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Bidirectional Counterregulation of Human Lung Mast Cell and Airway Smooth Muscle β2 Adrenoceptors

Rebecca J. Lewis,*1 Latifa Chachi,*1 Chris Newby,* Yassine Amrani,*2 and Peter Bradding*1,4,2

Human lung mast cells (HLMCs) play a central role in asthma pathogenesis through their relocation to the airway smooth muscle (ASM) bundles. β2 adrenoceptor (β2-AR)-agonists are used to relieve bronchoconstriction in asthma, but may reduce asthma control, particularly when used as monotherapy. We hypothesized that HLMC and human ASM cell (HASMC) responsiveness to β2-AR agonists would be attenuated when HLMCs are in contact with HASMCs. Cells were cultured in the presence of the short-acting β2-agonist albuterol, and the long-acting β2-agonists formoterol and olodaterol. Constitutive and FceR1-dependent HLMC histamine release, HASMC contraction, and β2-AR phosphorylation at Tyr589 were assessed. Constitutive HLMC histamine release was increased in HLMC–HASMC coculture and this was enhanced by β2-AR agonists. Inhibition of FceR1-dependent HLMC mediator release by β2-AR agonists was greatly reduced in HLMC–HASMC coculture. These effects were reversed by neutralization of stem cell factor (SCF) or cell adhesion molecule 1 (CADM1). β2-AR agonists did not prevent HASMC contraction when HLMCs were present, but this was reversed by fluticasone. β2-AR phosphorylation at Tyr589 occurred within 5 min in both HLMCs and HASMCs when the cells were cocultured, and was inhibited by neutralizing SCF or CADM1. HLMC interactions with HASMCs via CADM1 and Kit inhibit the potentially beneficial effects of β2-AR agonists on these cells via phosphorylation of the β2-AR. These results may explain the potentially adverse effects of β2-AR agonists when used for asthma therapy. Targeting SCF and CADM1 may enhance β2-AR efficacy, particularly in corticosteroid-resistant patients. The Journal of Immunology, 2016, 196: 55–63.

Asthma is a chronic, and persistent disorder that accounts for significant morbidity and mortality (1–4). Approximately 10% of patients have asthma that is resistant to current therapies (2, 3), and this group consumes 50–60% of health care costs attributed to asthma, reflecting a considerable unmet clinical need.

β2-Adrenoceptor (β2-AR) agonists are an important component of asthma therapy. In fast-acting (rapid-onset) form they are used to provide relief from bronchoconstriction, and in long-acting form are used as preventer medication in conjunction with inhaled corticosteroids (ICS) (5). β2-AR agonists principally target the airway smooth muscle (ASM) to induce bronchodilatation, and confer bronchoprotection against bronchoconstrictor stimuli. However, in many patients the bronchodilator response to a β2-AR agonist is poor (6), and there is a loss of bronchodilator activity during acute asthma exacerbations (7). Many studies have also indicated that the regular use of β2-AR agonists in the absence of an ICS, and sometimes in the presence of an ICS, have deleterious effects in patients with asthma. Thus regular β2-AR agonist use may enhance airway hyperresponsiveness (8–11) and eosinophilic airway inflammation (12, 13), accelerate lung function decline (14), reduce asthma control (15–17), and potentially contribute to asthma deaths in both short-acting and long-acting form (15, 17, 18). The mechanism(s) contributing to a poor therapeutic response and potential adverse effects on airway function are poorly understood (18). Targeting these mechanisms would have the potential to enhance both the efficacy and safety of β2-AR agonists in many patients.

Human lung mast cells (HLMCs) are fundamental to asthma pathogenesis (19). Importantly in asthma, increased mast cell numbers are found in the ASM bundles (20) where they are activated (21) and have the potential to interact intimately with ASM cells (22–25). Human ASM cells (HASMCs) maintain HLMC survival and induce HLMC proliferation through a co-operative interaction between membrane-bound stem cell factor (SCF) on HLMCs, and the SCF receptor Kit and cell adhesion molecule 1 (CADM1) expressed on HASMCs (23). CADM1 is a key molecule that facilitates initial HLMC–HASMC adhesion, and may present Kit to membrane-bound SCF (23, 26). HASMCs increase “constitutive” HLMC degranulation and the release of mediators such as histamine and tryptase (23). Tryptase release induces HASMC...
Tyr350 (31). In parallel, there was a dose-dependent increase in unexplained.

