

# $\beta$ -Arrestin-2 mediates the proinflammatory effects of proteinase-activated receptor-2 in the airway

Heddie L. Nichols<sup>a</sup>, Mahmoud Saffeddine<sup>b</sup>, Barbara S. Theriot<sup>c</sup>, Akhil Hegde<sup>c</sup>, Daniel Polley<sup>b</sup>, Tamer El-Mays<sup>d</sup>, Harissios Vliagoftis<sup>e</sup>, Morley D. Hollenberg<sup>b</sup>, Emma H. Wilson<sup>a</sup>, Julia K. L. Walker<sup>c</sup>, and Kathryn A. DeFea<sup>a,1</sup>

<sup>a</sup>Division of Biomedical Sciences, University of California, Riverside, CA 92521; <sup>b</sup>Department of Physiology and Pharmacology, and <sup>d</sup>Department of Pathology and Laboratory Medicine, University of Calgary, Calgary, AB, Canada T2N 1N4; <sup>c</sup>Pulmonary, Allergy and Critical Care Medicine, Duke University Medical Center, Durham, NC 27710; and <sup>e</sup>Pulmonary Research Group, University of Alberta, Edmonton, AB, Canada T6G 2S2

Edited by Robert J. Lefkowitz, Howard Hughes Medical Institute, Duke University Medical Center, Durham, NC, and approved September 4, 2012 (received for review May 24, 2012)

Proteinase-Activated receptor-2 (PAR<sub>2</sub>), a G-protein-coupled Receptor, activated by serine proteinases, is reported to have both protective and proinflammatory effects in the airway. Given these opposing actions, both inhibitors and activators of PAR<sub>2</sub> have been proposed for treating asthma. PAR<sub>2</sub> can signal through two independent pathways: a  $\beta$ -arrestin-dependent one that promotes leukocyte migration, and a G-protein/Ca<sup>2+</sup> one that is required for prostaglandin E<sub>2</sub> (PGE<sub>2</sub>) production and bronchiolar smooth muscle relaxation. We hypothesized that the proinflammatory responses to PAR<sub>2</sub> activation are mediated by  $\beta$ -arrestins, whereas the protective effects are not. Using a mouse ovalbumin model for PAR<sub>2</sub>-modulated airway inflammation, we observed decreased leukocyte recruitment, cytokine production, and mucin production in  $\beta$ -arrestin-2<sup>-/-</sup> mice. In contrast, PAR<sub>2</sub>-mediated PGE<sub>2</sub> production, smooth muscle relaxation, and decreased baseline airway resistance (measures of putative PAR<sub>2</sub> "protective" effects) were independent of  $\beta$ -arrestin-2. Flow cytometry and cytosins reveal that lung eosinophil and CD4 T-cell infiltration, and production of IL-4, IL-6, IL-13, and TNF $\alpha$ , were enhanced in wild-type but not  $\beta$ -arrestin-2<sup>-/-</sup> mice. Using the forced oscillation technique to measure airway resistance reveals that PAR<sub>2</sub> activation protects against airway hyperresponsiveness by an unknown mechanism, possibly involving smooth muscle relaxation. Our data suggest that the PAR<sub>2</sub>-enhanced inflammatory process is  $\beta$ -arrestin-2 dependent, whereas the protective anticonstrictor effect of bronchial epithelial PAR<sub>2</sub> may be  $\beta$ -arrestin independent.

Currently, 300 million people suffer from asthma resulting in nearly 250,000 asthma-related deaths reported annually, ~80% occurring in low- and lower-middle-income regions. The development of new medications that inhibit cellular inflammation may reduce morbidity rates, and attempts to manage this disease have identified proteinase-activated receptor-2 (PAR<sub>2</sub>) as an attractive new target (1). PAR<sub>2</sub> is a G-protein-coupled receptor (GPCR) that is widely expressed in bronchial epithelial cells, leukocytes, and airway smooth muscle, where it may be activated by proteinases secreted from invading pathogens, inhaled proteinases, or by locally released proteinases such as tissue kallikreins or tryptase (2–5). Serine proteinases activate PAR<sub>2</sub> by cleaving its N terminus, revealing a tethered ligand (SLIGRL/SLIGKV, human/mouse) that self-activates the receptor, leading to G-protein coupling and  $\beta$ -arrestin recruitment. Peptides corresponding to the tethered ligand and peptidomimetics such as 2-furoyl-LIGRL-ornithine-NH<sub>2</sub> (2fAP) are commonly used to activate PAR<sub>2</sub>, both in cultured cells and in vivo (6, 7). We previously reported that PAR<sub>2</sub> can activate two independent signaling pathways, one transduced by "classical" G-protein-coupled signaling and the other by a G-protein-independent,  $\beta$ -arrestin-mediated signaling pathway (8–12). Although the two signaling pathways can target common downstream effectors, the outcomes can be distinct and even opposing. For example,  $\beta$ -arrestins can scaffold the actin-severing protein, cofilin, with its upstream activator (Chronophin) while inhibiting its negative regulator (LIMK). This scaffold has been identified in fibroblasts, tumor cells, and primary mouse leukocytes, and is crucial for PAR<sub>2</sub>-

stimulated chemotaxis. Downstream of the G-protein-coupled pathway, this same process is inhibited (11, 12).

In keeping with its opposing signals in vitro, studies done in vivo suggest that PAR<sub>2</sub> activation can play diametrically opposed roles in allergic asthma. In favor of a proinflammatory role for PAR<sub>2</sub>, the recruitment of leukocytes to the lungs in a murine ovalbumin (OVA) model of allergic inflammatory airway disease was reduced in PAR<sub>2</sub><sup>-/-</sup> mice and increased in PAR<sub>2</sub>-overexpressing mice (13, 14). The inflammatory response to OVA is also enhanced by the intranasal administration of PAR<sub>2</sub> peptide agonists in wild-type (WT) mice (15). These inflammatory responses involve cell migration, leading to the hypothesis that they are  $\beta$ -arrestin dependent.  $\beta$ -Arrestins are also required for leukocyte chemotaxis downstream of a number of other GPCRs, including several chemokine receptors known to be involved in allergic asthma (11, 12, 16). In favor of a protective role, administration of PAR<sub>2</sub> agonists promotes prostanoinduced cytoprotection in rodent and human airways, and bronchoconstriction is elevated in PAR<sub>2</sub><sup>-/-</sup> mice (17). Prostaglandin E<sub>2</sub> (PGE<sub>2</sub>) production also inhibits eosinophil migration and degranulation (17, 18). PAR<sub>2</sub>-induced PGE<sub>2</sub> production uses a G $\alpha_q$ -Ca<sup>2+</sup>-coupled mechanism that we hypothesize is independent of  $\beta$ -arrestins (19, 20). Depending on the balance of PAR<sub>2</sub> signals between G-protein- and  $\beta$ -arrestin-dependent pathways, PAR<sub>2</sub> agonists may be capable of either compounding or curbing allergic asthma. This study examines the potential role of the  $\beta$ -arrestin signaling pathway in the proinflammatory and protective actions of PAR<sub>2</sub> in the airway.

## Results

**PAR<sub>2</sub> Induces Cellular Airway Inflammation in WT but Not  $\beta$ -Arrestin-2<sup>-/-</sup> Mice.** To assess a role for  $\beta$ -arrestin-2 in PAR<sub>2</sub>-induced cellular airway inflammation, we used a modification of a previously described OVA-induced murine model of allergic asthma (15). Mice were sensitized with an i.p. injection of saline (as a negative control) or OVA/alum, on days 1 and 6. This was followed on days 12 and 14 by an intranasal (i.n.) challenge of saline or OVA plus either a PAR<sub>2</sub> agonist (2fAP) or a scrambled negative control peptide [2-furoyl-OLRIGL-NH<sub>2</sub> (CP)]. Optimal 2fAP concentrations were determined by conducting a dose-response curve (Fig. S1). The mice were killed on day 15, bronchoalveolar lavage fluid (BALF) and lungs were collected, and cellular inflammation was assessed (Fig. 1A). In this short-term model, the response to OVA alone is less pronounced, and

Author contributions: M.D.H. and K.A.D. designed research; H.L.N., M.S., B.S.T., A.H., D.P., and T.E.-M. performed research; H.V., M.D.H., E.H.W., and J.K.L.W. contributed new reagents/analytic tools; H.L.N., E.H.W., J.K.L.W., and K.A.D. analyzed data; and H.L.N., M.D.H., J.K.L.W., and K.A.D. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

<sup>1</sup>To whom correspondence should be addressed. E-mail: kathryn.defea@ucr.edu.

