

RESEARCH ARTICLE

Protease-activated receptor-2 signaling through β -arrestin-2 mediates *Alternaria* alkaline serine protease-induced airway inflammation

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Yee MC, Nichols HL, Polley D, Saifeddine M, Pal K, Lee K, Wilson EH, Daines MO, Hollenberg MD, Boitano S, DeFea KA. Protease-activated receptor-2 signaling through β -arrestin-2 mediates *Alternaria* alkaline serine protease-induced airway inflammation. *Am J Physiol Lung Cell Mol Physiol* 315: L1042–L1057, 2018. First published October 18, 2018; doi:10.1152/ajplung.00196.2018.—*Alternaria alternata* is a fungal allergen associated with severe asthma and asthma exacerbations. Similarly to other asthma-associated allergens, *Alternaria* secretes a serine-like trypsin protease(s) that is thought to act through the G protein-coupled receptor protease-activated receptor-2 (PAR₂) to induce asthma symptoms. However, specific mechanisms underlying *Alternaria*-induced PAR₂ activation and signaling remain ill-defined. We sought to determine whether *Alternaria*-induced PAR₂ signaling contributed to asthma symptoms via a PAR₂/ β -arrestin signaling axis, identify the protease activity responsible for PAR₂ signaling, and determine whether protease activity was sufficient for *Alternaria*-induced asthma symptoms in animal models. We initially used in vitro models to demonstrate *Alternaria*-induced PAR₂/ β -arrestin-2 signaling. *Alternaria* filtrates were then used to sensitize and challenge wild-type, PAR₂^{−/−} and β -arrestin-2^{−/−} mice in vivo. Intranasal administration of *Alternaria* filtrate resulted in a protease-dependent increase of airway inflammation and mucin production in wild-type but not PAR₂^{−/−} or β -arrestin-2^{−/−} mice. Protease was isolated from *Alternaria* preparations, and select in vitro and in vivo experiments were repeated to evaluate sufficiency of the isolated *Alternaria* protease to induce asthma phenotype. Administration of a single isolated serine protease from *Alternaria*, *Alternaria* alkaline serine protease (AASP), was sufficient to fully activate PAR₂ signaling and induce β -arrestin-2^{−/−}-dependent eosinophil and lymphocyte recruitment in vivo. In conclusion, *Alternaria* filtrates induce airway inflammation and mucus hyperplasia largely via AASP using the PAR₂/ β -arrestin signaling axis. Thus, β -arrestin-biased PAR₂ antagonists represent novel therapeutic targets for treating aeroallergen-induced asthma.

airway inflammation; allergen-induced asthma; *alternaria alternata*; β -arrestin-signaling; biased G protein-coupled receptor signaling; protease-activated receptor-2

INTRODUCTION

Fungal exposure has long been linked to respiratory disease, including allergy and asthma (65). *Alternaria alternata* is a ubiquitous fungal allergen that has been associated with severe asthma and has been identified as a risk factor for the development and persistence of asthma (27, 36, 51, 55). Although most reports of *Alternaria*-associated asthma focus on outdoor exposure, including an increase in asthma exacerbations during thunderstorms (e.g., see Ref. 51), indoor exposure to *Alternaria* has also been associated with increased asthma symptoms (55). More than a dozen IgE-reacting proteins have been isolated from *Alternaria*, including the major antigen Alt a 1 (10, 22); however these proteins alone do not sufficiently explain the contribution of *Alternaria* to asthma symptoms and disease.

In addition to allergenic proteins, *Alternaria* secretes proteases that contribute to airway inflammation (8, 58). These heretofore unidentified proteases are thought to act through the G protein-coupled receptor (GPCR) protease-activated-receptor-2 (PAR₂) to induce airway inflammation. That PAR₂ is activated by trypsin-like serine proteases associated with allergy-inducing pathogens is not limited to *Alternaria* and also includes asthma-associated allergens from cockroach and house dust mites (e.g., see Refs. 1, 44, and 50). PAR₂ in the airway, presumably activated by either endogenous or allergen-derived proteases, has been implicated in the pathogenesis of allergic asthma. PAR₂ activation is associated with an increase in allergen-induced leukocyte lung infiltration, which is reduced in PAR₂-null animals (5, 15, 16, 44). We have shown that direct activation of PAR₂ using peptidomimetic agonists in ovalbumin-sensitized mice can cause inflammatory cell infiltrate, epithelial thickening, and heightened mucus production via a β -arrestin-2-dependent pathway (40). Interestingly, other studies have additionally implicated PAR₂ sig-

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naling in bronchodilation independent of the PAR₂/β-arrestin-2 signaling axis and via prostanoid release from PAR₂-activated bronchial epithelial cells (14, 29, 49). These data suggest that many of the detrimental effects of PAR₂ activation in the setting of allergic lung inflammation are β-arrestin dependent, whereas other putative protective effects can occur independent of β-arrestin signaling (40).

Our previous studies of filtrate products derived from *Alternaria* cultures demonstrated that they promote PAR₂ signaling via a serine protease-mediated stimulus (as measured with Ca²⁺ signaling downstream of a G protein-coupled pathway) and can induce inflammation in vivo (8). Others have shown that *Alternaria*-dependent protease activity in vivo leads to an increase in airway IL-33 and subsequent asthma exacerbations (58). However, the identity and numbers of *Alternaria* filtrate serine proteases that can activate PAR₂ have not been elucidated, and their mechanisms for regulating airway inflammation and remodeling have not been determined. We hypothesize that a serine protease-mediated PAR₂-β-arrestin signaling axis plays a key role in *Alternaria*-induced asthma symptoms in vivo and that this lung inflammation can be largely mimicked by the isolated protease(s). To test these hypotheses, we first sought to determine whether *Alternaria* could activate the PAR₂-β-arrestin-2 signaling axis. We next sought to determine whether the protease/PAR₂/β-arrestin-2 signaling pathway was necessary to induce asthma phenotype in *Alternaria* filtrate sensitized and challenged wild-type, PAR₂^{-/-} or β-arrestin-2^{-/-} mice. Finally, we set out to isolate and characterize the trypsin-like serine protease(s) produced by *Alternaria* and to evaluate the ability of the enzyme(s) so identified to activate PAR₂ to stimulate Ca²⁺ signaling and PAR₂-β-arrestin interactions as well as asthma symptoms in our mouse models.

MATERIALS AND METHODS

Materials. All chemicals were from Sigma-Aldrich (St. Louis, MO) unless otherwise noted.

***Alternaria* preparations.** *Alternaria* preparations used in cellular and animal experiments (cat. no. XPM1D3A2.5; Greer Laboratories) were suspended in Hanks' balanced saline solution buffered with 10 mM HEPES, pH 7.4. Two *Alternaria* preparations were used for isolation and sequencing of the single serine protease identified by the activity-based probe (below) and for isolation of a purified enzyme preparation for cell function studies: 1) the crude buffer extract of freeze-dried defatted *Alternaria* powder from above and 2) a culture filtrate from Strain ATCC 11680 (cat. no. XPM1C3A25; Greer Laboratories). The culture filtrate was used directly and the crude defatted *Alternaria* allergen reconstituted in 25 mM Tris-HCl buffer, pH 7.4, as a stock solution containing ~1.7 U/mg trypsin-like activity using the QAR-AMC substrate assay (Bachem, I1550; see below). Aliquots of the Tris buffer solution were used for activity-based probe (ABP) labeling and for isolation of the purified protease fractions, as described below.

Other reagents. The calcium-sensitive indicator Fluo-4 NW was from Life Technologies (Carlsbad, CA). Ion exchange columns were from Bio-Rad (Hercules, CA; Bio-Scale Mini Macro-Prep High Q Cartridge). Antibodies and their catalog numbers were as follows: anti-phospho-cofilin (no. 3311; Cell Signaling Technology), anti-phospho-cofilin-PE-Cy5.5 (no. bs-3099R-Cy5.5; BIOCSS), anti-total cofilin (no. 612144; BD Biosciences), anti-CCR3-PE (no. FAB729P; R & D Systems), anti-CD3-APC-780 (no. 47-0032-80; eBioscience); anti-CD4-APC (no. 17-0042-81; eBioscience), anti-CD8-PerCP-Cy5.5 (no. 551162; BD PharMingen), anti-CD19-PE (no. 12-0193-82; eBiosciences), anti-CD45-PerCP-Cy5.5 (no. 550994, Clone 30-F11;

BD PharMingen), anti-CD45-FITC (no. 11-0451-81; eBioscience), anti-GR1-APC (no. 17-5931-81; eBioscience), F4/80, and APC-eFluor 780 (no. 47-4801-82; eBioscience). NOVEX 4–20% polyacrylamide gels were from Life Technologies.

Animal studies. All animal procedures were in accordance with the guidelines on the use and care of laboratory animals set by the National Institutes of Health and approved by the Institutional Animal Care and Use Committees at the University of California Riverside (Riverside, CA) and the University of Calgary (Calgary, AB, Canada). β-Arrestin-2^{-/-} mice on a C57BL/6 background were provided by Robert Lefkowitz (Duke University Medical Center). PAR₂^{-/-} mice were provided by Dr. Robin Plevin (University of Strathclyde, Glasgow, Scotland) and were developed by KOWA Pharmaceuticals. WT C57BL/6 (wild-type) mice were from The Jackson Laboratory. All animals were bred in-house. Animals were between 6 wk and 1 yr of age, and animal study groups were matched by age and sex. Specific numbers in each group are listed in the text.

Bioluminescence resonance energy transfer measurements of recruitment of β-arrestin-2. Bioluminescence resonance energy transfer (BRET), in which the energy emitted when luciferase (Luc) oxidizes its substrate will excite an acceptor fluorophore [yellow fluorescence protein (YFP)] only if the two proteins containing the luciferase and YFP tags are in very close proximity (usually 2–6 nm), provides a means for monitoring direct actions in live cells. Briefly, cells transfected with PAR₂-YFP and luciferase-tagged β-arrestin-2 were plated in 96-well microplates, and either vehicle or agonist is added along with the Luc substrate coelenterazine. Readings were collected using a Multilabel Reader Tristar9640 from Berthold, and emission was detected at 480 nm (Luc) and 535 nm (YFP). Net BRET was determined from the ratio of emission in the YFP channel to the emission in the luciferase channel (E535/E488) minus the donor-only (β-arrestin-luc) control values. To eliminate the possibility that observed signals represented nonspecific interactions, we included a β-arrestin-Luc + YFP in every experiment, which gave values similar to donor-only controls. Dose curves, ranging from 6.5 to 650 μg/ml *Alternaria* filtrate, were used to evaluate agonist EC₅₀s; kinetic curves were performed with a single dose of agonist (65 μg/ml) and BRET values monitored over 20 min. For acceptor/donor ratio assays, β-arrestin-Luc (donor) concentration was held constant at 0.5 μg, and PAR₂-YFP concentration was varied from 0.5 to 1.5 μg. BRET values were determined after 15 min of incubation with 65 μg/ml *Alternaria* filtrate.

Preparation of spleen lymphocytes for migration and signaling assays. To isolate lymphocytes, age- and sex-matched wild-type and β-arrestin-2^{-/-} mice were euthanized and spleens immediately removed, minced, homogenized in 10 ml of PBS, and filtered through sterile nylon mesh. Cells were passed through 70-μm filters, pelleted at 250 g for 5 min, washed three times in modified Hanks' balanced saline solution (without Ca²⁺ and Mg²⁺), and resuspended in RPMI complete media for subsequent experiments. Cytospin analysis and flow cytometry revealed the prep was >80% lymphocytes.

Transwell migration assay. After RBC lysis, ~10⁵ cells (isolated as described above) were plated onto collagen-coated membrane inserts with 5-μm pores and treated with *Alternaria* filtrate (65 μg/ml) or 100 nM 2fAP. After 1 h, nonmigratory cells were removed from the top of the filter, and migration was analyzed two ways. In the first procedure, cells attached to the underside of the filter were stained with crystal violet, and stained cells and migrated lymphocytes (identified by cell morphology) were counted using a bright-field compound microscope in four fields of vision. Migrated cells were then expressed as a fold change over baseline migration to saline control. Saline solution here and throughout in vitro and in vivo experiments was Hanks' balanced saline solution buffered with 10 mM HEPES, pH 7.4 (HBSS). In the second procedure, migrated cells were gently scraped into 2 ml of FACS buffer, and CD4⁺ T cells were confirmed by flow cytometry using anti-CD3 and anti-CD4, antibodies. Migrated CD4⁺ T-cells were expressed as the number of cells per milliliter.