\[ b \]

flow cytometry at passage 2 to confirm a smooth muscle phenotype.

were examined for the expression of cell types to grow until 50% confluent and then passaged into T75 flasks. All cultures were supplemented with 10% FCS (Invitrogen), 1% antibiotic-antimycotic (con- 

\[ \text{Tyr350} \] (Santa Cruz Biotechnology, Santa Cruz, CA); purified rabbit IgG were transferred/selenium (ITS) supplement (Sigma-Aldrich).

\[ \text{Tyr350} \]

olodaterol (synthesized at BoehringerIngelheim); and Insulin/insulin (0.1% H2O for albuterol and 0.1% DMSO for formoterol and olodaterol). HLMC monocultures were set up in parallel in ITS medium. No exoge-

\[ \text{Tyr350} \]

muscle actin expression and contractility (24).

such a response potentially explains the lack of efficacy and loss of asthma control sometimes encountered with chronically administered \( \beta_2 \)-AR agonists.

In view of the above, we hypothesized that HASCs would impair HLMC sensitivity to \( \beta_2 \)-AR agonists through an SCF- dependent mechanism. We have therefore investigated the con-

\[ \beta_2 \-AR \]

2-AR agonist exposure.

Materials and Methods

Human subjects

All subjects were recruited from the Glenfield Hospital (Leicester, U.K.) and gave written informed consent. The use of lung resection tissue was approved by the National Research Ethics Service (reference number 07/ MRE08/42), and bronchoscopic studies were approved by the Leicester- 

\[ \text{Tyr350} \]

ing microscope. The small smooth muscle bundles were individually 

\[ \text{Tyr350} \]

from each sample using a glass knife and col-

\[ \text{Tyr350} \]

sation over 16 h at 60˚C. Polymerized samples were cut into 5-mm squares for freezing. The Transwells were fixed in 1% osmium tetraoxide for 90 min at room 

\[ \text{Tyr350} \]

sodium pyruvate, antibiotic/antimycotic, and DMSO (Sigma-Aldrich, 

\[ \text{Tyr350} \]

Materials

The following items were obtained from the sources indicated: sodium bicarbonate (7.5%; Life Technologies); collagen (3 mg/ml; INAMED Biomaterials); salbutamol hemisulfate salt, formoterol fumarate dihydrate, sodium pyruvate, antibiotic/antimycotic, and DMSO (Sigma-Aldrich, Poole, U.K.); DMEM, 10× DMEM, nonessential amino acids, Dyna-

\[ \text{Tyr350} \]

sections of HLMC–HASMC coculture on the responses of both cell types to \( \beta_2 \)-AR agonist exposure.

THE TRANSWELLS WERE IMMERSED IN ITS ASM MEDIUM OR EPITHELIAL CELL MEDIUM (BRONCHIAL EPITHELIAL CELL BASAL MEDIUM [Lonza, Basel, Switzerland], respectively, and incubated overnight at 37˚C. HLMCs (25,000) were then seeded onto HASC/EC or epithelial cell monolayers and incubated in ITS or epithelial cell medium, respectively. HLMC monocultures were set up on fibronectin-coated (40 

\[ \text{Tyr350} \]

Histamine was measured by radioenzymatic assay as described previously (33).

Assessment of HLMC morphology using electron microscopy

HASCs and undifferentiated primary bronchial epithelial cells were plated at a density of 100,000 cells on 0.4-μm pore-sized Transwells membranes (Corning) in duplicate. Transwells were immersed in ITS ASM medium or epithelial cell medium (bronchial epithelial cell basa 

\[ \text{Tyr350} \]

Collagen gel contraction assay

Spontaneous HASC contraction was examined in collagen gels as described previously (24, 25). For each individual collagen gel, 20 μl sodium bicarbonate, 37 μl 10× concentrated DMEM, and 144 μl cell suspension
(2.5 × 10⁵ cells resuspended in ITS medium either HASM alone or HLMC–HASM cocultures incubated together at a 1:4 ratio for 15 min at 37°C before casting of gels) were added to 299 μl collagen. The resulting suspension was then cast in a single well of a 24-well plate, which had been precoated with PBS/2% BSA for 1 h at room temperature. Collagen gels were left to polymerize for 90 min at 37°C.

