combined with CI-IB-MECA, dexamethasone demonstrated a striking synergy with the activated A3R in upregulating most of the tested A3R target genes (Fig. 2). In particular striking was the impact of this combination on IL8 and AREG, whose expression increased by 700 and 6000 fold, respectively (Fig. 2).

3.3. Dexamethasone increases both expression and secretion of IL8 and AREG

Consistent with earlier results of others (Feoktistov et al., 2003) and us (Baram et al., 2010) that failed to detect mediator secretion in response to CI-IB-MECA, we did not detect AREG and monitored only low levels of IL8 in the media of CI-IB-MECA-activated HMC-1 cells, despite the significant increase in their mRNA levels (Fig. 3). Analysis of their total cellular protein levels revealed no increase in AREG and a 4 fold increase in IL8 in the CI-IB-MECA treated cells (Fig. 3). However, in sharp contrast, the combined presence of CI-IB-MECA and dexamethasone resulted in a dramatic increase in both protein expression and secretion of AREG and IL8 (Fig. 3). Notably, the presence of CI-IB-MECA was essential, since dexamethasone alone stimulated only slightly release of IL8 and failed to induce secretion of AREG, despite the remarkable increase in AREG transcription by dexamethasone (Fig. 3).

3.4. Dexamethasone upregulates, whereas CI-IB-MECA down regulates expression of the A3R in HMC-1 cells

Dexamethasone was previously shown to upregulate the expression of the A3R in the rat mast cell line, RBL-2H3 (Ramkumar et al., 1993). This observation prompted us to analyze the influence of dexamethasone on the expression level of the A3R in HMC-1 cells. We rationalized that receptor upregulation might underlie the synergy of dexamethasone and CI-IB-MECA. Indeed, dexamethasone increased by 9 fold the amount of A3R mRNA (Fig. 4A). However, unexpectedly, we noticed that CI-IB-MECA significantly reduced the A3R transcript (Fig. 4A). A similar reduction in A3R mRNA was noted in cells treated with adenosine in the combined presence of the specific A2a and A2b adenosine receptor antagonists, establishing further the involvement of the A3R in the down-regulation of its own expression (Fig. 4B). Unfortunately, our attempts to quantify the protein level of the receptor in control and CI-IB-MECA treated cells by immunoblotting, using commercially available antibodies, have failed. We therefore aimed at obtaining functional evidence for the cell surface expression and ligand induced down-regulation of the A3R. To this end, we analyzed the capacity of CI-IB-MECA to stimulate phosphorylation of the ERK1/2 MAP kinases that reside downstream of the A3R (reviewed in Rudich et al., 2012). Indeed, consistent with our previous observations (Baram et al., 2010), CI-IB-MECA stimulated ERK1/2 phosphorylation when added for