Determination of phospho-cofilin in cultured leukocytes. Cells (10^5) were plated on 24-well culture dishes and grown overnight in serum supplemented RPMI when media was replaced with serum free media. Cells were allowed to recover from media exchange for 1 h, after which trypsin or *Alternaria* filtrate (65 μ g/ml) was added for 0–60 min. Cells were lysed in 1 $\times$ Laemmli sample buffer, sonicated, boiled, and analyzed by 15% SDS-PAGE, followed by Western blotting with anti-phospho-cofilin (rabbit, 1:1,000) and anti-total cofilin (mouse, 1:1,000) antibodies. Secondary antibodies anti-rabbit IR-800 (no. 611-732-127; Rockland) and anti-mouse Alexa 680 (no. A-21059; Invitrogen) were added at 1:30,000 and bands visualized on a LICOR Odyssey Infrared Imaging system. Band intensities were quantified with the LICOR Odyssey software, and phospho-cofilin levels were normalized to total cofilin levels for each blot.

Mouse inflammation models and measurement of airway inflammation. Paired 6-wk-old wild-type, β -arrestin-2^{-/-}, and PAR₂^{-/-} mice were exposed to either 25 μ l of HBSS alone (negative saline control) or 0.4 mg/kg of *Alternaria* filtrate (in 25 μ l HBSS), administered intranasally three times over an 8-day period (on days 1, 5, and 8). For trypsin inhibitor experiments, 232 pmol of soybean trypsin inhibitor (SBTI) were included with the *Alternaria* filtrate during each intranasal administration. For studies with purified AASP, we used 1.5 and 0.075 units of AASP in 25 μ l of HBSS, administered intranasally on the same schedule, as was done for the *Alternaria*. Mice were euthanized on day 9, and lungs were lavaged with 5 ml of PBS. Bronchioalveolar lavage fluid (BALF) was centrifuged, and supernatants were used for analysis of cytokine production. Cell pellets were resuspended in PBS, subjected to NH₄Cl lysis (0.83% ammonium chloride for 2 min at 4°C) to remove red blood cells (RBCs), and resuspended in 1 ml of PBS. Cell numbers were determined by hemocytometer, and samples were then divided into 2 (200 μ l was used for cytopins, and 300 μ l was used for flow cytometry). Lungs were simultaneously harvested and fixed for histological analysis.

Hematoxylin and eosin stain, Alcian blue stain, and quantification. Staining was performed on paraffin-embedded sections and analyzed as described in previous studies (40). Briefly, for general histological analysis, lung sections were stained with hematoxylin and eosin (H & E) and histological grading based on infiltration of white blood cells and perivascular thickness. Separate sections were stained with Alcian blue (to stain mucin) and fast nuclear red (to identify cell nuclei). Quantification of inflammation and mucin staining was performed by histological grading, as previously described (40). For histological grading of inflammation, a score of 1 to 4 was given, with 1 being no inflammation present, 2 being one ring of leukocytes around the vasculature and mild infiltration, 3 being two to three rings and moderate infiltration, and 4 being four or more rings and numerous leukocytes throughout the epithelial tissue. Epithelial thickness was quantified using National Institutes of Health (NIH) ImageJ to measure the distance from the basolateral surface of the epithelial cell to the muscular layer. Mucin-positive cells were quantified by calculating the percentage of total epithelial cells per millimeter that stained positive with Alcian blue. Because Alcian blue can also stain basal lamina matrix proteins, staining at the basal lamina was not considered positive. Mucin staining was calculated using a user-defined NIH ImageJ macro; images were inverted, and total red channel density (corresponding to blue by eye) per 100 cells was determined.

Cytospins and differential cell counts. Briefly, 100 μ l of BALF was spun on to glass slides using Shandon CytoSpin, and cells were stained using a Hema 3 stain kit (Fisher Scientific), following manufacturer's protocol. Differential counts were obtained by counting 200 cells/slide and categorized based on morphological criteria, as described in our previous study (40).

Flow cytometry. BALF cell pellets were resuspended in 1 ml of FACS buffer and stained with a panel of antibodies (group A: anti-CD45, anti-CD3, anti-CCR3, and anti-GR1; group B: anti-CD4, anti-CD19, anti-CD8, anti-CD4 and anti-F4/80), as described in our previous studies using a BD FacScan, and analyzed with FlowJo

version 6.1 (40). From CD45-positive population, FSC/SSC was used to identify granulocytes (high FSC/SSC), macrophages (high-FSC/mid-SSC) and lymphocytes (high-FSC/low-SSC). From the granulocyte population, eosinophils (CCR3+) and neutrophils (GR1) were further identified. T-lymphocytes were further confirmed as CD3+ and either CD4+ or CD8+. B cells were identified as the CD19-positive group. Macrophages were further confirmed as F4/80+. For in vivo cofilin dephosphorylation assays, resuspended BALF cells were fixed, permeabilized, and incubated with PE-Cy5.5-conjugated anti-phospho-cofilin and FITC-conjugated anti-CD45. Cells were first gated on CD45 staining to identify leukocytes, and granulocyte and lymphocytes were identified based on FSC/SSC. The population described as "granulocytes" in Fig. 8 was previously shown to be comprised primarily of eosinophils, and to a lesser extent, neutrophils and the population described as "lymphocytes" were primarily CD3 positive (see Fig. 7). Phospho-cofilin levels were then analyzed in granulocyte and lymphocyte populations. One sample from each treatment group received PE-Cy5.5 IgG isotype as a negative control (no. bs-0295P-Cy5.5; BISS). The number of cells with a mean fluorescence level above 5×10^3 and the mean fluorescence intensity in each treatment group were determined.

Quantifying serine protease. *Alternaria* enzyme samples were quantified in terms of their "trypsin-like" equivalents, using a micro-titer plate fluorogenic assay with Glu-Ala-Arg-aminomethylcoumarin (QAR-AMC; I-1550; Bachem, Bubendorf, Switzerland) as substrate. High-specific-activity trypsin was used as a standard (catalog no. T0303, 16,000 U/mg, Type IX-S porcine trypsin; Sigma). The filtrate suspended in Hanks' buffered saline solution (HBSS) with a protein content of ~650 μ g/ml bovine albumin equivalents routinely had a trypsin-like enzyme activity equivalent to 1.0–2.5 U/ml (as detected with the QAR substrate). *Alternaria* enzyme samples were also tested for serine and cysteine cathepsin substrate, Phenylalanine-Arginine-aminomethylcoumarin (FR; I-1160; Bachem) and urokinase substrate Glycine-Glycine-Arginine-minomethylcoumarin (GGR; 230914; Calbiochem).

Activity-based probe labeling of serine proteases for mass spectral sequencing. The serine protease activity-based probe (ABP) Biotin-Pro-Lys-diphenylphosphonate was synthesized and graciously provided for our studies by Dr. Brendan Gilmore, Queens University School of Pharmacy, Belfast, UK. Covalent labeling of enzymes in the *Alternaria* filtrates and crude cellular antigen, as well as in chromatographic column fractions isolated from these sources, was achieved essentially as described previously (28, 50), with minor modifications. The *Alternaria* samples were diluted to a final concentration of 1.5 U/ml and incubated with 100 μ M of the ABP in a 10- μ l reaction volume consisting of: 50 mM Tris-HCl, pH 8, 0.1% NP40, and 1.5 mM CaCl₂ for 2 h at room temperature to biotinylate the enzymes. The reaction was terminated with the addition of Laemmli sample buffer, SDS-PAGE analysis (4–20%), followed by Western blotting with horseradish peroxidase-conjugated streptavidin. Additionally, the total protein in the fractions was visualized by staining gels with SYPRO Ruby fluorescent protein stain (Life Technologies) and monitoring UV-activated fluorescence in a Kodak Image Station 4000 MM Pro gel doc apparatus.

For isolation of the biotinylated enzymes for mass spectral sequencing, volumes corresponding to 7.5–15 U total of trypsin-like activity (estimated to be ~0.5 to 1 μ g of enzyme) were incubated with 100 μ M of the ABP. The ABP-bound proteins were pulled down with Avidin-Sepharose beads for 1 h, washed five times with 2 M urea, and analyzed by SDS-PAGE (4–20%) and protein with Coomassie Blue stain (Bio-Rad). Bands in the molecular weight range of the ABP-labeled allergen enzyme (20–25 kDa) were excised from the gel and digested with chymotrypsin (10 μ g/ml) at 37°C overnight. Liquid chromatography-mass spectrometry/mass spectrometry (LC-MS/MS) analysis of in-gel chymotrypsin digested-proteins was carried out using a LTQ Orbitrap Velos mass spectrometer (Thermo Fisher Scientific, San Jose, CA) equipped with an Advion nanomate ESI

source (Advion, Ithaca, NY) following ZipTip (Millipore, Billerica, MA), as described previously (52, 56). Data-dependent scanning was performed by the Xcalibur version 2.2 SP1.48 software using a survey mass scan at 60,000 resolution in the Orbitrap analyzer scanning m/z 400–2,000, followed by collision-induced dissociation (CID) tandem mass spectrometry (MS/MS) of the six most intense ions in the linear ion trap analyzer (4). The MS/MS spectra of visible protein bands processed for LC-MS/MS as above were searched against a custom database made of 1) NCBI proteins that contained the word “trypsin” in their annotation entries and 2) proteins that contained the word “trypsin” found at the *Alternaria* genome database. This custom protein database contained 4,623 entries. Variable modifications considered during the search included methionine oxidation (15.995 Da) and cysteine carbamidomethylation (57.021 Da) as well as adduction of lysine or cysteine residues by 4HNE (156.115 Da). Proteins were identified at 95% confidence, with XCorr scores as determined by a reversed database search.

Ion exchange chromatography for isolation of *Alternaria* serine proteases. Proteases in the *Alternaria* culture filtrate and in the reconstituted *Alternaria* cellular antigen extract were subjected to anion and cation column chromatography. In brief, enzyme-containing preparations were dialyzed for 2 h at 4°C against 20 mM Tris-HCl buffer, pH 7.2, containing 10 mM NaCl. Dialyzed samples (1-ml volumes at 7.0–7.2 U/ml) were then applied to a 5-ml High Q ANION exchange column (40 × 12.6 mm) with a fast-phase liquid chromatography (FPLC) system (Pharmacia; Stockholm, Sweden) and 1-ml fractions were collected at a flow rate of 1 ml/min using a gradient/step NaCl elution protocol ranging from 10 mM to 1 M salt. The eluted fractions were monitored for both protein (absorption at 280 nm and SDS-PAGE) and trypsin activity (QAR substrate). Each fraction containing serine protease activity was analyzed by ABP assay, as described above.

Calcium signaling assay. Kirsten virus-transformed rat kidney cells (KNRK), which do not express functional endogenous PAR₂, were transfected with COOH terminally tagged YFP-rat-PAR₂ (3), seeded at 50% confluence in 96-well black cell culture plate (BD Falcon, Franklin Lakes, NJ), and grown overnight. Cells were loaded for 30 min at 37°C with the no-wash Ca²⁺-sensing dye Fluo-4 NW (25 µg/ml) in Ca²⁺ assay buffer [HEPES-fortified Hank's buffered saline, pH 7.4 (Gibco): 10 mM HEPES, 1.5 mM CaCl₂, 1.5 mM MgCl₂] along with 2.5 mM probenecid (Invitrogen, Carlsbad, CA). The plates were loaded into the Victor X4 2030 Multilabel plate reader (Perkin-Elmer, Waltham, MA), and agonists were applied using the plate reader sample injection system. Cells in the plate were excited at 480 nm, and kinetic traces of fluorescence emission at 530 nm, representing increased cytoplasmic Ca²⁺ levels, were recorded every 0.5 s for 1 min. Fluorescence levels were normalized to the signal generated by 2 µM Ca²⁺ ionophore A23187 (Sigma, St. Louis MO).