After polymerization, gels were detached from the well surface to allow free contraction. ITS medium, appropriate vehicle control, salbutamol (10⁻⁹ and 10⁻⁶ M), formoterol (10⁻⁷ M), or olodaterol (10⁻⁹ M) was added to appropriate wells and the gels incubated for 16 h at 37°C. Photographs were taken at 0 and 16 h, and percentage gel contraction was assessed by measuring the collagen gel surface area at each time point using ImageJ software (http://rsbweb.nih.gov/ij/, n = 5 independent experiments).

Assessment of β₂-AR phosphorylation in cocultured HLMCs

HASMCs were seeded onto wells of a Lab-Tek II 8-well chamber slide (Nunc) at a density of 40,000 cells/well in ASM medium and incubated for 24 h at 37°C. After 24 h, ASM cells were washed with HBSS (Invitrogen) and growth-arrested in ITS medium for an additional 24 h at 37°C. HLMCs were then seeded onto the growth-arrested ASM monolayers at a ratio of 1:4. Cells were cocultured together for 5 min at 37°C before fixation. HLMC monolayers in ITS were set up in parallel on chambers precoated with fibronectin (40 μg/ml; Sigma-Aldrich). HASMC monocultures served as further control. Neutralizing anti-SCF, anti-CADM1, and isotype control Abs were added for 15 min prior where appropriate.

Assessment of β₂-AR phosphorylation at Tyr³⁵⁰ was analyzed using immunofluorescent staining. The anti–β₂-AR-Tyr³⁵⁰ Ab was previously validated by Western blotting and flow cytometry (31). After incubation at 37°C, all chambers were rinsed with 200 μl PBS (Invitrogen), and the cells were fixed with 200 μl ice-cold methanol (Fisher Scientific) on ice for 20 min. Methanol was then removed from chambers, which were left to air-dry for 10 min at room temperature. Once dry, 200 μl PBS/3% BSA was added to each well for 30 min at room temperature, then removed, and followed by 200 μl appropriate primary anti–β₂-AR-Tyr³⁵⁰ Ab (4 μg/ml; Santa Cruz Biotechnology) or isotype control (rabbit IgG, 4 μg/ml; DakoCytomation) for 90 min at room temperature.

Chambers were then rinsed three times with PBS/0.05% Tween-20 before the addition of 200 μl secondary Ab (swine anti-rabbit, 25 μg/ml, Dako) for 90 min at room temperature. Chambers were rinsed three times with PBS/0.05% Tween-20 and then further three times with PBS. After rinsing, 200 μl DAPI (0.1 μg/ml; Sigma-Aldrich) was added to all chambers for 30 s, followed by six washes with PBS. Chambers were then removed from slides, which were mounted with prolong anti-fade medium (Invitrogen) and coverslipped for assessment via fluorescence microscopy.

Original images were captured using an epifluorescent microscope (Olympus BX50, Olympus U.K.) and analyzed using Cell F image analysis software (Olympus). Microscope settings were kept constant throughout and for each experiment matched exposures were used for isotype controls. Images were converted to grayscale and mean grayscale area values indicative of β₂-AR phosphorylation at Tyr³⁵⁰ in HLMC monoculture, ASM monoculture or in HLMC–HASMC coculture were obtained for each donor. HLMCs and HASMCs were readily identified by cellular and nuclear morphology. A minimum of 10 HLMCs and 10 HASMCs in two random high-power fields were assessed per condition in five independent experiments.

Immunostaining for tryptase and α-smooth muscle actin was included as positive controls.

**Statistical analysis**

Data are expressed as mean ± SEM unless otherwise stated. Data were tested for normality and compared using paired or unpaired parametric or nonparametric statistical tests. Significance was assessed using one-way ANOVA with Bonferroni’s post-hoc test. Data are expressed as mean ± SEM. **p < 0.01 compared with respective monoculture (Sidak’s multiple comparison test (MCT)).***p < 0.001 compared with respective monoculture (Sidak’s MCT).##p < 0.0001 compared with respective monoculture (Sidak’s MCT).###p < 0.0001 compared with DMSO monoculture (Sidak’s MCT).###p < 0.0001 compared with H2O or DMSO coculture control (Sidak’s MCT).‡p < 0.05, **p < 0.01 compared with isotype control (Sidak’s MCT). n = 7 independent experiments for all graphs.

