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Dissection of Alternaria-induced Asthma: Role of the PAR_2-beta-arrestin Signaling Axis

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DEDICATION

To my parents and grandparents

ABSTRACT OF THE DISSERTATION

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by

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Doctor of Philosophy, Graduate Program in Biomedical Sciences
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Dr. Kathryn A. DeFea, Chairperson

The goal of this dissertation project was to understand how activation of Protease-activated receptor-2 (PAR_2)-beta-arrestin-2-dependent signaling leads to promotion of inflammation in the allergic response to the airborne allergen *Alternaria alternata* (*A. alternata*) in the lung. Exposure to *A. alternata* spores is a major problem for people susceptible to the allergens, and is a leading cause of asthmatic symptoms. The current standard of care for treatment of acute asthma exacerbations is not sufficient for many patients, highlighting the need for alternative therapies. PAR_2 is of great interest as a therapeutic target because it promotes both pro- and anti-inflammatory responses in the airway, through opposing G-protein and β-arrestin signaling pathways, and it is activated by proteases released by household pathogens such as Alternaria. These studies examine: 1) the molecular mechanisms underlying

Alternaria-induced asthma, delineating the signaling pathways and identifying a new PAR_2 activating protease (AASP) present in *Alternaria* extracts and 2) characterizing a novel PAR_2 antagonist (C391) for potential β-arrestin bias, and investigating its ability to inhibit *Alternaria* –induced inflammation. In cultured cells, I examined key readouts of G-protein and β-arrestin-dependent PAR_2 signaling and demonstrate that *Alternaria* and AASP promote both pathways. *In vivo*, *Alternaria* and AASP promote pro-inflammatory responses in the lung that are dependent on PAR_2 and β-arrestin-2. However, the proinflammatory components of *Alternaria* also promote the protective PAR_2 effects in isolated bronchioles. My studies indicate that the PAR_2 antagonist, C391, inhibits *Alternaria*-induced airway inflammation *in vivo*. In cultured cells, C391 inhibits PAR_2-β-arrestin signaling in cells at an IC50 100-fold lower than that for inhibition of G-protein signaling, suggesting it may represent a β-arrestin biased antagonist. Altogether, I have found that PAR_2 activation via β-arrestin-2 plays a major role in mediating pro-inflammatory responses induced by several allergens in the airway, and that the use of the novel antagonist using C391 may be a new avenue for therapeutic intervention. Thus, PAR_2-βarrestin-2 activation acts as a central switch that allergens turn on but can be turned off therapeutically.

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Chapter 1:

Background and Introduction on Protease-Activated-Receptor-2 Activation via β-Arrestin-2 in models of allergic inflammation

Section 1.1

Protease-Activated-Receptor-2 Signaling via β-arrestin-2

a. **PAR_2-β-arrestin Scaffolds**

b. **β-arrestin biased agonists independent of G-protein signaling**

Protease-Activated-Receptor-2 (PAR_2) is a G-protein coupled receptor, also called a seven transmembrane protein, that plays an important role in many pathological and disease states. PAR2 is unique from the other PARs, PAR_1, PAR_3, and PAR_4, both in activation and downstream signaling. The extracellular N-terminus of PAR_2 is cleaved by trypsin-like proteases, while PAR_1 is cleaved by thrombin and PAR_3 acts as a cofactor for PAR_4, which is cleaved by both thrombin and trypsin-like proteases (1,2). This proteolytic cleavage unmasks the tethered ligand; this corresponds to SLIGRL in mouse or SLIGKV in human PAR2 that then binds to and activates the receptor irreversibly. Activation of PAR_2 leads to both canonical G-protein signaling and non-canonical signaling through β-arrestins (Fig 1). The most common G-protein target is G-α-q/11, which leads to hydrolysis of PIP_2 into DAG and IP_3, promoting intracellular calcium release, activation of conventional PKCs, ras-dependent nuclear ERK activation, PI3K activation, and further downstream signaling. In some cell types, G-α-i and G-α-12/13 are also activated and can trigger some of these same downstream events. The non-canonical pathway through β-arrestins will be the major focus of this dissertation.

This pathway operates independent of, and often in opposition to, the G-protein pathway and is responsible for a number of pathogenic effects of PAR_2, including inflammation and tumor cell migration. (3,4). This chapter will review PAR_2 signaling, from a discussion of molecular pathways to its role in disease and putative therapeutic strategies targeting PAR_2.

a. PAR_2-β-arrestin Scaffolds

β-arrestins were first identified as terminators of GPCR signaling through heterotrimeric G-proteins. There are two family members, β-arrestin-1 and 2, that share substantial sequence homology and functional overlap. Both β-arrestins can bind to activated GPCRs, uncouple them from their cognate G-proteins, and bind clathrin, and clathrin adaptor proteins (AP-1 and AP-2) to facilitate endocytosis and degradation or recycling of the receptor (5–7). However, a key discovery in 2000 was that not only do β-arrestins bind and terminate G-protein signaling, but they also trigger a separate set of signals by scaffolding sets of macromolecules and exerting spatio-temporal control over their signaling. Pushing this paradigm shift even farther were findings in 2003 that β-arrestin signaling can counteract and act independently of G-protein signaling. That β-arrestins could regulate signaling independent of G-proteins was first demonstrated using the biased agonist, SII, for Angiotensin II Receptor Type I (AT1R). SII was shown to activate β-arrestin-dependent MAPK but not G-protein coupling (8–10).

Other receptors were added to the list of GPCRs capable of eliciting signals independent of G-protein signaling including β2AR, dopamine receptor (D2R), and PAR_2 (10–14). Uncovering this feature of GPCR signaling revolutionized the field because GPCRs have long been popular targets for pharmaceutical intervention, but prior to the discovery of SII, drug discovery efforts focused only on readouts for G-protein signaling.

Over the past decade, over 100 binding partners for β-arrestins have been identified; association with β-arrestins leads to activation of some and inhibition of others. The first example of this was shown with β-arrestin scaffolding the entire ERK1/2 signaling module (Raf, MEK, ERK1.2) (15,16). PAR_2 was one of the first receptors shown to use β-arrestins to signal to ERK1/2, and the ability of β-arrestins to prolong ERK1/2 activity at the cell membrane was first characterized by our laboratory using PAR2 as the model receptor system. β-arrestin-dependent, membrane sequestered ERK1/2 was then shown to be important for cell polarization, pseudopodia formation, and cell migration (17). Sequestration of MAPK family members by β-arrestins was then shown for a number of other receptors (6,8,18–23). In the case of PAR_2, and other receptors such as AT1AR, there is also a G-protein dependent ERK1/2 activation pathway, but this results primarily in nuclear ERK1/2 activity and regulated gene expression, among them being COX2 which is important for Prostaglandin E2 (PGE2) production that mediates some anti-infammatory effects of PAR_2 discussed in Chapter 2 (15,16,24). Thus, the two pathways, even when converging on the same signaling moiety, direct distinctly different cellular events (Figure 1A).

Another major target of β-arrestin scaffolding downstream of PAR_2 is the cofilin pathway. Cofilin severs actin filaments and contributes to actin filament assembly by increasing availability of actin monomers and by creating new barbed ends for polymerization (25). Cofilin activation is regulated by opposing actions of LIMK, which inactivates it by phosphorylation, and the phosphatases slingshot (SSH) and chronophin (CIN), which dephosphorylate and activate it. Downstream of PAR_2, β-arrestins promote cofilin activation by the formation of two separate scaffolds: one contains β-arrestin-1 and LIM Kinase (LIMK), which suppresses LIMK activity thus favoring dephosphorylation of cofilin. A second complex containing β-arrestin-2, cofilin, and CIN facilitates dephosphorylation of cofilin by CIN. β-arrestins spatially regulate cofilin activity at the leading edge, leading to actin reorganization, the formation of actin rich membrane protrusions comprising and cell migration (Fig. 1B) (5,18,26,27).

Downstream of PAR_2, β-arrestins can also directly inhibit PI3K and CAMKKβ, both of which are promoted by the G-protein signaling arm (28). The G-protein mediated PI3K and nuclear ERK pathways are important for the anti-inflammatory effects of PAR_2 associated with prostaglandin E2 (PGE2) production by airway epithelial cells (12,24). Inhibition of these same pathways, as well as the membrane ERK and cofilin pathways that facilitate leukocyte migration are important for the pro-inflammatory effects of PAR_2, points that will be elaborated on in subsequent sections.

b. β-arrestin biased agonists independent of G-protein signaling

While β-arrestins are now known to mediate several pathways, the relative efficacies of receptor activation can differ and can be specifically modulated under the evolving paradigm of ligand bias. β-arrestin functions such as internalization and signaling can be completely independent of G-protein signaling, representing the extreme case of ligand bias, or "perfect bias." In the case of "imperfect bias," selectivity for different signaling pathways is a matter of degree or partial agonism (29).

