# Inhibition of ABCC1 Decreases cAMP Egress and Promotes Human Airway Smooth Muscle Cell Relaxation

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**Running title:** β-agonists evoke cAMP efflux through ABCC1

AJRCMB Articles in Press. Published October 14, 2021 as 10.1165/rcmb.2021-0345OC

**Text:** 4,641 Words

**Author Contributions:** G.C., S.S.A., and R.A.P. designed research; G.C, H.L., J.A.J., and N.K. performed research; J.A.J., M.K., W.J., C.K.-W., B.E.H., G.C., S.S.A., and R.A.P. contributed new reagents and analytic tools; G.C., H.L., J.A.J., N.K., C.K.-W., S.S.A, and R.A.P. analyzed data; G.C., S.S.A., and R.A.P. wrote the paper; S.S.A. and R.A.P. directed all studies.

**Competing Interest Statement:** The authors declare no competing interest.

**Funding:** This work was supported by the New Jersey Alliance for Clinical and Translational Science (UL1TR0030117) and the National Institutes of Health grants (P01HL114471 and R56HL155937).

This article has an online data supplement, which is accessible from this issue's table of content online at www.atsjournals.org.

AJRCMB Articles in Press. Published October 14, 2021 as 10.1165/rcmb.2021-0345OC

## ABSTRACT

In most living cells, the second messenger roles for 3',5'-cyclic adenosine monophosphate (cAMP) are short-lived, confined to the intracellular space, and tightly controlled by the binary switch-like actions of the stimulatory G protein ($G\alpha_s$)-activated adenylyl cyclase (cAMP production) and cAMP-specific phosphodiesterase (cAMP breakdown). Using human airway smooth muscle (HASM) cells in culture as a model, here we report that activation of the cell surface $\beta_2$-adrenoceptor ($\beta_2$AR), a $G_s$-coupled G protein-coupled receptor (GPCR), evokes cAMP egress to the extracellular space. Increased extracellular cAMP levels ($[cAMP]_e$) are long-lived in culture and induced by receptor-dependent and receptor-independent mechanisms in such a way as to define a universal response class of increased intracellular cAMP levels ($[cAMP]_i$). We find that HASM cells express multiple ATP-binding cassette (ABC) membrane transporters, with *ABCC1* being the most highly enriched transcript mapped to multidrug resistance associated proteins (MRPs). We show that pharmacological inhibition or downregulation of ABCC1 with small interfering RNA markedly reduces $\beta_2$AR-evoked cAMP release from HASM cells. Further, inhibition of ABCC1 activity or expression decreases basal tone and increases β-agonist-induced HASM cellular relaxation. These findings identify a previously unrecognized role for ABCC1 in the homeostatic regulation of $[cAMP]_i$ in HASM that may be conserved traits of the $G_s$-coupled family of GPCRs. Hence, the general features of this activation mechanism may uncover new disease-modifying targets in the treatment of airflow obstruction in asthma. Surprisingly, we find that serum cAMP levels are elevated in a small cohort of patients with asthma as compared with controls that warrants further investigation.


**Word Count:** 250 / 250

**Keywords:** $\beta_2$-adrenoceptor, multidrug resistance associated proteins, cAMP, airway smooth muscle, asthma

AJRCMB Articles in Press. Published October 14, 2021 as 10.1165/rcmb.2021-0345OC

**Abbreviations**

ABC membrane transporters, ATP-binding cassette membrane transporters

ABCC1, ABC subfamily member C 1

$\beta_2$AR, $\beta_2$-adrenoceptor

cAMP, 3',5'-cyclic adenosine monophosphate

E-C coupling, excitation-contraction coupling

EPAC, exchange proteins activated by cAMP

GPCR, G protein-coupled receptor

$G_s$-GPCR, $G_s$-coupled G protein-coupled receptor

HASM, human airway smooth muscle

MRP1, multidrug resistance-associated protein 1

PDE4, phosphodiesterase isoform 4

PKA, protein kinase A

**Clinical Relevance:**

We report that activation of $\beta_2$ARs, common druggable target for asthmatic bronchospasm, evokes cAMP release from human airway smooth muscle cells and show that cAMP egress is mediated by ABCC1 belonging to multidrug resistance associated proteins, implicating new disease-modifying targets in the treatment of airflow obstruction in asthma.

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## INTRODUCTION

As a cornerstone in the management of asthma or COPD, bronchodilators such as β-agonists reverse or prevent the airflow obstruction. In the constricted airways, the principal action of β-agonists is to stimulate $\beta_2$-adrenoceptors ($\beta_2$ARs), a class of 7-transmembrane spanning cell surface G protein-coupled receptors (GPCRs) expressed on the smooth muscle of human bronchi–the pivotal tissue regulating bronchomotor tone (1-3). Upon activation, $\beta_2$ARs signal to the stimulatory G protein ($G\alpha_s$) to activate adenylyl cyclase which generates a key second messenger, 3',5'-cyclic adenosine monophosphate (cAMP) (4). Increased intracellular cAMP levels ($[cAMP]_i$) consequently activate protein kinase A (PKA) that in turn modulates multiple downstream targets to promote human airway smooth muscle (HASM) relaxation and reverse airways obstruction (4-6).

Classically, the signal transduction evoked by the $G_s$-coupled family of GPCRs is short-lived; and, multiple mechanisms are in place to ensure homeostatic regulation of $[cAMP]_i$. These include PKA-mediated receptor uncoupling, as well as homologous or heterologous desensitization by β-arrestin, inhibition of adenylyl cyclase by the inhibitory G protein ($G\alpha_i$), and termination of cAMP signaling by phosphodiesterase (7, 8). In HASM, the enzymatic conversion of cAMP to its inactive 5'-AMP is fast and predominated by phosphodiesterase isoform 4 (PDE4) that functions to switch off $G\alpha_s$-activated increased $[cAMP]_i$ (8). Accordingly, significant research and drug discovery efforts are focused on the elements of these molecular pathways to not only improve functional efficacy of β-agonists, but also to develop new biased mechanisms toward $G\alpha_s$ and away from β-arrestin signaling in HASM for the treatment of obstructive lung diseases.

Apparently, the binary switch-like actions of $G\alpha_s$-activated adenylyl cyclase and cAMP-specific phosphodiesterase are prone to breakdown, perturbing cAMP homeostasis and dysregulating excitation-contraction (E-C) coupling in HASM shortening (9, 10). Further, $G\alpha_i$ and

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PDE4 expression and activity are upregulated by cytokines and mediators implicated in asthma, diminishing β-agonist-induced $[cAMP]_i$ and HASM relaxation (11, 12). Of note, genetic variants of *PDE4D* (13, 14) and *ADRB2* (15, 16) in patients with asthma and COPD have been associated with sub-optimal control and/or inter-individual difference in treatment response to β-agonists (17). Interestingly, there is an emergence of pharmacogenetics studies showing association of the variability in treatment response to asthma therapy with single nucleotide polymorphisms in *ABCC1* gene, which encodes multidrug resistance-associated protein 1 (MRP1) (18-20).

ABCC1/MRP1 (herein referred to as ABCC1), a member of the superfamily of ATP-binding cassette (ABC) membrane transporters, is ubiquitously expressed and functions to displace out of the cells a wide range of structurally diverse and physiologically important endogenous substrates (21-23). In human lungs, ABCC1 and ABCC4/MRP4 are expressed on the epithelium lining the proximal airways (24-26) and are reported to pump inflammatory mediators, lipid metabolites, and signaling molecules out of the cells (27-32). The molecular signature and biological function of multidrug resistance-associated proteins in HASM remain unclear, however. Here we find multiple *ABCC* transcripts to be expressed in HASM cells, with the ABCC1 subtype being the most highly expressed by RNA-seq, qPCR and proteomics analyses. We show that β-agonists evoke cAMP egress to the extracellular space and that inhibition of ABCC1 activity and/or expression decreases $\beta_2$AR-evoked cAMP efflux, resulting in increased $[cAMP]_i$ and HASM cell relaxation. This previously unrecognized mechanism of β-agonist-mediated cAMP homeostasis in HASM may provide new therapeutic strategies to promote functional efficacy of $\beta_2$ARs and/or mitigate the mechanical endotype of airflow obstruction in asthma.

## MATERIALS AND METHODS

**Reagents:** Unless otherwise stated, all chemicals and drugs were purchased from Sigma-Aldrich (St. Louis, MO), reconstituted in sterile distilled water or DMSO, frozen in aliquots, and serially diluted in the appropriate buffer or media on the day of use. HAM's F-12 medium, PBS, FBS,

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0.05% Trypsin and EDTA, and PAGE/western blotting were purchased from Life Technologies (Carlsbad, CA). All antibodies were purchased from Cell Signaling Technology (Danvers, MA). MK-571 was purchased from Abcam (Cambridge, MA). Cyclic AMP-Screen System ELISA was purchased from Applied Biosystems (Bedford, MA). The synthetic arginine-glycine-aspartic acid (RGD) containing peptide was purchased from American Peptide Company (Sunnyvale, CA).

**Isolation and culture of HASM cells:** Human lungs were received from the National Disease Research Interchange (Philadelphia, PA) and from the International Institute for the Advancement of Medicine (Edison, NJ). All tissues are obtained from de-identified donors, and their use does not constitute human subject research as described by the Rutgers Institutional Review Board. Primary HASM cells were derived from the tracheas, harvested, characterized, and grown in culture as described by us in detail elsewhere (33). In brief, cells were cultured in Ham's F-12 medium supplemented with 100 $U \cdot mL^{-1}$ penicillin, 0.1 $mg \cdot mL^{-1}$ streptomycin, 2.5 $mg \cdot mL^{-1}$ amphotericin B and 10% FBS. Medium was replaced every 48 hrs. For all experiments, HASM cells were serum-deprived for 24 hrs and used within the first 4 passages to ensure proper smooth muscle phenotype (2, 3, 33).

**siRNA transfection**: Small interfering RNAs (siRNAs) targeting ABCC1 (M-007308-01-0005) and ABCC4 (M-007313-01-0005) were purchased from Dhamacon. siRNA-mediated knockdown was performed using a reverse transfection procedure. Briefly, HASM cells were trypsinized, resuspended in plain media, and incubated with Hi-Perfect Transfection Reagent (#301705; Qiagen) and siRNAs, before plating for a final siRNA concentration of 10 $\mu$M. Cell suspension was added to the siRNA mixture and incubated for 30 min. The cell suspension and the siRNA mixture were then seeded into cell culture plates according to experimental design and incubated for 5 hrs. After 5 hrs, complete cell culture media (described above) were added to the cell culture plate wells in a 1:1 ratio and were incubated for 18 hrs. After 18 hrs, media were changed to

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complete media. Cells were serum deprived for 24 hrs before collection. Cells were collected 72 hrs post-transfection.