![Figure 1](http://www.jimmunol.org/) Constitutive histamine release in HLMC–HASMC coculture and its regulation by SCF, CADM1 and β₂-AR agonists. (A) Constitutive HLMC histamine release was significantly enhanced in coculture with HASMCs, and this was inhibited marginally by blocking SCF but not CADM1. p = 0.002 by repeated measures ANOVA, *p < 0.05, **p < 0.01 compared with HLMC monoculture (Dunnett’s multiple comparison test). *p < 0.0001 compared with respective monoculture (Sidak’s MCT). (B) Constitutive HLMC histamine release was enhanced further in coculture with HASMCs by β₂-AR agonists. p < 0.0001 compared with respective monoculture (Sidak’s MCT). (C and D) The enhancing effect of β₂-AR agonists in coculture was attenuated by blocking either SCF (C) or CADM1 (D). *p < 0.05, **p < 0.01 compared with isotype control (Sidak’s MCT). n = 7 independent experiments for all graphs.
nonparametric tests as appropriate. Across group differences were examined using ANOVA with selected post hoc testing for individual comparisons as appropriate. For analysis of β2AR agonist dose response in monoculture and coculture, two-way ANOVA was used. A p value < 0.05 was taken as statistically significant.

Results

HLMC and HASMC responses were similar irrespective of the source of HASMCs or their disease status. The following experiments contain HASMCs from both asthmatic and healthy donors.

**HLMC–HASMC coculture enhances constitutive HLMC mediator release**

Constitutive histamine release over 16 h was consistently greater in HLMC–HASMC coculture compared with HLMC monoculture (Fig. 1A, 1B). This was also evident using electron microscopy to study mast cell morphology, which demonstrated significant piecemeal degranulation in HLMC–HASMC coculture compared with HLMC monoculture or HLMC–human airway epithelial cell coculture (Supplemental Fig. 1A). HASMC-dependent HLMC histamine release was inhibited marginally by neutralizing SCF when compared with goat isotype control (p = 0.023; Fig. 1A) but not by neutralizing CADM1 (Fig. 1A).

**β2-AR agonists enhance HASMC-dependent HLMC histamine release through an SCF- and CADM1-dependent mechanism**

All β2-AR agonists tested significantly increased the enhanced constitutive histamine release seen in HLMC–HASMC coculture when compared with vehicle control (p = 0.0041 albuterol, p ≤ 0.0001 formoterol, p = 0.035 olodaterol) (Fig. 1B). This was particularly evident for formoterol, which increased histamine release in coculture significantly more than olodaterol (p < 0.0001) and which also significantly enhanced constitutive histamine release in HLMC monoculture compared with both DMSO control and olodaterol (p < 0.0001 for both). However, the net formoterol-dependent increase in constitutive histamine release was significantly greater in HLMC–HASMC coculture than HLMC monoculture (p = 0.001). Albuterol and olodaterol did not significantly increase constitutive release by HLMCs in monoculture.

Neutralizing either SCF or CADM1 significantly reduced β2-agonist-dependent increases in histamine release from HLMC-HASMC cocultures for all β2-agonists tested (Fig. 1C, 1D). Blocking SCF or CADM1 did not alter constitutive degranulation in monoculture.

**HLMC-HASMC coculture reduces β2-AR responsiveness in FcεRI-activated HLMCs through an SCF- and CADM1-dependent mechanism**

The effect of HLMC-HASMC coculture for 16 h on the responsiveness of FcεRI-activated HLMCs to olodaterol, formoterol, and albuterol was assessed (Fig. 2A). Net mean ± SEM FcεRI-dependent histamine release by monocultured HLMCs in buffer, 0.1% H2O, and 0.1% DMSO vehicle controls was 17.5 ± 3.6, 16.7 ± 3.7, and 16.1 ± 4.7%, respectively. In HLMC–HASMC coculture, net mean ± SEM FcεRI-dependent histamine release in buffer, 0.1% H2O, and 0.1% DMSO vehicle controls was 14.6 ± 4.1, 16.3 ± 5.2, and 13.1 ± 3.4%, respectively (not significantly different compared with monoculture). Total cell histamine content after overnight culture prior to activation with anti-FcεRIα was 1691 ± 345 ng/10^6 cells (monoculture) and 1786 ± 335 ng/10^6 cells (coculture).

In HLMC monoculture, all three β2-agonists tested demonstrated dose-dependent inhibition of FcεRI-dependent histamine release (p = 0.043, two-way ANOVA) (Fig. 2A). However, the ability of albuterol, formoterol, and olodaterol to inhibit FcεRI-dependent histamine release was significantly attenuated when HLMCs were cocultured with HASMCs (p < 0.0001, two-way ANOVA) (Fig. 2A).