Significant interest has been stimulated because of the potential implications for the use of these biased ligands. TRV120027, which is biased for β-arrestin for the angiotensin II receptor subtype Ia (AT1aR), is in clinical development for treating acute heart failure by increasing cardiac output and decreasing mean arterial pressure (30). On the other hand, TRV130, which is a G-protein biased ligand in first-in-human studies, was developed to maintain analgesic effects of opioids like morphine while reducing the β-arrestin-associated side effects of such as intestinal dysfunction and respiratory depression (31–35). Although niacin is an effective therapeutic for raising HDL-cholesterol and decreasing triglycerides in the treatment of dyslipidemia, unpleasant side effects of cutaneous flushing are dependent on β-arrestin-1. But the biased agonist, MK-6892, has been chosen as a preclinical candidate for its potent, selective antilipolytic actions while reducing cutaneous flushing (36–38). In regulating bone formation, β-arrestin-biased agonists have been developed to promote anabolic bone formation

without bone resorption, a potential strategy for treating osteoporosis (38,39). In testing antimaniac or antidepressant effects, mood stabilizers lamotrigine, lithium, and valproate exert behavioural effects by disrupting β-arrestin-2-mediated signaling by D2 dopamine receptors (38,40).

Section 1.2

The Paradoxical role of PAR_2 being both protective and pro-inflammatory

PAR_2 has been implicated in inflammation in a number of systems, including hyperalgesia, rheumatoid arthritis, inflammatory bowel disease, and asthma. This dissertation centers around asthma, but it is important to explain the overall roles that PAR_2 plays in inflammation. One of the first examples of PAR_2 in regulating inflammation was the observation that PAR_2 agonists activate nociceptive neurons and promotes the release of Substance P, leading to localized vasodilation, extravasation of plasma and granulocytes, and hyperalgesia (41–43). Intraplantar injection of PAR_2 agonists caused local paw edema (41,43,44). When administered to the lumen of the mouse colon, PAR_2 agonists provoked inflammation and disruption of the intestinal barrier integrity as observed by increased paracellular permeability to radioactively labeled chromium; this was also dependent on activation of myosin light chain kinase (45,46). PAR_2 has been reported to increase expression of various cytokines, chemokines, and growth factors including TNFα, IL-1β, IL-6, IL-8, GM-CSF, GROα, CCL20/MIP3A, and CCL17/TARC (47–

53). In addition to these pro-inflammatory responses, PAR_2 has also been reported to be protective in several examples of inflammatory diseases, such as colitis and asthma, via mechanisms involving epithelial prostaglandin production (54–56).

In the context of asthma, PAR_2 has a somewhat paradoxical role, being able to promote both bronchoprotective and pro-inflammatory responses (Fig. 1A) (57–60). The first studies on PAR_2 in the airway suggested a protective role, demonstrating that bronchioles from PAR_2-/- mice had increased bronchoconstriction and that adding PAR_2 agonists to isolated murine and human bronchioles promoted smooth muscle relaxation and bronchodilation. PAR_2-induced smooth muscle relaxation was then shown to be mediated by prostaglandin E2 (PGE2) release, derived from airway epithelial cells (24,61). PGE2 binds prostaglandin receptors on smooth muscle, which then promotes bronchorelaxation; it has also been shown to reduce eosinophil chemotaxis (62). PAR_2-induced PGE2 production requires Gαq-dependent mobilization of intracellular Ca2+, activation of PI3K and phosphorylation of Akt (which leads to release of PGE2), and nuclear ERK1/2 activation, which leads to expression of COX-1 and 2 (24,60,63). Thus, the protective effects of PAR_2 induced bronchorelaxation was shown to be G-protein dependent, and this led to the proposed use of PAR_2 agonists to treat asthma.

While the initial studies on PAR_2 in the airway had suggested it was protective, other studies running a common airway inflammation model in PAR_2 knockout and transgenic mice suggested the opposite. $PAR_2^{-/-}$ mice have a deficiency in inflammation in an OVA-induced model of airway inflammation and PAR_2 transgenic mice have

heightened airway hyperresponsiveness in the same OVA model. In these studies it reported that PAR_2-$^{/-}$ mice had reduced immune cell infiltrates primarily consisting of eosinophils and lymphocytes, epithelial thickening, and hyper-reactivity (64,65). In other studies, both OVA-induced and histamine-induced airway inflammation was exacerbated with administration of PAR_2 agonists (66,67). Recently, blockade of PAR_2 by administration of a PAR_2 antibody SAM-11 was shown to be effective in preventing airway hyperresponsivity and inflammation induced both by OVA and by cockroach frass (68). Together, these studies suggest that the initial allergic response may trigger release of endogenous PAR_2 activating proteases within the airways, along with other factors and that PAR_2 is part of the complex receptor response that results in IgE-mediated inflammation. However, localized activation of PAR_2 by intranasal administration of peptide agonists exacerbates asthma in both mouse and guinea pig models, even with short term exposure that results in IgE-independent inflammation. An underyling theory driving this work is that PAR_2 is involved in both localized acute and systemic components of the asthmatic response.

The conflicting information thus far on PAR_2 in asthma becomes clearer when considering the typical models used to study this disease. The typical asthma model starts with the “sensitization” phase, often administered intraperitoneally rather than intranasally, as is the case with Ovalbumin (OVA). The initiation of the allergic inflammatory response occurs with the uptake of allergens by professional antigen-presenting cells (APCs) including macrophages and dendritic cells that present peptides

on MHC class II molecules to naïve T cells, directing them to a T_H2 cell phenotype. Key features also include T-cells that upregulate expression of cytokines that are involved with class-switching of B cells to IgE synthesis (IL-4 and IL-13), recruitment of mast cells (IL-4, IL-9, and IL-13), and the maturation of eosinophils (IL-3, IL-5, and GM-CSF) and basophils (IL-3 and IL-4). In the second phase, the "challenge phase' administration is often intranasal or through nebulization to localize the inflammation to the airways. In asthma, the epithelium is more susceptible to oxidant stress and injury, and the presence of IL-13 and other growth factors leads to mucous cell metaplasia and remodeling of the airway walls (69). Studies in the OVA model have suggested that IgE levels are reduced in PAR_2-/- mice, consistent with the idea that PAR_2 plays an intrinsic role in this systemic immune response (64).

It is important to note that asthma symptoms can be exacerbated by environmental factors that induce an allergic response mediated by the IgE-FcεR1 complex on mast cells, leading to immediate hypersensitivity. Crosslinking of IgE-FcεR1 complexes on the mast-cell surface by allergens leads to the "early phase" allergic reaction within minutes involving mast cell degranulation and the release of cytokine and chemokines. The "late phase" peaks hours later involving recruitment and activation of inflammatory cells at the site of allergenic sensitivity. The populations of B cells and plasma cells in the airways are biased towards the production of high-affinity IgE, known as "class switching." Monocytes-macrophages and eosinophils are among the different IgE effector cells that are drawn to the site of allergic inflammation and function to clear

antigen-IgE complexes, killing, and phagocytosis of pathogens (70,71). The ability of anti-PAR_2 antibodies administered during the challenge phase to block aspects of inflammation suggests a role for PAR_2 in the acute local response as well as the systemic one.

There is often an underlying assumption that PAR_2 activation occurs when an allergen containing a PAR_2-activating protease cleaves and activates apically exposed epithelial PAR_2, leading to cytokine and chemokine production, which mediates the remainder of the response. While protease stimulation of airway epithelium does produce cytokines IL-6, GM-CSF, chemokines IL-8, eotaxin (CCL11), and platelet-derived growth factor (PDGF) and tryptase induces TNFα, IL-6, and IL-1β from human PBMCs (72), this assumption ignores a number of factors. First, PAR_2 is present on virtually all of the cells involved in asthma – epithelium, mast cells, eosinophils, neutrophils, monocytes-macrophages, lymphocytes, smooth muscle, endothelium fibroblasts, antigen presenting cells and neurons (62,73–76). While activation of epithelial PAR_2 promotes PGE2 production that then promotes smooth muscle relaxation, PAR2 agonists administered directly to smooth muscle itself cause contraction. PAR_2 agonists administered directly to leukocytes promote chemotaxis (17). Thus, dissecting the precise role for PAR_2 in the asthmatic response is complex. Throughout Chapters 2 and 3, I dissect the molecular mechanisms underlying PAR_2-induced inflammation in the airway and provide an explanation for how this receptor might orchestrate multiple portions of this complex inflammatory response.

Since we previously knew that PAR2 signaling pathways via β-arrestin led to leukocyte chemotaxis, which could contribute to recruitment of immune cells, while the G-protein-dependent pathway led to PGE2-mediated protective effects, we proposed that the proinflammatory pathway would be absent in β-arrestin knockout mice. We used the previously established model of ovalbumin (OVA)-induced model of airway inflammation in which a PAR2- peptidomimetic-ligand (2-furoyl-LIGRL-orn-NH2, aka 2fAP) is included in the sensitization phase. We found that the pro-inflammatory responses, including bronchoepithelial thickening, immune cell infiltration (eosinophils, neutrophils, T-cells, and B-cells) into the lung, and goblet cell hyperplasia are mediated by β-arrestin-2, while the bronchoprotective responses including airway relaxation and prostaglandin-E2 production are independent of β-arrestin-2. We also found that expression of IL-4, IL-6, IL-13, and TNFα was abolished in β-arrestin-2-/- mice (3). Altogether, these studies show that β-arrestin-2 regulates the pro-inflammatory responses in the lung and that the opposing PAR2-signaling arms lead to distinct physiological effects *in vivo* (Fig. 1).