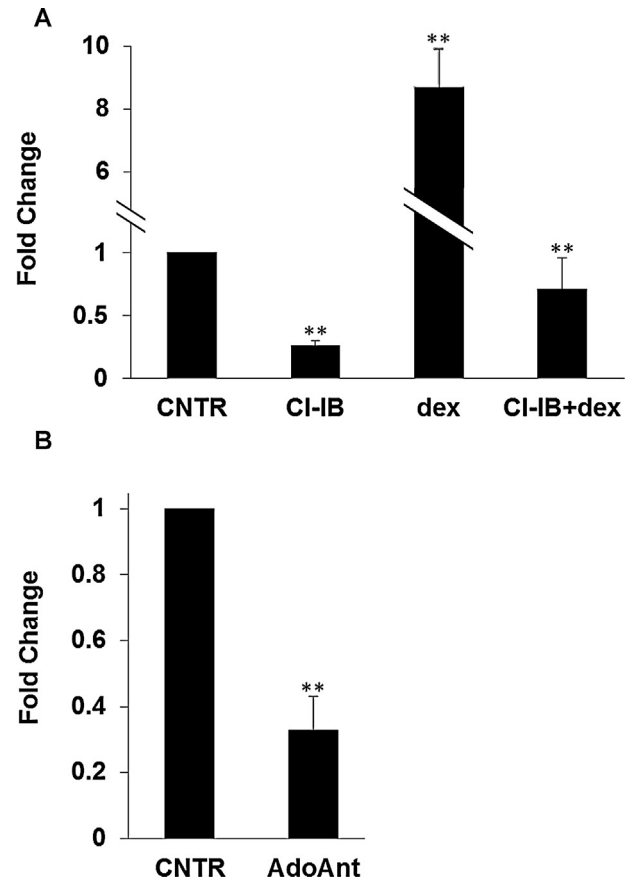


Fig. 4. Effect of CI-IB-MECA and dexamethasone on A3R expression. (A) HMC-1 cells (1×10^6 cells/mL) were either left untreated (CNTR) or preincubated for 18 h with 1 μ M dexamethasone (dex), as indicated. Cells were then incubated for the last 3 h of incubation with 100 nM of CI-IB-MECA (CI-IB), as indicated. The relative expression of the A3R was determined by quantitative real time PCR as described in Section 2. (B) HMC-1 cells (1×10^6 cells/mL) were either left untreated (CNTR) or preincubated for 30 min with 100 nM of MRS1754 and 100 nM of CSH58261 as indicated. Cells were then stimulated for 3 h with 100 μ M of adenosine (AdoAnt), as indicated. The relative expression of the A3R was determined by quantitative real time PCR as described in Section 2. The fold changes in A3R expression in treated cells relative to control untreated cells are presented. Data are means $\pm$ S.E.M. from 4 independent experiments. ** $P < 0.01$ compared with the control untreated cells.

1 min (Fig. 5). However, this response was completely abolished in cells that were subjected to a 3-h prior treatment with CI-IB-MECA (Fig. 5). Therefore, these results confirmed that HMC-1 cells express a functional cell surface A3R that signals in response to agonist binding and is down regulated following long-term exposure to its ligand. It warrants mention that down-regulation of the A3R following its ligand binding was not restricted to human mast cells. Analysis of other human cell lines that were shown to express the A3R, including the jurkat T cell lymphoma, the A549 lung epithelial cell line and the PC3 prostate cancer cell line (Gessi et al., 2001; Kamiya et al., 2012; Aghaei et al., 2011), revealed a similar pattern of CI-IB-MECA induced reduction of the A3R mRNA level (Supplemental Fig. S1). These results therefore imply the generality of this feedback mechanism in regulating the expression of the A3R in human cells.

3.5. Knockdown of the A3R upregulates tissue remodeling genes

The inverse relationship noted in CI-IB-MECA treated HMC-1 cells between the down-regulation of the A3R transcript and the upregulation of its target tissue remodeling genes has prompted us to analyze the functional impact of A3R knockdown on the

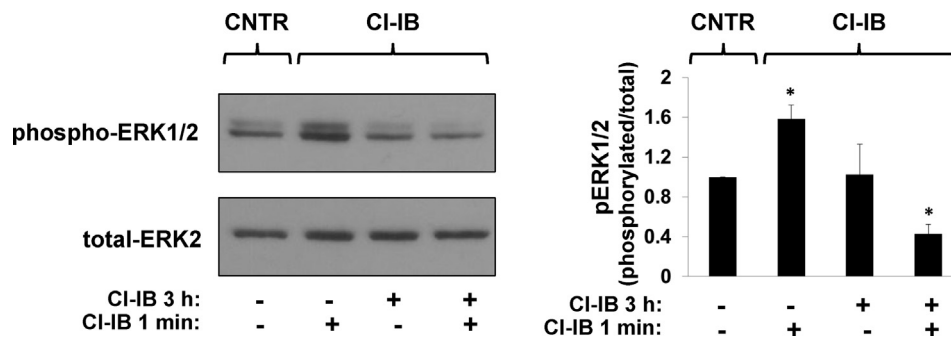


Fig. 5. CI-IB-MECA induced down-regulation of the A3R. HMC-1 cells (1×10^6 cells/mL) were either left untreated (CNTR) or incubated for 3 h with 100 nM of CI-IB-MECA (CI-IB) as indicated. Cells were then washed and incubated for 1 min with 100 nM of CI-IB-MECA (CI-IB), as indicated. Cell lysates were resolved by SDS-PAGE and immunoblotted with anti phospho-ERK1/2, followed by reprobing with anti ERK2 (Total ERK). A representative blot is shown. The intensities of the bands were quantified and relative (phosphorylated/total) pixel densities were calculated and are presented relative to the value of untreated cells. Data are means $\pm$ S.E.M. from 2 independent experiments. * $P < 0.05$.