This article contains supporting information online at [www.pnas.org/lookup/suppl/doi:10.1073/pnas.1208881109/-DCSupplemental](http://www.pnas.org/lookup/suppl/doi:10.1073/pnas.1208881109/-DCSupplemental).

the additive effect of PAR<sub>2</sub> on OVA-induced inflammation is more evident. Analysis of H&E-stained lung tissue sections reveals that influx of inflammatory cells into the perivascular and peribronchial regions from all WT mice treated with OVA (receiving either CP or 2fAP with the i.n. challenges) was increased compared with saline controls (Fig. 1B). Lung sections were scored for characteristics associated with acute and chronic inflammation as described in *Materials and Methods* revealing that the inflammation index was 1.5-fold greater for OVA+2fAP-treated WT compared with the OVA+CP-treated mice (Fig. 1C). In contrast, OVA+2fAP treatment was unable to exacerbate inflammation in  $\beta$ -arrestin-2<sup>-/-</sup> mice. Although OVA+CP treatment elevated total BALF cells for both WT and  $\beta$ -arrestin-2<sup>-/-</sup> mice relative to their respective saline controls, the effect was significantly less in  $\beta$ -arrestin-2<sup>-/-</sup> mice, consistent with previous reports (21). Challenge with OVA+2fAP in WT animals augmented the influx of inflammatory cells (16.6  $\pm$  3.5-fold increase over saline), but in  $\beta$ -arrestin-2<sup>-/-</sup> mice, no augmentation of total cells was observed with 2fAP (Fig. 2A). Differential counts, performed from cytospin preparations (Fig. S2), revealed challenge of WT mice with OVA+CP alone increased lung recruitment of both eosinophils (Fig. 2B) and lymphocytes (Fig. 2C) compared with saline controls. Challenge with OVA+2fAP further increased the numbers of both cell types in the BALF of WT mice by 3- and 2.5-fold, respectively, over those observed with OVA+CP. In contrast, the numbers of eosinophils and lymphocytes recovered from the BALF of  $\beta$ -arrestin-2<sup>-/-</sup> mice receiving OVA+CP was significantly lower than those in similarly treated WT mice, and the proinflammatory effect of 2fAP was not observed in  $\beta$ -arrestin-2<sup>-/-</sup> mice. In  $\beta$ -arrestin-1<sup>-/-</sup> mice, the inflammation induced by OVA alone and the PAR<sub>2</sub>-stimulated augmentation of inflammation were not significantly different from that of similarly treated WT mice, suggesting the requirement is specific to  $\beta$ -arrestin-2 (Fig. S3). Flow-cytometric analysis, using forward and side scatter (FSC/SSC) and expression of cell surface markers CCR3 and CD3 to calculate the percentage of CD45<sup>+</sup> BALF cells that were either eosinophils or lymphocytes, respectively (Fig. 2D and E), revealed that BALF from OVA+CP-treated WT mice contained ~50% eosinophils and 9% lymphocytes. In contrast, the inflammatory response to OVA+CP in  $\beta$ -arrestin-2<sup>-/-</sup> mice was significantly muted (19% CCR3<sup>+</sup>; <5% CD3<sup>+</sup>). Whereas PAR<sub>2</sub> activation by 2fAP elevated BALF levels of both eosinophils (72%) and lymphocytes (22%) in WT mice, no such effect was observed in similarly treated  $\beta$ -arrestin-2<sup>-/-</sup> mice. Similar results were observed in

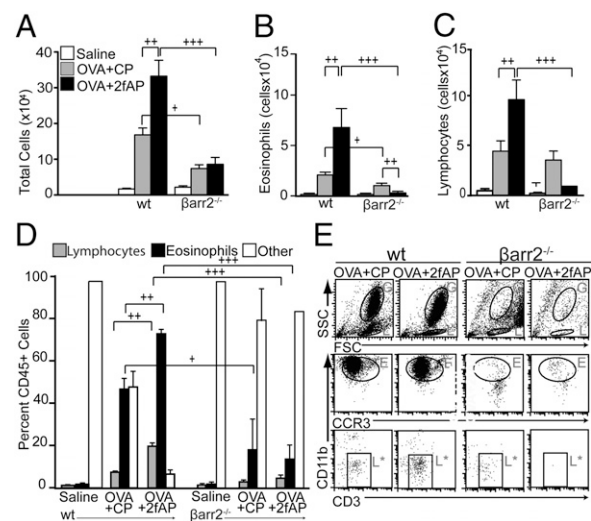


**Fig. 2.** PAR<sub>2</sub>-induced recruitment of leukocytes to the lungs requires  $\beta$ -arrestin-2. (A–C) Bar graphs depict cell numbers in BALF: total cells (A), eosinophils (B), and lymphocytes (C). Numbers from all OVA-treated mice were statistically different from saline controls except eosinophils in  $\beta$ -arrestin-2<sup>-/-</sup> treated with OVA+2fAP. (D) Flow-cytometric determination of the percentage of CD45<sup>+</sup> cells that were lymphocytes, eosinophils, or other cells. Significant differences between bracketed groups are indicated as follows: \* $P$  < 0.05, \*\* $P$  < 0.01, and \*\*\* $P$  > 0.001. (E) Representative scatter plots of flow-cytometric analysis of BALF cells. High FSC/SSC, CD45<sup>+</sup> granulocyte populations (G) were analyzed for the presence of CD11b and CCR3 [eos (E)]. Low SSC, CD45<sup>+</sup> lymphocyte (L) populations were further analyzed for the presence of the T-lymphocyte marker, CD3 (L\*) ( $n$  = 18 for saline,  $n$  = 24 each for OVA+CP and OVA+2fAP).

digested lung tissue (Fig. S4). Lymphocytes, identified by SSC, were further analyzed for expression of lineage-specific markers (Fig. S4). When 2fAP treatment replaces that of CP in the context of OVA, a significant elevation in BALF CD4<sup>+</sup> and CD8<sup>+</sup> cells occurs, but only in WT mice. Consistent with the other BALF data, OVA+CP treatment has a significantly reduced inflammatory effect on  $\beta$ -arrestin-2<sup>-/-</sup> mice relative to WT mice (Fig. 3B). Both WT and  $\beta$ -arrestin-2<sup>-/-</sup> mice, challenged with either OVA+CP or OVA+2fAP, showed a similar increase in production of OVA-IgE, compared with saline-treated controls (Table 1), suggesting that the differences in cellular inflammation observed in  $\beta$ -arrestin-2<sup>-/-</sup> mice were not due to their inability to become sensitized to ovalbumin. Measurement of surface PAR<sub>2</sub> levels in the airways of WT and  $\beta$ -arrestin-1 and -2<sup>-/-</sup> mice demonstrated that there was no significant difference in surface receptor levels, suggesting the effect was not due to decreased PAR<sub>2</sub> expression (Fig. S5).

To determine the extent to which 2fAP is able to access cells expressing PAR<sub>2</sub> within the pleural cavity, we examined the labeling of airway cells with rhodamine-conjugated 2fAP (Rh-2fAP) 24 h after i.n. administration. We observed Rh-2fAP-labeled cells distributed along both the basement membrane and apical surface of airway epithelia in WT mice, but not PAR<sub>2</sub><sup>-/-</sup> mice (Fig. S6). These data raise the possibility that PAR<sub>2</sub> expressed on leukocytes might mediate their chemotaxis into the lungs. This hypothesis was further supported by the observation that the cellular inflammation observed in response to OVA+2fAP is partially mediated by hematopoietic PAR<sub>2</sub>. We observed that OVA+2fAP-induced airway inflammation was reduced after adoptive transfer of PAR<sub>2</sub><sup>-/-</sup> bone marrow cells into WT mice (Fig. S7). Conversely, when WT bone marrow was transplanted into PAR<sub>2</sub><sup>-/-</sup> mice and these mice were challenged with OVA+2fAP, recruitment of immune cells to the lungs was partially restored.