Measurement of *Alternaria* enzyme-stimulated bronchorelaxation. After euthanasia, second-order bronchioles were excised and trimmed for mounting in a wire myograph for bioassay measurements at resting tension in a 37°C Krebs buffer bath. After 60 min of equilibration at ~0.5 g of resting tension, tissues were treated with 50 mM KCl (to test responsiveness), washed, and precontracted with 500 nM carbachol. After stable force was achieved (100% on the waveforms in response to carbachol), tissues were treated with substance P (SP), a neurokinin-1 receptor agonist, to demonstrate viable G_{αq} signaling. Tissues were washed, followed by treatment with *Alternaria* enzyme (0.6 U/ml). The relaxation response was recorded as done previously (40), with relaxation expressed as a percentage reduction in tension (%) relative to the tension observed with carbachol.

Data and statistical analysis. All graphs and statistical analyses were performed using Microsoft Excel 2011 or GraphPad Prism 6.0. Experiments were performed a minimum of three times; specific numbers are indicated in the figure legends. Statistical significance was determined using one-way ANOVA and Tukey *t*-tests (to compare between treatment groups).

RESULTS

***Alternaria*-activated PAR₂ signaling invokes β-arrestin signaling.** Existing cellular studies demonstrating *Alternaria*-PAR₂ activation have relied on the PAR₂/Gq/Ca²⁺ signaling as a cellular readout (8, 39). We have shown using the PAR₂-activating peptide 2-furoyl-LIGRL-Orn-NH₂ (2fAP) that PAR₂-stimulated leukocyte cell migration, activation of cofilin, and subsequent actin reorganization and airway inflammation were dependent upon PAR₂/β-arrestin-2 signaling (71, 72). To determine whether *Alternaria* could induce full PAR₂ signaling (i.e., G protein and β-arrestin signaling), we first used bioluminescence resonance energy transfer (BRET), a cellular assay to examine the recruitment of luciferase-tagged β-arrestin-2 to PAR₂-YFP, in response to receptor activation by *Alternaria* filtrate or purified trypsin (Fig. 1A) (38). Concentration-effect curves (0–650 µg/ml protein tested) showed that *Alternaria* filtrate stimulated BRET with an EC₅₀ of 65 µg/ml protein (Fig. 1A). Comparison experiments using the PAR₂-activating serine protease trypsin (0.00045–4.5 U tested in a 200-µl reaction) resulted in an EC₅₀ of 2.0 U/ml (Fig. 1B). Because β-arrestin interactions are dependent upon receptor conformation, this steric requirement can translate into differences in the apparent rate of recruitment, the length of receptor/β-arrestin interactions, and/or differences in the apparent affinity of β-arrestin for PAR₂. Differences in the apparent affinity of β-arrestin for PAR₂ can be assessed by determining the acceptor/donor ratio at which BRET₅₀ is obtained. Time-based capture of BRET shows that the kinetics of recruitment were slightly more rapid in response to trypsin than with *Alternaria* filtrates (Fig. 1C). However, both maximal BRET values (BRET_{max}) and half-maximal BRET values (BRET₅₀) for β-ar-

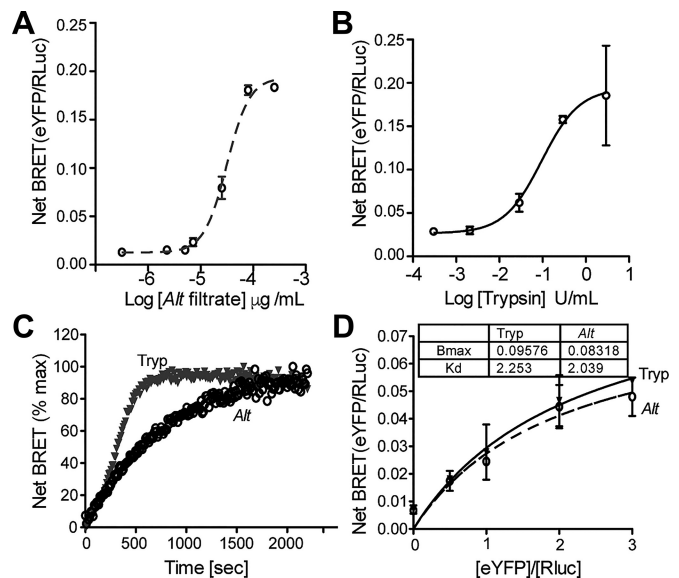


Fig. 1. *Alternaria* promotes recruitment of β-arrestins to protease-activated receptor-2 (PAR₂). A and B: bioluminescence resonance energy transfer (BRET) was measured upon addition of increasing concentrations of *Alternaria* (Alt) filtrate (A) or trypsin control (B). C: the time to reach maximal BRET signal was determined in response to Alt or trypsin. D: half-maximal (BRET₅₀) and maximal BRET values (BRET_{max}) were determined for recruitment of β-arrestin-2 to PAR₂ in response to 65 µg/ml Alt or 2 U/ml of trypsin. Alt triggered β-arrestin recruitment, similar to trypsin controls, albeit with a slightly reduced time constant; *n* ≥ 3 for all experimental time points; see MATERIALS AND METHODS for experimental details.

restin-2/PAR₂ interactions were similar in response to either 65 μ g/ml *Alternaria* filtrate or 2.0 U/ml trypsin (Fig. 1D).

We have demonstrated that 2fAP-activated PAR₂ promotes dephosphorylation and activation of cofilin by a β -arrestin-dependent, G protein-independent mechanism (71). We used this cellular assay to verify activation of *Alternaria*-induced, β -arrestin-dependent PAR₂ signaling. Naïve lymphocytes isolated from spleens of wild-type or β -arrestin-2^{-/-} mice were treated with *Alternaria* filtrate or trypsin (as a positive enzyme control) for 0–60 min. Using cytospin preparations, we determined that the preparation was predominantly lymphocytes, and there were no differences in basal numbers of lymphocytes between wild-type and β -arrestin-2^{-/-} mice (not shown). Lysates were analyzed by SDS-PAGE, followed by Western blotting with anti-phospho(p)-cofilin and anti-total(t)-cofilin antibodies (Fig. 2, A and B). Both *Alternaria* filtrate and trypsin promoted cofilin dephosphorylation ($\leq 45\%$ of control values). This dephosphorylation response was abolished in cells prepared from β -arrestin-2^{-/-} mice. We next performed Transwell chemotaxis assays with immune cells isolated from spleens of wild-type or β -arrestin-2^{-/-} mice. Addition of *Alternaria* filtrate or 2fAP to the bottom chamber promoted an increase in wild-type cell migration compared with HBSS controls (6.1 ± 1.1 -fold and 2.5 ± 0.3 -fold recruitment, respectively; Fig. 3A). Migrated cells were collected from the Transwell filters and further analyzed by flow cytometry using CD4⁺ staining to specifically quantify T cell migration. Controls using saline without PAR₂ activators resulted in recruitment of 165 ± 23 CD4⁺ cells/ml (Fig. 3B). Addition of *Alternaria* filtrates (510 ± 54 CD4⁺ cells/ml) or 2fAP ($290 \pm$

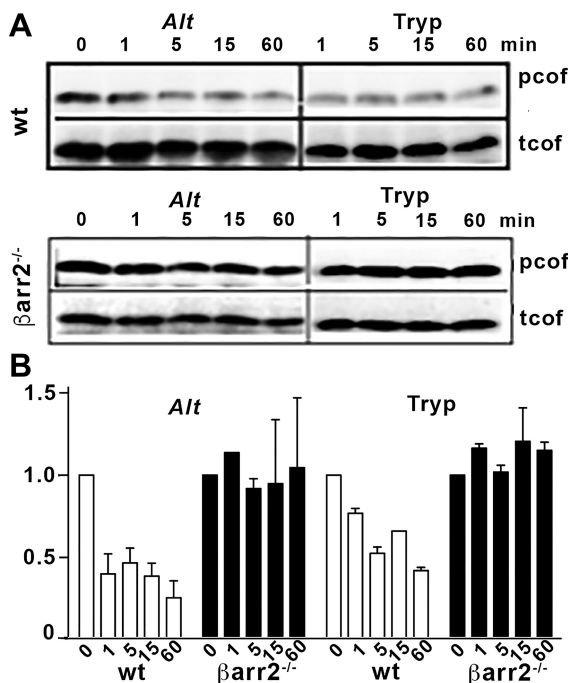


Fig. 2. *Alternaria* promotes β -arrestin dependent activation of cofilin. Adherent cells from spleens of wild-type (WT) and β -arrestin-2^{-/-} (β -arr2^{-/-}) mice were treated with 65 μ g/ml *Alternaria* (Alt) or 2 U/ml trypsin (Tryp; control). A: Western blots of cell lysates detecting phosphorylated (p) or total (t) cofilin (cof) are shown. B: quantification of normalized p-cofilin levels (fold change from untreated cells); $n = 3$ for each experiment, and data are shown as means $\pm$ SE. Alt activated cofilin is similar to the trypsin control.

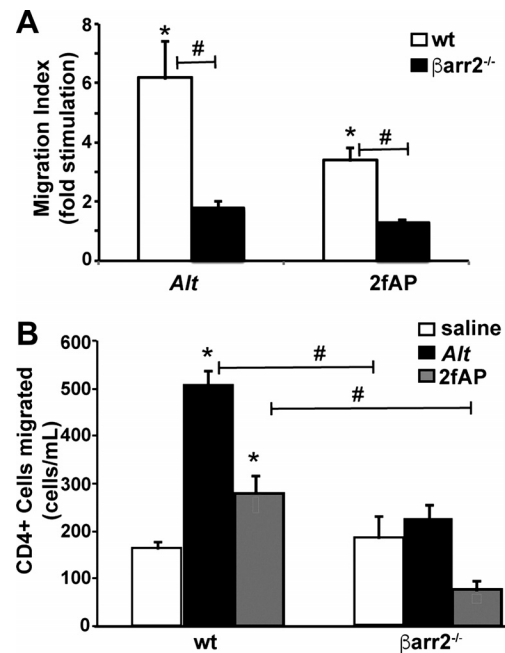


Fig. 3. *Alternaria* promotes β -arrestin-dependent lymphocyte migration. Lymphocytes were seeded onto Transwell filters, and basal medium was supplemented with Hanks' balanced saline solution (HBSS), *Alternaria* (Alt), or the protease-activated receptor-2 (PAR₂) agonist 2-furoyl-LIGRL-Orn-NH₂ (2fAP). After 1 h, migrated cells were stained with Crystal Violet and counted (A) or recovered into 0.5 ml of FACS buffer, incubated with antibody to CD4, and analyzed by flow cytometry (B); $n = 3$ for each experiment, and data are shown as means $\pm$ SE. *Significant difference between HBSS and Alt-treated groups; #significant difference between bracketed groups ($P < 0.01$). Both Alt- and 2fAP-induced lymphocyte migration was abrogated in β -arrestin2^{-/-} mice.

48 CD4⁺ cells/ml) significantly increased the numbers of recruited T cells (Fig. 3B) when compared with the saline controls. In contrast, there was no increased migration of CD4⁺ T cells from β -arrestin-2^{-/-} mice to *Alternaria* filtrate or 2fAP (Fig. 3, A and B). Taken together, we conclude that *Alternaria* filtrates promote protease-dependent PAR₂ signaling through β -arrestin pathways. Coupled with previous results with Ca²⁺ signaling responses (8, 39), *Alternaria* filtrates can fully activate PAR₂ signaling.

Protease-dependent, Alternaria-induced airway inflammation and goblet cell hyperplasia in vivo requires PAR₂ and β -arrestin-2. To determine the role of the PAR₂/ β -arrestin signaling axis in *Alternaria*-induced inflammation, *Alternaria* filtrate was administered to mice intranasally on days 1, 4, and 8 (Fig. 4A). Lung tissue and bronchoalveolar lavage fluid (BALF) were collected 24–48 h after the final administration for analysis. Epithelial damage and cellular inflammation were first assessed by examination of hematoxylin and eosin- (H & E)-stained lungs (Fig. 4, B–F). In wild-type mice, *Alternaria* filtrate induced inflammation that was largely reduced if SBTI was administered with the filtrate (Fig. 4, B–F). *Alternaria* filtrate treatment of PAR₂^{-/-} or β -arrestin-2^{-/-} mice, however, resulted in only minor inflammatory response (Fig. 4, C, D, and F). Examination of bronchial epithelium in the exposed samples indicated that *Alternaria* promoted a twofold increase in epithelial thickness in the wild-type mice that was significantly abated by SBTI in the wild types as well and not observed in either PAR₂^{-/-} or β -arrestin-2^{-/-} mice (Fig. 4G).