**Quantitative reverse transcription PCR (qRT-PCR):** RNA was collected and converted to cDNA using the Superscript IV CellsDirect cDNA Synthesis Kit (Invitrogen) in accordance with manufacturer protocol. No-enzyme control was included to ensure fidelity of amplification and was made in parallel with other samples (reverse-transcriptase was replaced with nuclease free water). Real-Time PCR was subsequently conducted using pre-designed TaqMan probes for ABCC1 (Assay ID: Hs.PT.58.26515729) and ABCC4 (Assay ID: Hs.PT.58.26860542) from Integrated DNA Technologies using a two-step real-time PCR system (Applied Biosystems).

**Proteomics of HASM cells:** One mg lysate from each donor lung-derived HASM cells (n=6 donors) was digested with trypsin following standard FASP protocol (34). Fractions of the digests were labeled with TMT10 Plex reagent (Thermo Scientific, Rockford, IL) following manufacturer's instructions, combined, and dried. The peptides in combined samples were desalted and fractionated by high-pH reverse-phase HPLC using a C18 Xbridge column (Waters, Milford, MA) connected to Agilent HP1100. For the total proteome analysis, 14 fractions (between 27 and 40 min) were chosen, desalted with stage tip, and analyzed by nano-LC-MS/MS (Q Exactive™ HF, Thermo Scientific, Rockford, IL). MS data were analyzed with MaxQuant version 1.6.2.6 (MaxPlanck Institute of Biochemistry, Munich, Germany) and Andromeda search engine (35). Uniprot database was used to identify proteins and potential contaminants. Proteins with a false discovery rate (FDR) < 1% are reported.

**Immunohistochemistry:** HASM cells were seeded in 4-well chamber slides and cultured for 3-5 days until 60% confluence. Adherent cells were serum-deprived overnight and then incubated with 1μg/ml wheat germ agglutinin-Alexa 594 (WGA-594, Invitrogen) for 10 min at 37°C. Cells were washed 2X with PBS, and fixed with 1% paraformaldehyde in PBS for 10 min. Fixed, nonpermeabilized cells were washed 5X with PBS for 5 min, incubated with 1% BSA/PBS

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containing FcR Blocking reagent (Mat# 120-000-442, MACS Miteny Biotec) for 30 min at room temperature, and incubated with either 1 μg/ml of rabbit anti-human ABCC1 antibody (Cat# 72202, Cell Signaling Technology) or rabbit anti-IgG Isotype Control (Cat# 3900, Cell Signaling Technology) for 2 hrs at room temperature. Subsequently, cells were washed 3X with PBS and incubated with 1:500 donkey anti-rabbit-Alexa 488 (Code 711-545-152, Jackson ImmunoResearch Labs) for 50 min at room temperature. Cells were then permeabilized with 0.01% Triton-X100 in PBS for 5 min, and incubated with DAPI for 5 min. Slides were washed, mounted with coverslip, and imaged under fluorescent microscope (Nikon-80i, 40x objective lens).

**cAMP assay:** HASM cells were seeded in a 24-well plate with F12 medium containing 10% FBS until ~80-90% confluent, and then serum deprived overnight. Cells were washed with fresh serum-free media before agonist treatments. After indicated times, the media (infranatant) in each sample-well was collected, and the cells were treated with lysis buffer from cAMP-Screen System ELISA kit (Catalog number: 4412182, Applied Biosystems, Bedford, MA, USA) for 30min at 37°C. For each sample-well, 150μl of the infranatant and 150μl of the cell lysate were applied for cAMP ELISA assay to quantitate the extracellular and intracellular cAMP levels, respectively. Experiments were conducted according to the manufacturer's protocol. Unless otherwise noted, assays were performed in duplicate for each sample.

For measurements of cAMP in patient samples, study participants were recruited from the Yale Center for Asthma and Airways Disease (YCAAD) at Yale New Haven Hospital in New Haven, Connecticut using an institutional review board approved protocol. The inclusion and exclusion criteria and the YCAAD phenotyping protocol have been previously described (36). Patients underwent asthma phenotyping that included a medical history, lung function testing, and blood draw. All participants provided informed consent.

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**Magnetic twisting cytometry (MTC):** We used MTC to measure dynamic changes in the cytoskeletal stiffness as a surrogate for agonist-induced single-cell contractility, as we have validated previously (2, 3). Briefly, an RGD-coated ferrimagnetic microbead (4.5 μm in diameter) functionalized to the cytoskeleton through cell surface integrin receptors was magnetized, twisted by an external magnetic field that varied sinusoidally in time, and forced bead motions were detected with a spatial resolution of ~5 nm (2, 3). Unless otherwise stated, the ratio of specific torque to lateral bead displacements was computed and expressed as the cell stiffness in units of Pascal per nm (Pa/nm).

**Statistical Analysis:** Unless otherwise stated, HASM cells from at least 5 donors were used. When applicable, the experimental readouts were first normalized to vehicle control in each donor to obtain the fold change. The fold changes from individual donors were used to obtain group mean graphs. The data are expressed as Mean ± SE. For these studies, GraphPad Prism 6.0 was used for statistical analysis using One-Way ANOVA with Dunnett's multiple comparison test, and the means were considered significantly different when p-values less than 0.05. For MTC studies on multiple donor-derived HASM cells, we used nested design analysis to control for random effects from repeated measurements of multiple cells in the same subject (3). To satisfy the normal distribution assumptions associated with the Analysis of Variance (ANOVA), stiffness data were converted to log scale prior to analyses. Tukey-Kramer method was applied for multiple comparisons adjustment using SAS V.9.2 (SAS Institute Inc., Cary, NC), and 2-sided *p*-values less than 0.05 were considered significant.

## RESULTS

### HASM cells express multiple MRPs

To define the gene expression signature of multidrug resistance-associated proteins (MRPs) in HASM, we surveyed our RNA-seq data from a previously published study that is available in the

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Gene Expression Omnibus under accession number GSE94335 (37). In this dataset that consisted of primary HASM cells derived from 17 white, nonsmoking lung donors (nine who died of fatal asthma and eight with no chronic illness or medication use, see Table S1), we detected transcripts for 12 of the 14 known ABC subfamily member C (ABCC) genes, based on having non-zero normalized read counts. As shown in Fig. 1A, levels for the 12 expressed ABCC genes varied in HASM cells, but none differed by asthma status. *ABCC1* was the most highly expressed ABCC transcript with mean normalized counts of 8102 in asthma-donor-derived HASM cells and 8136 in non-asthma-donor-derived HASM cells (Fig. 1A). In both groups, the next most expressed ABCC was *ABCC4*, with normalized counts of 2785 and 2723 in asthma- and non-asthma-donor-derived HASM cells, respectively, which are approximately a third of the *ABCC1* levels (Fig. 1A). Using qPCR, we confirmed the relative mRNA expression levels of *ABCC1* and *ABCC4* in HASM cells derived from 8 additional lung donors with and without asthma (Fig. 1B and Table S2). In both groups, mRNA expression levels of *ABCC1* were ~5-fold higher than *ABCC4*, and there were no differences by asthma status (Fig. 1B).

**ABCC1 is highly expressed in HASM cells**

Concordant with a rank order based on ABCC transcripts detected by RNA-seq and qPCR, nanoscale liquid chromatography coupled to tandem mass spectrometry (nano LC-MS/MS) detected high abundance of ABCC1 protein levels in the tryptic digests of HASM cells (Fig. 1C). In this dataset that consisted of 6 non-asthma-donor-derived HASM cells (Table S3), protein levels of ABCC4, ABCC3, and ABCC10 were approximately 33%, 14%, and 2% of the ABCC1 levels; other ABCC members were not detected. Immunohistochemistry studies on non-permeabilized HASM cells showed a strong fluorescent signal (Alexa 488) for ABCC1 that clustered and localized with wheat germ agglutinin (WGA-594), which stains glycoproteins and glycolipids at the cell membrane (Fig. 1D); a weak, diffuse fluorescence (Alexa 488) was detected with IgG isotype control (Fig. S1). In contrast, we were unable to verify the cell surface expression

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of ABCC4 using commercially purchased antibodies against ABCC4. These results established that ABCC1, a member of the ABC transporter superfamily, is highly expressed in HASM cells.

**Activation of the $G_s$-GPCRs evokes cAMP efflux**

In a number of cellular models, ABCC1 and ABCC4 show substrate specificities to a broad array of signaling molecules, including cAMP (21-23, 27-30). These studies prompted us to ask 1) whether HASM cells are able to pump out intracellular cAMP and 2) whether these events are mediated by ABCC1/ABCC4. In initial studies, we stimulated HASM cells with a long-acting $\beta_2$-agonist formoterol (half-life, ~8-10 hrs) and measured intracellular/extracellular cAMP levels ($[cAMP]_{i/e}$) using a highly sensitive ELISA method. For these studies, we used non-asthma-donor-derived HASM cells and studied them under identical experimental conditions, without phosphodiesterase inhibitors, in standard culture media for 24 hrs. As expected, formoterol rapidly increased intracellular cAMP levels ($[cAMP]_i$) in HASM cells (from basal levels of 6$\pm$2 fmol to 432$\pm$46 fmol within 5 min) (Fig. 2A). Formoterol-evoked $[cAMP]_i$ was transient, however, and declined to a lower but suprabasal levels within 30 min (81$\pm$9 fmol); the suprabasal $[cAMP]_i$ was maintained over 4-24 hrs (in keeping with a typical half-life of formoterol). Further, forskolin that directly activates adenylyl cyclase also evoked a similar time course of intracellular cAMP generation in HASM cells (Fig. 2A).

Strikingly, exposure of HASM cells to formoterol or forskolin culminated in a slow accumulation of cAMP in the extracellular space (Fig. 2B). Increased extracellular cAMP levels ($[cAMP]_e$) were substantial at 15 min (from basal levels of 32$\pm$6 fmol to 169$\pm$30 fmol in response to formoterol; from 25$\pm$6 fmol to 184$\pm$35 in response to forskolin), equi-effective to peak $[cAMP]_i$ within 30 min (498$\pm$9 fmol for formoterol; 414$\pm$109 fmol for forskolin), and reached a peak by 4-24 hrs. Of note, HASM cells showed expected between donor variations in basal $[cAMP]_e$, but all showed significant increased $[cAMP]_e$ in response to $\beta$-agonists (Fig. 2C, Fig. S2A, and Table

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S4). This long-lived increased $[cAMP]_e$ was also induced by forskolin that activates adenylyl cyclase and independent of $\beta_2$AR activation (Fig. 2D).

In order to test whether the observed $\beta_2$AR-evoked cAMP efflux is a shared mechanism of the $G_s$-coupled family of GPCRs, we next stimulated HASM cells with prostaglandin $E_2$ ($PGE_2$). $PGE_2$ activates prostaglandin $E_2$ receptor subtypes (EP1-4) that display multifunctional signaling. In a previous study, we have shown that HASM cells express high abundance of EP2 and EP4 transcripts, the receptor subtypes that largely signal to the G$\alpha$s to activate adenylyl cyclase: $PGE_2$ is a strong generator of $[cAMP]_i$ and a potent inducer of HASM relaxation (38). HASM cells stimulated for 4 hrs with $PGE_2$ showed a dose-dependent increase in $[cAMP]_e$ (Fig. S2B). Of note, $PGE_2$-evoked cAMP egress was appreciably greater than that of formoterol and non-selective β-agonist isoproterenol (Fig. S2B). Taken together, these results suggest that cAMP egress is a universal response class of increased $[cAMP]_i$ in HASM cells. The findings also support the presence of intracellular cAMP sensors or additional homeostatic regulation of $[cAMP]_i$ mediated by the $G_s$-coupled family of GPCRs, involving the export of the second messenger from HASM cells.