In further experiments, we assessed whether neutralizing SCF and CADM1 in coculture could restore the responsiveness of HLMCs to β2-ARs. Following FcεRI-dependent activation, the mean percent inhibition using albuterol (10⁻⁸ M), formoterol (10⁻⁹ M), and olodaterol (10⁻⁹ M) in HLMC monoculture was 70.8 ± 7.6, 81.2 ± 6.2, and 91.0 ± 3.1%, respectively (Fig. 2B). This inhibition was significantly reduced in HLMC–HASMC coculture.
coclure to 6.5 ± 4.2, 2.1 ± 1.8, and 8.1±5.3%, respectively (p < 0.0001 by ANOVA and p = 0.0005, p = 0.0006, p = 0.0007 for monoculture versus coculture for each drug respectively, Sidak’s MCT; Fig. 2B). SCF neutralizing Ab significantly improved the responsiveness of cocultured HLMCs to albuterol, formoterol, and olodaterol, increasing agonist-induced inhibition of FcεRI-dependent histamine release to 47.6 ± 5.6, 60.08 ± 5.8, and 75.4 ± 8.4%, respectively (p = 0.0036, p = 0.0015, p = 0.0023, respectively, compared with isotype control, Sidak’s MCT; Fig. 2B).

In separate CADM1 neutralization experiments, the mean percent inhibition of FcεRI-dependent histamine release by albuterol (10⁻⁵ M), formoterol (10⁻⁶ M), and olodaterol (10⁻⁷ M) in HLMC monoculture was 32.7 ± 14.1, 70.5 ± 10.1, and 81.6 ± 7.5%, respectively (Fig. 2C). Again, the ability of β₂-agonists to inhibit FcεRI-mediated histamine release was significantly reduced in HLMC–HASMC coculture (Fig. 2C). CADM1 neutralization improved β₂-AR responsiveness to formoterol and olodaterol but not albuterol (Fig. 2C).

**FIGURE 3.** HLMC–HASMC coculture increases phosphorylation of the β₂-AR at Tyr³⁵⁰. (A) Representative immunofluorescent staining for p-β₂-AR (Tyr³⁵⁰) in HLMC monoculture, HASMC monoculture, and HLMC–HASMC coculture, n = 5 independent experiments. p < 0.001 by repeated measures ANOVA. *p < 0.05, **p < 0.01 compared with respective monoculture (Sidak’s MCT). **p < 0.01 compared with HASMCs in coculture (Sidak’s MCT).

**FIGURE 4.** SCF and CADM1 neutralization prevents phosphorylation of the β₂-AR at Tyr³⁵⁰. (A) Representative immunofluorescent staining for p-β₂-AR (Tyr³⁵⁰) in HLMCs and HASMCs in coculture and its attenuation by SCF and CADM1 neutralization. (B and C) Quantification of p-β₂-AR (Tyr³⁵⁰) expression in HLMCs and HASMCs in coculture and the effect of SCF (B) and CADM1 (C) neutralization, n = 5 independent experiments. p < 0.0001, p = 0.0002, respectively, by repeated measures ANOVA. *p < 0.05, **p < 0.01 compared with respective isotype control (Sidak’s MCT).
The $\beta_2$-AR is phosphorylated at Tyr$^{350}$ in HLMC–HASMC coculture

Phosphorylation of the $\beta_2$-AR at Tyr$^{350}$ by receptor tyrosine kinases (RTKs) including Kit results in receptor uncoupling and internalization (31, 36). We therefore examined whether $\beta_2$-AR phosphorylation at Tyr$^{350}$ occurs in HLMC-HASMC coculture using immunofluorescent staining.

Minimal p-$\beta_2$-AR (Tyr$^{350}$) expression was detectable in HLMC and HASMC monocultures (Fig. 3A, 3B). However, p-$\beta_2$-AR (Tyr$^{350}$) expression was significantly enhanced in both HASMCs and HLMCs following 5 min of coculture (Fig. 3A, 3B). However, p-$\beta_2$-AR (Tyr$^{350}$) expression was significantly greater in cocultured HLMCs than in cocultured HASMCs (Fig. 3B). Isotype control staining was negative.