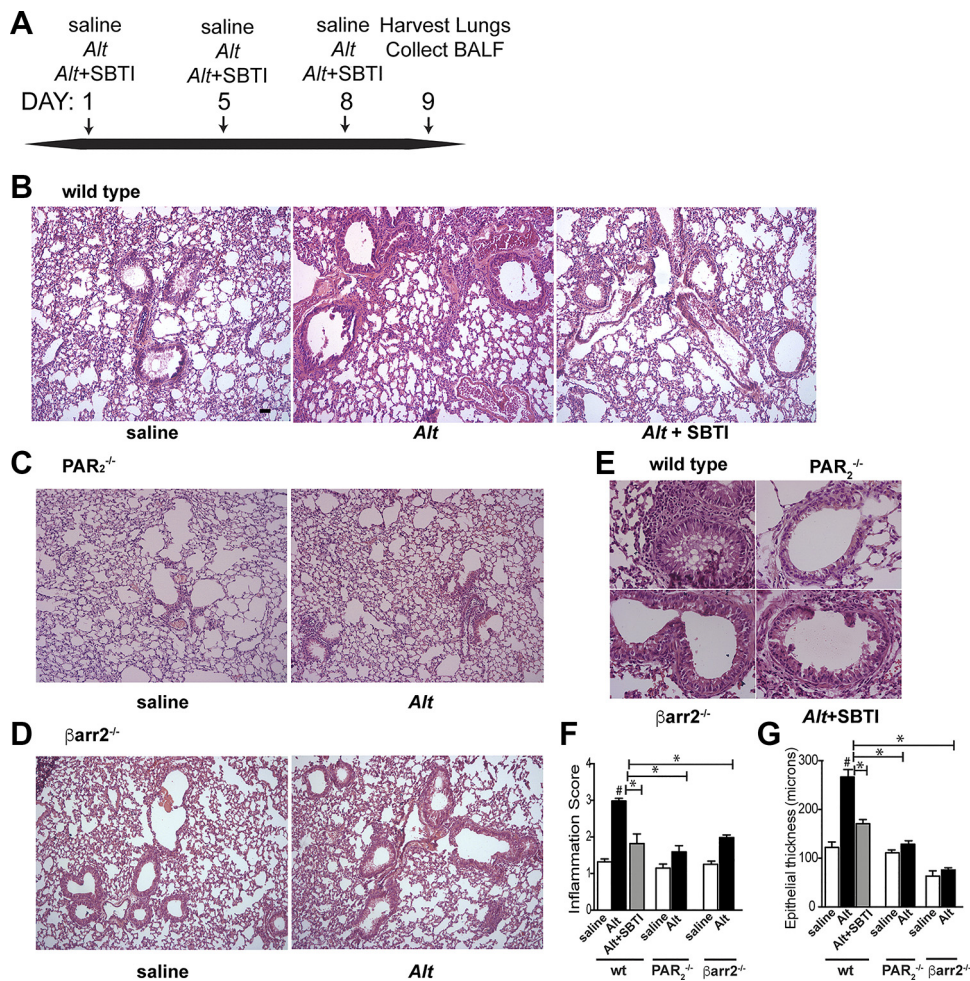


Fig. 4. *Alternaria* induces protease-activated receptor-2 (PAR₂) and β -arrestin-2-dependent inflammation of the airway epithelium. **A**: timeline of *Alternaria* filtrate (Alt) exposure included 4 mice/treatment group, which was repeated 3 times. **B–D**: representative images ($\times 10$) of hematoxylin and eosin-stained lungs after Hanks' balanced saline solution control, Alt, or Alt + soybean trypsin inhibitor (SBTI) in wild-type (**B**), PAR₂^{-/-} (**C**), or β -arrestin-2^{-/-} mice (**D**). Scale bar in **B** = 100 μ m and is representative of images in **B–D**. **E**: higher magnification of lungs from Alt-treated mice are shown for emphasis of differences in inflammation. **F** and **G**: histological grading of slides, determined from 10–15 images quantified/mouse. Each group includes data from ≥ 4 and ≤ 8 mice. Significant differences between Alt-treated and saline controls or between bracketed Alt-treated groups are indicated as follows: * $P \leq 0.05$. Alt-induced inflammation is reduced by protease and requires expression of PAR₂ and β -arrestin-2; # $P < 0.01$, significant difference from saline-treated control.

We next examined the effect of *Alternaria* filtrate exposure on both the amount of mucin produced and the number of goblet cells in wild-type, PAR₂^{-/-}, and β -arrestin-2^{-/-} mice using Alcian blue (AB) to mark acidic proteoglycans present in mucin and goblet cells (Fig. 5, A–C). Both increased mucus staining and goblet cell hyperplasia in response to *Alternaria* were abolished when *Alternaria* was administered with SBTI and were not observed in either the PAR₂^{-/-} or β -arrestin-2^{-/-} mice. Semiquantification of the AB-stained lungs reveals a 15-fold increase in mucin staining (Fig. 5D) and a ninefold increase in mucin-positive goblet cells (Fig. 5E) in response to *Alternaria* exposure in wild-type mice. In contrast, *Alternaria* exposure of wild-type mice in the presence of SBTI resulted in AB staining and goblet cell numbers that were not different from the saline-treated controls (Fig. 5, D and E). Similarly, *Alternaria* filtrate exposure of PAR₂^{-/-} or β -arrestin-2^{-/-} mice only slightly increased mucin staining and goblet cell number. Taken together, these data strongly support that *Alternaria* filtrate-mediated airway inflammation and mucus hyperplasia requires proteolytic activation of PAR₂ and subsequent signaling through β -arrestins to induce asthma symptoms.

A major hallmark of airway inflammation, and one that was apparent in histological analyses above, is the infiltration of leukocytes into the lung. Both the total number of leukocytes present and the relative distributions of each cell type are

indicative of the inflammatory response. *Alternaria*-induced cell infiltration into the airways can be seen in BALF cytospin preparations from wild-type mice and is markedly reduced when SBTI is included in the intranasal challenges (Fig. 6A). Examination of cytospin preparations from PAR₂^{-/-} and β -arrestin-2^{-/-} mice also show reduced cell recruitment when comparing *Alternaria* filtrate exposure to HBSS-exposed controls (Fig. 6, B and C). Differential counts of cytospins (Fig. 6, D–G) revealed that exposure of wild-type mice to *Alternaria* filtrate increases eosinophil concentration in the BALF almost 150-fold in the wild-type animals (600 cells/ml in saline-treated mice to 88,800 cells/ml in the *Alternaria*-treated mice; Fig. 6D). Similar, albeit less dramatic, increases were observed for lymphocyte concentrations (14.5-fold change from 3,400 to 49,300 cells/ml; Fig. 6E), neutrophil concentrations (41-fold change from 1,110 to 45,100 cells/ml; Fig. 6F), and macrophage concentrations (11-fold change from 2,600 to 28,600 cells/ml; Fig. 6G). When compared with the *Alternaria*-treated wild type, both *Alternaria* filtrate/SBTI-treated wild type mice and *Alternaria* filtrate-treated PAR₂^{-/-} mice showed similar reductions in airway inflammation. Eosinophil BALF concentrations (1,600 cells/ml in the wild-type *Alternaria*/SBTI-treated animals and 7,800 cells/ml in the *Alternaria*-treated PAR₂^{-/-} animals) and lymphocyte BALF concentrations (3,800 and 2,500 cells/ml, respectively) were reduced by >90% (Fig. 6D). Neutrophil BALF concentrations (16,200 cells/ml in

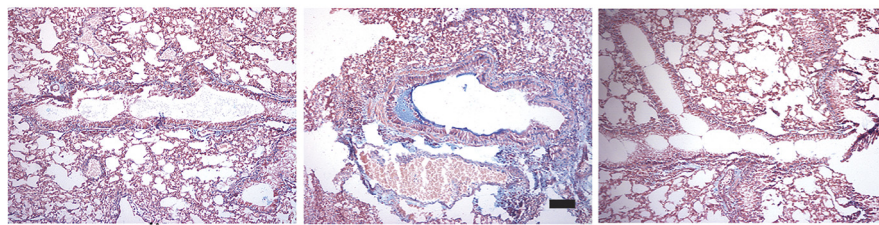
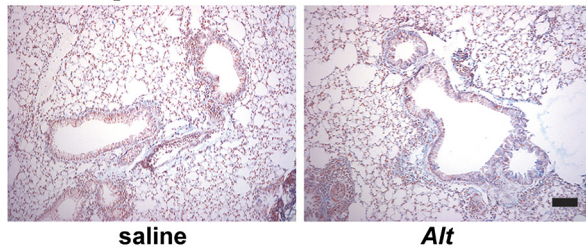
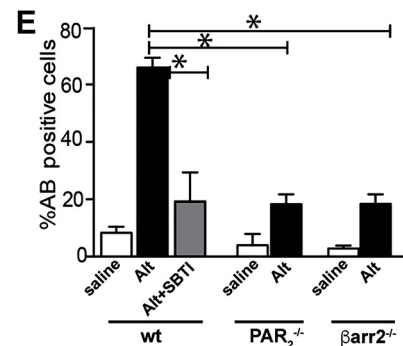
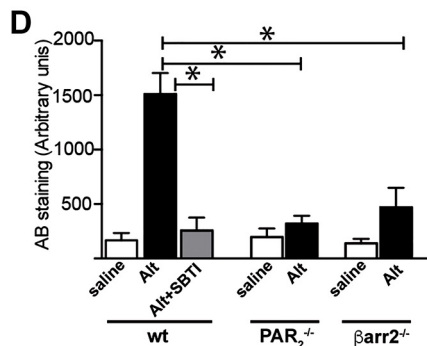
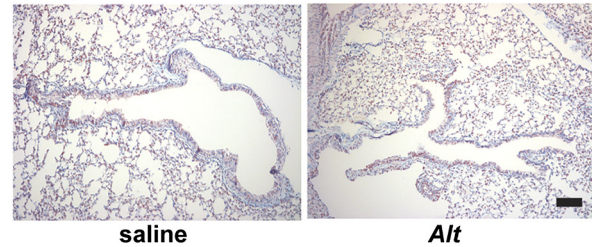
A wild type**B** PAR₂^{-/-}**C** βarr2^{-/-}

Fig. 5. *Alternaria*-induced mucus production and goblet cell hyperplasia require protease-activated receptor-2 (PAR₂) and β-arrestin-2. A–C: representative images of Alcian blue (AB)-stained paraffin-embedded lung sections from wild-type (A), PAR₂^{-/-} (B), and β-arrestin-2^{-/-} (C) mice (×10 mag). D and E: quantification of mucin staining (D) and %goblet cells (AB positive/10 mm² section of epithelium; E). Each group includes data from ≥4 and ≤8 mice. Significant differences between *Alt*-treated and saline controls or between bracketed *Alt*-treated groups are indicated as follows: **P* ≤ 0.01. *Alt*-induced mucus enhancement is reduced by protease and requires expression of PAR₂ and β-arrestin-2.