**PAR<sub>2</sub>-Induced Cytokine Production Is Partially Dependent on  $\beta$ -Arrestin-2.** Allergic asthma involves a Th2 response orchestrated by cytokines and chemokines secreted by epithelial cells, T cells, and other

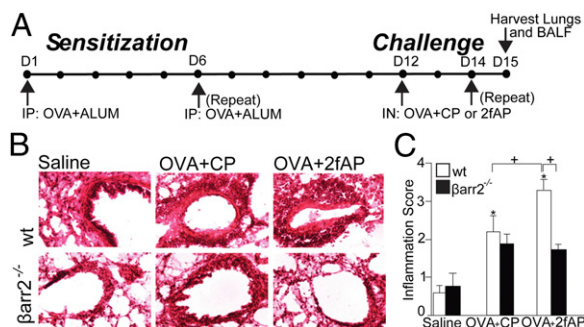


**Fig. 1.** PAR<sub>2</sub>-induced lung inflammation is reduced in  $\beta$ -arrestin-2<sup>-/-</sup> mice. (A) Timeline of OVA-induced sensitization and PAR<sub>2</sub> challenge in WT and  $\beta$ -arrestin-2<sup>-/-</sup> mice (24 per OVA treatment group, 18 per saline treatment group, 128 total mice). (B) Representative images of H&E-stained frozen lung sections from mice treated as described in Fig. 1A. (C) Histological inflammation scoring for peribronchial thickness and leukocyte invasion on a scale from 0 to 4 (see *SI Materials and Methods* for details; 20 sections from each mouse, four separate experiments, scored double-blinded). \*Significant difference between groups ( $P$  < 0.01). \*All groups differed significantly from saline-treated mice ( $P$  < 0.05,  $n$  = 15 for saline,  $n$  = 20 for OVA+CP and OVA+2fAP). Statistics in this and all subsequent figures were determined by ANOVA with Tukey HSD posttests.



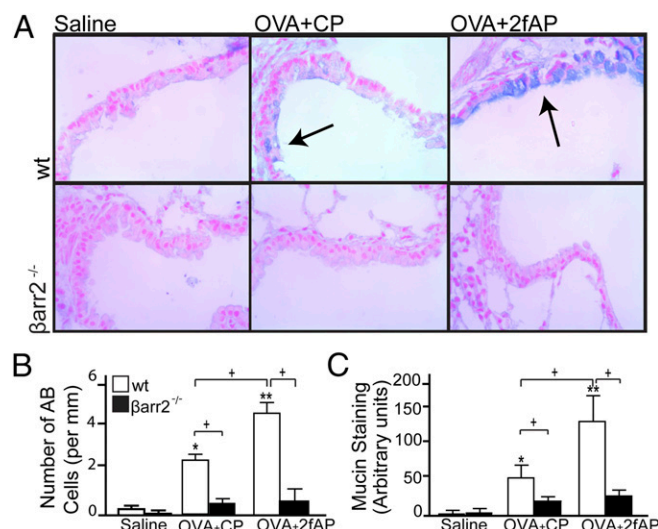


**Fig. 5.** Goblet cell hyperplasia and mucin production in response to PAR<sub>2</sub> is abolished in β-arrestin-2<sup>-/-</sup> mice. (A) Lung tissue from each of the treatment groups shown in Fig. 1A was stained with Alcian blue to reveal acidic mucins and counterstained with neutral red. The arrows indicate mucin-producing goblet cells. (B) Double-blind quantification of the number of mucin-producing cells per millimeter of basement membrane. (C) Quantification of intensity of Alcian blue stain. Significant difference from saline treated mice (\**P* < 0.05; \*\**P* < 0.005). Significant differences between bracketed groups are indicated as follows: \**P* < 0.05. Twenty images of at least two mice from three independent experiments (120 total) were analyzed for B and C.

debate as to whether PAR<sub>2</sub> is a protective or proinflammatory receptor in allergic inflammatory airway disease resembles the continued controversy surrounding the function of the β<sub>2</sub>-adrenoceptor (β<sub>2</sub>-AR), the prototypical GPCR, in asthma. Although β-agonists are bronchodilators of choice to alleviate acute bronchospasm, their chronic use is associated with loss of bronchoprotection, worsening of asthma control, and asthma-related death (26). In murine models, the asthma phenotype is significantly promoted by chronic β-agonist treatment (27, 28) or impaired in mice having genetic or pharmacologic ablation of β<sub>2</sub>-ARs (29, 30). Similarly, mice lacking β-arrestin-2 do not develop the asthma phenotype (21), placing β-arrestin-2 downstream of GPCRs in the proasthmatic signaling pathway. Thus, the seemingly paradoxical effects of PAR<sub>2</sub> and β<sub>2</sub>-AR agonists on the asthma phenotype can likely be explained by the activation of dual signaling pathways, a proinflammatory β-arrestin-dependent signaling pathway in addition to the classical G-protein-mediated bronchorelaxation pathway.

Our studies provide insight to explain the apparently opposing responses of PAR<sub>2</sub> activation. We, and others, have previously shown that, in a 25-d murine multiple-OVA challenge model, development of the asthma phenotype is significantly impaired in mice lacking either β-arrestin-2 or PAR<sub>2</sub> (13, 14, 21). To differentiate between PAR<sub>2</sub>-specific effects that are mediated by β-arrestin-2, and the general β-arrestin-2-OVA-induced allergic responses also we used a 15-d OVA model in the current study. At this time point, the magnitude of the asthma phenotype induced by OVA is low, whereas the PAR<sub>2</sub> agonist exacerbating effects are high (15), allowing the asthma phenotype-inducing effects of PAR<sub>2</sub> to be highlighted. We show that the “inflammatory” leukocyte infiltration response in our asthma model depends on β-arrestin-2-mediated signaling, whereas many of the protective bronchodilator effects of PAR<sub>2</sub> activation (such as PGE<sub>2</sub> production and subsequent bronchiolar smooth muscle relaxation) do not. The results shown here are consistent with previous studies demonstrating two independent signaling pathways downstream of PAR<sub>2</sub>: a β-arrestin-2-dependent pathway leading to actin cytoskeletal changes and cell migration, and a G-protein-dependent one that promotes Ca<sup>2+</sup> mobilization

and diacylglycerol formation (10, 11). We have previously shown formation of a β-arrestin scaffold containing the actin filament-severing protein cofilin and its upstream activator in primary bone marrow leukocytes, and have demonstrated that this signaling pathway is crucial for PAR<sub>2</sub>-stimulated cell migration (11, 12). PAR<sub>2</sub> has also been reported to increase PGE<sub>2</sub> levels by multiple mechanisms, some resulting in rapid, and others delayed, PGE<sub>2</sub> release, all of which appear to occur through Gαq-dependent, β-arrestin-independent signaling (8–11, 18, 20). Our data suggest that both COX1 and COX2 appear to be interlinked to generate the relaxant response, because a selective inhibition of either enzyme blocked the relaxant response. Taken together, these results indicate that this protective signaling axis is independent of β-arrestin-2, despite reports that IL-1 and angiotensin II can increase PGD<sub>2</sub> and PGE<sub>2</sub> levels via a β-arrestin-dependent pathway in vitro (31, 32). The signaling pathway activated by a PAR<sub>2</sub> mechanism that does not involve β-arrestin-2 merits further investigation. Taken together, these studies support the hypothesis that these two independent PAR<sub>2</sub> signaling pathways may direct opposing responses in vivo.