SCF and CADM1 neutralization inhibits phosphorylation of the $\beta_2$-AR at Tyr$^{350}$ in HLMC-HASMC coculture

Because neutralizing SCF or CADM1 restored $\beta_2$-AR function in HLMCs cultured with HASMCs, we examined the effect of SCF and CADM1 neutralization on phosphorylation of the $\beta_2$-AR at Tyr$^{350}$ in HLMC-HASMC coculture (Fig. 4). $\beta_2$-AR phosphorylation at Tyr$^{350}$ was again evident in both HLMCs and HASMCs following 5-min coculture (Fig. 4). Phosphorylation was also evident in HLMC–HASMC cocultures incubated for 5 min with appropriate isotype control Abs (Fig. 4). However, incubation of HLMC–HASMC cocultures with either anti-SCF or anti-CADM1 Ab significantly reduced $\beta_2$-AR phosphorylation at Tyr$^{350}$ in both HLMCs and HASMCs (Fig. 4).

HLMC–HASMC coculture reduces $\beta_2$-AR responsiveness in primary HASMCs

Because HASMCs in coculture with HLMCs also demonstrated phosphorylation of the $\beta_2$-AR at Tyr$^{350}$, we assessed whether HLMC–HASMC coculture also impairs $\beta_2$-AR responsiveness in HASMCs. For this, we used the collagen gel contraction assay, a recognized in vitro model that is useful for examining the mechanisms of cellular cytoskeletal reorganization and stress fiber formation (24).

**FIGURE 5.** $\beta_2$-AR agonists do not inhibit spontaneous HASMC contraction in HLMC–HASMC coculture. (A) $\beta_2$-AR agonists attenuated the spontaneous contraction of collagen gels containing HASMCs alone. *$p < 0.05$, **$p < 0.01$ compared with respective controls (paired $t$ tests). (B) $\beta_2$-AR agonists increased the contraction of collagen gels containing both HLMCs and HASMCs. *$p < 0.05$, **$p < 0.01$ compared with respective controls (paired $t$ tests). (C) The net change (drug minus vehicle control) in gel contraction with $\beta_2$-AR agonist exposure for monocyte contration compared with coculture. $n = 5$ independent experiments for albuterol and formoterol, $n = 4$ for olodaterol. *$p = 0.01$, **$p = 0.006$, ***$p = 0.0006$ compared with monoculture (paired $t$ tests). (D) Initial experiments ($n = 2$) investigating the effects of cetirizine and leupeptin on HASMC contraction in coculture showed that in the presence of formoterol, both reduced coculture contraction to values seen in monoculture. (E) Further experiments ($n = 4$) showed that there was similar inhibition with cetirizine and leupeptin alone, with no interaction between formoterol and either cetirizine or leupeptin.
Incubation of HASMC-embedded collagen gels for 16 h with 10^{-8} M albuterol, 10^{-7} M formoterol, and 10^{-3} M olodaterol reduced spontaneous gel contraction compared with vehicle controls (p = 0.041 [log transformed], p = 0.002, p = 0.011, respectively; Fig. 5A, Supplemental Fig. 2). In contrast, incubation of HLMC–HASMC-embedded gels with β_{2}-AR agonists failed to inhibit spontaneous gel contraction compared with vehicle controls. In the experiments involving all three β_{2}-AR agonists studied in parallel, there was some enhancement of spontaneous gel contraction (Fig. 5B). In this study, for all β_{2}-AR agonists tested, there was a significant difference in the change in spontaneous gel contraction (compared with relevant vehicle control) between gels embedded with HASMCs alone and those embedded with HLMCs plus HASMCs (Fig. 5C). In further experiments using formoterol to study the effects of the H1 receptor blocker cetirizine and the tryptase inhibitor leupeptin on coculture contraction, formoterol failed to inhibit contraction (Fig. 5D, 5E). Both cetirizine and leupeptin markedly inhibited background contraction in coculture (Fig. 5D, 5E), but not monoculture (not shown), and no additive effect of formoterol in coculture was evident.

Corticosteroids restore β_{2}-AR responsiveness in HASMCs in the presence of HLMCs

The corticosteroid fluticasonepropionate did not affect HASMC contraction in collagen gels on its own, but restored the ability of β_{2}-AR agonists to attenuate spontaneous HASMC contraction in the presence of HLMCs, and to values similar to those seen in HASMC monoculture (Fig. 6, Supplemental Fig. 3).