**β-agonist-evoked cAMP egress is mediated through ABCC1 transporter**

To explore the roles for ABCC1/ABCC4 in β-agonist-induced cAMP egress, we employed a well-established MRP inhibitor MK-571 (39-40). MK-571 decreased isoproterenol-induced cAMP release from HASM cells, in a dose-dependent manner (Fig. S3). Compared with vehicle treated cells, HASM cells treated with MK-571 (10 μM) produced markedly lower $[cAMP]_e$ in response to isoproterenol (Fig. 3A-B and Table S5). As shown in Fig. 3B, while the magnitude of decreases varied between donor-derived HASM cells, MK-571 treatment effectively inhibited β-agonist-induced cAMP efflux.

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To determine the relative contribution of ABCC1 and ABCC4 in regulating cAMP efflux, we used siRNA-mediated knockdown of ABCC1 in HASM cells (Table S5). Here we focused on ABCC1 because, unlike human airway epithelial cells (24-26), ABCC4 mRNA and protein levels were markedly lower than that of ABCC1 in HASM cells (Fig. 1). Further, consistent with previous report (30), we were unable to verify the cell surface expression of ABCC4 in HASM cells. Transfection of HASM cells with siRNA directed against *ABCC1* decreased ABCC1 protein levels compared with the respective non-targeting siRNA controls; Western blots showed variable reductions of ABCC1 protein levels in 7 donor-derived HASM cells (Fig. 4A). Compared with non-targeting siRNA (Control) transfected cells, however, HASM cells transfected with *ABCC1* siRNAs showed ~40% reduction in cAMP efflux in response to isoproterenol (Fig. 4B-C and Fig. S4); there were no group differences in basal $[cAMP]_e$. These results suggest that β-agonist-induced accumulation of cAMP in the extracellular space is predominantly mediated by ABCC1 expressed on HASM cells.

**Inhibition of ABCC1 enhances β-agonist-induced HASM cellular relaxation**

Of note, in multiple donor-derived HASM, isoproterenol stimulation markedly increased $[cAMP]_i$ in MK-571 treated cells than untreated cells (Fig. 5A). To address the physiological consequences of ABCC1 inhibition, magnetic twisting cytometry (MTC) was applied to quantify the stiffness changes of HASM cells in response to isoproterenol (3). For these studies, we used HASM cells derived from 3 different lung donors (Table S6) and applied mixed effect models to control for the random effects due to multiple cell measurements from the same donor as we have done previously (3). Compared with untreated cells, HASM cells treated with MK-571 exhibited significant decreases in baseline stiffness (basal tone) and, moreover, showed further stiffness decreases in response to isoproterenol (Fig. 5B and Fig. S5A). Interestingly, levels of stiffness after MK-571 treatment were comparable to that induced by isoproterenol in untreated cells; there

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were no significant differences between the two groups. These data suggest that endogenous activity of ABCC1 may regulate cAMP homeostasis in HASM cells.

Similar to pharmacological inhibition of ABCC1 with MK-571, HASM cells transfected with ABCC1 siRNAs showed appreciable increases in peak $[cAMP]_i$ in response to isoproterenol (Fig. 5C and Fig. S4C). Of note, *ABCC1* siRNA transfected cells exhibited markedly lower baseline stiffness (basal tone) than cells transfected with non-targeting siRNAs (Fig. 5D and Fig. S5B). As a negative control, we also transfected HASM cells with siRNA targeting *ABCC4*. There were no significant differences in baseline stiffness or isoproterenol-induced stiffness decreases in HASM cells transfected with *ABCC4* siRNA vs. non-targeting siRNA (Fig. 5D). Compared with non-targeting or *ABCC4* siRNA transfected cells, however, HASM cells transfected with *ABCC1* siRNA showed markedly lower basal tone and isoproterenol-induced cellular relaxation (Fig. 5D and Fig. S5B). These findings suggest that ABCC1 directly regulates $[cAMP]_i$ in HASM and that this previously unrecognized mechanism of cAMP homeostasis may provide new therapeutic strategies to promote functional efficacy of β-agonists and/or inhibit excitation-contraction coupling in HASM shortening.

**Serum cAMP levels are increased in patients with asthma**

Patients with asthma express increased circulating levels of chitinase and chitinase-like protein YKL-40, effector molecules implicated in immune inflammatory responses of the airways (41, 42). Further, it has been shown that serum YKL-40 levels are positively correlated with airway remodeling, airway hyperresponsiveness, and asthma severity (36, 42). As a proof-of-concept that extracellular cAMP levels may provide new markers for asthma, we measured the levels of circulating cAMP in a small cohort of patients with (n=16) and without (n=8) asthma (as defined by GINA criteria). The demographics of the asthma and non-asthma patients are shown in Table S7. As shown in Fig. 6, we were able to detect cAMP in patient samples and found increased serum cAMP levels in patients with asthma than controls. Further investigations are warranted,

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however, to establish its relationship to asthma severity and determine the clinical utility of extracellular cAMP in health and diseases.

## DISCUSSION

In 1971, Earl Sutherland won a Nobel Prize in *Physiology or Medicine* for his work on the *'mechanisms of the action of hormones'* which led to the identification of a "heat-stable factor", cAMP, and established the founding principles on the second messenger system (43, 44). The second messenger role of cAMP is widespread and is one of the most studied intracellular signaling cascades of the $G_s$-coupled family of GPCRs ($G_s$-GPCRs). Classically, the multi-function cAMP transduction evoked by $G_s$-GPCRs is short-lived, confined to the intracellular space, and tightly controlled by the binary switch-like actions of the adenylyl cyclase (cAMP production) and phosphodiesterase (cAMP breakdown), with many built-in nodal points for signal amplification, modification, and attenuation.

For example, in this signaling pathway, the production of cAMP from ATP by adenylyl cyclase is triggered or repressed by the activation GPCRs that coupled to the stimulatory G protein ($G\alpha_s$) or the inhibitory G protein ($G\alpha_i$), respectively. Once synthesized, cAMP acts on its primary effector(s), including protein kinase A (PKA) and exchange proteins activated by cAMP (EPAC), which in turn modulate further downstream signal transduction. Signal attenuation occurs by enzymatic degradation of cAMP to AMP by phosphodiesterase (PDE), compartmentalization, desensitization, and/or extrusion of cAMP to the extracellular space. The latter is cell-type dependent, however, and is mainly mediated by multidrug resistance-associated proteins (MRPs), belonging to the ATP-binding cassette (ABC) membrane transporters (45).

Here we find that β-agonists, most widely used bronchodilators for the treatment of asthmatic bronchospasm, evoke cAMP release from HASM cells. We show that cAMP is exported from HASM cells in response to agonists/agents that stimulate different

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family/component of the $G_s$-coupled GPCRs ($\beta_2$ARs, EPs, and adenylyl cyclase) and is long-lived in culture. These general features of the receptor-dependent and receptor-independent mechanisms of cAMP egress suggest a universal response class of increased $[cAMP]_i$ mediated by the $G_s$-coupled family of GPCRs expressed on HASM cells.

We sought to understand the mechanism(s) for this previously unrecognized $\beta_2$AR-evoked cAMP secretion from HASM cells. Because ABCC4/MRP4, ABCC5/MRP5, and ABCC11/MRP8 have substrate specificities to cyclic nucleotides (46), we first screened for the expression of 14 known ABC subfamily member C (ABCC) genes in HASM cells. In our own RNA-seq dataset (GSE94335) (37), which profiled primary HASM cells derived from 17 white, nonsmoking lung donors, we detected transcripts of 12 of 14 ABCCs (based on having non-zero normalized read counts), including *ABCC4* (encoding MRP4), *ABCC5* (encoding MRP5), and *ABCC11* (encoding MRP8). We did not detect protein expression of MRP5 and MRP8 in our proteomics dataset, however. In addition, consistent with the findings of Huff and colleagues (30), we were unable to verify the cell surface expression of MRP4 in isolated HASM cells. Strikingly, ABCC1 was highly expressed in both RNA-seq and proteomics datasets. Applying qPCR in 8 additional donor-derived HASM cells, we confirmed the relative mRNA levels of *ABCC1*, which were ~5-fold higher than that of *ABCC4* (second highest in both RNA-seq and proteomics). Further, using commercially purchased antibodies against ABCC1, we detected cell surface expression of ABCC1 in isolated HASM cells. These results established that HASM cells manifest restricted expression of MRPs and that ABCC1/MRP1 is highly expressed in HASM cells.

Of note, single nucleotide polymorphisms in *ABCC1* gene (rs119774) have been associated with the variability in montelukast (leukotriene inhibitor) treatment responses in patients with asthma (18, 19). Further, ABCC1 is reported to have substrate specificity to lipid metabolites, including leukotriene C4 and sphingosine-1-phosphate (21-23, 27, 28, 46). We pursued the role for ABCC1 in cAMP secretion from HASM cells. We showed that

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pharmacological inhibition or downregulation of ABCC1 with small interfering RNA markedly decreased $\beta_2$AR-evoked cAMP release from multiple donor-derived HASM cells. On the other hand, inhibition of ABCC1 activity and/or expression increased β-agonist-induced intracellular cAMP levels ($[cAMP]_i$) and associated HASM cell relaxation. These findings identify a previously unrecognized role for ABCC1 in the homeostatic regulation of $[cAMP]_i$ in HASM that may be conserved across the $G_s$-coupled family of GPCRs. Hence, the general features of this activation mechanism may uncover new disease-modifying targets in the treatment of airflow obstruction in asthma.

Characterized by airway inflammation and reversible airflow obstruction, asthma is a common debilitating lung disease that afflicts ~4.5% of the world's population. Despite advances in treatment, asthma remains poorly controlled in most patients. Recently, for patients with difficult-to-control and severe asthma, the use of biologics that target type 2 (Th2) inflammation has substantially impacted asthma management. Surprisingly, Th2-high (IL-4, IL-5, and IL-13) endotype-based approaches provide therapeutic improvements in only a minority of patients (47). Although multiple factors contribute to asthma pathobiology, the principal mechanism of increased airways resistance to airflow is aberrant shortening of airway smooth muscle (2, 3, 48, 49). The results presented in this study showed new molecular pathways invoked by β-agonists involving the extrusion of intracellular cAMP through ABCC1. Of note, extracellular cAMP levels ($[cAMP]_e$) remained elevated in culture for 24 hrs. This despite finding appreciable transcript levels of ectonucleotidases (i.e. ENTPD1-8, NT5E, ENPP1-6, and ALPL) in our RNA-seq dataset (37), suggesting a limited negative feedback through adenosinergic signaling mechanisms.