Section 1.3

The role of allergens in airway activation of PAR_2

Several studies have suggested a role for PAR_2 in asthma models using common household allergens. Several studies have shown that allergens from the cockroach *B.*

germanica activate PAR_2-dependent calcium signaling and that *B. germanica*-dependent airway inflammation, including recruitment of eosinophils, is abolished in $PAR_2^{-/-}$ mice and by addition of trypsin inhibitors (77,78). In a cockroach model of asthma, $PAR_2^{-/-}$ mice have decreased IgE and airway inflammation (64,79). In another study using house dust mite (HDM) allergens, the HDM-induced inflammation including eosinophil, neutrophil, and lymphocyte recruitment and bronchiolar inflammation seen in wild-type mice was abrogated in $PAR_2^{-/-}$ mice (80).

A wide variety of allergens have been known to contain proteases that can directly activate PAR_2 (46). House dust mite *Dermatophagoides pteronyssinus* (D. pteronyssinus) contain serine proteases which have been identified and activate PAR_2 (81). Bacterial proteases from *P. gingivallis*, which is responsible for periodontitis in humans, can cleave PAR_2 and induce expression of IL-6 and CCL20 (53,82). The cockroach *periplaneta americana* contains proteases that upregulated PAR expression on mast cells, and its activity was inhibited by serine protease inhibitors (83,84). Cockroach extracts from both *P. americana* and *Blattella germanica* were able to activate PAR_2-dependent Ca2+ signaling that was abolished with serine protease inhibitors (85). *B. germanica* increased IL-8 expression and ERK activation that was lost with serine protease inhibitors (86,87). A host of fungal species are known to contain a variety of proteases, and one of these, *Alternaria alternata*, contains aspartate proteases that cleave PAR_2 and was shown to induce expression of GM-CSF, IL-6, and IL-8 via PAR_2 (88–91). *A. alternata* also induced IL-33 that was reduced using serine

protease inhibitors, suggesting that they activate PAR_2 (92). In a related species, a 42 kDa serine protease was purified and identified from *Alternaria solani* (93). These studies suggest that serine proteases in allergens play a key role in activating PAR_2-dependent responses in the airway.

In a previous study, our collaborators showed evidence that *Alternaria alternata* promotes airway inflammation and suggested that these proteases present in *Alternaria* specifically activate PAR_2 but not the other PARs (94). Using a human bronchoepithelial cell line, 16HBE14o, and calcium mobilization assay to measure cellular activation, they found that *Alternaria*-induced calcium signaling was lost with either AEBSF, a serine protease inhibitor, or heat inactivation. Using a desensitization assay, they found that pretreatment of cells with trypsin desensitized *Alternaria*'s ability to induce calcium signaling, while thrombins did not. Using PAR_2-transfected cells, they also found that *Alternaria* induced Calcium mobilization dependent upon PAR_2 expression. Furthermore, when wild-type mice were treated with *Alternaria*, infiltration of macrophages, neutrophils, eosinophils, and lymphocytes was increased, but this was reduced with *Alternaria* that was pretreated with AEBSF. Altogether, these experiments highly suggest that a protease within *Alternaria* allergens were activating PAR_2, and that could be responsible for the airway inflammation in this *Alternaria*-induced model of allergic asthma.

One mechanism by which allergen-activated PAR_2 plays a role in the airway is by regulating chemokine and cytokine expression. CCL20 (MIP3α) was found induced by

cockroach extracts dependent on PAR_2 (95). Administration of the mold allergen *Alternaria alternata* induced expression of IL-1β and IL-33 in mouse lung that was ablated by administration of *Alternaria* pretreated with a serine protease inhibitor, thus suggesting activation via PAR_2. *Alternaria* potently induced production of thymic stromal lymphopoietin (TSLP) in epithelial cells, but this was found to be partially blocked by siRNA against PAR_2 (96). *Alternaria also* induces expression of chemokines such as RANTES/CCL5, Eotaxin/CCL11, MIP-1a/CCL3 in the mouse lung (97), IP-10/CXCL10 and MCP-1/CCL2 in bronchoepithelial cells (98). *Alternaria* was also shown to induce GM-CSF, IL-4, IL-6, IL-8, IL-18, and IL-33 release in bronchial epithelial cells or dendritic cells.

Section 1.4

The Requirement of Hematopoietic PAR_2 and Accessibility of Allergens

One question that none of these studies on PAR_2 in the airways have addressed is at what point might agonists such as PAR_2 peptides or proteases be activating PAR_2? As mentioned previously, there is a prejudice in the field that they only access lumenally exposed PAR_2 and all other events are mediated by the classical mediators such as cytokines, chemokines, and IgE. Yet several studies have shown that the agonist gained access to serosal tissue and occurs through disruption of the epithelial barrier by allergens.

Based on a number of studies from our laboratory, we proposed that both lumenally-exposed epithelial PAR_2, as well as PAR_2 expressed on circulating leukocytes, are directly exposed to these exogenous proteases. The most compelling evidence that PAR_2 expressed on more than just epithelial cells is important is that in the exacerbation model of OVA-induced allergic inflammation with PAR_2 agonists, adoptive transfer of $PAR_2^{-/-}$ bone marrow into wild-type mice eliminated eosinophil and CD4+ T-cell recruitment to the lungs (3). This means that if there is no PAR_2 on the infiltrating cells, they are not able to be recruited to the airways. Similar results were observed with cockroach-induced allergic inflammation (Figure 2). Furthermore, it is possible that endogenous proteases such as mast cell tryptase, human airway trypsin, and Coagulation Factor Xa may also be released in response to these environmental allergens, further contributing to PAR_2 activation in the airway.

Supporting the idea that exogenous PAR_2 agonists might gain access to the serosal side of the airway epithelium, rhodamine-labeled PAR_2 agonist 2fAP, administered intranasally, was able to label both apical and basolateral regions of the lung epithelium as well as in cervical lymph nodes (Fig. 3A-B) (3). However, a protease is much larger than a peptide, and it would be more difficult for it to readily gain access to serosa. Yet other labs have shown that PAR_2 activation of epithelia increases permeability for enzymes with a molecular weight of 44 kilodaltons (99). The house dust mite allergen Der p 1 with a molecular weight of 25 kilodaltons was able to cross the epithelial cell layer and was also shown to directly cleave occludin via cysteine protease

activity (100). *Alternaria*, which is known to cause necrosis and leaf spot disease in plants, was shown to induce morphological changes in an airway-derived epithelial cell-line, A549, including cell shrinkage and desquamation without affecting cell viability (101). A recent study showed that *Alternaria* promoted a dose-dependent change in transepithelial electrical resistance in donor bronchial epithelial tissue from asthmatic patients but not tissue from healthy individuals, indicating a greater susceptibility to disruption of barrier function in asthmatic epithelia (102). Similarly, cockroach allergens promoted a decrease in epithelial electrical resistance (103). Furthermore, our lab found that administration of 2fAP, *Alternaria*, or *B. germanica* promotes allows transport of FITC-dextran across the airway epithelial monolayer (Fig. 3C). Altogether, these studies suggest that *Alternaria* allergens such as small proteases can not only activate PAR_2 at the epithelium but might cross and directly activate PAR_2 expressed on cells beyond the epithelial barrier such as immune cells and smooth muscle cells within the lung. However, it is likely that the entirety of proteolytic activation of PAR_2 in the airway, in response to exposure to *Alternaria*, is not explained solely by access to the pathogenic protease, but involves endogenous PAR_2 activating proteases as well.

Given the role of β-arrestin-2 in regulating pro-inflammatory effects of PAR_2, the ability of proteases found in common household allergens to activate PAR_2, and direct consequences of PAR_2 activation by proteases found in allergens, I proposed that the *Alternaria* proteases might activate PAR_2 signaling via β-arrestin-2 leading to the allergic, inflammatory response found in the lung (addressed in Chapter 2). As Alternaria is a

major cause of asthma worldwide and is particularly prevalent in the inland regions of Southern California, I focused on this asthma model and investigated the ability of a PAR_2 antagonist to block the inflammatory effects promoted by *Alternaria*.

Section 1.5

The Epidemiological Significance of *Alternaria*-induced Allergic Asthma

Asthma is a global and national problem and reduces the quality of life for 235 million people worldwide (104). 1 in 11 children, and 1 in 12 adults, has asthma (105). Common symptoms include coughing, wheezing, chest tightening, and shortness of breath that compound and result in the person's inability to breathe efficiently that impacts his or her ability to function. A common cause of asthma is the exposure to allergens such as the household fungus, *Alternaria alternata*, and the feces of the common cockroach, *Blattella germanica*. Extracts from these sources have been found to contain proteases that activate Protease-Activated-Receptor-2 (59,106).