Discussion

Infiltration of ASM bundles by activated mast cells is a key pathological feature of asthma (20, 21, 37), occurring across inflammatory phenotypes and the spectrum of asthma severity (38–40). This study demonstrates that when HLMCs and HASMCs are cultured together in vitro, HASMCs induce mast cell degranulation [as described previously (23)], and this is enhanced in the presence of β_{2}-AR agonists. Furthermore, HLMCs are less susceptible to inhibition of degranulation by β_{2}-AR agonists following FceRI-dependent activation. These changes in β_{2}-AR agonist responsiveness in HLMCs were associated with phosphorylation of the β_{2}-AR at Tyr^{350}, a process which uncouples the β_{2}-AR and promotes its internalization (36, 41). Surprisingly, HLMC–HASMC coculture also induced phosphorylation of the β_{2}-AR at Tyr^{350} in HASMCs, with loss of protection by β_{2}-ARs on HASMC contraction (summarized in Supplemental Fig. 4).

Cross-talk between GPCRs such as the β_{2}-AR and RTKs is well described, particularly for insulin, which acts via its receptor to rapidly inhibit β_{2}-AR function (36, 41). This occurs through phosphorylation at Tyr^{350}, which uncouples the β_{2}-AR and also promotes β_{2}-AR sequestration through the creation of an SH2 binding site to which molecules such as Src and Grb2 bind to induce β_{2}-AR internalization (41). SCF, acting via its RTK Kit, exerted similar effects on the β_{2}-AR in monocultured HASMCs previously (31). In consequence, β_{2}-AR agonists no longer inhibited FceRI-dependent HLMC degranulation or leukotriene C_{4} production (31) and actually enhanced HLMC histamine release in the presence of SCF and monomeric IgE (31).

The effects of SCF on HLMC β_{2}-AR agonist responses described previously are very similar those found here when HLMCs were cocultured with HASMCs. Indeed, the loss of β_{2}-AR responsiveness, the enhancement of histamine release by β_{2}-AR agonists and the Tyr^{350} phosphorylation induced by HASMCs was reversed largely by neutralizing either SCF or CADM1. CADM1 interacts cooperatively with Kit and likely orientates it with membrane-bound SCF at points of cell–cell adhesion (23, 26). Thus, both Kit and CADM1 or their ligands are potential targets that might enhance β_{2}-AR responsiveness in HLMCs and HASMCs and attenuate the potential proteocerebro effect of β_{2}-AR agonists.

Perhaps surprisingly, HLMC–HASMC coculture also lead to rapid phosphorylation of the β_{2}-AR at Tyr^{350} in HASMCs. This was also dependent on SCF and CADM1, suggesting that release of a mast cell–derived product(s) stimulated by cell contact is involved. This would probably need to be present preformed and could include one of a number of mediators including histamine, tryptase, TGF-β1, basicFGF and adenosine nucleotides. Alternatively, if Kit docking is also important for CADM1 function, then a signal delivered via the CADM1 counterreceptor on HASMCs, most likely nectin-3, might also play a role (42). Additional work is required to elucidate the mechanism(s) at work. However, the consequences of this β_{2}-AR phosphorylation in HASMCs appear functionally important in that β_{2}-AR agonists are no longer able to prevent HASMC contraction in the presence of cocultured HLMCs.

The ability of β_{2}-AR agonists to enhance constitutive histamine release in coculture is interesting but not easily explained. A po-

**FIGURE 6.** Fluticasone restores β_{2}-AR function in HLMC–HASMC coculture. (A) In HASMC monoculture, fluticasone (10^{-7} M) did not alter HASMC contraction, and the fluticasone/β_{2}-AR(10^{-7} M) combination inhibited spontaneous HASMC contraction. *p < 0.05 compared with DMSO control (paired t tests). (B) In HLMC–HASMC coculture, fluticasone (10^{-7} M) did not alter spontaneous gel contraction, but β_{2}-ARs (10^{-7} M) in combination with fluticasone were able to reduce spontaneous gel contraction. *p < 0.05. ***p < 0.001 compared with DMSO control (paired t tests). (C) The net change in gel contraction (drugs minus 0.1% DMSO control) in HLMC–HASMC coculture for formoterol alone (n = 5), olodaterol alone (n = 4) (data from Fig. 5C), or the combination of formoterol or olodaterol with fluticasone (n = 6 for each, data calculated from B). ***p < 0.001, ****p < 0.0001 compared with respective combination, unpaired t tests.
tential mechanism whereby β2-agonists increase constitutive HLMC mediator release in coculture is through interference with HLMC organic cation transporters (43, 44). These transporters are inhibited by albuterol and formoterol, and this may result in reduced histamine reuptake (43). However, perhaps with the exception of formoterol, a second and relatively mild stimulus is also required for the potentiating effects of β2-AR agonists to become evident. Previously, both SCF and monomeric IgE were required, although in this study, a consistent effect across all β2-AR agonists was only evident when a stimulus from HASMCs was present. Alternative mechanisms must therefore be considered, and there are potential parallels with the paradoxical effects of β2-AR agonists in other cell types. For example, in HASMCs, β2-AR agonists enhance HASMC contraction though the inhibition of regulator of G protein signaling 5 (45). In airway epithelial cells, the β2-AR agonists salmeterol and albuterol enhanced both rhinovirus- and IL-1β-induced IL-6 production, through a cAMP-dependent mechanism which potentiated rhinovirus-induced IL-6 promoter activation (46). Additional work is clearly required to delineate the potential mechanisms behind the augmentation of HLMC histamine release by β2-AR agonists.