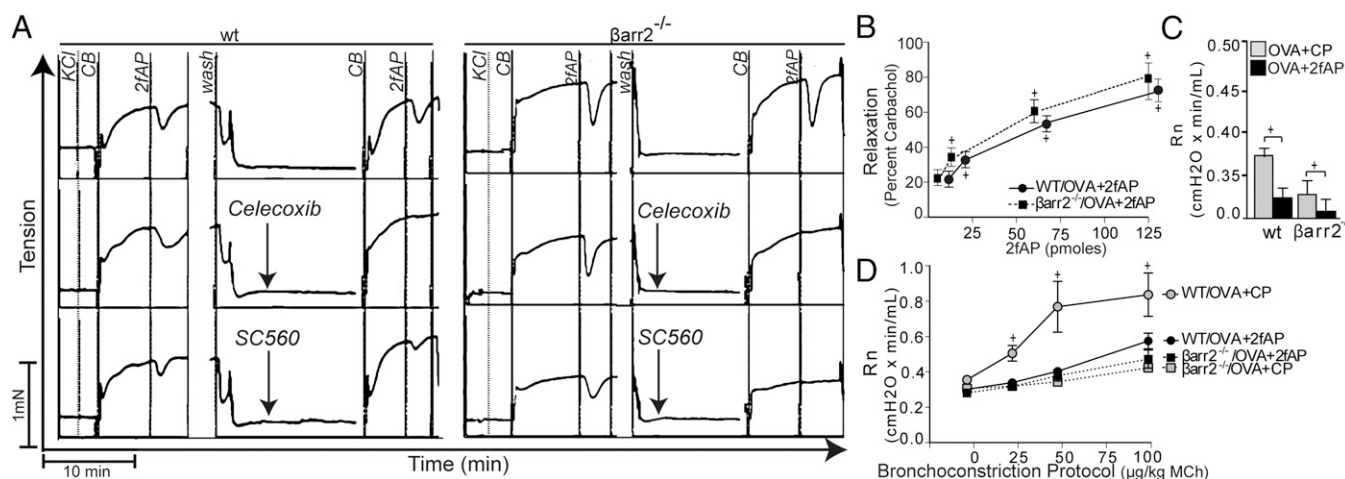
Another important hallmark of cellular airway inflammation is the production of cytokines. Both the resident airway cells and invading eosinophils and CD4<sup>+</sup> T cells can secrete these factors. Because the lung level of both cell types was reduced in β-arrestin-2<sup>-/-</sup> mice, it is not surprising that the 2fAP-induced increases in cytokines such as IL-6, IL-13, and TNFα were also reduced. IL-13 is important for goblet cell hyperplasia and mucin production such as was observed in the WT mice receiving OVA+2fAP. Consistent with β-arrestin dependence of IL-13 production, mucin production and goblet cell number were significantly reduced in OVA-treated β-arrestin-2<sup>-/-</sup> mice. Increased acidic mucin is clinically relevant, as mucus plugging is often associated with severity of disease in humans.

Like human asthma, one of the defining features of allergic inflammatory airways disease in mice is AHR, a measure of the sensitivity and reactivity of airway narrowing to a bronchoconstrictor. We assessed airway reactivity, the more clinically relevant component of AHR (33), by measuring the resistance response to multiple increasing concentrations of the bronchoconstrictor, methacholine. WT mice treated with 2fAP in the context of OVA displayed a statistically significant and physiologically relevant decrease in airway reactivity relative to those treated with CP. Because the effect of OVA treatment on AHR in β-arrestin-2<sup>-/-</sup> mice is so muted, it is difficult to assess whether this protective effect of PAR<sub>2</sub> in vivo requires β-arrestins; however, a significant reduction in baseline resistance was observed in mice receiving OVA+2fAP compared with OVA+CP in both WT and β-arrestin-2<sup>-/-</sup> animals, suggesting that PAR<sub>2</sub>-mediated airway smooth muscle relaxation is independent of β-arrestin-2. The protective effect of 2fAP on AHR in mice appears to be dominant over the proinflammatory effect because AHR decreased despite the concomitant enhanced airways inflammation and mucin phenotypes, which are typically associated with in-

**Table 2.** PGE<sub>2</sub> levels in BALF after OVA+2fAP-induced airway inflammation model

| Mouse strain         | Sensitization           | Challenge | PGE <sub>2</sub> levels, pg/mL |
|----------------------|-------------------------|-----------|--------------------------------|
| WT                   | None                    | Saline    | 1.4 ± 0.23                     |
| WT                   | OVA+Al(OH) <sub>3</sub> | OVA+CP    | 2.2 ± 0.18                     |
| WT                   | OVA+Al(OH) <sub>3</sub> | OVA+2fAP  | *3.4 ± 0.15                    |
| βarr2 <sup>-/-</sup> | None                    | Saline    | 1.8 ± 0.31                     |
| βarr2 <sup>-/-</sup> | OVA+Al(OH) <sub>3</sub> | OVA+CP    | 2.2 ± 0.29                     |
| βarr2 <sup>-/-</sup> | OVA+Al(OH) <sub>3</sub> | OVA+2fAP  | **3.2 ± 0.06                   |

BALF supernatants were assayed for prostaglandin (PGE<sub>2</sub>) levels using PGE<sub>2</sub> Express AChE tracer ELISA. Values are mean ± SEM (*n* = 8). Statistically significant differences from control group are indicated as follows: \**P* = 0.0003, \*\**P* = 0.0001 (*n* = 15 for saline, *n* = 20 for OVA+CP and OVA+2fAP).



**Lung Histology.** Extracted lungs were either flash frozen in OCT or fixed and embedded in paraffin. Five-micrometer sections were stained using standard H&E protocol and imaged with an upright Nikon Eclipse E600 using PAXIT! software. Sections were scored for epithelial and perivascular inflammation using a 0–4 point scale, as described in *SI Materials and Methods*. For analysis of mucin, formalin-fixed lung tissue was paraffin embedded and 5- $\mu$ m sections were cleared, hydrated, and stained with Alcian blue for 30 min (to identify mucin proteins) and neutral red for 5 min (to identify cell nuclei). Sections were then dehydrated and mounted with resinous mounting media, and slides were imaged as described above.

**Smooth Muscle Relaxation and AHR.** These methods have been described previously (20, 27, 45, 46), and experimental details are provided in *SI Materials and Methods*. Briefly, airway responsiveness in mice was measured using the

forced oscillation technique, an invasive method that measures lung impedance. From this measure, Newtonian resistance, an indicator of airway luminal diameter, was calculated according to the constant-phase model.

**Data and Statistical Analysis.** Statistical significance was determined using one-way ANOVA and Tukey honestly significant difference (HSD) posttests to compare treatment groups. Data, graphs, and statistical analyses were performed using FACS DIVA, FlowJo, Microsoft Excel 2003, or GraphPad Prism 5.0.

**ACKNOWLEDGMENTS.** We thank Dr. Robert Lefkowitz for  $\beta$ -arrestin-2<sup>−/−</sup> mice and Dr. Robin Plevin for PAR<sub>2</sub><sup>−/−</sup> mice. This work was funded by National Institutes of Health Grants 1R21HL092388 (to K.A.D.), R01HL084123 and R01HL093103 (to J.K.L.W.), and R01NS072298 (to E.H.W.), and the Canadian Institutes of Health Research (M.D.H.).