Alternaria alternata is found as an aeroallergen in the outdoor environment as well as in households throughout the United States (107–109). Exposure to *Alternaria alternata* has long been recognized in association with increased incidence of the risk of fatal asthma attacks for both children and elderly patients (110–113). In a study of asthmatic children in the central Los Angeles area, a correlation was found between the amount of pollutants in the air, including *Alternaria*, with asthma severity (114).

Section 1.6

Targeting β-arrestin-dependent PAR_2 biased signaling as a therapeutic strategy

One strategy of treating asthma is the use of short acting or long-acting agonists of β2 adrenergic receptor to induce bronchorelaxation. However, repeated doses result in decreased bronchodilation, leading to patients' increased use of β-agonists, the increased health risks associated with higher sympathetic activation and decreasing effectiveness of inhalers during an asthma attack, and a higher risk of fatal asthma attacks (115).

PAR_2 exhibits biased signaling, being able to engage G-proteins or β-arrestins for distinctly different outcomes. As discussed earlier, downstream of β-arrestin, a number of molecular scaffolds are formed leading to activation of the actin assembly protein, cofilin, as well as activation and membrane sequestration of ERK1/2, leading to cell migration. This pathway is particularly important in leukocytes and metastatic tumor cells. Downstream of Gαq, PAR_2 promotes nuclear ERK1/2 activation and PI3K activation, leading to transcription of anti-inflammatory mediators, such as PGE2, as well as release of preformed PGE2. We have demonstrated a role for PAR_2 signaling through β-arrestins in the pathogenesis of airway inflammation and allergic asthma, which likely involves the ability of PAR_2 to promote leukocyte migration. However, it can also promote protective responses such as bronchorelaxation via a β-arrestin-independent, G-protein mediated pathway, involving the release of PGE2 (3). Thus, the ideal antagonist would exhibit bias

toward the β-arrestin signaling pathway and would not block the protective responses at doses that inhibit inflammation.

Because of its widespread involvement in pain and inflammation, PAR_2 has been an attractive therapeutic target by taking advantage of this property of "biased antagonism" (116). Antagonists targeting PAR_2, including the weak antagonist ENMD-1068, the peptidomimetics K-14585 and the novel C391, and the non-peptide GB88, have been developed to reduce inflammation and pain (116). GB88 can effectively attenuate inflammation in a rat model of colitis and is a signal pathway-specific antagonist that inhibits PAR_2-induced intracellular calcium release, cyclic AMP stimulation, receptor internalization and pro-inflammatory cytokine release without affecting PAR_2-mediated MAPK (117,118). Using a fluorometric calcium assay, ENMD-1068, has been shown to antagonize trypsin-induced Ca2+ mobilization. Using a mouse model, they found that it reduced knee-joint swelling (119). K-14585 blocked PAR_2-induced Ca2+ mobilization and IL-8 production. It also inhibited PAR_2-induced vascular permeability and contractile tone (120).

Antagonists targeting PAR_2 including GB88, ENMD1068, K-14585, and others have been at the discovery stage (116). Patents have been filed for several derivatives of these small molecule antagonists, but the development of effective and selective drugs for modulating PAR_2 has been challenging. Not many potent PAR_2 antagonists have been described recently. For example, ENMD-1068 and K-14585 have a high IC50 and are relatively unstable, making them unattractive therapeutics, and most PAR_2 antagonists

patented to date have been small molecules with high nanomolar or high micromolar potencies (121).

More recently, C391, also a weak PAR_2 agonist, was shown to effectively block PAR_2-induced Ca2+ mobilization by both proteolytic and peptide agonists in cultured immortalized human bronchial epithelial cells at a low micromolar effective concentration, and to block PAR_2-mediated pain in a thermo-analgesic mouse model (122). C391 contains a 2-furoyl group that protects it from endogenous proteases, azabicycloalkane that provides a scaffold with an isobutyrl side chain, a lysline-like side chain, and C-terminal Leu and Tyr, making it more stable (122). It orthosterically and effectively outcompetes the high affinity peptidomimetic ligands, 2-furoyl-LIGRL, reducing PAR_2 activation. Based on these characteristics, C391 may be a viable PAR_2 antagonsit in the context of allergic asthma. In this study, I have investigated whether it may be able to preferentially block the proinflammatory β-arrestin pathway.

Section 1.7

Dissertation Synthesis

Given the role of PAR_2 and β-arrestin-2 in regulating pro-inflammatory effects in the lung, a better understanding of the regulation of this pathway in a physiologically relevant model is essential. I proposed that the *Alternaria* proteases might activate PAR_2 signaling via β-arrestin-2 that would lead to the allergic, inflammatory response found in

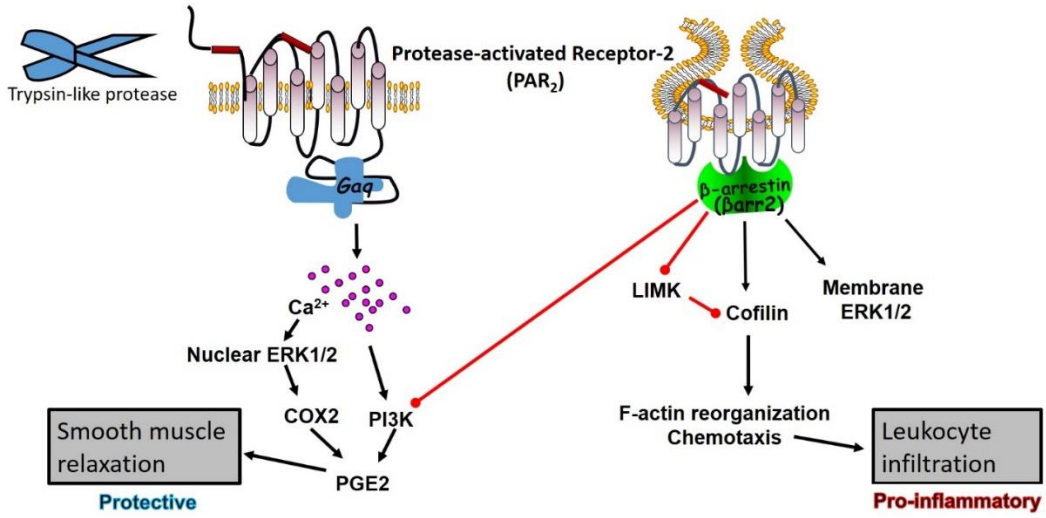
the lung (addressed in Chapter 2). Using $PAR_2^{-/-}$ and β-arrestin-$2^{-/-}$ mice, histological and cellular analyses of inflammation, and key molecular readouts for β-arrestin signaling, I examined the molecular mechanisms underlying *Alternaria*-induced asthma, delineating the role of PAR_2 signaling via β-arrestin-2, and identifying the PAR_2 activating protease. This leads to the conclusion that a single *Alternaria* protease contributes to allergic inflammation in the airways by activating PAR_2 signaling via β-arrestin-2. Thus, my dissertation brings together the biochemical importance of this signaling pathway with the essential understanding of the role of PAR_2 in the airway using this asthma model that has a high physiological and clinical relevance.

Furthermore, testing the effects of a newly developed PAR_2 antagonist, C391 (covered in Chapter 3), in targeting the inflammatory pathway in *Alternaria* signaling revealed that it was effective at inhibiting *Alternaria*-induced inflammation and β-arrestin signaling at a lower concentration than it inhibited Ca2+ signaling. This suggests that it is able to preferentially block β-arrestin signaling and indicates that this is a good candidate for the study of a biased antagonist that could pave the way for targeting β-arrestin-dependent PAR_2 signaling. Therefore, this strategy that takes advantage of biased antagonism may be used as a new avenue for therapeutic intervention.

Section 1.8

Figures

A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Protease-activated Receptor-2
(PAR2)
Gaq
β-arrestin
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Ca2+
Nuclear ERK1/2
COX2
PI3K
PGE2
Smooth muscle relaxation
Protective
LIMK
Cofilin
Membrane ERK1/2
F-actin reorganization
Chemotaxis
Leukocyte infiltration
Pro-inflammatory

B

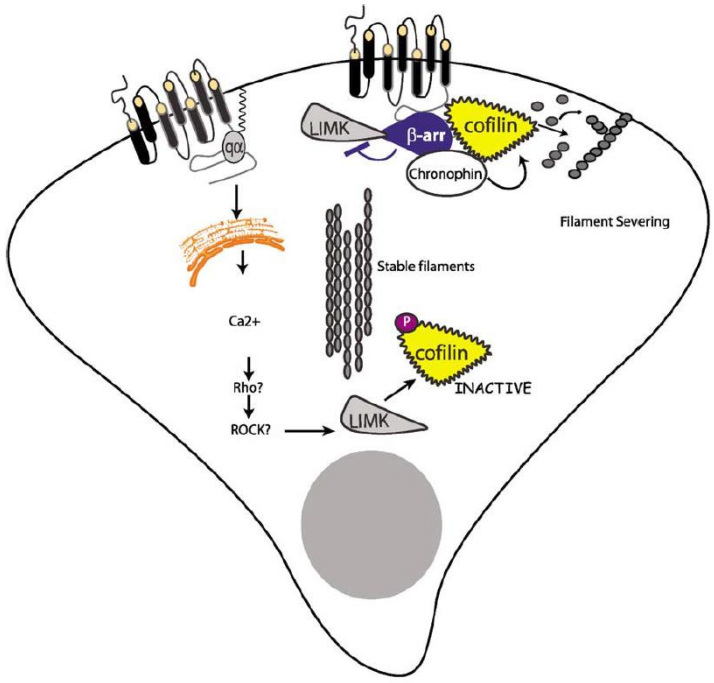
LIMK
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Figure 1.1. Model for Opposing arms of PAR_2 activation through G-protein-coupled signaling and β-arrestin-2-dependent signaling. **A.** PAR_2 activation in the lung leads to paradoxical signaling arms: G-protein as opposed to β-arrestin-dependent signaling. **B.** Localized activation and inactivation of cofilin through recruitment of β-arrestin, its activating phosphatase (chronophin) and its inhibitory kinase (LIMK). β-arrestin inhibits LIMK, allowing for cofilin activation, and this results in actin reorganization and cell migration. PAR_2 coupling to Gαq activates LIMK, leading to stable actin filaments and localized inhibition of cofilin (Adapted from Dr. DeFea) (10).