Alternaria/SBTI animals and 14,900 cells/ml in *Alternaria*-treated PAR₂^{-/-} animals) were reduced by ~65% (Fig. 6E). Macrophage BALF concentrations (14,400 cells/ml in *Alternaria*/SBTI-treated animals and 13,400 cells/ml in *Alternaria*-treated PAR₂^{-/-} animals) were reduced by ~50% (Fig. 6F). Whereas airway inflammation was also significantly reduced in the *Alternaria*-treated β-arrestin-2^{-/-} mice, the pattern of inflammation reduction was distinct. Eosinophil BALF concentration in *Alternaria*-treated β-arrestin-2^{-/-} mice (11,400 cells/ml) was reduced by 87% compared with *Alternaria*-treated wild-type mice (Fig. 6D); lymphocytes (12,200 cells/ml) and neutrophils (10,700 cells/ml) were reduced by ~75% compared with *Alternaria*-treated wild type (Fig. 6, E and F), and macrophage recruitment (4,800 cells/ml) was decreased by 83% in β-arrestin-2^{-/-} mice (Fig. 6G). In summary, *Alternaria*-induced eosinophil and lymphocyte recruitment was largely limited by protease inhibition, loss of PAR₂ signaling, or β-arrestin signaling, whereas macrophage recruitment and, to a lesser extent, neutrophil recruitment were more sensitive to the β-arrestin signaling pathway. These data suggest an interplay between multiple signaling pathways for full *Alternaria*-induced inflammation.

To better determine any signaling-biased differential inflammatory recruitment, we subjected the BALFs collected from *Alternaria*-exposed animals as described above to flow cytometry (Fig. 7, A–D). In saline control-treated wild-type mice, <10% of the live cells in the BALF were CD45+. The CD45+ cells in saline control-treated BALF were comprised of 0.3% eosinophils [high forward scatter (FSC)/high side scatter (SSC)/CCR3+], 2.2% neutrophils (high FSC/high SSC/GR1+), 2.5% lymphocytes (high FSC/low SSC, CD3+), and 35% alveolar macrophages (high FSC/mid SSC, F4/80+). In contrast, BALF from *Alternaria*-treated wild-type mice displayed an inflammatory phenotype that included the following CD45+ cell distribution: 38% eosinophils, 19% neutrophils, 14% lymphocytes, and 24% macrophages (Fig. 7D). Although *Alternaria* also increased the relative amounts of eosinophils, neutrophils, and lymphocytes in PAR₂^{-/-} mice and β-arrestin-2^{-/-} mice when compared with HBSS control, the specific values were significantly different from those observed in *Alternaria*-treated wild type mice. In PAR₂^{-/-} mice, *Alternaria*-treatment resulted in CD45+ cells in the BALF that were comprised of 4% eosinophils, 14% neutrophils, 10% lymphocytes, and 37% macrophage, whereas in β-arrestin-2^{-/-} ani-

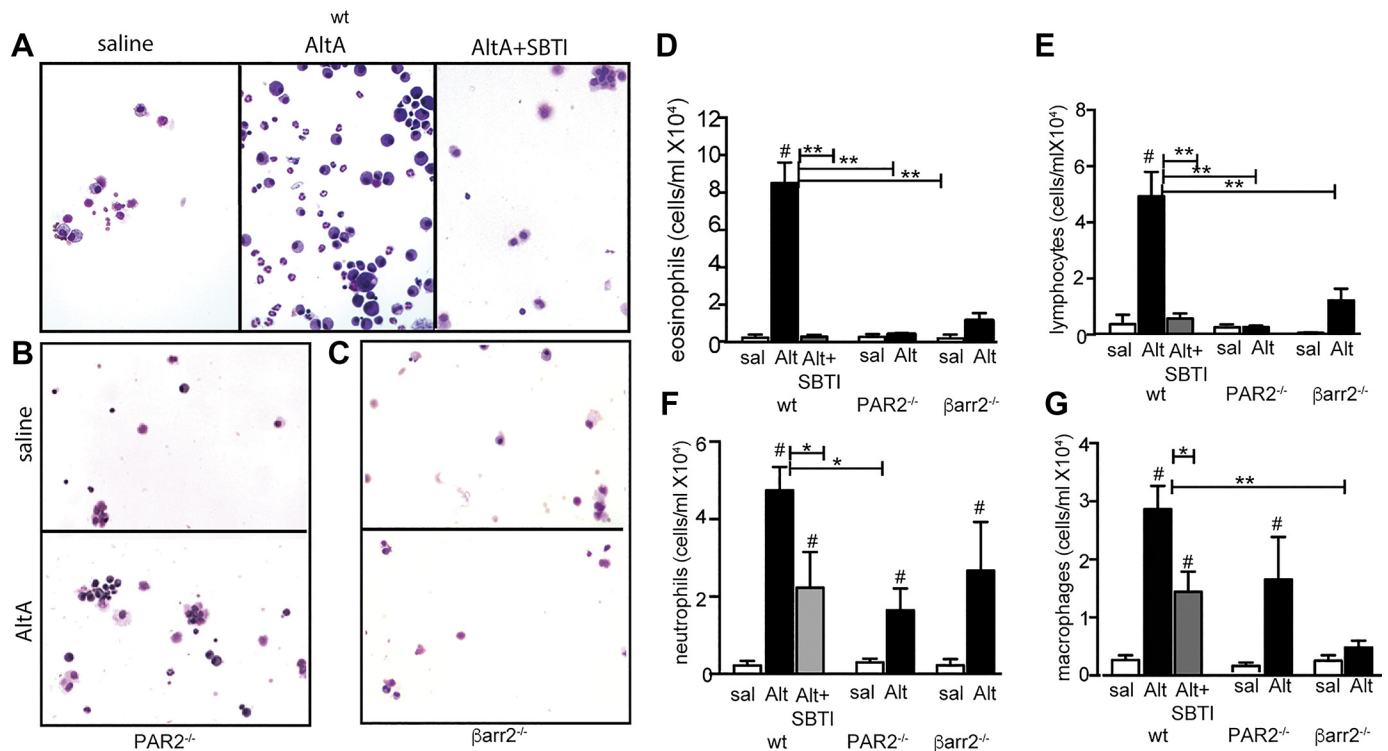


Fig. 6. *Alternaria* (Alt)-induced airway leukocyte migration requires protease-activated receptor-2 (PAR₂) and β-arrestin-2. A–C: representative Giemsa-stained cytopins from bronchoalveolar lavage fluid (BALF) of wild-type (A), PAR₂^{-/-} (B), and β-arrestin-2^{-/-} (C) mice. D–G: differential counts of eosinophils (D), lymphocytes (E), neutrophils (F), and macrophages (G) in BALF cytopins; *n* = 8 animals with 4 slides/treatment and 2 fields of vision/slide. #Significant differences between matching saline (sal)-treated controls. Significant difference between bracketed Alt-treated groups are indicated as follows: **P* ≤ 0.05; ***P* ≤ 0.01. Alt-induced eosinophil and lymphocyte recruitments were abolished, and neutrophil recruitment reduced by protease inhibition or loss of PAR₂ or β-arrestin expression. Alt-induced macrophage recruitment was partially reduced by protease inhibition and loss of PAR₂ signaling but completely abolished by absence of β-arrestin. SBTI, soybean trypsin inhibitor.

mals, the BALF cell composition was 19% eosinophils, 10% neutrophils, 6% lymphocytes, and 29% macrophages (Fig. 7D). Furthermore, flow cytometric analysis of the individual lymphocyte populations demonstrated that CD4⁺ T cells were the primary lymphocyte subtype recruited to the airways (Fig. 7, E and F). These data suggest that *Alternaria*-induced PAR₂/β-arrestin signaling is important for increasing inflammation of the airways and also mediates recruitment of specific cell types to the airways.

To demonstrate the role of *Alternaria*-induced, β-arrestin-dependent PAR₂ signaling in vivo, we employed a phospho-flow assay, using Cy5.5-conjugated anti-phospho-cofilin. We determined cofilin dephosphorylation in BALF cells after induction of lung inflammation with *Alternaria* filtrate. Granulocyte and lymphocyte populations were identified from the CD45⁺ cells by SSC versus FSC (Fig. 8A), and the mean Cy5.5 fluorescence for each population was determined by histogram analysis (Fig. 8, B and C). *Alternaria* treatment of wild-type mice resulted in leftward shift in mean fluorescence intensity from that observed in HBSS control-treated mice in both granulocytes (Fig. 8B) and lymphocytes (Fig. 8C), consistent with a 2.1-fold increase in cofilin dephosphorylation in granulocytes (Fig. 8D, left) and a 1.6-fold increase in lymphocytes (Fig. 8E, left). No significant cofilin dephosphorylation was observed in β-arrestin-2^{-/-} mice in either cell type (Fig. 8, C and D). The percentage of wild-type granulocytes (Fig. 8D, right) and lymphocytes (Fig. 8E, right) in the BALF that stained positive for phospho-cofilin was also significantly re-

duced after *Alternaria* treatment. These data demonstrate for the first time that allergen treatment activates the PAR₂/β-arrestin signaling axis in the invading inflammatory cells during airway inflammation in vivo. As expected, in β-arrestin-2^{-/-} mice, cofilin dephosphorylation was diminished (e.g., decreased overall p-cofilin and decreased numbers of p-cofilin-positive cells). No differences in total cofilin levels were observed in BALF cells from each treatment group. Thus, *Alternaria* promotes robust β-arrestin-dependent dephosphorylation of cofilin in vivo.

Purification and sequence identification of a PAR₂-activating serine protease from *Alternaria alternata*. Our data above, similar to previous studies (8, 58), shows that both *Alternaria* filtrate-induced lung inflammation in vivo and PAR₂ activation in cultured cells were inhibited by serine protease inhibitors [including soybean trypsin inhibitors (SBTI) and 4-(2-Aminoethyl)benzenesulfonyl fluoride hydrochloride (AEBSF)], implying the action of a serine protease. Airway inflammation in response to *Alternaria* has been reported using several commercial and noncommercial sources, including spores, filtrate, and cellular extract. Using a biotinylated, serine protease-selective activity-based probe (ABP) (46, 50), we found that both the unfractionated, defatted *Alternaria* cellular extract and the filtrate (see MATERIALS AND METHODS) contained only a single biotinylated enzyme with an apparent molecular mass of ~24 kDa (Fig. 9A). Consistent with the protease capture experiments, labeling of the enzyme with the ABP was abrogated by treating the samples with SBTI. The results suggest that a