tissue remodeling gene expression. A3R targeting siRNA reduced the expression level of the A3R by 60%, without affecting the expression levels of the A2a or A2b receptors (Fig. 6A). Strikingly, the same siRNA significantly increased the expression of all the tested tissue remodeling genes, including IL6, IL8, VEGF, SPP1 and AREG (Fig. 6B). This effect was specific as indicated by the fact that control siRNA had no effect (Fig. 6B). Combining the A3R targeting siRNA with CI-IB-MECA resulted in a further increase in these genes expression (Fig. 6B). Therefore, reducing the expression of the A3R was clearly linked with the upregulation of its target tissue remodeling genes.

4. Discussion

The A3R is implicated in numerous diseases including inflammatory conditions, autoimmune and neurodegenerative diseases and cancer (Gessi et al., 2008). Yet, the literature evidence is split between reports revealing pro-inflammatory functions of this receptor, while others demonstrate anti-inflammatory and protective outcomes (Gessi et al., 2008). In human cancer as well as in chronic inflammatory diseases such as rheumatoid arthritis and chronic obstructive pulmonary diseases (COPD) the expression of the A3R is upregulated (Walker et al., 1997; Varani et al., 2006, 2011; Montinaro et al., 2013). However, whether this upregulation is associated with the induction of the disease, or whether it reflects a protective mechanism aimed at counteracting inflammatory signals remains unclear. In view of the implicated role of this receptor in mediating adenosine responses in mast cells in rodent animal models for asthma (Young et al., 2004), we set up to characterize the activation profile and mode of regulation of the A3R in human mast cells. Because of the difficulty in obtaining sufficient human lung derived mast cells for genetic profiling, we have chosen to employ the HMC-1 human mast cell line as model. Our choice of these cells rather than the LAD2 human mast cell line or human cord blood derived mast cells was based on the following considerations: HMC-1 cells express endogenously the A2a, A2b and A3 adenosine receptors, which are also expressed by mast cells from most species (Hua et al., 2011); others (Feoktistov et al., 2003) and us (Baram et al., 2010) have previously shown that HMC-1 cells express a functional A3R; in a similar fashion to human lung mast cells, HMC-1 cells produce VEGF in response to NECA, unlike the LAD2 cells (Detoraki et al., 2009); HMC-1 and human lung mast cells interact with human lung fibroblasts (Moiseeva et al., 2013) and migrate towards Th2 stimulated airway smooth muscle that is derived from asthmatic, but not non-asthmatic, subjects (Sutcliffe et al., 2006); both cell types produce tissue-plasminogen-activator in response to adenosine (Sereda et al., 2011) and display similar anti-microbial activity (Cruse et al., 2010). Taken together with the findings that neither the LAD2 cells nor human cord blood derived

mast cells recapitulate the potentiation of Fc ϵ R1 induced degranulation by adenosine or A3R agonists that occurs in human lung mast cells (Suzuki et al., 1998; Hua et al., 2011; Gomez et al., 2011; Leung et al., 2014), we rationalized that despite their limitations arising from the expression of a constitutively active kit receptor and lack of expression of the Fc ϵ R1 (Nilsson et al., 1994), HMC-1 cells are instrumental as model for deciphering the functional signature of the A3R in human lung mast cells.