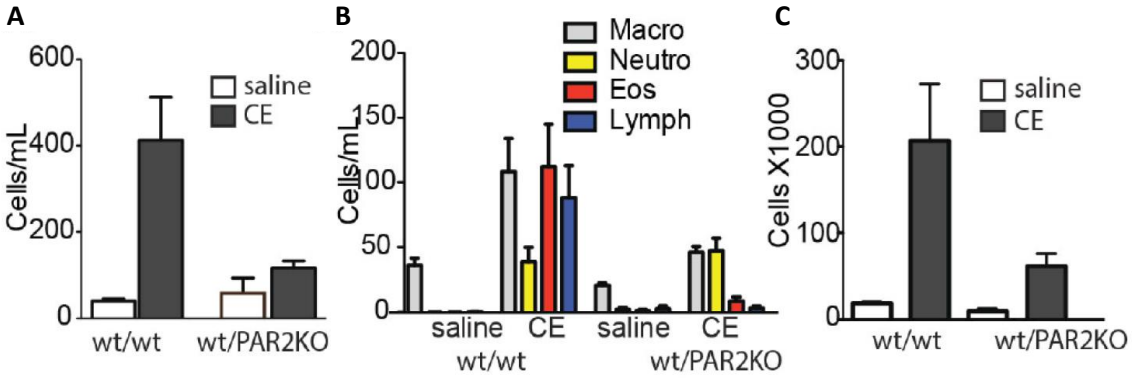

Figure 1.2. Hematopoietic PAR_2 is required for *B. germanica*-induced immune cell recruitment. Adoptive transfer of bone marrow from wt/GFP or $PAR_2^{-/-}$/GFP mice by retro-orbital injection into wt mice. After *B. germanica* (CE) treatment, **(A)** total cells, **(B)** BALF differential cell counts from cytospins stained with Hema 3, and **(C)** flow cytometric quantification of GFP-positive cells was determined (Courtesy of Heddie Nichols).

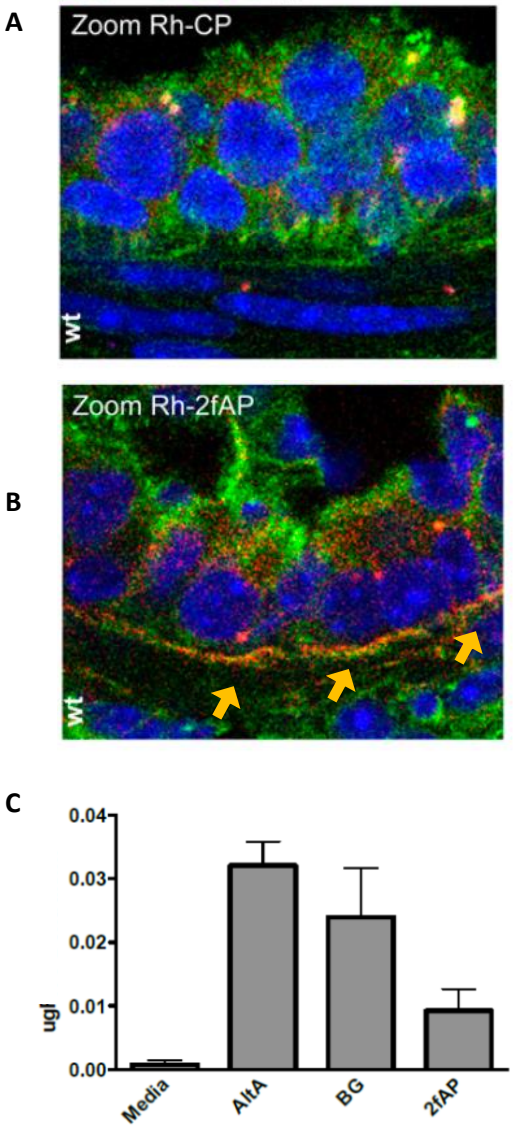

Figure 1.3. PAR_2 agonists access basolateral side of airway and promote barrier disruption. Wild-type mice were given **(A)** rhodamine-labeled control peptide (Rh-CP) or **(B)** rhodamine-labeled PAR_2 agonist 2fAP (Rh-2fAP) in red. Lung were stained with antibody against lung surfactant protein (LSP) in green, TOPRO-3 to visualize cell nuclei, and imaged using the Zeiss LSM510 100x objective (3). **C.** FITC-dextran (4 kDa) was added to the apical side of BEAS-2B cell monolayers, grown on a collagen-coated permeable support. After administration of media alone, 2fAP, the PAR_2 agonist, Alternaria, or cockroach allergens, the presence of FITC-dextran was quantified on the basolateral side by absorbance at 488 nm (Courtesy of Heddie Nichols) (3).

Section 1.9

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Chapter 2:

Protease-activated-Receptor-2-Induced Signaling through β-Arrestin-2 Mediates *Alternaria* Serine Protease-induced Airway Inflammation

Section 2.1

Abstract

PAR_2 can be activated by proteases found in household allergens, including *Alternaria alternata*, which is a common exacerbating factor in fatal asthma attacks. As discussed in Chapter 1, PAR_2 plays both protective and proinflammatory roles in the airway, and work from our laboratory demonstrated that administration of PAR_2 peptide agonists exacerbates airway inflammation via β-arrestin-2. In this chapter, I describe the isolation and sequence-identification of the single trypsin-like serine protease, AASP (Alternaria Alkaline Serine Protease) found in *Alternaria* filtrates and show that intranasal administration of the enzyme-active filtrate in wild-type (but not β-arrestin-$2^{-/-}$ or $PAR_2^{-/-}$ mice) increases airway epithelial damage, mucin production, and recruitment of eosinophils, $CD4^+$T-cells and macrophages. Furthermore, I demonstrate that the majority of these acute inflammatory responses are promoted by the purified AASP and blocked by Soybean Trypsin Inhibitor. I demonstrate that, in cultured cells, both filtrates and isolated enzyme promote PAR_2 interaction with β-arrestins as well as downstream cofilin dephosphorylation and chemotaxis. Thus, my work demonstrates that a single serine protease in an aeroallergen like *Alternaria* can activate PAR_2 signaling through β-arrestin-2 to cause an inflammatory asthma phenotype.

Section 2.2

Introduction

PAR_2 is activated not just by endogenous serine proteases such as trypsin and tryptase, but also by serine proteases associated with allergy-inducing pathogens including dust mites, the German cockroach, *B. Germanica*, and the several species of *Alternaria* fungi, such as *Alternaria alternata* (1–8). Recent studies show that exposure to *Alternaria* is highly correlated with the increase in symptomatic epidemic asthma observed in the United States over the past decade (9–12). Furthermore, *Alternaria* is thought primarily to promote lower airway inflammation, as opposed to the rhinitis observed in response to the isolated *Alternaria* Alt a 1 glycoprotein or to other pollen allergens (13–15). Recently, culture filtrate components from *Alternaria* have been shown to promote PAR_2-dependent airway inflammation in a murine model (4). While the commonly used ovalbumin (OVA)-based mouse models of allergic airway inflammation reflect the eosinophilic inflammation also seen in humans, *Alternaria* also promotes fibrosis and an inflammation with neutrophilic as well as an eosinophilic component (16–19). This model is thus more reflective of moderate to severe human asthma, which leads to airway remodeling and is less well controlled by current standard of care. PAR_2 in the airway, presumably activated by either endogenous or allergen-derived proteases, has been implicated in the pathogenesis of allergic asthma

(3,20). Its activation is associated with an increase in allergen-induced leukocyte lung infiltration, which is reduced in PAR_2 null animals. Activation of PAR_2 can cause inflammatory cell infiltrate, epithelial thickening and heightened mucus production in asthma models via a β-arrestin-2-dependent pathway (21). However, studies have also shown that it can trigger bronchodilation independent of β-arrestin-2 signaling via prostanoid release from PAR_2-activated bronchial epithelial cells (21–24). Thus, some of the inflammatory effects of PAR_2 in the setting of allergic lung inflammation are β-arrestin-dependent, whereas other putative protective effects are not.