Caution is always required in extrapolating in vitro findings to the in vivo clinical situation, but we believe that our results have potentially important clinical implications and provide a plausible mechanistic explanation for many of the paradoxical effects of β2-AR agonists observed in human asthma. Many papers have described potential adverse effects of β2-AR agonists in asthma, and examples are highlighted in the introduction to this manuscript. Of particular note, when short-acting β2-agonists (SABAs) or long-acting β2-agonists (LABAs) are given regularly in the absence of an ICS, mast degranulation and the early-phase bronchoconstrictor response following allergen challenge or exercise are increased, implying that not only is there a loss of protection on mast cell degranulation but also loss of inhibition of ASM contraction (9, 10, 30, 47). These latter observations are similar to those we have observed in HLMC–HASMC coculture. We therefore propose that the regular use of β2-AR agonists in the absence of an ICS may in some patients enhance HLMC mediator release within the ASM bundles, which in turn enhances airway hyperresponsiveness and uncouples the HASMC β2-ARs. Thus background asthma control would likely deteriorate, and with a superimposed airway insult, for example during viral infection or allergen exposure, the subsequent exacerbation might be worse.

Treatment guidelines for asthma recommend that LABAs are never used in the absence of an ICS, yet it is still considered reasonable to use SABAs in isolation with perceived mild disease. However, a significant number of patients with so-called mild disease still die of asthma (48). In addition, many patients with asthma of all severities adhere poorly to corticosteroid therapy and are overreliant on SABAs for symptom relief (49, 50). Importantly, in this study, we found that the corticosteroid fluticasone propionate restored the protective effects of β2-AR agonists on HASMC contraction in the presence of HLMCs. We believe this reinforces the view put forward by others (18), that all β2-AR agonist inhalers used for asthma, whether SABAs or LABAs, should contain an ICS. However, in patients with relative corticosteroid resistance, targeting the pathways identified through the inhibition of SCF-Kit or CADM1-nectin 3, has the potential to reverse this β2-AR deregulation, and thus enhance both the efficacy and safety of β2-AR agonists.

Disclosures
The authors have no financial conflicts of interest.

References


A) Piecemeal degranulation was evident in HLMCs in co-culture with HASMCs but not control media or in co-culture with human airway epithelial cells when viewed by electron microscopy. Scale bar = 2 μm. B) Quantification of HLMC piecemeal degranulation in monoculture and HASMC or epithelial cell co-culture. p=0.0006 by ANOVA, *p=0.0002 compared to HLMCs alone in HASMC media, #p=0.004 compared to HLMC-ep.cell co-culture (Sidak’s multiple comparison test). Mean±SEM for 10 HLMCs per condition, from 3 HLMC donors.
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Representative images of collagen gel contraction under the conditions indicated.
Figure E3

Representative images of collagen gel contraction under the conditions indicated.
A) When HASMCs and HLMCs are not in contact, β₂-AR stimulation induces relaxation and prevents contraction of HASMCs, and prevents FcεRI-dependent HLMC degranulation. B) When HASMCs and HLMCs are engaged through CADM1 and Kit and their respective HASMC ligands, phosphorylation of the β₂-AR at Tyr350 by Kit enhances constitutive HLMC mediator release on β₂-AR agonist exposure, and attenuates the inhibitory effect of β₂-ARs on FcεRI-dependent HLMC degranulation. Simultaneously, phosphorylation of the β₂-AR at Tyr350 in HASMCs through an unknown mechanism prevents HASMC relaxation on β₂-AR agonist exposure.