- Ramachandran R, Noorbakhsh F, Defea K, Hollenberg MD (2012) Targeting proteinase-activated receptors: Therapeutic potential and challenges. *Nat Rev Drug Discov* 11:69–86.
- Cocks TM, Moffatt JD (2001) Protease-activated receptor-2 (PAR2) in the airways. *Pulm Pharmacol Ther* 14:183–191.
- Oikonomopoulou K, et al. (2006) Proteinase-mediated cell signalling: Targeting proteinase-activated receptors (PARs) by kallikreins and more. *Biol Chem* 387:677–685.
- Arizmendi NG, et al. (2011) Mucosal allergic sensitization to cockroach allergens is dependent on proteinase activity and proteinase-activated receptor-2 activation. *J Immunol* 186:3164–3172.
- Boitano S, et al. (2011) Potent agonists of the protease activated receptor 2 (PAR2). *J Med Chem* 54:1308–1313.
- Hollenberg MD, et al. (2008) Derivatized 2-furoyl-LIGRLO-amide, a versatile and selective probe for proteinase-activated receptor 2: Binding and visualization. *J Pharmacol Exp Ther* 326:453–462.
- McGuire JJ, Saifeddine M, Triggler CR, Sun K, Hollenberg MD (2004) 2-Furoyl-LIGRLO-amide: A potent and selective proteinase-activated receptor 2 agonist. *J Pharmacol Exp Ther* 309:1124–1131.
- DeFea KA, et al. (2000) beta-Arrestin-dependent endocytosis of proteinase-activated receptor 2 is required for intracellular targeting of activated ERK1/2. *J Cell Biol* 148:1267–1281.
- Ge L, Ly Y, Hollenberg M, DeFea K (2003) A beta-arrestin-dependent scaffold is associated with prolonged MAPK activation in pseudopodia during protease-activated receptor-2-induced chemotaxis. *J Biol Chem* 278:34418–34426.
- Wang P, DeFea KA (2006) Protease-activated receptor-2 simultaneously directs beta-arrestin-1-dependent inhibition and Galphaq-dependent activation of phosphatidylinositol 3-kinase. *Biochemistry* 45:9374–9385.
- Zoudilova M, et al. (2007) Beta-arrestin-dependent regulation of the cofilin pathway downstream of protease-activated receptor-2. *J Biol Chem* 282:20634–20646.
- Zoudilova M, et al. (2010) beta-Arrestins scaffold cofilin with chronophin to direct localized actin filament severing and membrane protrusions downstream of protease-activated receptor-2. *J Biol Chem* 285:14318–14329.
- Schmidlin F, et al. (2002) Protease-activated receptor 2 mediates eosinophil infiltration and hyperreactivity in allergic inflammation of the airway. *J Immunol* 169:5315–5321.
- Takizawa T, et al. (2005) Abrogation of bronchial eosinophilic inflammation and attenuated eotaxin content in protease-activated receptor 2-deficient mice. *J Pharmacol Sci* 98(1):99–102.
- Ebeling C, et al. (2005) Proteinase-activated receptor 2 activation in the airways enhances antigen-mediated airway inflammation and airway hyperresponsiveness through different pathways. *J Allergy Clin Immunol* 115:623–630.
- DeFea KA (2007) Stop that cell! Beta-arrestin-dependent chemotaxis: A tale of localized actin assembly and receptor desensitization. *Annu Rev Physiol* 69:535–560.
- Cocks TM, et al. (1999) A protective role for protease-activated receptors in the airways. *Nature* 398:156–160.
- Henry PJ (2006) The protease-activated receptor2 (PAR2)-prostaglandin E2-prostanoid EP receptor axis: A potential bronchoprotective unit in the respiratory tract? *Eur J Pharmacol* 533:156–170.
- Kawao N, et al. (2005) Signal transduction for proteinase-activated receptor-2-triggered prostaglandin E2 formation in human lung epithelial cells. *J Pharmacol Exp Ther* 315:576–589.
- Nagataki M, Moriyuki K, Sekiguchi F, Kawabata A (2008) Evidence that PAR2-triggered prostaglandin E2 (PGE2) formation involves the ERK-cytosolic phospholipase A2-COX-1-mitochondrial PGE synthase-1 cascade in human lung epithelial cells. *Cell Biochem Funct* 26:279–282.
- Walker JKL, et al. (2003)  $\beta$ -Arrestin-2 regulates the development of allergic asthma. *J Clin Invest* 112:566–574.
- Izuhara K, et al. (2009) The mechanism of mucus production in bronchial asthma. *Curr Med Chem* 16:2867–2875.
- McGuire JJ, Hollenberg MD, Andrade-Gordon P, Triggler CR (2002) Multiple mechanisms of vascular smooth muscle relaxation by the activation of proteinase-activated receptor 2 in mouse mesenteric arterioles. *Br J Pharmacol* 135:155–169.
- Hollingsworth JW, et al. (2010) Both hematopoietic-derived and non-hematopoietic-derived beta-arrestin-2 regulates murine allergic airway disease. *Am J Respir Cell Mol Biol* 43:269–275.
- Vergnolle N, Hollenberg MD, Sharkey KA, Wallace JL (1999) Characterization of the inflammatory response to proteinase-activated receptor-2 (PAR2)-activating peptides in the rat paw. *Br J Pharmacol* 127:1083–1090.
- Walker JKL, Penn RB, Hanania NA, Dickey BF, Bond RA (2011) New perspectives regarding  $\beta_2$ -adrenoceptor ligands in the treatment of asthma. *Br J Pharmacol* 163(1):18–28.
- Lin R, et al. (2012) Chronic treatment in vivo with  $\beta$ -adrenoceptor agonists induces dysfunction of airway  $\beta_2$ -adrenoceptors and exacerbates lung inflammation in mice. *Br J Pharmacol* 165:2365–2377.
- Nguyen LP, et al. (2008) Chronic exposure to beta-blockers attenuates inflammation and mucin content in a murine asthma model. *Am J Respir Cell Mol Biol* 38:256–262.
- Nguyen LP, et al. (2009) Beta2-adrenoceptor signaling is required for the development of an asthma phenotype in a murine model. *Proc Natl Acad Sci USA* 106:2435–2440.
- Callaerts-Vegh Z, et al. (2004) Effects of acute and chronic administration of beta-adrenoceptor ligands on airway function in a murine model of asthma. *Proc Natl Acad Sci USA* 101:4948–4953.
- Mathurin K, et al. (2011) An interaction between L-prostaglandin D synthase and arrestin increases PGD2 production. *J Biol Chem* 286:2696–2706.
- Kendall RT, et al. (2011) The beta-arrestin pathway-selective type 1A angiotensin receptor (AT1A) agonist [Sar1,Ile4,Ile8]angiotensin II regulates a robust G protein-independent signaling network. *J Biol Chem* 286:19880–19891.
- Busse WW (2010) The relationship of airway hyperresponsiveness and airway inflammation: Airway hyperresponsiveness in asthma: Its measurement and clinical significance. *Chest* 138(2 Suppl):45–105.
- Barrios VE, Jarosinski MA, Wright CD (2003) Proteinase-activated receptor-2 mediates hyperresponsiveness in isolated guinea pig bronchi. *Biochem Pharmacol* 66:519–525.
- Knight DA, et al. (2001) Protease-activated receptors in human airways: Upregulation of PAR-2 in respiratory epithelium from patients with asthma. *J Allergy Clin Immunol* 108:797–803.
- Kanke T, et al. (2009) Novel antagonists for proteinase-activated receptor 2: Inhibition of cellular and vascular responses in vitro and in vivo. *Br J Pharmacol* 158(1):361–371.
- De Campo BA, Henry PJ (2005) Stimulation of protease-activated receptor-2 inhibits airway eosinophilia, hyperresponsiveness and bronchoconstriction in a murine model of allergic inflammation. *Br J Pharmacol* 144:1100–1108.
- D'Agostino B, et al. (2007) Activation of protease-activated receptor-2 reduces airways inflammation in experimental allergic asthma. *Clin Exp Allergy* 37:1436–1443.
- Alshurafa HN, et al. (2004) A protease activated receptor-2 (PAR-2) activating peptide, tc-LIGRLO-NH<sub>2</sub>, induces protease release from mast cells: Role in TNF degradation. *BMC Pharmacol* 4:12.
- Ramachandran R, et al. (2009) Agonist-biased signaling via proteinase activated receptor-2: Differential activation of calcium and mitogen-activated protein kinase pathways. *Mol Pharmacol* 76:791–801.
- Ramachandran R, et al. (2011) Neutrophil elastase acts as a biased agonist for proteinase-activated receptor-2 (PAR2). *J Biol Chem* 286:24638–24648.
- Sevigny LM, et al. (2011) Interdicting protease-activated receptor-2-driven inflammation with cell-penetrating pepducins. *Proc Natl Acad Sci USA* 108:8491–8496.
- Remme WJ, et al. (2007) Effect of carvedilol and metoprolol on the mode of death in patients with heart failure. *Eur J Heart Fail* 9:1128–1135.
- Wisler JW, et al. (2007) A unique mechanism of beta-blocker action: Carvedilol stimulates beta-arrestin signaling. *Proc Natl Acad Sci USA* 104:16657–16662.
- Schuessler TF, Bates JH (1995) A computer-controlled research ventilator for small animals: Design and evaluation. *IEEE Trans Biomed Eng* 42:860–866.
- Proskocil BJ, Fryer AD (2005) Beta2-agonist and anticholinergic drugs in the treatment of lung disease. *Proc Am Thorac Soc* 2(4):305–310; discussion 311–312.

# Supporting Information

Nichols et al. 10.1073/pnas.1208881109

## SI Materials and Methods

**Rhodamine-Labeled 2fAP in Airway Epithelium.** Tetramethyl-rhodamine conjugated to the C-terminal ornithine residue of either 2fAP (Rh-2fAP) was synthesized by Genemed. Naïve WT and PAR<sub>2</sub><sup>-/-</sup> mice were administered Rho-2fAP intranasally for 0, 2, and 24 h after which mice were killed and pulmonary lobes were extracted and flash frozen in OCT. Five-micrometer sections were cut with Microm HM500 OMV Motorized Cryostat and were fixed with 4% (wt/vol) PFA for 20 min and blocked and permeabilized with goat serum (2 × 5 min). Sections incubated with 200 ng/mL Lung Surfactant Protein antibody (Neomarkers; ms-704-PO). Sections were washed with PBS+Tween 20 [0.05% (vol/vol)] and stained with secondary antibody (Gt-anti-Mo 530abs) diluted 1:500 in PBS for 40 min in dark at room temperature. Nuclei were stained with TOPRO-3 (1:1,000) for 20 min. Sections were mounted with V mounting media and imaged with Zeiss 510 confocal microscope.

**Lung Digest Preparations.** Lung single-cell suspension obtained by incubating lung samples with glass beads and collagenase 3 at 37 °C for 60 min, shaking them every 7 min during the incubation. Red blood cells were lysed with ammonium chloride, and samples were prepared for flow cytometry.