Our previous studies of filtrate products derived from *Alternaria* cultures demonstrated that they promote PAR_2 calcium signaling via a serine-protease-mediated stimulus involving a G-protein-coupled pathway. The activation of PAR_2 signaling by the *Alternaria* filtrate was blocked by trypsin inhibitors, implicating a trypsin-like proteolytic process (4). However, the identity and numbers of *Alternaria* filtrate serine proteases that can activate PAR_2 were not identified, and their mechanisms for regulating cell migration and airway inflammation were not determined. We hypothesized that a serine-protease-mediated PAR_2/β-arrestin signaling axis plays a key role in *Alternaria*-induced lung inflammation. To test this hypothesis, we first sought to isolate and characterize the trypsin-like serine protease(s) released by *Alternaria* into the culture filtrate and to evaluate the ability of the enzyme(s) so identified to activate PAR_2 to stimulate both calcium signaling and PAR_2-β-arrestin interactions, as well as subsequent downstream signaling events. We then sought to determine whether *Alternaria* filtrates

were able to trigger airway inflammation in $PAR_2^{-/-}$ or β-arrestin-$2^{-/-}$ mice in comparison with their wild-type littermates.

Section 2.3

Methods and Materials:

Materials: All chemicals were from Sigma unless otherwise noted.

Alternaria preparations: 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. a culture filtrate from Strain ATCC 11680 (Greer Laboratories Cat. No. XPM1C3A25) and 2. a crude buffer extract of freeze-dried defatted *Alternaria* powder (Greer Laboratories Cat. No. XPM1D3A2.5). The culture filtrate was used directly and the crude defatted *Alternaria* allergen was reconstituted in 25 mM Tris-HCl buffer, pH 7.4 as a stock solution containing approximately 1.7 U/mg trypsin-like activity using the QAR-AMC substrate (Bachem, I1550: see below). Aliquots of the Tris-buffer solution were used for activity-based-probe 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 Biorad, Hercules CA (Bio-Scale Mini Macro-Prep High Q Cartridge). Antibodies were from the following companies: phospho-cofilin (Cell Signaling), Total cofilin (BD Biosciences), PE-Cy5.5-conjugated-anti-

phospho-cofilin (BIOSS), FITC-CD45, APC-GR1, PE-CCR3, FITC-CD4, APC-CD8, PE-CY5-CD19 (BD Biosciences), actin (Santa Cruz). NOVEX 4-20% polyacrylaminde gels were from Life Technologies.

Quantifying serine protease Alternaria enzyme samples were quantified in terms of their 'trypsin-like' equivalents, using a microtiter plate chromogenic assay with Glu-Ala-Arg-aminomethylcoumarin (QAR-AMC) as substrate. High-specific-activity trypsin was used as a standard (Type IX-S porcine trypsin; Sigma, catalog # T0303, 16,000U/mg). The filtrate, having a protein content of about 650 µg/ml bovine albumin equivalents (in Hanks Buffered Saline Solution/ HBSS), routinely had a trypsin-like enzyme activity equivalent to 1-2.5 U/ml using the QAR substrate).

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 (25), with minor modifications. The *Alternaria* samples were diluted to a final concentration of 1.5 U/ml and incubated with 100 µM of the activity-based probe in a 10 µl reaction volume consisting of: 50 mM Tris-HCl, pH 8, 0.1% NP40 and 1.5 mM $CaCl_2$ 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

HRP-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 - 1 µg enzyme) were incubated with 100 µM of the activity-based probe. The ABP bound proteins were pulled down with Avidin-Sepharose beads for 1 h, washed 5X with 2 M urea, 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 µg/mL) at 37^{o}C overnight. 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 (26,27). Data dependent scanning was performed by the Xcalibur v 2.2 SP1.48 software using a survey mass scan at 60,000 resolution in the Orbitrap analyzer scanning *m/z* 400-2000, followed by collision-induced dissociation (CID) tandem mass spectrometry (MS/MS) of the six most intense ions in the linear ion trap analyzer (28). 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 http://arrakis.vbi.vt.edu:8080/Alternaria_alternata/Info/Index. This custom protein database contained 4,623 entries. Variable modifications considered during the search included methionine oxidation (15.995 Da), 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 4C 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 mm X 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 sodium chloride elution protocol, ranging from 10 mM to 1 M salt, as illustrated in the Figure legends. The eluted fractions were monitored for both protein (absorption at 280nm 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 endogenous PAR_2, were transfected with N-terminally tagged rat-PAR_2

(29,30), 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 calcium-sensing dye Fluo-4 NW (25mg/ml) in calcium assay buffer (HEPES-fortified Hanks buffered saline, pH 7.4 (Gibco): 10 mM HEPES, 1.5 mM $CaCl_2$, 1.5 mM $MgCl_2$) 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's sample injection system. Cells in the plate were excited at 480 nm and kinetic traces of fluorescence emission at 530 nM, representing increased cytoplasmic calcium levels, were recorded every 0.5 seconds for 1 min. Fluorescence levels were normalized to the signal generated by 2µM calcium ionophore A23187 (Sigma, St. Louis MO).

Bioluminescence resonance energy transfer (BRET) measurements of recruitment of β-arrestin-2: BRET, in which the energy emitted when luciferase 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 PAR_2YFP 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 (Berthold Technologies, Oak Ridge, Tennessee), 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 possibility that observed signals represented non-specific interactions, we included a β-arrestin-Luc + YFP in every experiment, which gave values similar to donor only controls. Dose curves, ranging from 6.5μg/ml to 650μg/ml *Alternaria* filtrate, were used to evaluate agonist EC_{50}s; kinetic curves and were performed with a single dose of agonist (65μg/ml) and BRET values monitored over 20 minutes. For acceptor/donor ratio assays, β-arrestin-Luc (donor) concentration was held constant at .5μg and PAR2-YFP concentration was varied from .5-1.5μg. BRET values were determined after 15 minutes of incubation with 65μg/ml *Alternaria* filtrate.

Preparation of spleen lymphocytes for signaling and migration assays: To isolate lymphocytes**,** age and sex-matched wild type and β-arrestin-$2^{-/-}$ mice were sacrificed and spleens immediately removed, minced into small pieces, homogenized in 10mls of PBS, filtered through sterile nylon mesh. Cells were passed through 70μ filters, pelleted at 250g for 5 minutes, washed three times in modified Hank's Balanced Salts Solution (HBSS, without Ca^{2+} and Mg^{2+}) and resuspended in RPMI complete media for subsequent experiments. Cytospin analysis and flow cytometry revealed the prep was >80% lymphocytes. or into 24 well dishes for cofilin activation assays.

Transwell Migration assay: After RBC lysis, approximately 10^5 cells (isolated and plated as described above) were plated onto collagen-coated membrane inserts with 5μm pores and treated with *Alternaria* filtrate (65μg/ml) or 100nM 2fAP. After 1 hour, non-migratory cells were removed from the top of the filter and migration was analyzed two

ways: 1) Cells attached to the underside of the filter were stained with crystal violet; stained cells and migrated lymphocytes (identified by cell morphology) were counted using a bright field compound microscope in 4 fields of vision. Migrated cells were then expressed as a fold change over baseline migration to HBSS 2) Cells were gently scraped into 2mls of FACS buffer and CD4+ T-cells were confirmed by flow cytometry, using anti-CD3 and CD4, antibodies. Migrated CD4T-cells were expressed as the number of cells per ml.

Determination of Phospho-cofilin in cultured leukocytes: 10^5 cells, plated on 24 well culture dishes, were grown overnight in serum supplemented RPMI and then media was replaced withserum free media. Cells were allowed to recover from media exchange for 1 hour, after which trypsin or *Alternaria* filtrate (650µg/ml) was added for 0-60 minutes. Cells were lysed in 1X Laemmli sample buffer, sonicated, boiled and analyzed by 15% SDS-PAGE followed by western blotting with anti-phospho-cofilin (rabbit, 1:1000) and anti-total cofilin (mouse, 1:1000). Secondary antibodies (Anti-rabbit IR-800 and anti-mouse Alexa680) were added at 1:45000 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: 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, USA, and the

University of Calgary, Calgary, Canada. β-arrestin-$2^{-/-}$ mice in a C57BL/6 background were provided by Robert Lefkowitz (Duke University Medical Center) $PAR_2^{-/-}$ 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.

Paired 6 week old wild type, β-arrestin-$2^{-/-}$ and $PAR_2^{-/-}$ mice were exposed to either 25 µl HBSS alone (negative control) or .4 mg/kg of *Alternaria* filtrate (in 25 µl HBSS), administered intranasally 3 times over an 8 day period (on days 1, 5 and 8). Mice were sacrificed on Day 9 and lungs were lavaged with 5 mls of PBS. Bronchio-alveolar lavage fluid (BALF) was centrifuged and supernatants used for analysis of cytokine production. Cell pellets were resuspended in PBS, subjected to NH_4Cl lysis (0.83% ammonium chloride, 2 min, at 4^oC) to remove red blood cells (RBCs), and resuspended in 1ml of PBS. Cell numbers were determined by hemocytometer and samples were then divided into 2 (200 µl were used for cytospins and 300 µl were used for flow cytometry). Lungs were simultaneously harvested and fixed for histological analysis.