Three important results emerged from our studies. First, significant upregulation of genes that affect severe asthma was demonstrated in A3R-activated HMC-1 cells. The latter included the cytokines IL6, IL8 and VEGF, which stimulate angiogenesis and AREG and SPP1 that are linked with pulmonary fibrosis (Pardo et al., 2005; Zhou et al., 2012). In contrast, no changes were detected in the expression levels of IL1 β or IL10. Therefore, taken together with previous reports that demonstrated the upregulation of angiopoietin-2, a cytokine that primes endothelial cells to the effects of VEGF, in A3R activated HMC-1 cells (Feoktistov et al., 2003), our results strongly assign the human mast cell A3R a role in regulating inflammatory tissue remodeling. Second, in agreement with previous results by others (Feoktistov et al., 2003) and us (Baram et al., 2010), which documented significant changes in gene transcription in A3R activated cells that are not necessarily accompanied by cytokine secretion, we show only modest secretion of IL8 and no secretion of AREG despite the significant increase in their transcripts in response to A3R activation. These results strongly suggest that the primary function of the A3R is to prime the human mast cells towards a tissue remodeling activity by altering their gene signature as to increase their responsiveness to additional signals that would also promote cytokine secretion. In this respect, the A3R in human mast cells behaves differently from its rodent counterpart that activates mast cell secretion alongside its potentiating impact on IgE-mediated responses. Therefore, unlike the rodent A3R, the human receptor should be regarded as modulator rather than trigger of mast cell responses. These different patterns of activity are not unexpected. The rodent and human A3R share only 74% homology (Fredholm et al., 2001) and display distinct antagonist specificities and different modes of desensitization [reviewed in Klaasse et al. (2008)]. These receptors may therefore couple to different G proteins or display distinct crosstalk with their co-expressed adenosine receptors or other co-expressed GPCRs (Palmer et al., 1995), which would eventually lead to the transmission of distinct signaling outputs and culminate in diverse functions. Surprisingly, and counter intuitively, a remarkable synergistic signal that increased CI-IB-MECA induced gene upregulation and facilitated cytokine secretion was provided by dexamethasone, a commonly prescribed asthma medication. It is therefore tempting to speculate that this synergy between