What are the roles for extracellular cAMP in the lungs? Could the levels of cAMP in the lung and/or circulation provide other clinical determinants or mechanisms of airflow obstruction in asthma? Almost 40 years ago, Scavennec et al. (50) observed that cAMP concentration in urine was higher in patients with solid tumors than in healthy volunteers. Therefore, cAMP levels in

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urine, or even plasma, could eventually serve as an efficacy biomarker to assess the patient's response to MRP-targeted therapy. Based on these previous studies, we asked 1) whether cAMP can be reliably measured in the serum of patients, and 2) whether serum cAMP levels can differentiate patients with obstructive lung diseases. Surprisingly, we find that serum concentration of cAMP is increased in a small cohort of patients with asthma than controls. This proof-of-concept study now allows us to explore further clinical utility of serum cAMP levels as potential endotype of obstructive lung diseases and/or markers of $\beta_2$AR tachyphylaxis in a large clinical studies.

**ACKNOWLEDGEMENTS AND FUNDING**

This work was supported by the New Jersey Alliance for Clinical and Translational Science (UL1TR0030117) and the National Institutes of Health grants (P01HL114471 and R56HL155937).

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## FIGURE LEGENDS

**Fig. 1. HASM cells express ABC transporter subfamily member C.** (**A**) Expression of ABCC genes in HASM cells as assessed in a publically available RNA-seq dataset (GSE94335) (37). RNA-seq results are expressed as normalized read counts (read counts were obtained by HTSeq and normalized by DESeq2). (**B**) RT-PCR cross-validation of the two most abundant ABCC genes, *ABCC1* and *ABCC4*, in asthma- and non-asthma-donor-derived HASM cells. The characteristics of the lung donors (n=4 asthmatics and n=4 non-asthmatics) are provided in Table S2. Results are normalized to endogenous GABARAP to account for template quantity discrepancies between samples, and expressed as relative fold changes to *ABCC1* expression levels detected in HASM-derived from one non-asthmatic donor. Analyses were done by one-way ANOVA, followed by Tukey's multiple comparisons test. ****, $p<0.0001$. (**C**) Tryptic digests from cell lysates were screened by nano-LC-MS/MS, and the reporter ion intensities of ABCC isoforms were expressed as the relative percentage of ABCC1 (set at 100%) for each non-asthma-donor-derived HASM (n=6). Analyses were done by one-way ANOVA, followed by Tukey's multiple comparisons test. ***, $p<0.001$; ****, $p<0.0001$. **(D)** A representative (merged) immunofluorescent images of HASM cells probed with wheat germ agglutinin (red) and anti-ABCC1 antibody (green). Nuclei are identified by the blue color from DAPI fluorescence. Image was acquired using Nikon-80i microscope under 40x objective lens. Individual fluorescent signals, including IgG isotype control for ABCC1 antibody, are shown in Fig. S1.

**Fig. 2. Activation of the $G_s$-GPCRs evokes cAMP efflux.** (**A-B**) Representative levels of intracellular cAMP ($[cAMP]_i$) and extracellular cAMP ($[cAMP]_e$) detected by ELISA. HASM cells were stimulated with formoterol (1 $\mu$M) or forskolin (10 $\mu$M) for 24 hrs. For each time point, assays were performed in duplicate. Data are presented as mean $\pm$ SE (data are stacked in relations to each agonist, showing the relative level changes with time). (**C-D**) formoterol- and forskolin-induced $[cAMP]_e$ from HASM cells derived from 5 lung donors (4 hrs). The characteristics of the

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5 lung donors are provided in Table S4. For each donor-derived HASM, agonist-induced $[cAMP]_e$ levels were superimposed in relations to basal $[cAMP]_e$ levels. Individual donor-derived cellular responses to isoproterenol are shown in Fig. S2A.

**Fig. 3. Pharmacologic inhibition of ABCC1 decreases cAMP egress.** (**A**) HASM cells were first treated for 20 min with or without 10 μM MK-571, followed by incubation with 10 μM isoproterenol for additional 4 hrs. Data are presented as mean ± SE (n=5 independent samples). Analyses were done by one-way ANOVA, followed by Dunnett's multiple comparisons test. *, $p<0.05$. (**B**) Isoproterenol-induced extracellular cAMP levels were measured in HASM cells derived from 7 lung donors (Table S5). For each donor-derived HASM, isoproterenol-induced extracellular cAMP levels in MK-571 treated cells were normalized to the respective vehicle treated cells. Data are presented as mean ± SE. p-value from paired t-test.

**Fig. 4. siRNA-mediated knockdown of ABCC1 decreases $\beta_2$AR-evoked cAMP release from HASM cells**. HASM cells were transfected with siRNAs directed against *ABCC1* and non-targeting siRNAs. (**A**) Expression of ABCC1 was detected by western blot (n=7 donor-derived HASM cells, Table S5). Tubulin was used as loading controls. (**B**) Extracellular cAMP levels were detected by ELISA before and after stimulation with 10 μM isoproterenol (4 hrs). Data are presented as box and whiskers plot. Analyses were done by one-way ANOVA, followed by Tukey's multiple comparison tests. *, $p<0.05$; **, $p<0.005$. (**C**) Individual donor-derived HASM cellular responses to isoproterenol. Colors indicate different donor-derived HASM cells. Circles indicate cells transfected with non-targeting siRNAs. Squares indicate cells transfected with *ABCC1* siRNAs. Dotted lines indicate matching donor lungs. P-value from paired t-test.

**Fig. 5. Inhibition of ABCC1 activity or expression increases β-agonist-induced $[cAMP]_i$ and promotes HASM cell relaxation.** (**A-B**) Individual HASM cells were treated for 20 min with or without MK-571, and then stimulated with β-agonist isoproterenol for 5 min. (**A**) Intracellular cAMP

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levels were detected by ELISA (n=7 donor-derived HASM cells). For each donor-derived HASM, isoproterenol-induced intracellular cAMP levels in MK-571 treated cells were normalized to the respective untreated cells. P-value indicates paired t-test. (**B**) Cell stiffness measured by MTC. HASM cells were derived from 3 different lung donors. Data are presented as geometric mean ± 95% CI (n=351-486 individual cell measurements from each donor-derived HASM cells for each condition). Mixed effect model was applied to test the group differences in stiffness with multiple comparisons adjusted by Tukey-Kramer method. *, $p<0.05$, ***, $p<0.001$. (**C**) Isoproterenol-induced $[cAMP]_i$ in HASM cells transfected with *ABCC1* siRNAs vs non-targeting siRNAs (n=7 donor-derived HASM cells). P-value indicates paired t-test. (**D**) MTC was applied in HASM cells derived from one donor lung transfected with *ABCC1*, *ABCC4*, and non-targeting siRNAs. Data are presented as geometric mean ± 95% CI (n=465-612 individual cell measurements from three independent experiments with multiple-cell wells transfected with siRNAs). Analyses were done by one-way ANOVA, followed by Tukey's multiple comparison tests. ****, $p<0.0001$. To satisfy the normal distribution assumptions associated with the Analysis of Variance (ANOVA), stiffness data were converted to log scale prior to analyses.

**Fig. 6. Serum cAMP levels are increased in patients with asthma.** ELISA was applied to detect cAMP levels in blood drawn from a small cohort of patients with and without asthma. The demographics of the 16 asthma and 8 non-asthma patients are shown in Table S7. P-value from Mann Whitney test.