Haematoxylin & Eosin (H&E) stain and quantification: Staining was performed on paraffin embedded sections and analyzed as described in previous studies (Nichols et al., 2012). Briefly, for general histological analysis, lung sections were stained with 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. For histological grading of inflammation, a score of 1-4 was given, with 1 being no inflammation present, 2 being 1 ring of leukocytes around the vasculature and mild infiltration, 3 being 2-3 rings and moderate infiltration, and 4 being 4 or more rings and numerous leukocytes throughout the epithelial tissue. Epithelial thickness was quantified using 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 mm that stained positive with Alcian blue. Mucin staining was calculated using a user-defined NIH Image J macro: images were inverted and total red channel density (corresponding to blue by eye) per 100 cells was determined.

Cytospins and Differential Cell Count: Briefly, 100ul of bronchio-alveolar lavage fluid (BALF) was spun on to glass slides using Shandon Cytospin and cells stained using Hema 3 stain kit following manufactures protocol. Differential counts were obtained by counting 200 cells per slide and categorized based on morphological criteria, as described in our previous study.

Flow Cytometry: Bronchioalveolar lavage fluid (BALF) cell pellets were resuspended in 1ml of FACS buffer as previously described 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-F480) as described in our previous studies(Nichols et al., 2012). 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 positive. B-cells were identified as the CD19 positive group. Macrophages were further confirmed as F480 positive. 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 CD-45. Cells were first gated on CD45 staining to identify leukocytes and granulocyte and lymphocytes were identified based on FSC/SSC. 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. The number of cells with a mean fluorescence level above $5X10^3$ and the mean fluorescence intensity in each treatment group were determined.

Measurement of Alternaria enzyme-stimulated bronchodilation. After sacrifice, 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 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 Substance P, (SP), an agonist for a different GPCR (neurokinin-1 receptor), to demonstrate viable Gαq signaling. Tissues were washed, followed by treatment with *Alternaria* enzyme (.6U/ml). The relaxation response was recorded as done previously

(Nichols et al., 2012) and relaxation was expressed as a percentage reduction in tension (%) relative to the tension observed with carbachol.

Data and statistical analysis: All graphs and statistical analyses performed using Kaleidagraph Version 4.0, Microsoft Excel 2003 or GraphPad Prism 5.0. Experiments were performed a minimum of 3 times. Statistical significance was determined using one-way ANOVA and Tukey t-tests (to compare between treatment groups).

Section 2.4

Results

Purification and sequence identification of a PAR_2 activating serine protease from Alternaria alternata: Previous studies showed that *Alternaria alternata* filtrate-induced lung inflammation *in vivo* and PAR_2 activation in cultured cells were both inhibited by soybean trypsin inhibitors (SBTI), implying the action of a serine protease (31). Airway inflammation in response to *Alternaria* has been reported using a number of commercial and non-commercial sources, including spores, filtrate and cellular extract. Using a biotinylated, serine protease-selective activity-based probe (ABP) (25,32), we found that both the filtrate and unfractionated, defatted *Alternaria* cellular extract contained only a single biotinylated enzyme, with an apparent molecular mass of about 24 KDa (Figure 1A). Labeling of the enzyme with the ABP was abrogated by treating the samples with SBTI. The results indicated that a single serine protease is responsible for proteolysis at the

canonical PAR_2 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 (Figure 1B). 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). We thus concluded that both the *Alternaria* culture filtrate and the cellular extract contain the same serine protease and do not contain other enzymes that can account for the PAR_2 cleavage. As shown in Table 1, although the *Alternaria* enzyme is sensitive to inhibition by SBTI (Ki ~ 25 pM), it displays biochemical characteristics distinct from those of mammalian trypsin in terms of relative Km's 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 as shown in Table 2. As indicated by the underlined sequences in the table, the peptide sequences obtained by mass spectral analysis matched the predicted amino acid sequence of the *Alternaria* enzyme found in the Est database. The 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 refer to this newly identified protease as *Alternaria* Alkaline Serine Protease (AASP).

Activation of PAR_2 calcium signaling by Alternaria proteases. We next evaluated the ability of the enzymes from both preparations to promote calcium mobilization, a hallmark of the canonical PAR_2/G-protein signaling. We previously showed that the crude Alternaria filtrate promoted Ca^{2+} mobilization in KNRK-PAR_2 cells and this activity was serine-protease and PAR_2-dependent, as it was eliminated by treatment with SBTI, and by prior desensitization of PAR_2 with the PAR_2-activating agonist peptide, 2-furoyl-LIGRL-Orn-amide (2fAP) (31). Similarly, here we showed that the *Alternaria* filtrate and partially purified AASP (fraction 4 from High Q column) promoted PAR_2-dependent Ca^{2+} mobilization (Figure 2); as demonstrated by the fact that it was not observed in the untransfected KNRK cells (31) and was eliminated when the receptor was first desensitized with 2fAP or cells were incubated with SBTI (Figure 2).

Our previous data had demonstrated that PAR_2-stimulated leukocyte cell migration, activation of cofilin and subsequent actin reorganization and airway inflammation were dependent upon β-arrestin-2. We next examined recruitment of luciferase-tagged β-arrestin-2 to PAR_2-YFP in response to *Alternaria* treatment, using Bioluminescence Resonance Energy Transfer (BRET) (Figure 3) (33). BRET provides a means for monitoring direct actions in live cells in response to agonist stimulation and there are several parameters that can be determined from BRET assays. First, concentration-effect curves revealed EC50 values of 65 μg/ml protein for *Alternaria* filtrate (Fig 3A). The EC50 values were similar for both AASP (2.5U/ml) (Figure 3B) and trypsin (Figure 3C) (2U/ml). β-arrestin interactions are dependent upon receptor

conformation, and 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 PAR_2. Differences in the apparent affinity of β-arrestin for PAR_2 can be assessed by determining the acceptor/donor ratio at which half maximal BRET signal ($BRET_{50}$) is obtained. Both maximal BRET values (BRETmax) and $BRET_{50}$ for β-arrestin-2/PAR_2 interactions were similar in response to either 65μg/ml *Alternaria* filtrate or 2.5U/ml trypsin (Figure 3E), but the kinetics of recruitment were more rapid in response to trypsin (Figure 3F).

Next, we examined cofilin dephosphorylation and cell migration, as read-outs for activation of β-arrestin signaling, as we have previously demonstrated that PAR_2 promotes dephosphorylation and activation of cofilin by a β-arrestin-dependent, G-protein-independent mechanism (34,35). Naïve lymphocytes, isolated from spleens of wild type and β-arrestin-$2^{-/-}$ mice, were treated with *Alternaria* or trypsin (as a positive enzyme control) for 0-60 minutes and lysates were analyzed by SDS-PAGE followed by western blotting with anti phospho(p)-cofilin and anti-total(t)-cofilin. That the preparation was predominantly lymphocytes, and there were no differences in basal numbers of lymphoyctes between wild-type and β-arrestin-$2^{-/-}$ mice was determined by analyzing cytospin preparations (Fig. S1). *Alternaria* filtrate promoted cofilin dephosphorylation (to approximately 45% of control values) as did the trypsin and the partially purified enzyme from *Alternaria* extracts. This dephosphorylation response was abolished in cells prepared from β-arrestin-$2^{-/-}$ mice (Fig. 4A, B). We next performed

Transwell chemotaxis assays with T-cells isolated from spleens of wild type or β-arrestin-$2^{-/-}$ mice. Addition of *Alternaria* filtrate or the PAR_2-activating peptide, 2fAP, to the bottom chamber promoted an increase in wild type cell migration compared to saline controls (6.1±1.1 fold and 2.5±.32-fold, respectively), as determined by counting crystal violet stained cells present on the filter undersides (Figure 5A). We also used flow cytometry to quantify migrated cells and observe 510±54 and 290±48 CD4+ T-cells per mL in response to *Alternaria* and 2fAP, respectively, compared to 165±23 in saline treated controls (Figure 5B). In contrast, migration of CD4+T-cells from β-arrestin-$2^{-/-}$ mice was abolished (Figure 5A, B). In summary, our data showed that the filtrates from *Alternaria alternata* allergen preparations contain a single serine protease activity, AASP, responsible for PAR_2 activation, with a unique amino acid sequence, and having catalytic properties quite distinct from those of mammalian trypsin. Both the filtrate and the isolated enzyme promote PAR_2 signaling through both G-protein and β-arrestin pathways. We chose to use the *Alternaria* filtrate, which contains both the PAR_2-activating serine protease and the Alt a 1 allergen, to evaluate role of β-arrestin-2 in Alternaria-induced pulmonary inflammation *in vivo.*

Alternaria filtrate-induced airway inflammation requires PAR_2 and β-arrestin-2 and is sensitive to inhibition of trypsin activity. To determine the role of the PAR_2/β-arrestin signaling axis in Alternaria-induced inflammation, filtrate from *Alternaria* was administered to mice intranasally on days 1, 4 and 8 (Figure 6A). 24-48 hours after the final intra-nasal administration, BALF and lung tissue were collected for further analysis.