**Adoptive Transfer.** To allow easy identification of transplanted cells, donor mice were crossed with GFP mice so that the transplanted cells were GFP positive (genotypes: GFP<sup>+/+</sup>-PAR<sub>2</sub><sup>-/-</sup> and GFP<sup>+/+</sup>-PAR<sub>2</sub><sup>+/+</sup>).

**Smooth Muscle Relaxation.** Primary first-order mouse bronchiolar rings with intact epithelium were, and 2 mm segments were mounted in a myograph at resting tension, in a 37 °C Krebs buffer bath. After 60 min of equilibration at ~0.5 g of resting tension, tissues were tested treated with 50 mM KCl (tests 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 increasing concentrations of 2fAP or CP, and the relaxation response was recorded. Relaxation was expressed as a percentage reduction in tension (%) relative to the tension measured in the presence of carbachol.

**AHR Measurements.** Airway hyperresponsiveness was measured as described previously (1). In brief, lung mechanics was measured in anesthetized (i.p. 65 mg/kg sodium pentobarbital), paralyzed (0.25 mg/kg pancuronium bromide), and tracheotomized mice 24 h after the last OVA challenge. The forced oscillation technique was used, using a 3-s optimized pseudorandom signal containing frequencies ranging from 0.25 to 19.63 Hz. Mice were ventilated with 100% oxygen at 150 breaths per minute at a constant volume of 8 mL/kg with a positive end-expiratory pressure of 3 cmH<sub>2</sub>O, using a computer-controlled small animal ventilator (flexiVent; SCIREQ). The cardiovascular function of the mice was inferred from heart rate data acquired using EKG electrodes and transmitted via loudspeaker. Following ~5 min of regular

ventilation, a standard lung volume history was established followed by the acquisition of three baseline respiratory input impedance measurements. Bronchospasm was induced by jugular vein administration of increasing doses of MCh (25, 50, 100 µg/kg), and the impedance measurements were made every 12 s and averaged over 2 min. A 5-min washout period was included between each MCh challenge that included two deep inspirations. The resultant total lung impedance signal contains information about the resistance and elastance properties of the lung from which Newtonian resistance was calculated using the constant-phase model. In an idealized lung model, Newtonian resistance is a good indicator of the luminal diameter of the conducting airways.

**Buffer Compositions.** For smooth muscle relaxation assay, Krebs buffer (pH 7.4) consists of the following (in mmol/L): NaCl, 120; NaHCO<sub>3</sub>, 25; KCl, 4.8; NaH<sub>2</sub>PO<sub>4</sub>, 1.2; dextrose, 11.0; and CaCl<sub>2</sub>, 1.8, aerated with 95% O<sub>2</sub> and 5% CO<sub>2</sub>.

FACS buffer consists of the following: 1% BSA and 0.1 mM EDTA in PBS.

**Flow Cytometry Gating Strategy.** Other cells (which presumably includes alveolar macrophages and dendritic cells) were not further characterized. Group A received conjugated antibodies against CD11c, CCR3, CD45, GR1, CD11b, and CD3. Group B received conjugated antibodies against CD11c, CD19, CD8, CD4, CD11b, and F480 (all antibodies were from eBioscience). Cells were washed and analyzed with BD FACSCANTO II flow cytometer and FlowJo analysis software, version 8.7.3. Isolated lung tissue samples were analyzed with an identical protocol. For quantification by flow cytometry, cells were gated as follows: CD45<sup>+</sup> cells were selected, and live granulocytes and lymphocytes were identified by forward versus side scatter (FSC vs. SSC). For Group A (antibodies to CD11c, CCR3, CD45, GR1, CD11b, and CD3), from the CD45<sup>+</sup> gate, low side scatter cells were scored as lymphocytes (L\*), CD11b<sup>+</sup>/CD3<sup>+</sup> cells were scored as T lymphocytes (L\*). From the CD45<sup>+</sup>/high side scatter gate, populations that were CD11b<sup>+</sup>/CCR3<sup>+</sup>/GR1<sup>-</sup> were scored as eosinophils. Cells that were CD11b<sup>+</sup>/GR1<sup>+</sup> were scored as neutrophils. To identify specific populations of lymphocytes, Group B samples (antibodies to CD11c, CD19, CD8, CD4, CD11b, and F480) were used and low SSC cells were scored as CD4<sup>+</sup>/CD8<sup>-</sup> (T helper), CD8<sup>+</sup>/CD4<sup>-</sup> (cytotoxic T cells), and CD19<sup>+</sup> (B cells).

**Histological Scoring.** H&E-stained lung samples were scored for presence of cellular inflammation and perivascular infiltration as follows: 0, no inflammatory cells present in epithelial or perivascular tissue; 1, scattered inflammatory cells present in epithelium and around vessels, with no thickening of the vessel wall; 2, many inflammatory cells present in epithelium with one to two rings of cells around vessels; 3, 100+ inflammatory cells found in clusters throughout epithelium with three or more rings of cells around vessels; 4, nearly continuous layer of inflammatory cells extending from vasculature throughout epithelium, loss of distinction between perivascular and epithelial tissue.

1. Lin R, et al. (2012) Chronic treatment in vivo with  $\beta$ -adrenoceptor agonists induces dysfunction of airway  $\beta_2$ -adrenoceptors and exacerbates lung inflammation in mice. *Br J Pharmacol* 165:2365–2377.

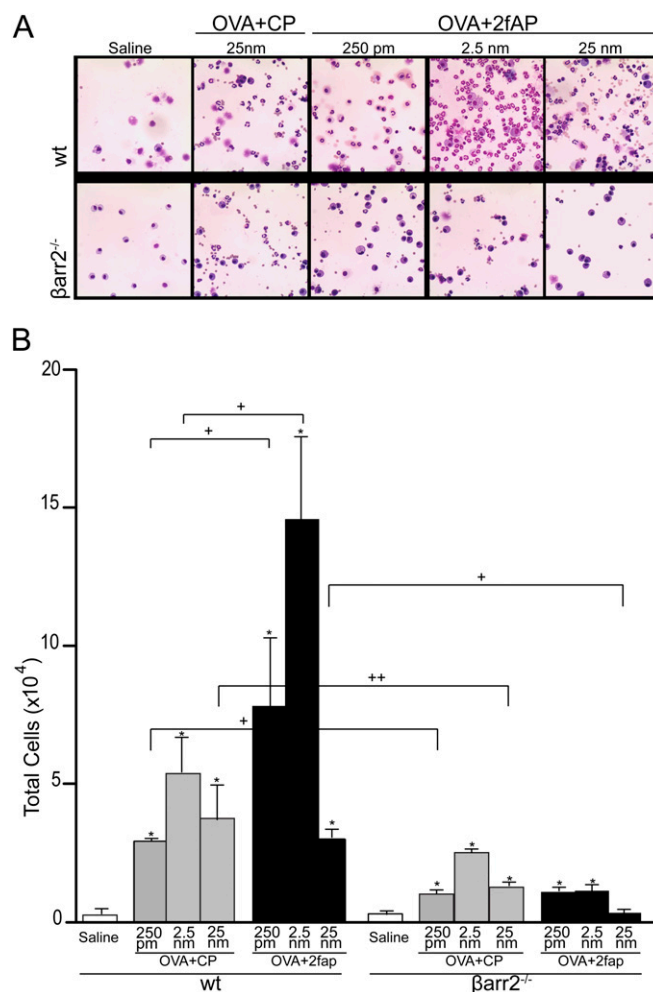


**Fig. S1.** Dose-dependent augmentation of OVA-induced asthma by 2fAP. WT and  $\beta$ -arrestin-2<sup>-/-</sup> mice were given an i.p. injection of OVA+alum on days 1 and 6 followed by two intranasal challenges on days 12 and 14 containing OVA+ increasing doses (250 pmol, 2.5 nmol, or 25 nmol) of PAR<sub>2</sub>-activating peptide (2fAP) or a negative control peptide (CP). As a negative control for OVA-induced inflammation, mice were given saline in place of both the i.p. sensitization and intranasal challenges. The schematic for this experiment is depicted in Fig. 1A of the main text. On day 15, BALF was collected for analysis of leukocyte infiltration. (A) Representative cytopins of BALF cells collected from saline-treated controls, OVA+CP (25 nmol), and OVA+2fAP (all three concentrations) shows increased eosinophils and lymphocytes in WT mice receiving 2.5 and 25 nmol of 2fAP during the challenge phase. Maximal inflammatory cell recruitment was observed with 2.5 nmol of 2fAP. (B) Total cell count of BALF cells reveals that 25 nmol of 2fAP administered during the challenge phase gives the maximal augmentation of cell recruitment to the BALF. A statistically significant increase is observed with 2.5 nmol and no significant increase in cells is observed with 25 nmol. For all subsequent studies, 2.5 nmol of 2fAP is the amount used in the challenges. \* ( $P > 0.05$ ) illustrates statistical significance from saline-treated controls. \* $P > 0.05$  and \*\* $P > 0.01$  above brackets demonstrate significant difference between OVA-treated groups.