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Figure 1

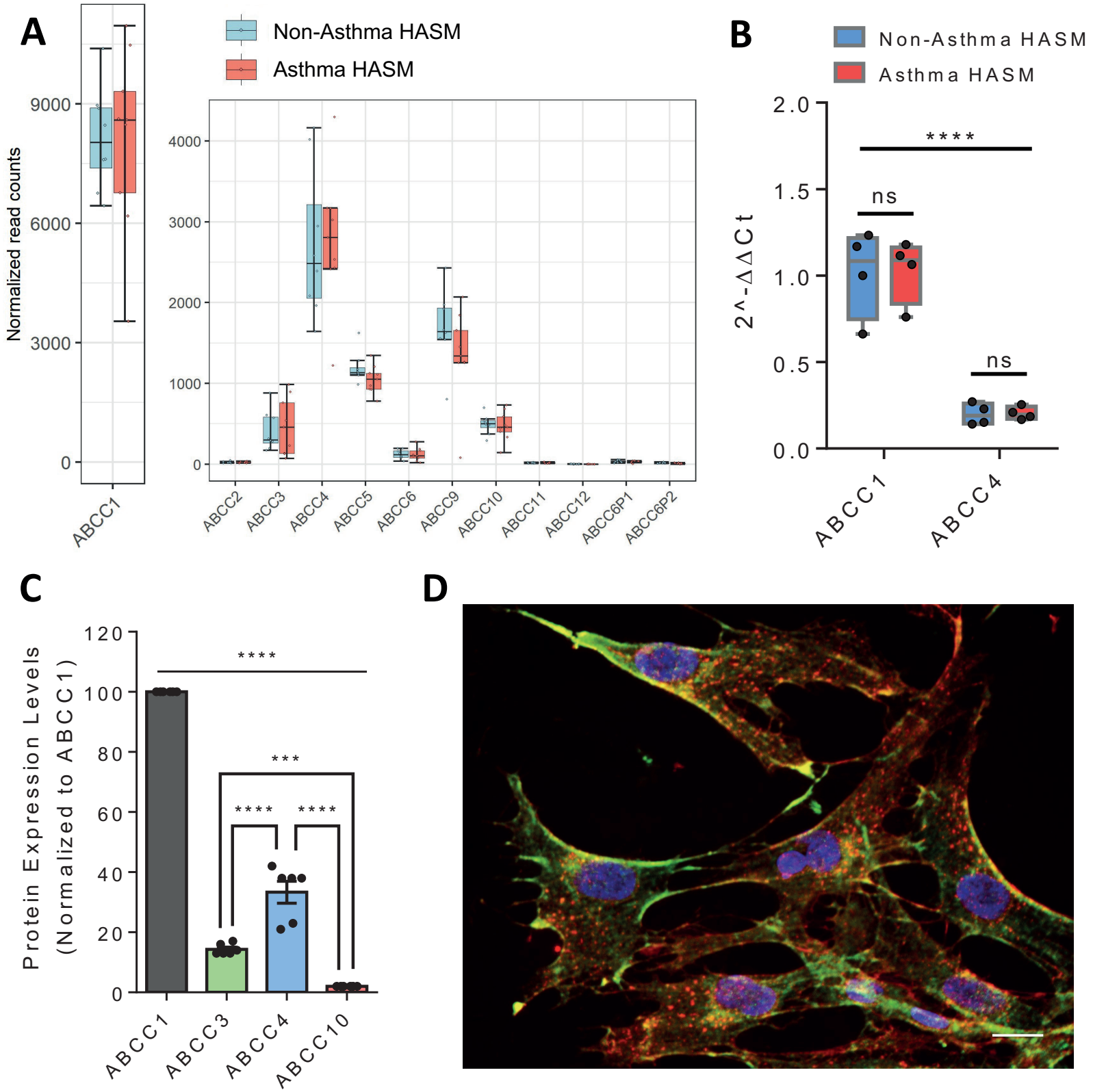


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Figure 2

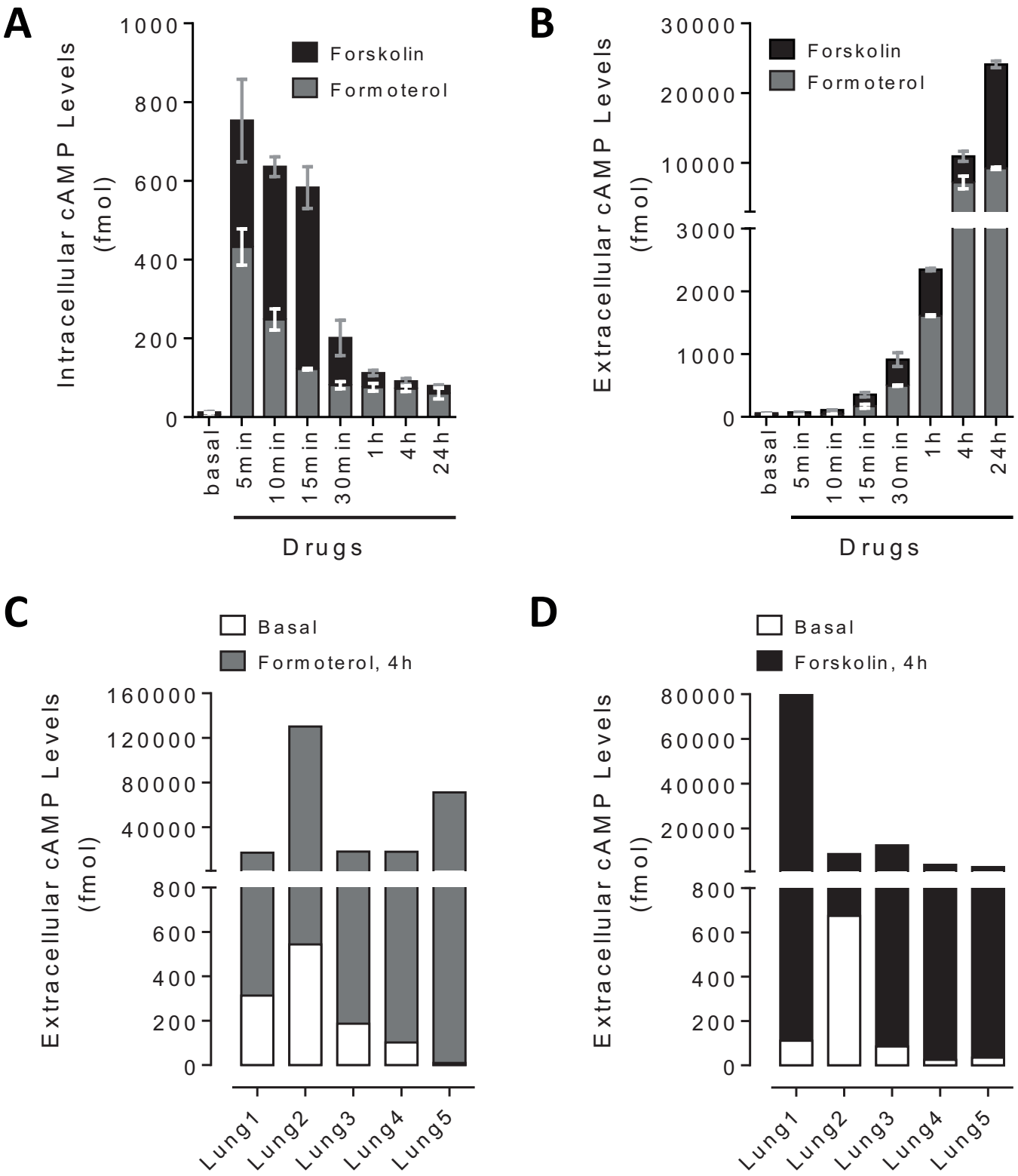


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Figure 3

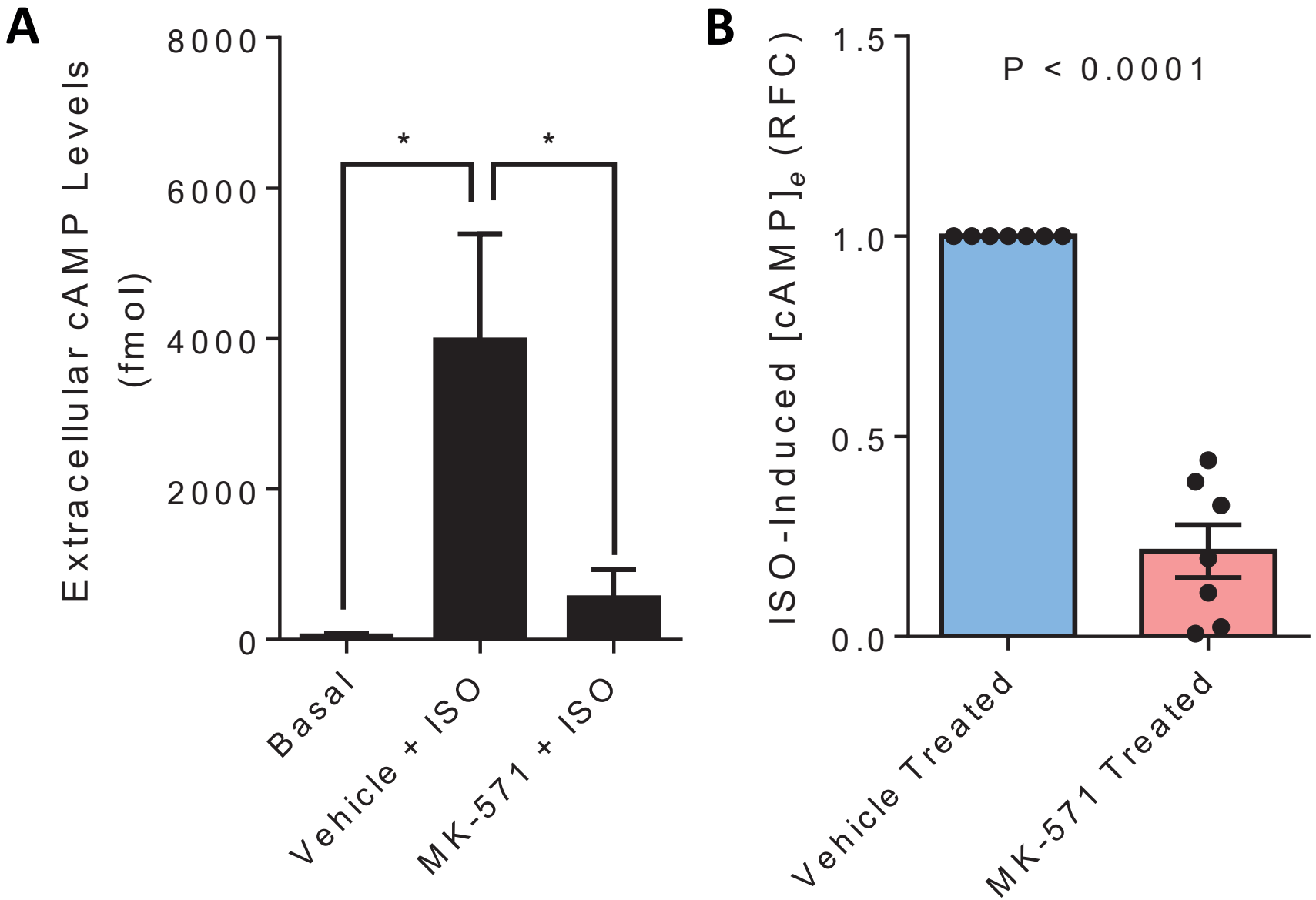


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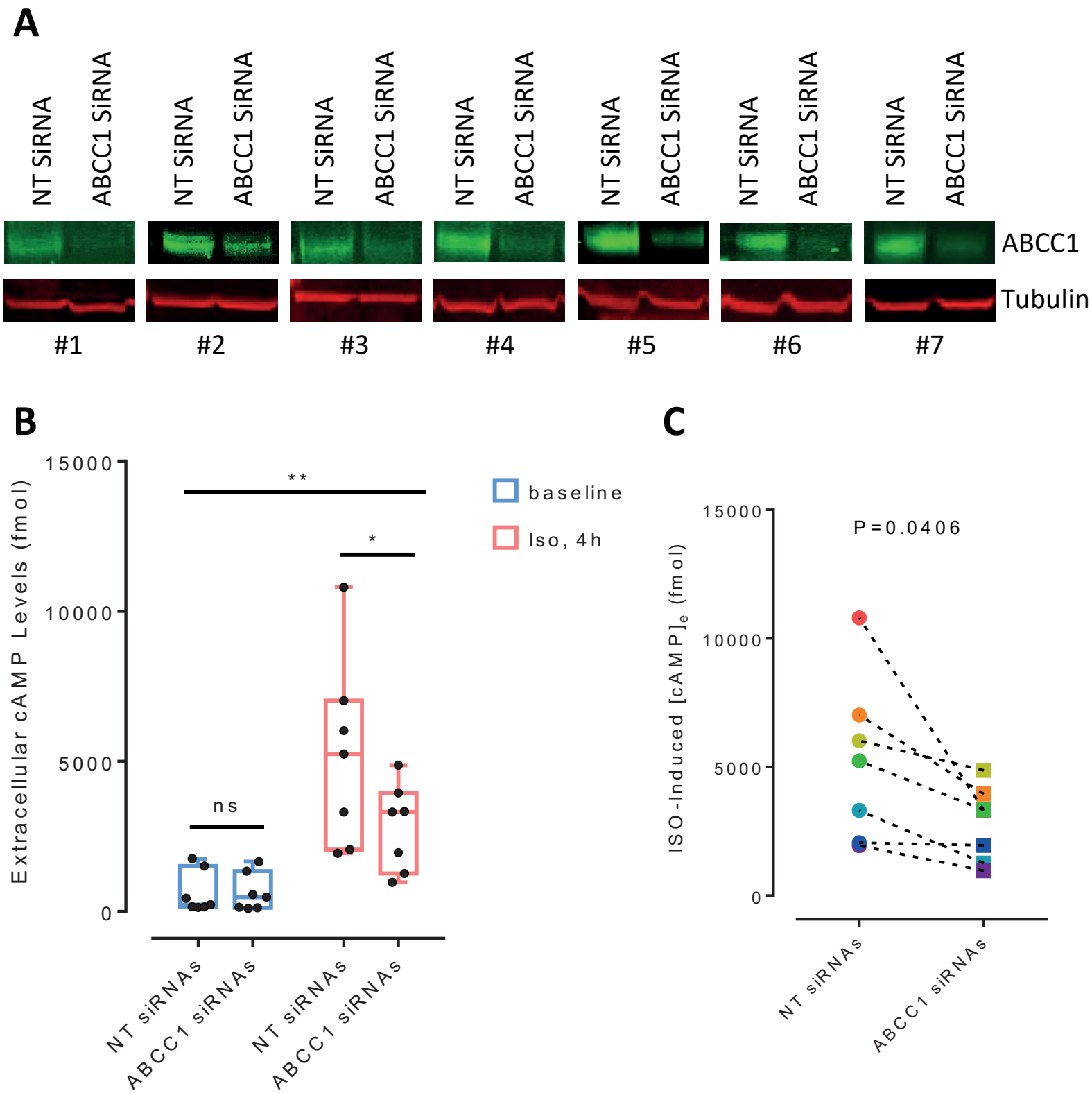
Figure 4