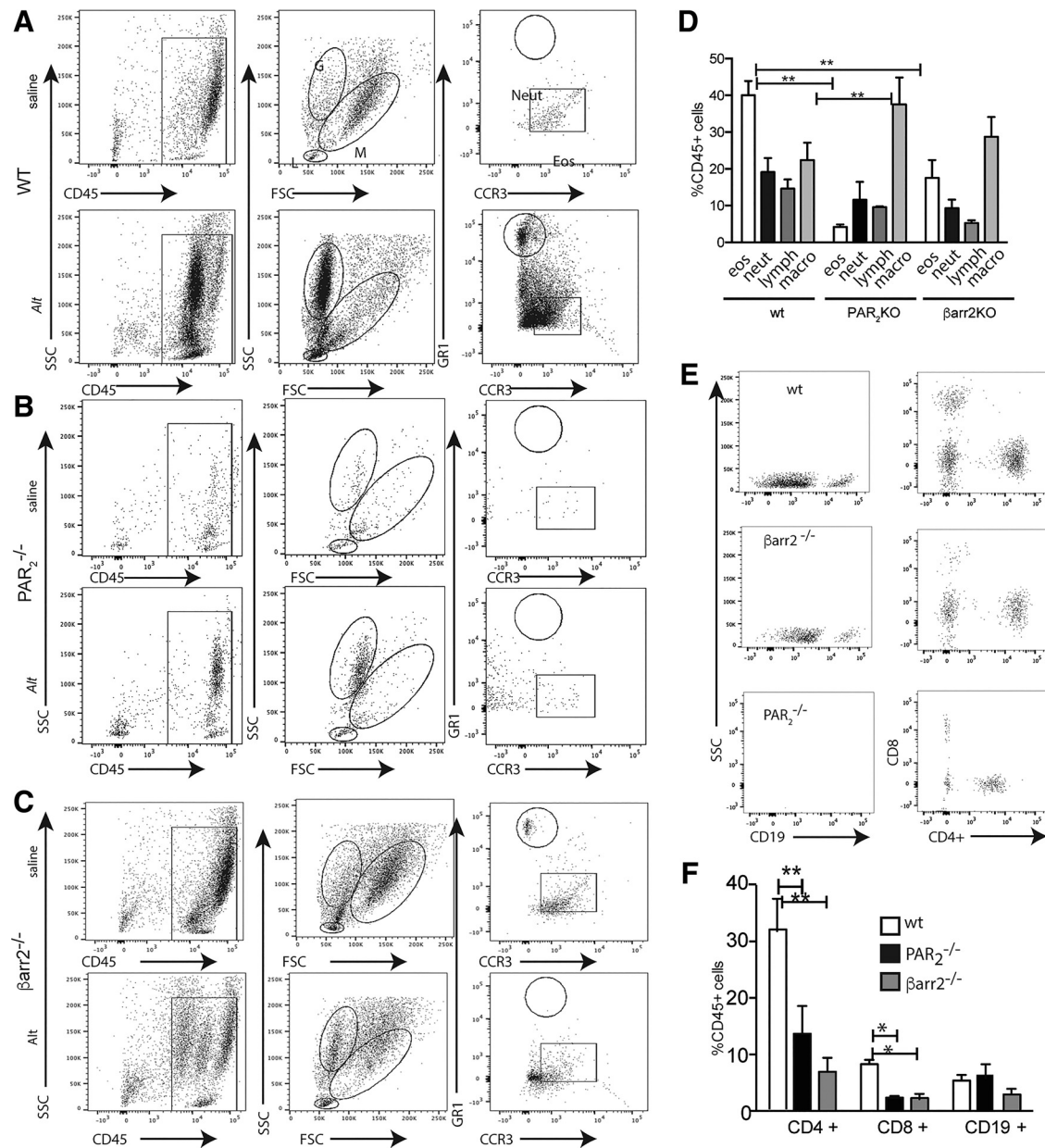


Fig. 7. *Alternaria* treatment increases the percentage of eosinophils (eos) and CD4+ lymphocytes (lymph) in the bronchoalveolar lavage fluid (BALF) by a protease-activated receptor-2 (PAR₂)/β-arrestin-2-dependent mechanism. A–C: representative histograms showing the gating strategy for identification of BALF cell types in wild-type (WT; A), PAR₂^{-/-} (B), and β-arrestin-2^{-/-} (C) mice. D: %CD45+ cells that are scored as eosinophils [high side scatter (SSC), mid-forward scatter (FSC), CCR3+], neutrophils (neut; high SSC, high FSC, GR1+), lymphocytes (low SSC, mid-FSC, CD3+) or macrophages (macro; high SSC/high FSC) by flow cytometric analysis of BALF from *Alt*-treated mice. E and F: %CD45+ cells from *Alt*-treated mice that are CD4+ (Th2), CD8+ (Natural Killer cells) or CD19+ (B cells). Each group includes cell isolations from at least 4 and up to 7 mice. Significant differences between bracketed *Alt*-treated mice are denoted by **P* ≤ 0.05 or ***P* ≤ 0.01.

single serine protease from *Alternaria* is responsible for proteolysis at the canonical PAR₂ cleavage site, leading to receptor activation.

To isolate the active enzyme for biochemical characterization and sequencing, the filtrate was subjected to High Q-anion exchange chromatography. Serine protease activity was isolated from elution fraction 4, free from many of the other contaminating proteins and well separated from the *Alt* a 1 antigen (Fig. 9B). Similar purification of a single protease was achieved by cation exchange chromatography, and the same enzyme was isolated from crude *Alternaria* cellular extract

using a DEAE column (not shown). Although the *Alternaria* enzyme is sensitive to inhibition by SBTI (*K_i* ~25 pM), it displays biochemical characteristics distinct from those of mammalian trypsin in terms of relative *K_m* for a variety of peptide substrates and optimal pH for activity (pH 10 for *Alternaria* protease compared with pH 8 for trypsin; Table 1). Sequencing of the ABP-labeled enzyme from the *Alternaria* culture filtrate and crude cellular extract yielded the same peptide sequence (Table 2). The peptide sequences obtained by mass spectral analysis matched the predicted amino acid sequence of an *Alternaria* enzyme found in the Est database. The

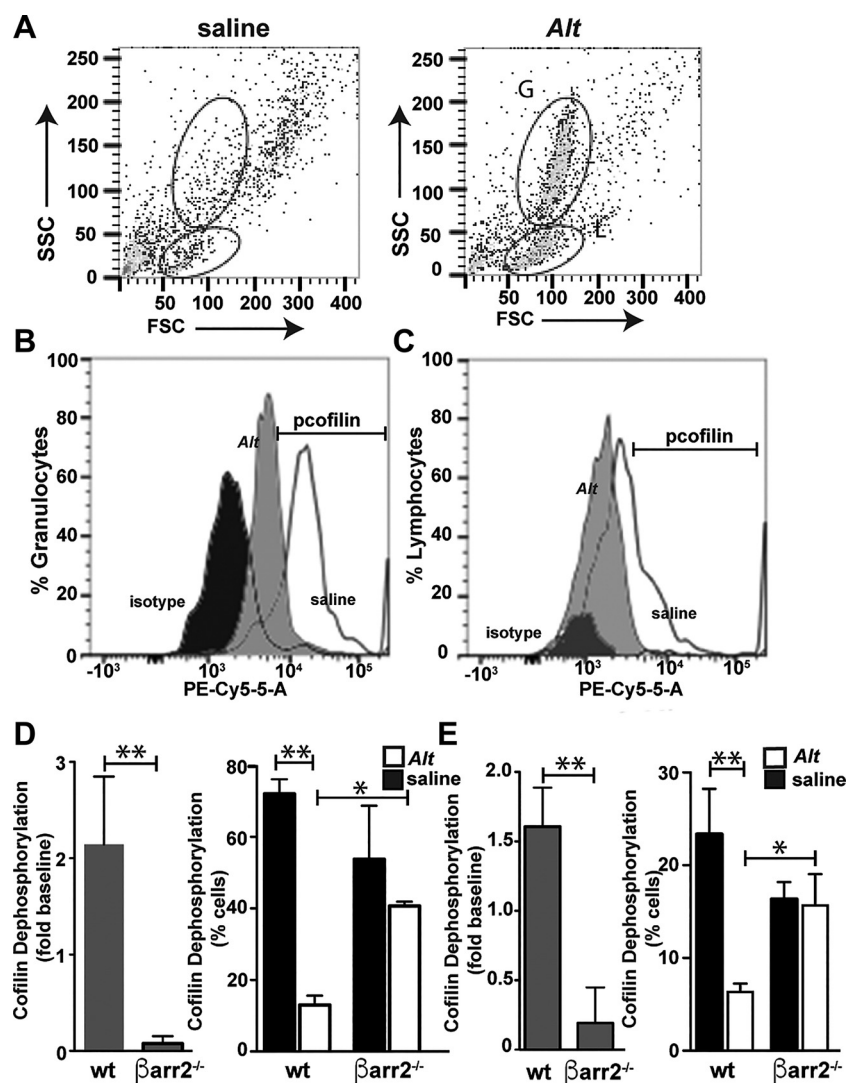


Fig. 8. *Alternaria* (Alt) induces cofilin dephosphorylation in granulocytes and lymphocytes in vivo. Cells were recovered from the bronchioalveolar lavage fluid (BALF) isolated from Alt-treated wild-type (WT) and β -arrestin-2^{-/-} mice. Cells were incubated with anti-CD45 and anti-phospho (p)-cofilin antibodies and analyzed by flow cytometry. Representative dot plot of CD45+ cells analyzed for forward (FSC) and side scatter (SSC). A: granulocytes (G) and lymphocyte (L) populations are indicated. B and C: histogram analysis of p-cofilin fluorescence in the granulocyte (B) and lymphocyte (C) population after treatment with Alt (gray) or Hanks' balanced saline solution control (white). Isotype saline-treated controls (black) are included to show background fluorescence levels. The positive p-cofilin gate is indicated by the bracket. D and E: overall cofilin dephosphorylation (left) and %cells (right) showing reduced cofilin dephosphorylation are shown for granulocytes (D) and lymphocytes (E). Each group includes data from ≥ 4 and ≤ 7 mice. Significant differences between bracketed Alt-treated mice are denoted as follows: * $P \leq 0.05$ or ** $P \leq 0.01$. Alt-dependent cofilin dephosphorylation in vivo was dependent on β -arrestin expression.

sequence is entirely in keeping with a trypsin-like serine protease containing a serine-histidine-aspartic acid catalytic triad with a key ABP-targeted serine. We conclude that both the *Alternaria* culture filtrate and the cellular extract contain the same singular serine protease, with catalytic properties quite distinct from those of mammalian trypsin. We refer to this newly identified protease as *Alternaria* alkaline serine protease (AASP).

Activation of PAR₂ signaling by AASP. We first evaluated the ability of AASP from either of the chromatographic preparations noted above to promote Ca²⁺ mobilization, a hallmark of the canonical PAR₂/G protein signaling. For comparison, we used the *Alternaria* filtrate to induce a Ca²⁺ change in PAR₂-transfected KNRK cells (Fig. 10A). The primary Ca²⁺ response was eliminated by SBTI and by desensitization of PAR₂ with preactivation by the PAR₂ agonist 2fAP (8, 32), consistent with a protease-dependent, PAR₂-specific Ca²⁺ activation. It is notable in this preparation that a secondary Ca²⁺ response appeared to be PAR₂ independent. We next tested the partially purified AASP for PAR₂-dependent Ca²⁺ signaling (fraction 4 from High Q column; Fig. 10B). AASP induced the typical, rapid Ca²⁺ transient in the PAR₂-expressing KNRK cells

associated with PAR₂ activation (19). The Ca²⁺ transient was fully eliminated in desensitized cells, confirming PAR₂-dependent Ca²⁺ mobilization. To examine whether AASP could induce β -arrestin recruitment, we repeated the BRET experiments described in Fig. 1 (see Fig. 10C). The EC₅₀ for AASP-induced β -arrestin recruitment (2.5 U/ml) was similar to that observed for trypsin (Fig. 1C). In summary, the isolated AASP was sufficient to fully activate PAR₂ Ca²⁺ and β -arrestin signaling in our cellular assays.

AASP-induced, PAR₂-mediated inflammation. We next tested whether localized activation of PAR₂ by AASP was sufficient to replicate some or all of the inflammatory effects of the *Alternaria* extract. Wild-type and β -arrestin-2^{-/-} mice were intranasally exposed to either AASP with 0.5 U/kg trypsin-like activity (equivalent to the trypsin-like activity measured in *Alternaria* filtrates above) or the reduced 0.05 U trypsin-like activity in 25 μ l saline. BALF was collected and analyzed for the presence of immune cell infiltrates. Although both doses of the purified AASP induced recruitment of eosinophils and lymphocytes to the airways of wild type mice (Fig. 11A), neutrophils and macrophages were recruited only by the higher dose and were <50% of that observed with total

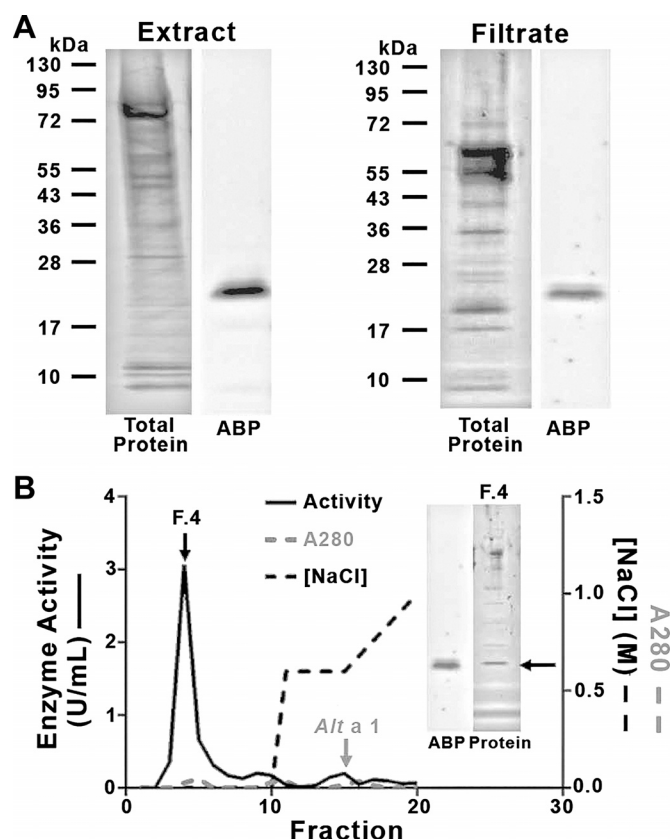


Fig. 9. *Alternaria* extract or filtrates contain a single protease-activated receptor-2 (PAR₂)-activating protease. A: crude extract (left) or culture filtrate (right) was labeled with the biotinylated activity-based probe (ABP), as outlined in MATERIALS AND METHODS. Total proteins are visualized in gel (left) or after Western blot detection of biotinylated enzymes (ABP; right). B: filtrate protein was applied to the High-Q anion exchange column; nonadsorbed fractions (1–10) and those eluted with a 2-step NaCl gradient (dashed black line) were collected. Fractions were monitored for enzyme activity (U/ml; left y-axis, solid black lines) and UV absorption (A280; gray lines, right ordinate). Alt a 1 was detected with ELISA. Protein in fraction 4 was run out on a gel as in A (inset at right and compared with ABP Western blot of whole filtrate from A, left). A single serine protease was labeled, isolated, and separated from the Alt a 1 antigen.