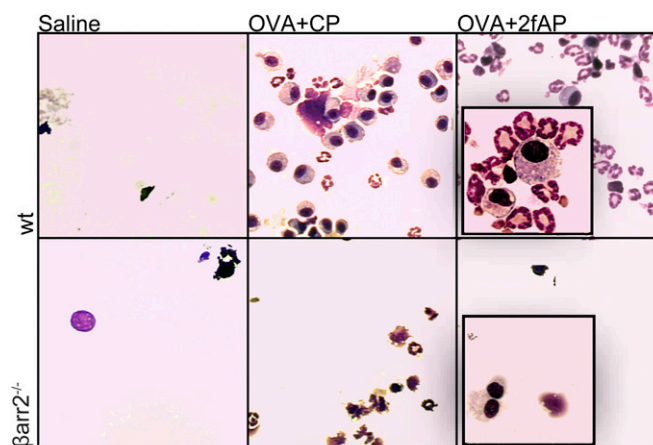


**Fig. S2.** Representative cytopsin images of BAL fluid. Images illustrate increase in inflammatory cells recruited to lungs in WT mice challenged with OVA+2fAP. Of equal importance is the augmented recruitment of eosinophils, an indicator of allergic disease, in the aforementioned mice (see zoom inlay). Note the reduced total cell number and absence of eosinophils in  $\beta$ -arrestin-2<sup>-/-</sup> mice also challenged with OVA+2fAP (see zoom inlay).

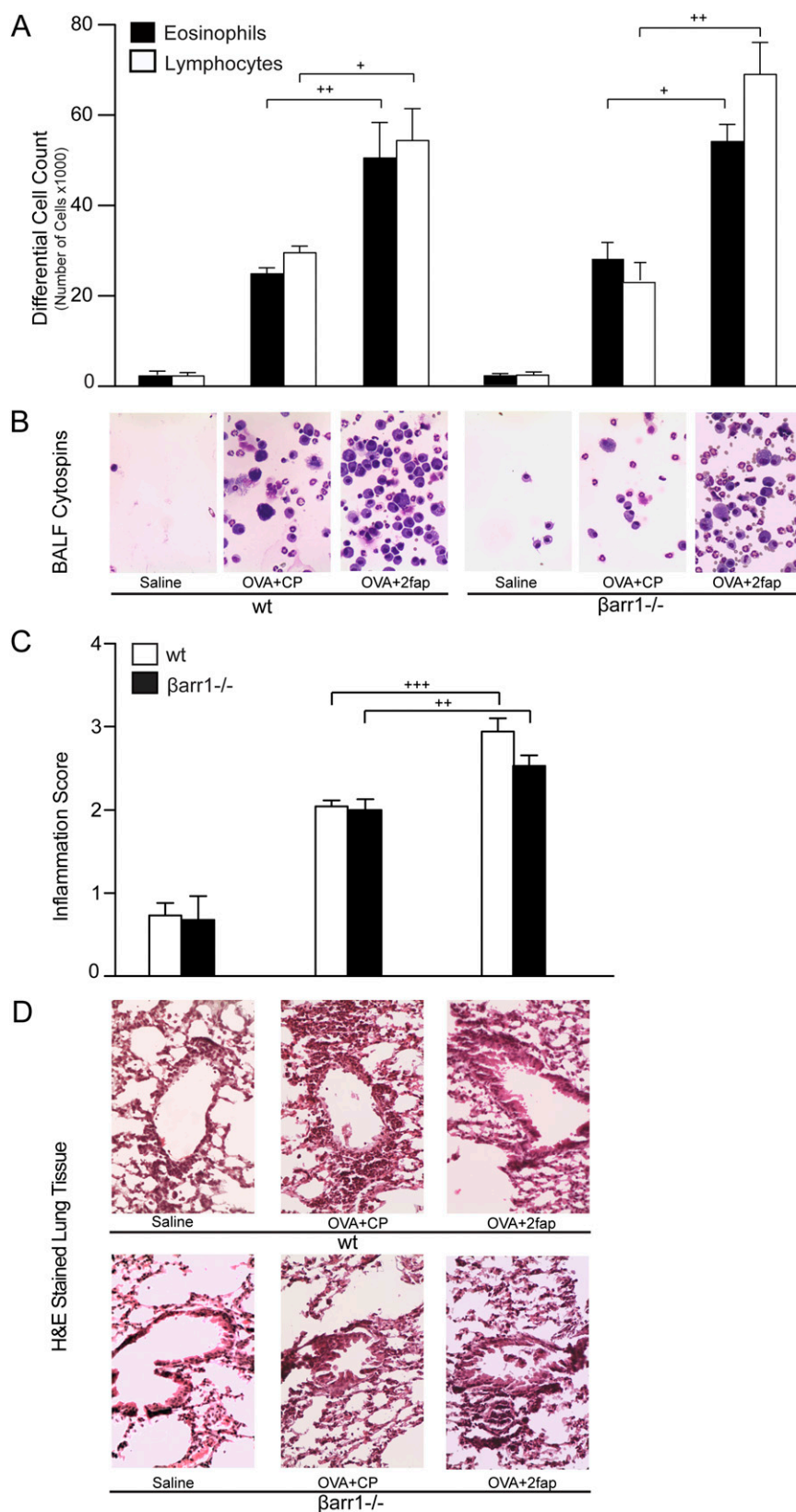


**Fig. S3.** OVA-induced and 2fAP-augmented inflammation is independent of  $\beta$ -arrestin-1. The same model of airway inflammation was run in WT versus  $\beta$ -arrestin-1 mice (4 mice per treatment, 24 mice total), and both BALF cell distribution and lung histology were examined. (A) Bar graph of eosinophils and lymphocytes found in BALF, determined from differential count of cytospin images. Differential cell determined in 10 different images from each mouse. Both the moderate increase in eosinophil and lymphocyte recruitment observed with OVA+CP and the augmented recruitment observed in the mice receiving 2fAP were maintained in the  $\beta$ -arrestin-1<sup>-/-</sup> mice, indicating that both are independent of  $\beta$ -arrestin-1. (B) Representative cytospins of BALF from each experimental condition are shown. (C) Histological scoring was performed on a 0–4 scale, as described for Fig. 1, where 0 represents no inflammation and 4 represents a high inflammation. Legend continued on following page



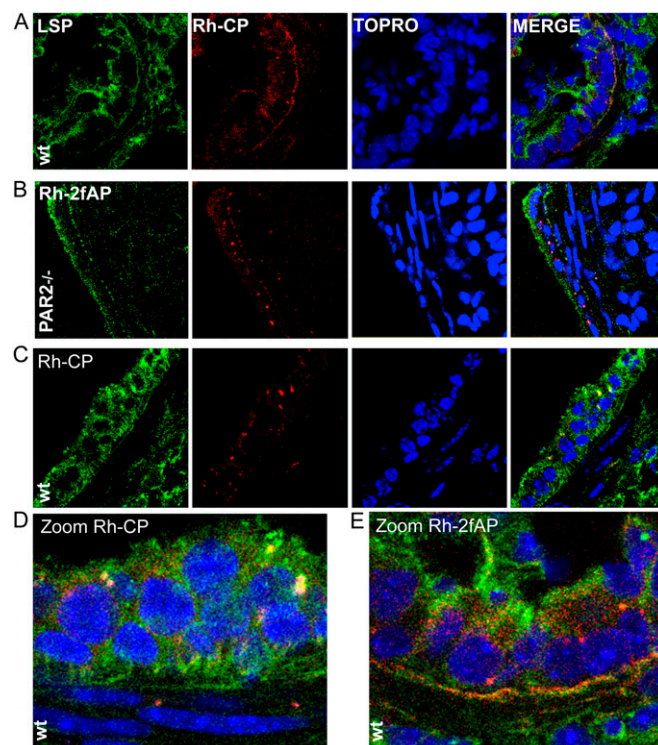


**Fig. S6.** Intranasally administered PAR<sub>2</sub> activating peptide can access the basolateral side of the airways. WT and PAR<sub>2</sub><sup>-/-</sup> mice were given rhodamine-labeled 2fAP (Rh-2fAP), or rhodamine-labeled control peptide (Rh-CP), intranasally, and lungs were harvested 2 h after administration. Frozen sections were stained with lung surfactant protein (LSP) (in green) and TOPRO 3 to visualize nuclei (in blue) and then imaged by confocal microscopy using a Zeiss LSM510, 100× objective. Representative images showing Rh-2fAP labeling (red) throughout the airway epithelium and serosa in WT (A) but not PAR<sub>2</sub><sup>-/-</sup> mice (B) or WT mice given Rh-CP (C) are shown. Zoomed images (3×) of Rh-CP-treated WT (D) and Rh-2fAP-treated WT (E) lungs are shown.