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# Figure 5

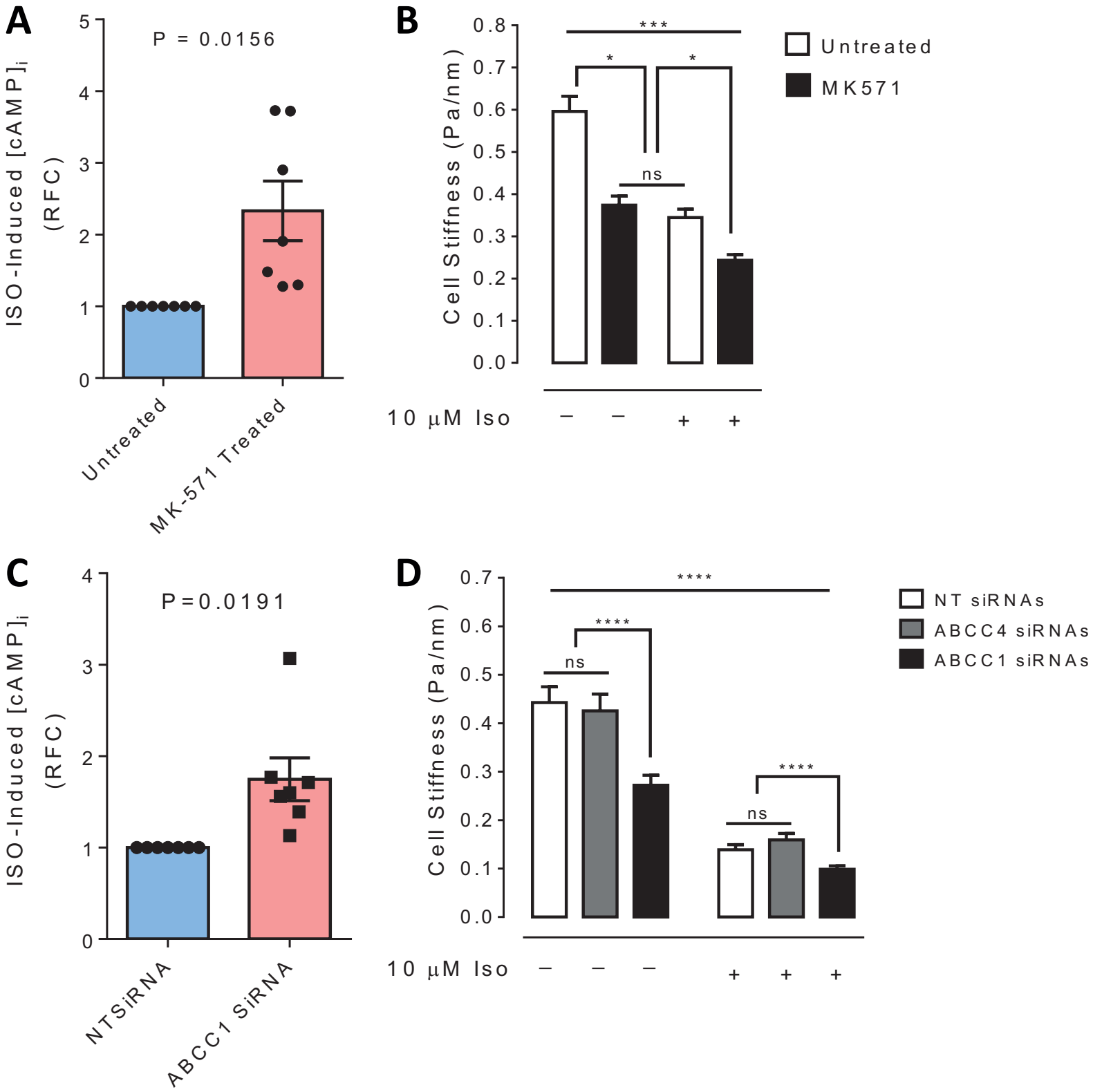


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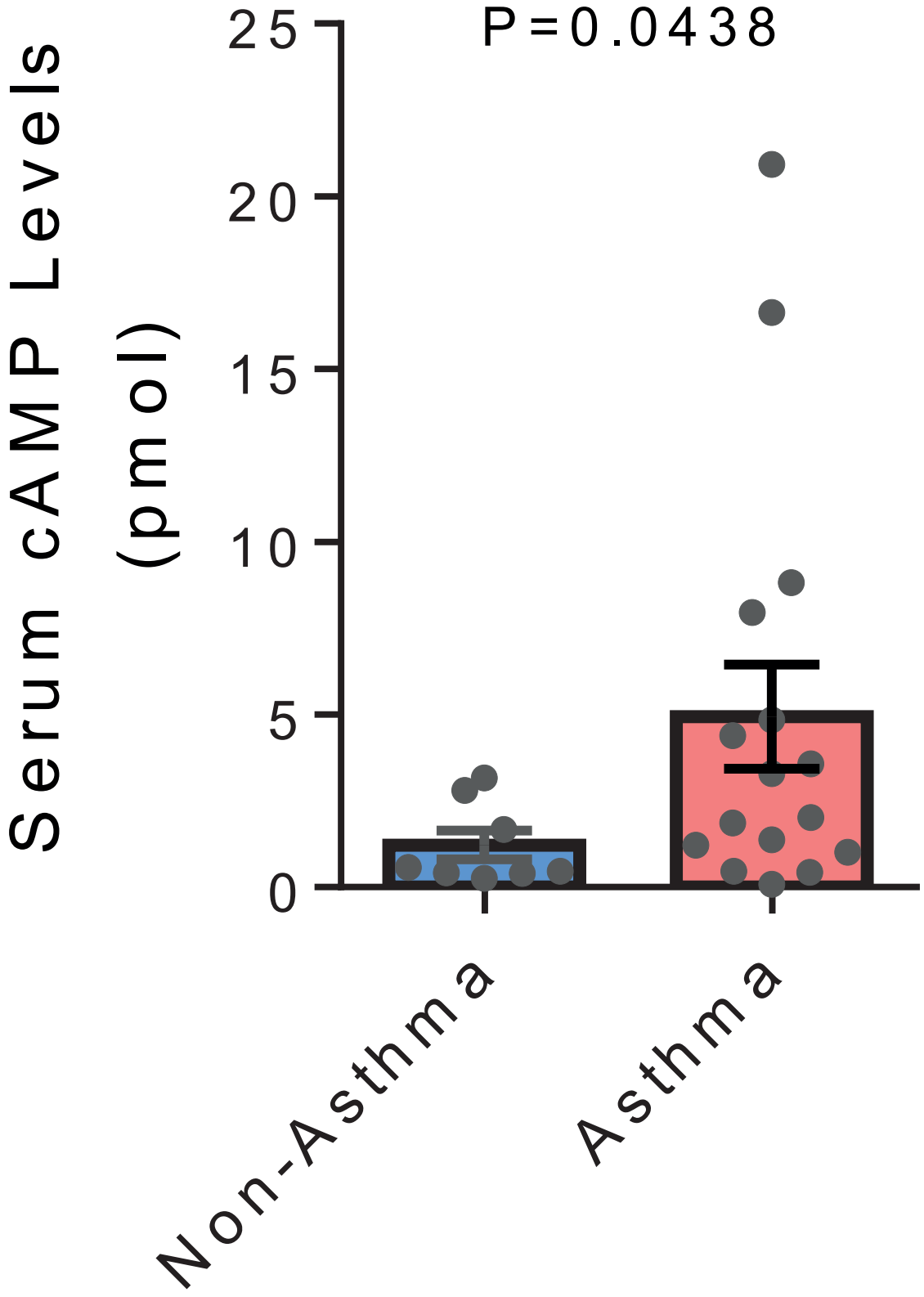
Figure 6

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**Online Data Supplement**

**Supplementary Materials**

**Supplementary Figures:**



**Supplementary Tables:**



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**Table S1:** Characteristics of donor lungs for RNA-seq dataset

| Donor | Age | Gender | Race and Ethnicity | Cause of Death |
|---|---|---|---|---|
| ***Non-asthma*** | | | | |
| ***1*** | 18 | F | White, not Hispanic | Head Trauma |
| ***2*** | 17 | M | White, not Hispanic | Head Trauma |
| ***3*** | 24 | F | White, not Hispanic | Cerebrovascular Accident |
| ***4*** | 23 | M | White, not Hispanic | Head Trauma |
| ***5*** | 52 | F | White, not Hispanic | Anoxia |
| ***6*** | 26 | M | White, not Hispanic | Head/Chest Trauma |
| ***7*** | 21 | M | White, not Hispanic | Anoxic Brain Injury |
| ***8*** | 52 | F | White, not Hispanic | Arteriovenous Malformation/Rupture |
| ***Asthma*** | | | | |
| ***9*** | 48 | F | White, not Hispanic | Anoxia |
| ***10*** | 44 | F | White, not Hispanic | Respiratory Arrest |
| ***11*** | 44 | F | White, not Hispanic | Respiratory Arrest |
| ***12*** | 14 | M | White, not Hispanic | Anoxia |
| ***13*** | 9 | M | White, not Hispanic | Asthma Attack |
| ***14*** | 21 | F | White, not Hispanic | Anoxia |
| ***15*** | 38 | M | White, not Hispanic | Anoxia |
| ***16*** | 14 | F | White, not Hispanic | Anoxia |
| ***17*** | 25 | F | White, not Hispanic | Anoxia |

**Table S2:** Characteristics of donor lungs for qPCR

| Donor | Age | Gender | Race and Ethnicity | Cause of Death |
|---|---|---|---|---|
| ***Non-asthma*** | | | | |
| ***24*** | 39 | M | White, not Hispanic | Anoxia |
| ***25*** | 47 | F | White, not Hispanic | Head Trauma |
| ***26*** | 38 | F | White, not Hispanic | Head Trauma |
| ***27*** | 52 | M | White, not Hispanic | Anoxia |
| ***Asthma*** | | | | |
| ***a*** | 48 | F | White, not Hispanic | Anoxia |
| ***28*** | 44 | M | White, not Hispanic | Anoxia |
| ***29*** | 37 | F | White, not Hispanic | Anoxia |
| ***30*** | 54 | M | White, not Hispanic | Anoxia |