Alternaria filtrate. Significantly, both eosinophil and lymphocyte recruitment were abrogated in the β -arrestin-2^{-/-} mice independent of AASP dose. We conclude that AASP is sufficient to promote acute inflammation in the airway.

AASP-stimulated, prostaglandin-dependent bronchorelaxation. In addition to the β -arrestin-2-dependent inflammatory response to PAR₂ activation observed in our work with the ovalbumin sensitized/2fAP challenged mouse model of asthma, we also observed epithelium-dependent, prostanoid-mediated bronchorelaxation following PAR₂ activation (40). We thus evaluated whether AASP was sufficient to cause bronchorelaxation using a tracheal ring assay (Fig. 12). Following carbachol-induced contraction, the tracheal rings were first subjected to substance P as a control bronchorelaxant. After washout, the experiment was repeated with AASP and a similar bronchorelaxation observed (Fig. 12A). This bronchorelaxation was blocked by the cyclooxygenase inhibitor indomethacin (Fig. 12B). These data are consistent with AASP being sufficient to initiate the “protective” bronchorelaxation response due to the epithelial cell-generated production of

cyclooxygenase-produced agonists (presumably, prostaglandins).

DISCUSSION

Alternaria alternata is a prominent fungal allergen that has been associated with asthma development and cases of severe asthma (10, 27, 51). Extensive work with cellular and animal models has implicated serine-protease(s) from *Alternaria* and their activation of protease-activated receptor-2 (PAR₂) in airway inflammation. PAR₂ signals through a canonical G_q pathway that leads to activation of phosphatidylinositol 3-kinases (PI3K), LIM kinases (LIMK), nuclear mitogen-activated kinases (MAPK), prostaglandin E₂ (PGE₂) synthesis and release, and proliferation (18, 69, 71). PAR₂ can also signal independent of G proteins via recruitment of β -arrestins and subsequent activation of membrane-retained MAPK (i.e., extracellular signal-regulated kinase 1 and 2), cofilin, and inhibition of PI3K and LIMK, all of which lead to reorganization of the actin cytoskeleton and chemotaxis in multiple cell types, including neutrophils and lymphocytes (24, 69, 71, 72). We have demonstrated that in vivo activation of airway PAR₂ with a synthetic peptide agonist (2fAP) promotes leukocyte recruitment, epithelial thickening, and mucus production that requires β -arrestin-2, whereas bronchodilation and PGE₂-mediated smooth muscle relaxation occur independent of β -arrestin-2 signaling (40). These findings were consistent with the in vitro pathways mediated by each signaling arm. Given that PAR₂ is upregulated in both human respiratory epithelium and peripheral blood monocytes in asthmatics (33, 57), PAR₂ signaling is of great interest in asthma development and severity. Previous reports on *Alternaria*-induced signaling in cellular and asthma models have relied on the use of protease inhibitors and/or the monitoring of Ca²⁺ signaling to detail specific roles for PAR₂ (8, 39, 58). It is now apparent that both small molecular ligands (61) and proteases (53) can activate PAR₂ independently of Ca²⁺ signaling. Here, we expand these observations to a more physiologically relevant model and show that proteolytic activation of PAR₂ is sufficient to promote the recruitment of eosinophils and lymphocytes, epithelial thickening, and mucous accumulation observed with exposure to *Alternaria*. Furthermore, we have isolated and characterized a single serine protease from *Alternaria*, *Alternaria* alkaline serine protease (AASP), that is sufficient to induce inflammation in the mouse models. These inflammatory responses require the presence of β -arrestin-2, strongly suggesting that PAR₂ signaling through

Table 1. Biochemical characteristics of AASP

	Substrate for K _m , μ M			Inhibitor (K _i)	
	QAR (trypsin)	FVR (thrombin)	GGR (urokinase)	SBTI, pM	TLCK, nM
Alt	16 $\pm$ 2	366 $\pm$ 57	98 $\pm$ 31	18 $\pm$ 4	12,879 $\pm$ 842
Trypsin	6 $\pm$ 0.6	24 $\pm$ 5	1054 $\pm$ 211	4 $\pm$ 0.4	2 $\pm$ 0.2

AASP, *Alternaria* alkaline serine protease; Alt, *Alternaria*; FR, FR-aminomethylcoumarin; GGR, GGR-aminomethylcoumarin; QAR, QAR-aminomethylcoumarin; SBTI, soybean trypsin inhibitor; TLCK, N α -p-tosyl-L-lysine-chloromethyl ketone. K_m values were determined for partially purified AASP (Alt) and purified trypsin with 3 substrates: QAR (trypsin specific), FVR (thrombin specific), and GGR (urokinase specific). K_i values to trypsin-specific inhibitor SBTI and chymotrypsin-specific inhibitor TLCK were determined; n = 3, K_m and K_i values are graphed as means $\pm$ SE.

Table 2. AASP amino acid sequence

AASP sequence
MRFQSIIAIALPALVLAAPTPQDDPYEFPEDAPADDIVGGTTASAGEFFP <u>IVSLQRSGSHFCGGSLDDSTTVITA</u> AHCSVSSVIGSVSGLRVRAGSLKSSGGTLVG
<u>VSSVTVHPSYRSGQDFDVAIWKLSTAVPTSSTIGYATLPASGSDPAAGSTATVAGWGALTEGGSSPSTLYKVSVP</u> IVSRTECRSSYGTSAITNNMFCAGYTT
<u>GGRDSCQDSSGPIVNSAKTLIGLVSWNGCAQPNFPGVYARTAAALLSFINSV</u>
99% Confidence
IVSLQRSGSHF
RVRAGSLNKSSGGTLVGVSSVTVHPSY
NKSSGGTLVGVSSVTVHPSY
VGVSSTVHPSY
RSSGQDFDVAIW
KLSTAVPTSSTIGY
ATLPASGSDPAAGSTATVAGW
GALTEGGSSPSTL
GALTEGGSSPSTLY
KVSVP
IVSRTECRSSY
GTSAITNNMF
GTSAITNNMFCAGY
TTGGRDSCQDSSGPIVNSAKTL
GNGCAQPNFPGVY
SFINSV
95% Confidence
IGLVSW
ARTAAALL

AASP, *Alternaria* alkaline serine protease. The AASP sequences obtained by mass spectral procedures, as outlined in MATERIALS AND METHODS, were deconvoluted by comparison with the translated amino acid sequence of an *Alternaria*-expressed sequence tag library, also as outlined in MATERIALS AND METHODS. Sequences that were identified with 99% confidence (underlined) and 95% confidence (boldface) are noted in the table and are shown below.

β -arrestin is sufficient to promote airway inflammation. *Alternaria* and isolated AASP are capable of promoting both G protein-dependent signals (as demonstrated by Ca^{2+} mobilization) and β -arrestin-dependent signals (as demonstrated by β -arrestin recruitment to PAR₂ and activation of cofilin). The slightly different immune cell recruitment induced by *Alternaria* filtrates and AASP confirms that roles for alternate proteins/antigens from *Alternaria* are required for full airway inflammation; nonetheless, the protease alone is able to promote significant inflammation.

While PAR₂^{-/-} mouse models have been used to evaluate the role for house dust mite- (15) or cockroach-induced asthma (16, 45), examination of β -arrestin-2 signaling arm for PAR₂ activation has provided further clues in understanding the mechanisms that underlie allergic asthma. That GPCR/ β -arrestin signaling pathways are important mediators of inflamma-

tion, and airway hyperresponsiveness is corroborated by other studies in ovalbumin-induced and cigarette smoke-induced allergic asthma animal models (11, 68) and by studies showing that some of the adverse effects associated with β_2 -adrenergic receptor activation are β -arrestin dependent (20, 64). Our studies suggested that PAR₂-stimulated, β -arrestin-dependent cell migration is a major contributing factor to the cellular inflammation observed during asthma. These findings led to the prediction that β -arrestin-dependent activation of cofilin might be increased in the *Alternaria*-induced asthma model, a theory that is supported by the observation here that the cells recruited to the BALF by administration of *Alternaria* have more active (dephosphorylated) cofilin compared with cells found in the BALF in control mice. Furthermore, the observation that *Alternaria*-induced dephosphorylation of cofilin is not observed in β -arrestin-2^{-/-} mice suggests that the PAR₂- β -

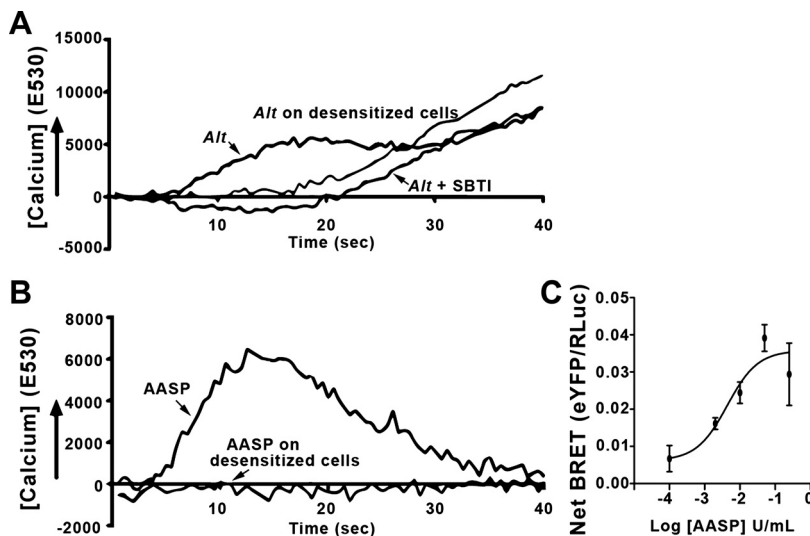


Fig. 10. *Alternaria* (Alt) alkaline serine protease (AASP) induces protease-activated receptor-2 (PAR₂)-dependent Ca^{2+} and β -arrestin signaling. A and B: Kirsten virus-transformed rat kidney cells (KNRK) cells transfected with PAR₂ were loaded with the Ca^{2+} sensitive dye Fluo-4 and treated with Alt filtrate (A) or AASP from High-Q column fraction F4 (B) from Fig. 9B. Alt filtrate induced a protease-sensitive Ca^{2+} transient that required functional PAR₂, as both soybean trypsin inhibitor (SBTI) and desensitization prevented the primary Ca^{2+} signaling. AASP induced a Ca^{2+} transient that required functional PAR₂ as desensitization eliminated the Ca^{2+} signal. C: bioluminescence resonance energy transfer (BRET) was measured upon addition of increasing concentrations of AASP, as described in Fig. 1C. Half-maximal BRET value (BRET₅₀) for AASP (2.0 U/ml) was similar to what was determined for trypsin (2.5 U/ml). AASP is sufficient to fully induce signaling pathways via PAR₂; $n \geq 3$ for all experiments (A and B) and experimental time points (C).