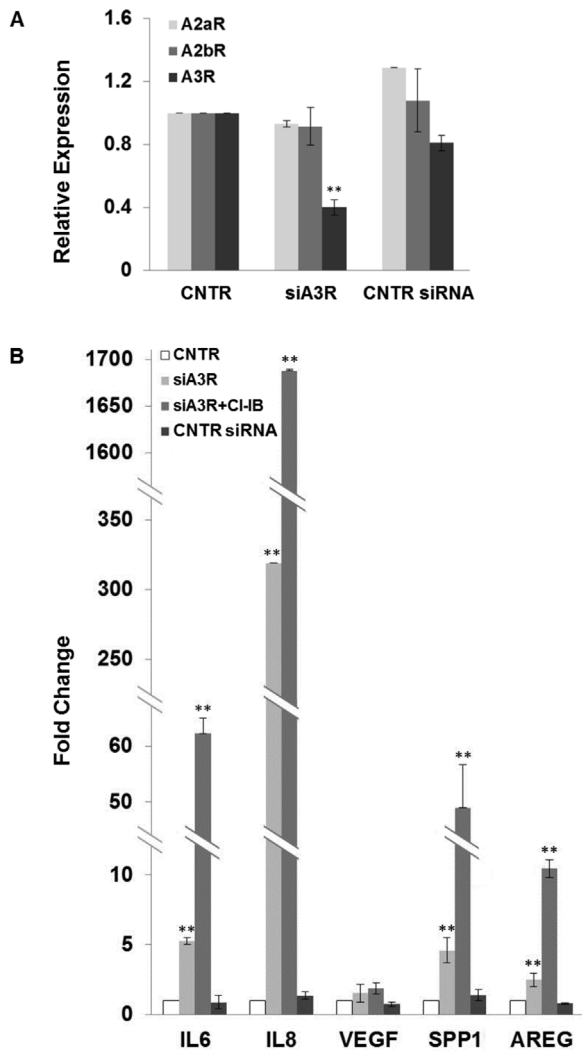


Fig. 6. Effect of A3R knockdown on the relative expression of tissue remodeling genes. (A) HMC-1 cells (5×10^5 cells/sample) were either left untreated (CNTR) or transfected with A3R targeting siRNA (siA3R), or with control siRNA (CNTR siRNA) as described in Section 2. The relative expression levels of the A2aR, A2bR and A3R were determined by quantitative real time PCR as described in Section 2. The fold changes in expression in the transfected cells relative to control non-transfected cells are presented. (B) HMC-1 cells (5×10^5 cells/sample) were either left untreated (CNTR) or transfected with A3R targeting siRNA (siA3R), or with control siRNA (CNTR siRNA) as described in Section 2. Cells were then left untreated or incubated for 3 h with 100 nM of CI-IB-MECA (CI-IB) as indicated. The relative expression levels of IL6, IL8, VEGF, SPP1 and AREG were then determined by quantitative real time PCR as described in Section 2. The fold changes in expression in transfected cells relative to control non-transfected cells are presented. Data are means $\pm$ S.E.M. from 3 independent experiments. ** $P < 0.01$ compared with the control untreated cells.

dexamethasone and A3R activation might underlie the resistance to corticosteroids that is experienced in severe refractory asthma. Consistent with this notion is the tight association between severe refractory asthma and intense inflammatory fibrosis that is linked with increased levels of angiopoietin, VEGF, IL8 and SPP1 in the sputum supernatant (Simpson et al., 2008; Delimpoura et al., 2010; Tselioui et al., 2012; Hantera et al., 2014).

Third, we demonstrate for the first time a feedback mechanism, whereby the activated A3R suppresses its own expression both at a protein and a transcriptional level. Hence an inverse relationship exists between the expression level of the A3R and the expression of its tissue remodeling target genes. Furthermore, siRNA targeting of the receptor replicates the consequences of ligand binding by increasing the expression of the A3R target genes. Therefore,

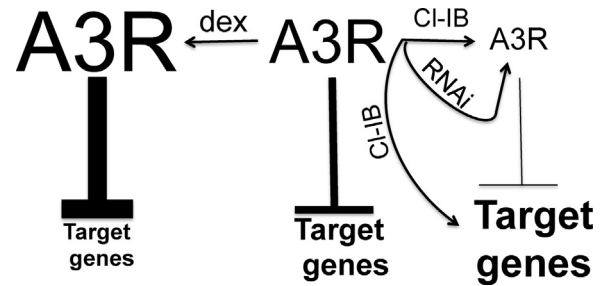


Fig. 7. A model for A3R mediated regulation of tissue remodeling gene expression. According to this model, by a mechanism that still needs to be resolved, the A3R represses the expression of genes involved in tissue remodeling (i.e. IL6, IL8, VEGF, SPP1 and AREG) in its basal state. Dexamethasone (dex) increases the expression of the A3R and consequently reduces further the basal expression level of the A3R target genes, except for VEGF and AREG that are upregulated by dexamethasone. Signaling elicited by the CI-IB-MECA (CI-IB)-bound A3R leads to receptor down-regulation thereby relieving the repression constraint allowing A3R target gene upregulation. A similar effect is achieved by siRNA-mediated receptor knockdown. Parallel signaling elicited by the activated receptor may also stimulate gene expression independently of the receptor expression level. Therefore, the combined outcome of ligand binding to the A3R is a marked increase in the expression of its target tissue remodeling genes. Consistent with this proposed model is the finding that the combination of CI-IB-MECA with either dexamethasone or A3R targeting siRNA is more effective than CI-IB-MECA alone in increasing the relative expression of the tissue remodeling genes.

our results are compatible with a model whereby the A3R functions under basal conditions, as a repressor of human mast cell gene transcription. According to this model, signals that are elicited by the ligand bound, activated receptor lead to receptor down-regulation, which in turn relieves the suppressing constraint thus giving rise to gene upregulation (Fig. 7). Coherent with this model is the observation that CI-IB-MECA stimulated signaling of the A3R, evidenced by the enhanced phosphorylation of the ERK1/2 MAP kinases, is completely abolished in cells that were preconditioned with CI-IB-MECA. Also compatible with this model is the influence of dexamethasone that by increasing the expression of the A3R may further repress the A3R target gene expression, which would be reflected in a larger increase in their relative expression, upon removal of this constraint by ligand binding (Fig. 7). Notably, our results do not exclude the possibility of a parallel signaling network that is elicited by the activated A3R and that positively regulates gene expression. In fact, such a dual-arm and time dependent mode of function may account for the paradox linked with A3R function that often displays opposite effects (Gessi et al., 2008).