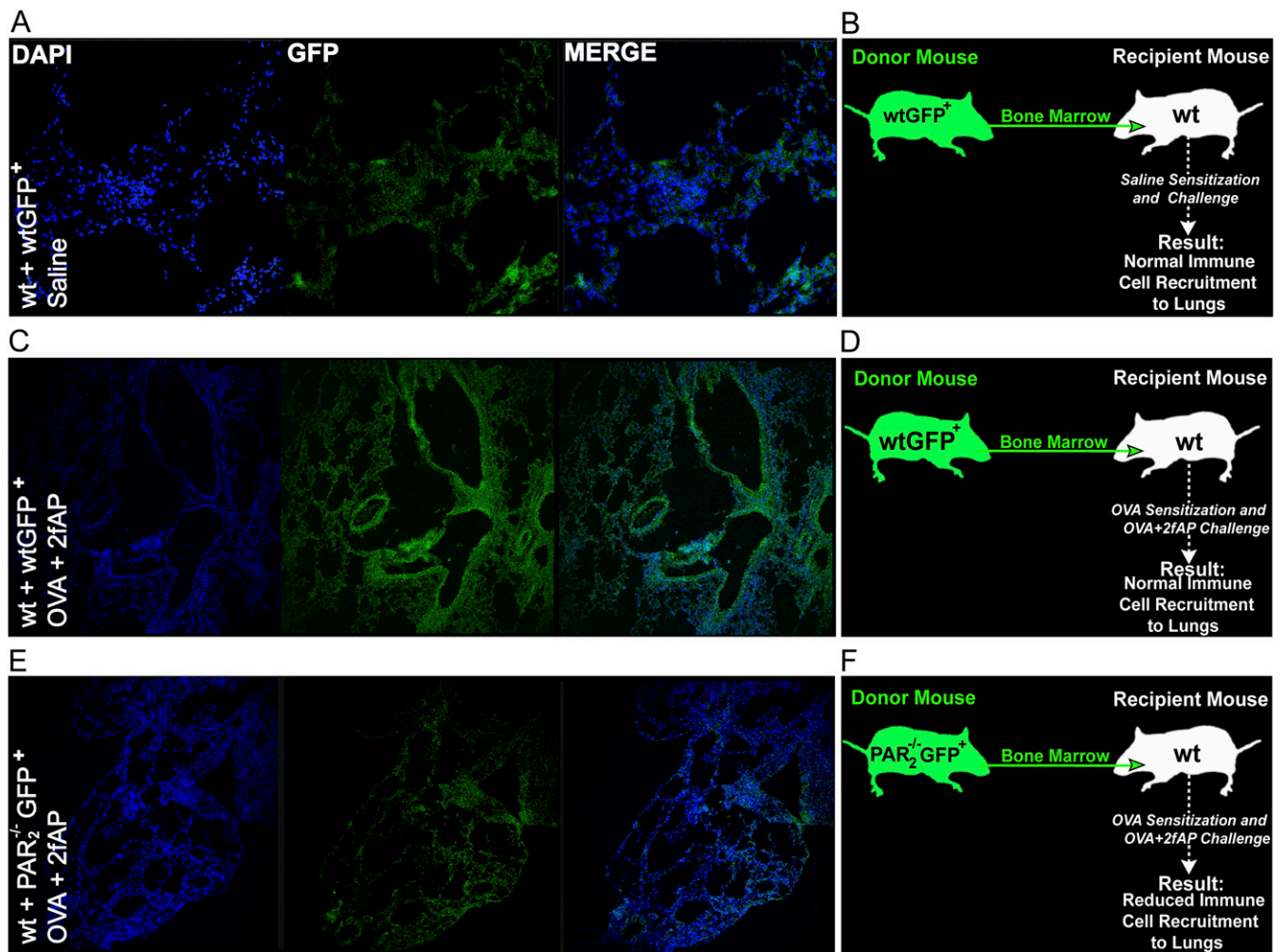


**Fig. S7.** Adoptive transfer of wtGFP<sup>+</sup> hematopoietic cells restores normal inflammatory response. (A) wtGFP<sup>+</sup> mice saline-treated have little inflammation in perivascular or peribronchial regions. (B) Cartoon illustration of adoptive transfer protocol identifying donor and recipient. (C and D) WT recipients of wtGFP<sup>+</sup> hematopoietic cells and challenged with OVA+2fAP show increased inflammation in the region of interest. (E and F) WT recipients of PAR<sub>2</sub><sup>-/-</sup>/GFP<sup>+</sup> hematopoietic cells show little inflammation even after OVA+2fAP treatment.

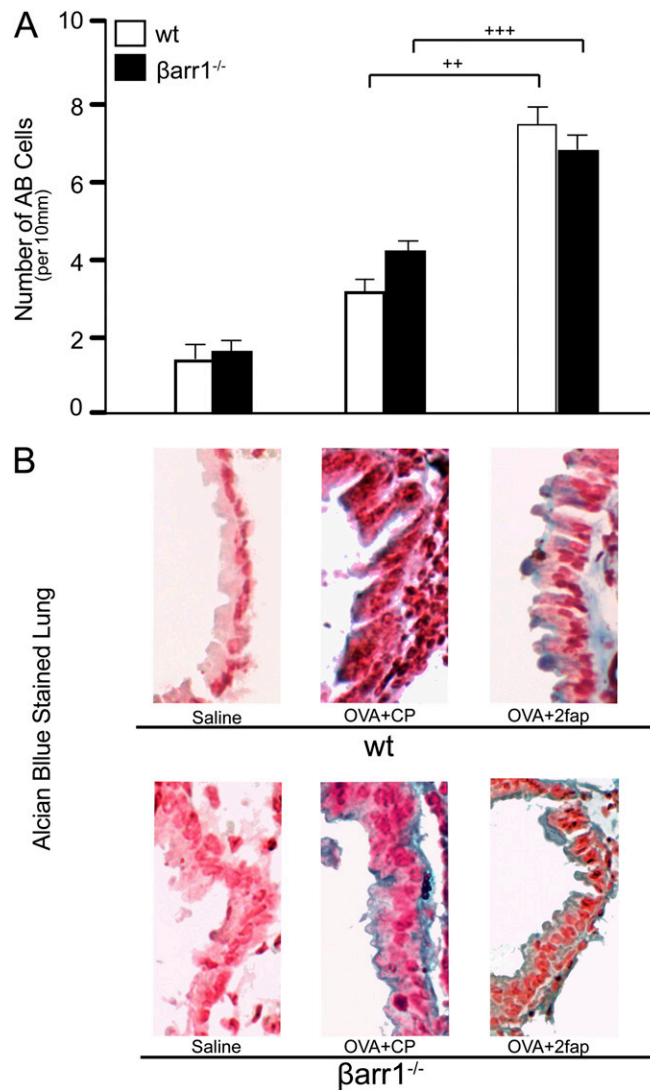


**Fig. S8.** Mucin production induced by PAR<sub>2</sub> is independent of  $\beta$ -arrestin-1. WT and  $\beta$ -arrestin-1<sup>-/-</sup> mice from the experiment described in Fig. S3 were stained with Alcian blue (as described in Fig. 5), and the number of mucin-producing cells per 10 mm was determined using Adobe Photoshop. (A) Quantification of mucus-producing cells. (B) Representative images of Alcian blue-stained airways. All treatment groups were significantly different from saline-treated controls ( $P > 0.005$ ).  $^{**}P > 0.01$  and  $^{***}P > 0.001$  above brackets demonstrate difference between OVA-treated groups.