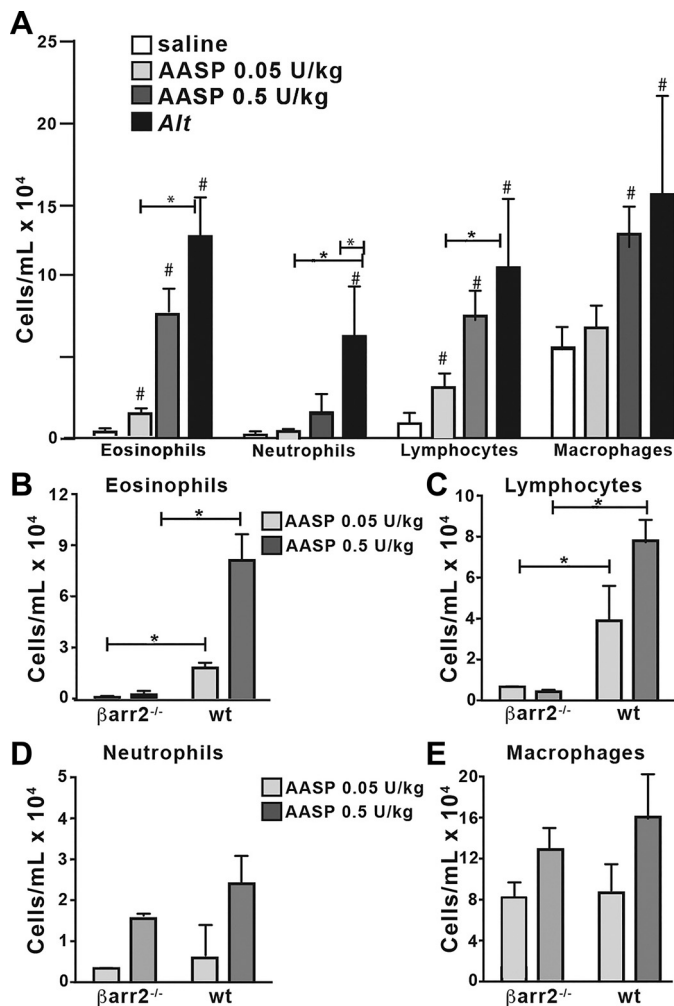


Fig. 11. *Alternaria* (Alt) alkaline serine protease (AASP) promotes β -arrestin-2-dependent recruitment of eosinophils and lymphocytes to the airways. Two doses of AASP were administered intranasally to wild-type (WT) or β -arrestin-2^{-/-} mice ($n \geq 3$). Negative controls (saline) and positive controls (Alt) were included. Bronchioalveolar lavage fluid (BALF) was collected and differential counts performed. Quantification of cell types in WT mice. A: AASP exposure elicited airway inflammation of all cell types, with the exception of neutrophils. B–E: direct comparison between WT and β -arrestin-2^{-/-} mice of eosinophil (B), lymphocyte (C), neutrophil (D), and macrophage (E) recruitment following exposure of high and low doses of AASP. Both eosinophil and lymphocyte recruitment require β -arrestin-2 expression. $P \leq 0.05$, *significant differences between bracketed experiments; #significant differences from saline control.

arrestin signaling axis is associated with the inflammatory process induced by *Alternaria* in vivo. Whether T cells directly encounter *Alternaria* proteases or whether the PAR₂ response in vivo is a result of other serine proteases and chemokines released in response to PAR₂ is still unclear. However, both *Alternaria* and 2fAP-mediated PAR₂ activation promote barrier damage in cultured airway epithelial cells (Refs. 25 and 35 and Nichols HL and DeFea KA, unpublished observations), raising the possibility that some of the apically acting proteases might gain access to the serosa. Nonetheless, these studies are the first to demonstrate activation of β -arrestin-dependent PAR₂ signaling by a ubiquitous allergen and the first to show an in vivo correlation of β -arrestin-dependent cofilin activation and leukocyte migration with lung inflammation.

Although many of the parameters of lung inflammation were similar in the *Alternaria*-asthma model we used herein and the ovalbumin/2fAP asthma model used in previous studies (40), there were some notable differences. First, we see a striking increase in neutrophil infiltration in the *Alternaria* asthma model. Although airway eosinophilia often characterizes allergic asthma, it is widely recognized that neutrophils are found in patients with acute severe asthma (17, 43). It has been shown in BALB/C mice that *Alternaria* induces a neutrophilic response in STAT6^{-/-} animals rather than the more typical eosinophilic response (66). In that report, the authors postulated that neutrophil recruitment is a normal response that is exacerbated when mechanisms for eosinophil migration are eliminated. In our study, several lines of evidence suggest that neutrophil infiltration was slightly less dependent upon PAR₂/ β -arrestin-2 signaling and trypsin-like protease activity than were eosinophils and leukocytes. First, whereas eosinophil and CD4⁺-T cell infiltration into the airway was reduced by 80–90% in PAR₂^{-/-} and β -arrestin-2^{-/-} or by pretreatment with SBTI, neutrophils were only reduced by 50%. Second, the purified AASP failed to promote significant neutrophil recruitment to the airways. These findings suggest a more complex signaling network at play, perhaps involving direct activation of immune cells by PAR₂ agonists as well as release of chemokines that then act to recruit additional cells. Because AASP was insufficient to promote the neutrophilia observed with exposure to complete *Alternaria* extract, the recruitment of different cell types is likely mediated by nonproteolytic components of the *Alternaria* allergen. Taken together with our previous evidence that IgE production is not dependent upon the β -arrestin pathway but that PAR₂ contributes to the process, these data suggest that AASP may mediate the localized PAR₂ exacerbation of asthma in the airways via PAR₂/ β -arrestin signaling, whereas the systemic responses may be mediated by other components.

A main finding of our study is that the asthma allergen *Alternaria alternata* contains a single trypsin-like serine protease, AASP, that can activate PAR₂ to promote airway inflammation via a β -arrestin-dependent mechanism. Serine proteases are a recurring theme among asthma-associated allergens (2), including those from other molds (13), house dust

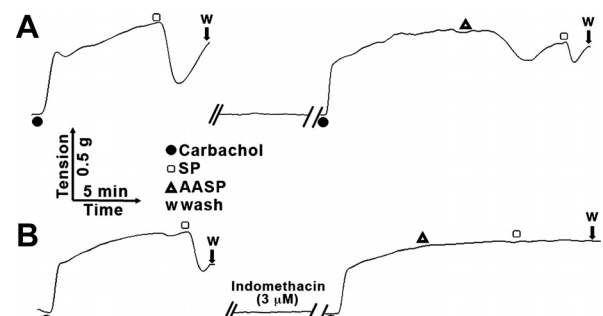


Fig. 12. *Alternaria* alkaline serine protease (AASP)-mediated bronchorelaxation. Second-order mouse bronchiolar ring segments were first contracted with 1 μ M carbachol and then exposed to substance P (SP; 20 nM) to demonstrate a prostanoind-generated indomethacin-blocked epithelium-dependent relaxation. Following wash with buffer (A) or buffer with 3 μ M indomethacin (B), carbachol was reapplied, followed by AASP (0.6 U/ml) and a 2nd SP wash. AASP induced an indomethacin-sensitive bronchorelaxation. $n \geq 3$ for all experiments.

mite (59, 63), and cockroach (42, 50, 60). The protease described here differs considerably from the recently described vacuolar serine protease from *Alternaria*, Alt a 15 (21). Similarly to the *Alternaria* filtrate, AASP was able to activate PAR₂ and initiate both Ca²⁺ and β -arrestin signaling. However, a close examination of the Ca²⁺ experiments shows a secondary Ca²⁺ signal invoked by the *Alternaria* filtrate that is absent in the AASP experiments. It has been shown that *Alternaria* exposure of epithelial cells can induce metabolite release, including ATP (41, 54, 58), and such metabolites could extend the Ca²⁺ signal. However, the Ca²⁺ signal induced by AASP was more similar to what is typically seen by specific PAR₂-activating peptides (19). As noted above, AASP was sufficient for sensitization and initiation of airway inflammation, albeit with the notable change in neutrophil cell recruitment when compared with the *Alternaria*-induced asthma model. From these findings, we conclude that AASP is itself a potent asthma allergen that defines much but not all of the *Alternaria*-induced asthma response.

Although we and others have demonstrated a major role for PAR₂ in the progression of airway inflammation, PAR₂ activation has also been reported to orchestrate protective effects in the airway by promoting prostaglandin E₂ (PGE₂)-mediated bronchiolar smooth muscle relaxation, leading to bronchodilation (14, 29, 34, 40). We observed a similar indomethacin-sensitive bronchorelaxing action of AASP, indicating the “dual” effect of PAR₂ activation on the airways, on the one hand promoting the influx of inflammatory cells and on the other triggering what might be considered as a “protective effect.” Thus, chronically, the overall effect of PAR₂ activation may enhance the pathogenicity of the allergen and promote irreversible airway remodeling, which worsens the pathology. That said, the acute action of the allergen protease activating PAR₂ on the airway epithelium to enhance bronchorelaxation represents an intrinsic beneficial action of PAR₂ stimulation.

Triggered by infection, environmental allergens, or other stimuli, asthma remains poorly understood and difficult to manage due to the heterogeneity of the disease. In addition, there are concerns surrounding the side effects from long-term use of the most frequently prescribed asthma medications that represent different therapeutic classes currently used clinically as FDA-approved therapies. Treatments that have been the mainstay of asthma medications for almost 50 yr include corticosteroids (inflammation reduction), short-acting β_2 -agonists (fast response bronchorelaxant), long-acting β_2 -agonists (bronchorelaxant for preventative management), mast cell stabilizers (inflammatory control), anticholinergics (supplemental bronchorelaxant), and leukotrienes (supplemental anti-allergen and anti-inflammatory) (37, 47). Although asthma treatments have been largely unchanged, it is an unfortunate reality that asthma remains uncontrolled in more than half the patients receiving standard asthma medication (48). Given its role(s) in asthma sensitization and exacerbation, PAR₂ represents a viable target for drug intervention in asthma. Enhancing this view are the findings that PAR₂-neutralizing antibodies were recently shown to reduce asthma symptoms in acute and chronic allergic asthma mouse models (6, 7). There are only a handful of PAR₂ antagonists that have been proposed (reviewed in Ref. 70). These include K-14585 (26, 31), GB88 (30, 61, 62), C391 (9), two small molecules isolated from a large screening library (AC-55541 and AC-264613) (23), and two recently described

PAR₂ allosteric antagonists (AZ8838 and AZ3451) (12). To our knowledge, none of these antagonists have been tested in asthma animal models. C391 is distinct from other antagonists in that it requires minimal pre-incubation for inhibition of peptide activation of PAR₂-dependent Ca²⁺ signaling, it acts as an antagonist for both Ca²⁺ and β -arrestin signaling pathways, and it is the only known antagonist shown to be effective in blocking both natural protease and peptidomimetic activation of PAR₂ in vitro (Ref. 9 and Yee MC, DeFea KA, and Boitano S, unpublished results). When considering the biphasic effects with respect to PAR₂ signaling in the airway (i.e., inflammation and bronchorelaxation), there is a growing interest in developing both PAR₂-signaling biased agonists and antagonists as putative therapeutic agents for asthma (2, 67). Because our previous studies suggest that the “protective effects” of PAR₂ activation are independent of β -arrestin signaling (40), we suggest that it will be of therapeutic value to develop biased PAR₂ antagonists that target the β -arrestin-dependent PAR₂ signaling pathway, but which do not affect the β -arrestin-independent PAR₂ signaling pathways that may be of benefit in the setting of asthma induced by PAR₂-activating household allergens such as *Alternaria*.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

M.C.Y., H.L.N., D.P., M.S., K.P., and K.L. performed experiments; M.C.Y., H.L.N., D.P., M.S., K.P., K.L., E.H.W., M.O.D., M.D.H., and K.A.D. analyzed data; M.C.Y., H.L.N., D.P., E.H.W., M.O.D., M.D.H., S.B., and K.A.D. interpreted results of experiments; M.C.Y., H.L.N., D.P., M.S., K.P., K.L., E.H.W., M.D.H., S.B., and K.A.D. prepared figures; M.C.Y., S.B., and K.A.D. drafted manuscript; M.C.Y., H.L.N., D.P., M.S., K.P., K.L., E.H.W., M.O.D., M.D.H., S.B., and K.A.D. approved final version of manuscript; S.B. and K.A.D. edited and revised manuscript.

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