In conclusion, we demonstrate a tissue remodeling gene signature that is acquired in A3R activated human mast cells and that is coupled to down-regulation of the receptor expression, both at protein and mRNA levels. Future research will examine how the active receptor controls its own expression and how does the non-occupied receptor repress the expression of its tissue remodeling target genes.

Conflict of interest

The authors declare no conflict of interest.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.molimm.2014.12.015>.

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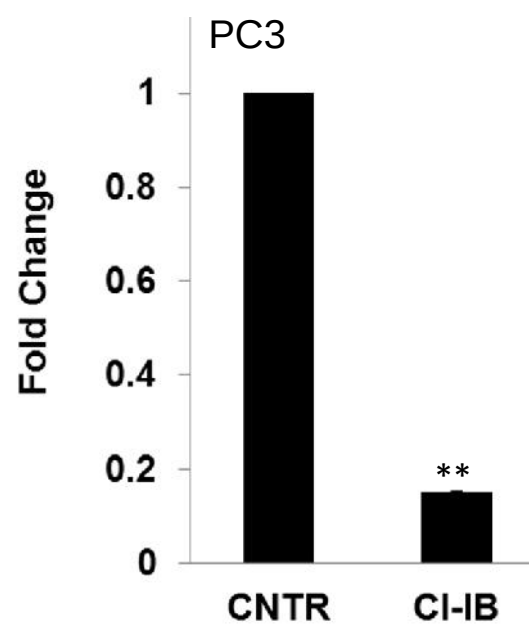
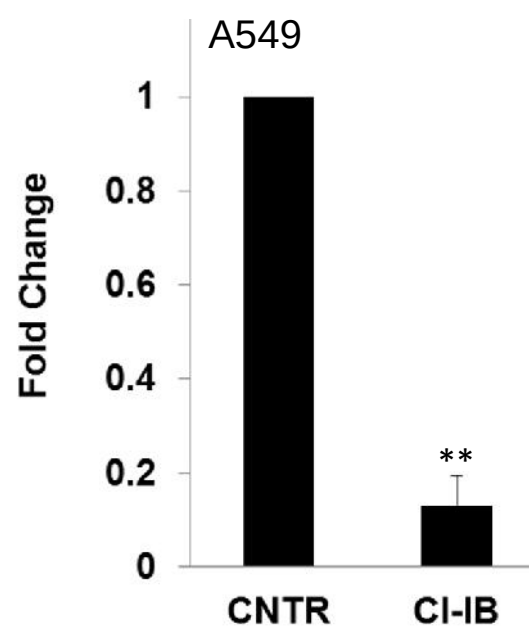
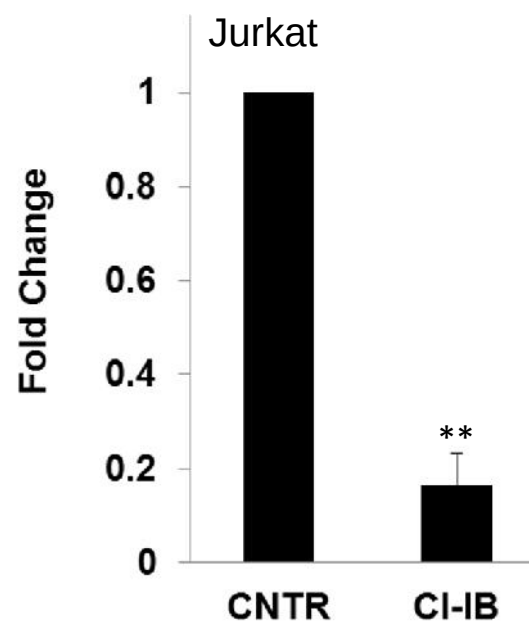


Fig. S1, Rudich et al.