Note: a = lung donor #9

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**Table S3:** Characteristics of donor lungs for proteomics dataset

| Donor | Age | Gender | Race and Ethnicity | Cause of Death |
|---|---|---|---|---|
| ***Non-asthma*** | | | | |
| ***18*** | 55 | F | White, not Hispanic | Central Nervous System Tumor |
| ***19*** | 52 | F | White, not Hispanic | Cerebrovascular Accident |
| ***20*** | 22 | F | Black, not Hispanic | Head Trauma |
| ***21*** | 52 | F | White, not Hispanic | Stroke |
| ***22*** | 50 | F | White, not Hispanic | Cerebrovascular Accident |
| ***23*** | 34 | F | Black, not Hispanic | Cerebrovascular Stroke |

**Table S4:** Characteristics of donor lungs for Gs-GPCR evoked cAMP studies

| Donor | Age | Gender | Race and Ethnicity | Cause of Death |
|---|---|---|---|---|
| ***Non-asthma*** | | | | |
| ***31*** | 19 | M | Hispanic | Cerebrovascular Stroke |
| ***32*** | 12 | F | White, not Hispanic | Anoxia |
| ***33*** | 44 | M | Black, not Hispanic | Cerebrovascular Stroke |
| ***34*** | 33 | F | White, not Hispanic | Cerebrovascular Stroke |
| ***35*** | 25 | M | White, not Hispanic | Head Trauma |

**Table S5:** Characteristics of donor lungs for MK-571 and ABCC1 siRNA studies

| Donor | Age | Gender | Race and Ethnicity | Cause of Death |
|---|---|---|---|---|
| ***Non-asthma*** | | | | |
| ***b*** | 19 | M | Hispanic | Cerebrovascular Stroke |
| ***c*** | 12 | F | White, not Hispanic | Anoxia |
| ***d*** | 44 | M | Black, not Hispanic | Cerebrovascular Stroke |
| ***36*** | 31 | M | White, Unknown | Anoxia |
| ***37*** | 12 | F | White, not Hispanic | Anoxia |
| ***38*** | 20 | M | White, not Hispanic | Head Trauma |
| ***39*** | 22 | M | Black, not Hispanic | Head Trauma |

Note: b = lung donor #31, c = lung donor #32, and d = lung donor #33

**Table S6:** Characteristics of donor lungs for MTC studies

| Donor | Age | Gender | Race and Ethnicity | Cause of Death |
|---|---|---|---|---|
| ***Non-asthma*** | | | | |
| ***d*** | 44 | M | Black, not Hispanic | Cerebrovascular Stroke |
| ***e*** | 31 | M | White, Unknown | Anoxia |
| ***f*** | 12 | F | White, not Hispanic | Anoxia |

Note: d = lung donor #33, b = lung donor #36, and c = lung donor #37

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**Table S7:** Characteristics of patients for serum cAMP studies

| Demographics | Asthma (N=16) | Control (N=8) |
|---|---|---|
| Age (years) | 48.7(23-64) | 29.6(16-56) |
| Sex, Female: Male | 13:3 | 3:5 |
| Atopy, n% | 13 (81.25) | 3 (37.5) |
| BMI (Kg/m2) | 33.08 (19.6 – 64.4) | 27.38 (20.7 – 40.6) |
| **Race** | | |
| White/Caucasian | 8 | 5 |
| Black/African American | 4 | 0 |
| Asian | 0 | 1 |
| Hispanic | 3 | 0 |
| **PFTs** | | |
| FEV1 Pre (%) PRD | 72 (42 – 98) | 98 (83 – 117) |
| FEV1/FVC Ratio PRD | 0.799 (0.77 - 0.85) | 0.83 (0.76 – 0.87) |
| **Asthma Severity** | | |
| Mild/Mod/Severe | 3/9/1 | N/A |
| **Medication** | | |
| Inhaled Corticosteroids in Combination with LABA, n% | 9(56.25) | 0 (0) |

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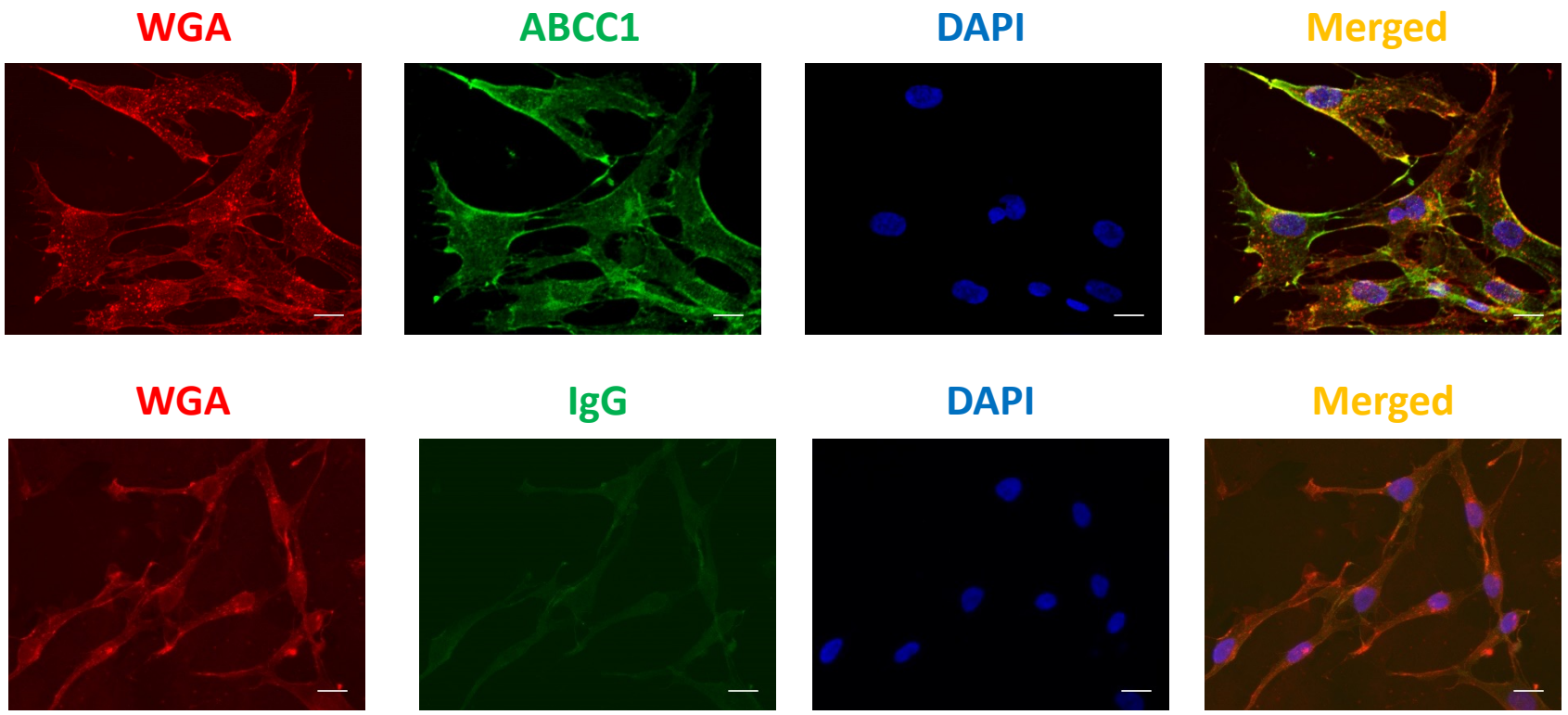


**Fig. S1. Cell surface expression of ABCC1 in HASM cells.** A representative immunofluorescent images of HASM cells probed with wheat germ agglutinin (red, WGA-Alexa-594) and rabbit anti-human ABCC1 antibody (green, donkey anti-rabbit-Alexa 488). As a negative control, we applied rabbit anti-IgG isotype control (bottom). Nuclei are identified by the blue color from DAPI fluorescence. Image was acquired using Nikon-80i microscope under 40x objective lens. Scale, 10 μm.

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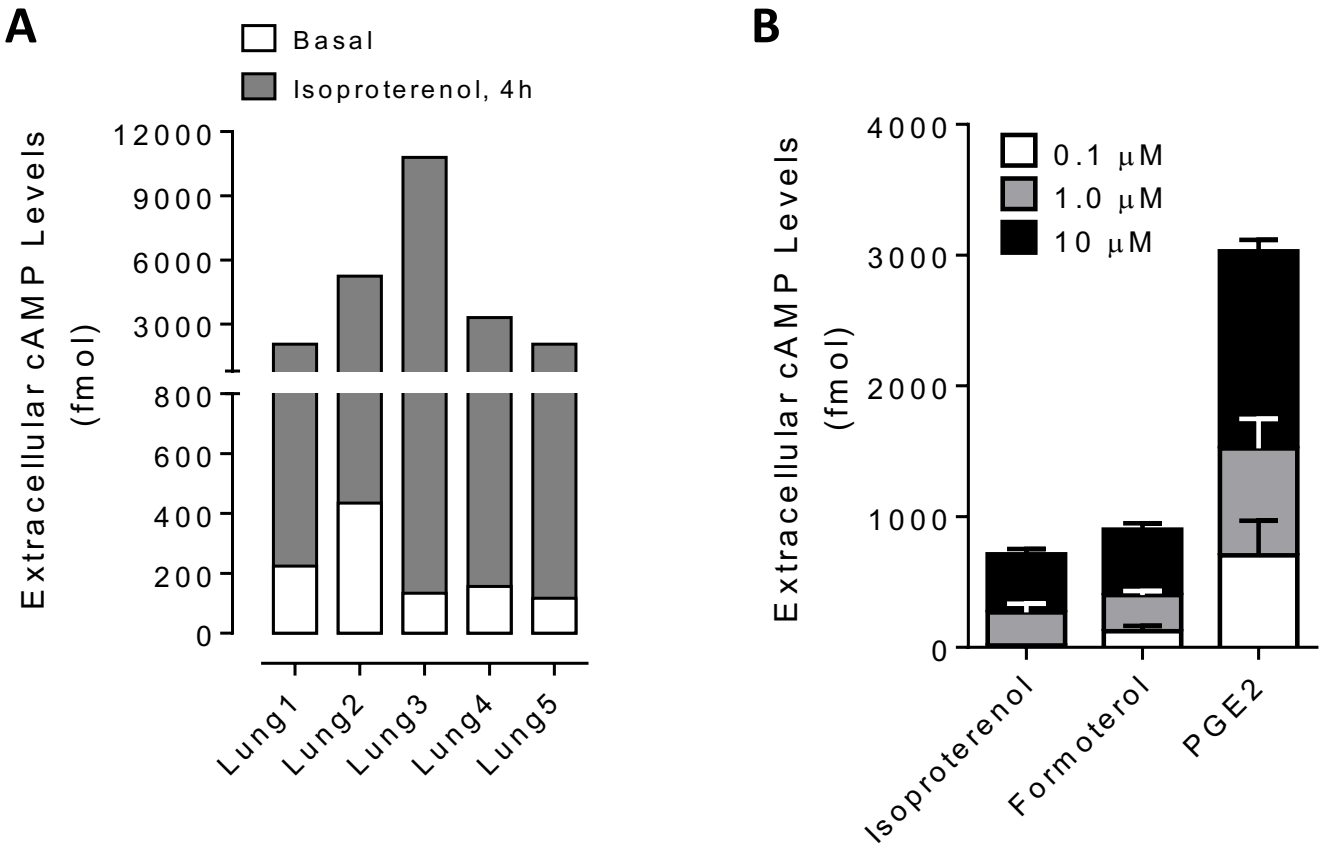


**Fig. S2. Activation of the Gs-coupled family of GPCRs evokes cAMP efflux. (A)** HASM cells derived from 5 lung donors were stimulated with 10μM isoproterenol for 4 hrs. The characteristics of the 5 lung donors are provided in Table S4. For each donor-derived HASM, isoproterenol-induced $[cAMP]_e$ levels were superimposed in relations to basal $[cAMP]_e$ levels. **(B)** Representative dose response of cAMP efflux to isoproterenol, formoterol, and $PGE_2$. For each agonist, $[cAMP]_e$ were detected at 4 hrs, in duplicate (data are stacked in relations to each dose).

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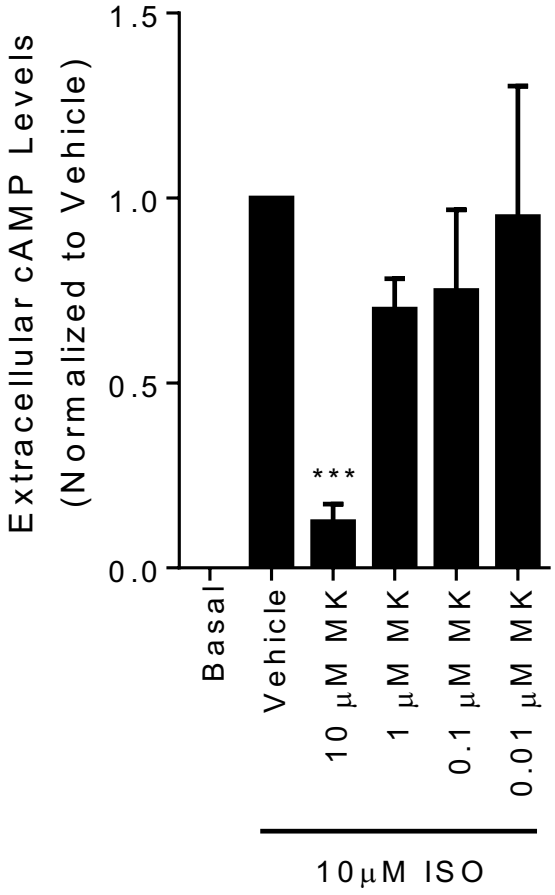


**Fig. S3. β-agonist-induced cAMP egress is inhibited by MK-571.** HASM cells were first treated with or without MK-571 (0, 0.01, 0.1, 1, and 10 μM) for 20 min, and then incubated with 10 μM isoproterenol for additional 4 hrs. Basal, without isoproterenol incubation. Vehicle, without MK-571 treatment. Data are presented as mean ± SE (n=4 independent samples). Analyses were done by one-way ANOVA, followed by Dunnett's multiple comparisons test. ***, $p<0.001$.

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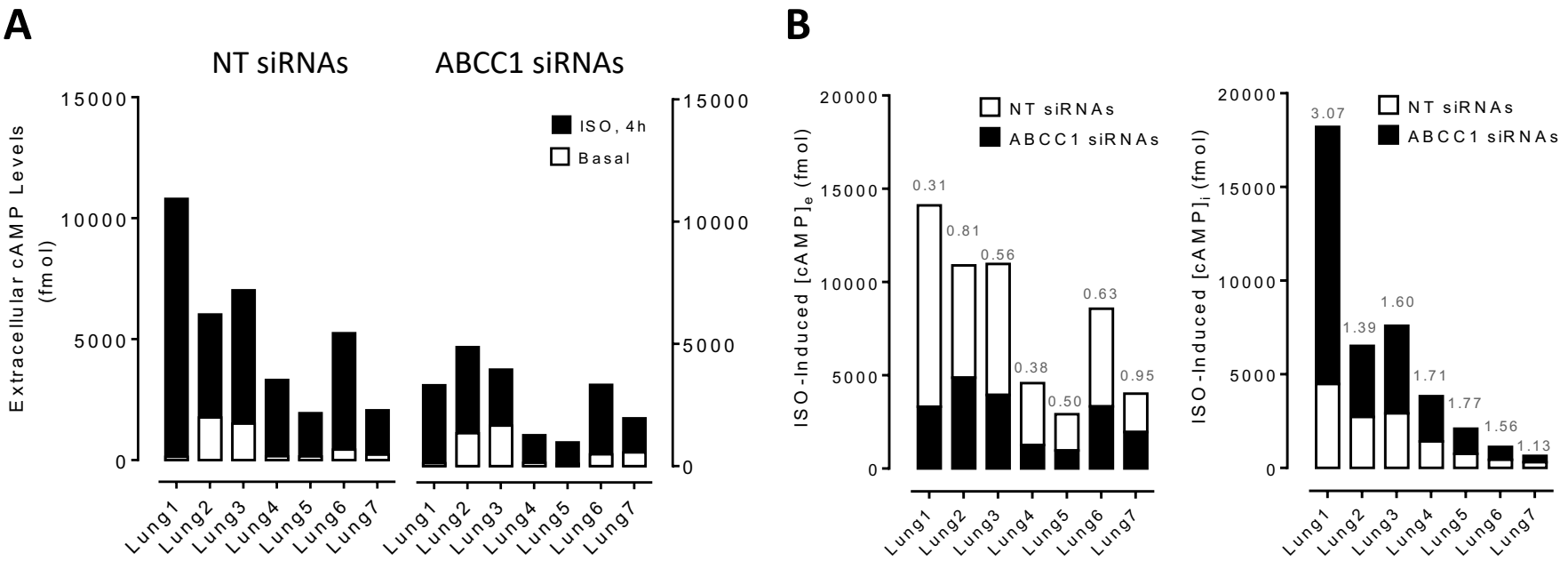


**Fig. S4. β-agonist-induced cAMP egress is mediated by ABCC1 expressed on HASM cells.** (**A-C**) HASM cells derived from individual donor lungs were transfected with *ABCC1* siRNAs or non-targeting (NT) siRNAs (Controls). (**A**) Extracellular cAMP levels ($[cAMP]_e$) were detected by ELISA before and after 4-hr stimulation with 10 μM isoproterenol. Isoproterenol-induced $[cAMP]_e$ are superimposed in relations to basal $[cAMP]_e$. (**B**) Levels of extracellular cAMP after 4-hr isoproterenol stimulation of HASM cells (*ABCC1* silenced vs Control). For each lung donor, isoproterenol-induced $[cAMP]_e$ of *ABCC1* silenced cells are stacked in relations to that of Control cells, with numerical values indicating their ratio. (**C**) Levels of intracellular cAMP after 5-min isoproterenol stimulation in individual HASM cells transfected with *ABCC1* siRNA and non-targeting siRNA. For each lung donor, isoproterenol-induced $[cAMP]_i$ of *ABCC1* silenced cells are stacked in relations to that of Control cells, with numerical values indicating their ratio.

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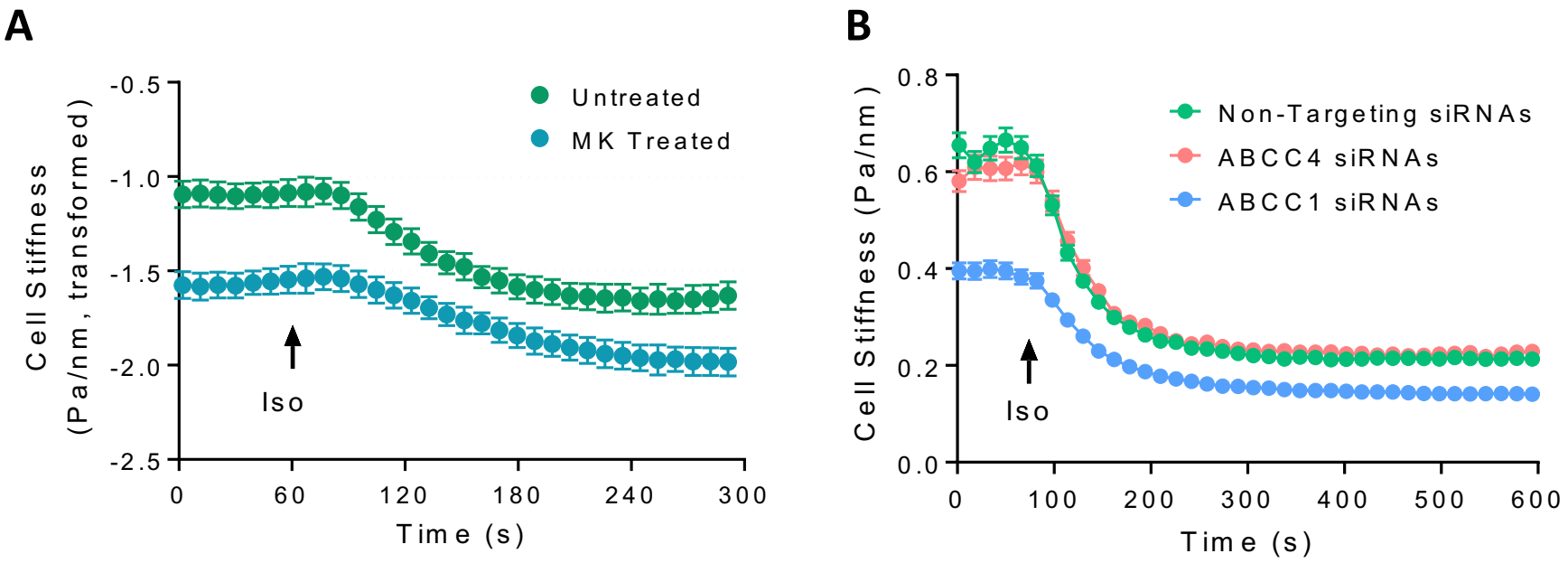


**Fig. S5. Inhibition of ABCC1 promotes β-agonist-induced HASM cellular relaxation.** (**A**) HASM cells derived from 3 lung donors were first treated for 20 min with or without MK-571. MTC was then applied to measure dynamic changes in cell stiffness in response to isoproterenol; isoproterenol was added at t=60s. Data are presented as estimated mean ± SE (n=351-486 individual cell measurements from each donor-derived HASM cells for each condition). (**B**) MTC was applied to measure isoproterenol-induced cellular relaxation in HASM cells transfected with *ABCC1*, *ABCC4*, and non-targeting siRNAs (one donor-derived HASM). Data are presented as mean ± SE (n=465-612 individual cell measurements from three independent experiments with multiple-cell wells transfected with siRNAs).

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