Epithelial damage and cellular inflammation were assessed by examination of H&E stained lungs (Fig. 6B-L). Quantification of epithelial hyperplasia indicated that *Alternaria* promoted a 2-fold increase in epithelial thickness and this increase was significantly abated in both $PAR_2^{-/-}$ and β-arrestin-$2^{-/-}$ mice and with SBTI pretreatment (Figure 6M). Histological grading revealed that *Alternaria* treatment of wild type, but not $PAR_2^{-/-}$ or β-arrestin-$2^{-/-}$ mice produced an increased lung inflammation score (Fig. 6N, defined by infiltration of leukocytes into the bronchioles). When SBTI was included in the intranasal administration of filtrates, inflammation score was decreased, similar to what was observed in $PAR_2^{-/-}$ and β-$arr2^{-/-}$ mice (Fig. 6N).

Increased mucin production by airway goblet cells is associated with severe asthma and results in narrowing of bronchioles, impeding airflow (36). Using Alcian blue as a marker of acidic proteoglycans present in mucin and goblet cells, we demonstrated that wild-type mice exhibit a significant increase in both the number of goblet cells and the amount of mucin produced after *Alternaria* treatment (Fig. 7A). Semi quantification reveals a 15-fold increase in mucin staining (Fig. 7B) and a 9-fold increase in goblet cells (Fig. 7C) in response to *Alternaria*. Both goblet cell hyperplasia and increased mucin staining in response to *Alternaria* were abolished in both the $PAR_2^{-/-}$ and β-arrestin-$2^{-/-}$ mice, as well as by treatment with SBTI (Fig. 7). These data strongly suggest that *Alternaria*-mediated airway inflammation requires proteolytic activation of PAR_2 and subsequent signaling through β-arrestins.

A major hallmark of airway inflammation, and one that was apparent in histological analysis, is the infiltration of leukocytes into the lung. The increased production of mucus traps these inflammatory cells in the airways and their presence can be quantified by analyzing the A major hallmark of airway inflammation, and one that was apparent in histological analysis, is the infiltration of leukocytes into the lung. The increased production of mucus traps these inflammatory cells in the airways and their presence can be quantified by analyzing the bronchio-alveolar lavage fluid (BALF). 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 clearly seen in BALF cytospins from wild type mice and is markedly reduced when SBTI is included in the intranasal challenges (Figure 8A). Similarly, the number of recruited cells is decreased in β-arrestin-$2^{-/-}$ and $PAR_2^{-/-}$ mice (Figure 8B, C). Differential counts of cytospins (Figure 8D-G) revealed that exposure to *Alternaria* increases the total numbers of eosinophils (88,800 cells/ml) (Figure 8D), lymphocytes (49,300 cells/ml) (Figure 8E) and neutrophils (40,000 cells/ml) (Figure 8F) compared to saline-treated mice (755, 1100 and 3700 cells/ml, respectively). In both *Alternaria*-treated $PAR_2^{-/-}$ mice, and wt mice treated with SBTI, recruitment of eosinophils was reduced by greater than 90% (7800/1600 cells/ml), neutrophils by approximately 60% (14,900/12,700 cells/ml) and lymphocytes by over 90% (2700/5700 cells/ml) compared to the wild type mice treated with *Alternaria* alone. Similarly, in β-arrestin-$2^{-/-}$ mice, recruitment of eosinophils was reduced by 88% (11,400 cells/ml), neutrophils by 67% (13,300 cells/ml) and lymphocytes

by 75% (12,200 cells/ml). Macrophage recruitment was also increased by treatment with *Alternaria* (27,000 cells/ml compared to 2600 in saline treated controls) and but more dependent upon β -arrestin-2 than PAR_2 or protease activity (Figure 8G). In β-arrestin-$2^{-/-}$ mice macrophage recruitment was decreased by 84%; in $PAR_2^{-/-}$ mice or mice treated with SBTI macrophage recruitment was only reduced by 50% (14,000 and 16,000 cells/ml, respectively). A major difference between this *Alternaria*-model and our previous ovalbumin-based mode is the significant presence of neutrophils in the BALF, and it is noteworthy that neutrophil infiltration was less dependent upon the PAR_2/β-arrestin signaling axis than was eosinophil or lymphocyte recruitment, suggesting interplay between multiple signaling pathways. Flow cytometry confirmed the differential counts, and revealed a change in the relative distributions of cell types in the BALF in the absence of PAR2 or β-arrestin-2 (Fig. 9A-D). We observed in saline –treated wild type mice treated, less than 10% of the live cells were CD45+ and of those eosinophils (high forward scatter (FSC)/high side scatter (SSC)/CCR3+) comprised 0.3%, neutrophils (high-FSC/high-SSC/GR1+) comprised 2.2%, lymphocytes (high FSC/low SSC, CD3+) comprised 2.5% and alveolar macrophages (high FSC/mid SSC, F4/80+) comprised 35% of the total cells. Approximately 60% of the cells were classified as “other” and were predominantly cells with very high forward scatter. In BALF from *Alternaria*-treated wild type mice, CD45+ cell distribution was 38% eosinophils, 19% neutrophils, 14%lymphocytes and 24% macrophages. Only 3% of the cells were characterized as “other.” While the relative amounts of eosinophils, neutrophils and lymphocytes was also increased in *Alternaria*-

treated $PAR_2^{-/-}$ mice and β-arrestin-$2^{-/-}$ mice, they were significantly different from that observed in wt mice, suggesting that PAR_2/b-arrestin signaling is important not just for increasing the total number of cells in the airway, but preferentially mediates recruitment of specific cell types. In $PAR_2^{-/-}$ mice, the BALF was 4% eosinophils, 14% neutrophils, 10% lymphocytes and 37% macrophage; whereas in β-arrestin-$2^{-/-}$, the BALF cell composition was 19% eosinophils, 10% neutrophils 6% lymphocytes and 29% macrophages (Fig. 9D). Further, flow cytometric analysis of the individual lymphocyte populations demonstrated that CD4+ T-cells were the primary lymphocyte sub-type recruited to the airways (Fig. 9E,F). Thus, in the $PAR_2^{-/-}$ and β-arrestin-$2^{-/-}$ mice, not only were the total numbers of each of these cell types reduced in the BALF, but their relative distributions were altered. Consistent with the increased lymphocyte recruitment, epithelial thickening and increased mucus production, we observed increased IL-6 and IL-13 levels in BALF supernatants after *Alternaria* treatment. This increased cytokine level was significantly reduced in β-arrestin-$2^{-/-}$ mice, consistent with our observations in the OVA+ 2fAP airway inflammation model (22) (Table 3).

Typically, the overall airway response to environmental allergens involves a systemic as well as local factors. Our previous studies suggested that PAR_2 does play a contributing role to the overall immune response, including IgE production, but that β-arrestins are only required for the acute localized response and are dispensable for production of IgE (21). Since the *Alternaria* model described here is a short-term model reflecting acute inflammation, we tested whether localized activation of PAR_2 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 exposed to 2 doses of AASP administered intranasally (40mU and 4mU trypsin-like activity in 25uL HBSS), which is equivalent to the activity found in the purified *Alternaria* extract and a 10-fold lower dose as is described for *Alternaria* in Figure 8A. BALF was collected and analyzed for the presence of immune cell infiltrates. Both doses of AASP induced recruitment of eosinophils and lymphocytes to the airways of wild type mice (Fig. 10A), but neutrophils and macrophages were only induced by the higher dose and at less than 50% of that observed with total *Alternaria* filtrate. Eosinophil and lymphocyte recruitment at both doses was abrogated in the β-arrestin-2$^{-/-}$ mice (Fig. 10B, C). Thus, AASP is sufficient to promote acute inflammation in the airway.

To demonstrate the role of β-arrestin-dependent PAR_2 signaling in the airway in response to *Alternaria*, we employed a phospho-flow assay, using Cy5.5-conjugated anti-phospho-cofilin to monitor cofilin dephosphorylation in BALF cells after induction of lung inflammation with *Alternaria* as described in Fig 6A. Granulocyte and lymphocyte populations were identified by SSC vs FSC (Fig. 11A) and the mean Cy5.5 fluorescence for each population were determined by histogram analysis (Fig. 11B, C). Cofilin phosphorylation levels (calculated as the mean fluorescence levels (MFL) observed with anti-p-cofilin minus the background fluorescence observed with isotype controls) were decreased with *Alternaria* treatment in both granulocytes (Fig. 11E) and lymphocytes (Fig. 9F) from wild type mice, indicating cofilin dephosphorylation. Furthermore, the

percentage of wild-type granulocytes and lymphocytes in the BALF that stained positive for phospho-cofilin was significantly reduced after *Alternaria* treatment (Fig. 11E, F). These data demonstrate, for the first time, that the PAR_2/β-arrestin signaling axis is activated in the invading inflammatory cells during airway inflammation induced by *Alternaria*. As expected, in β-arrestin-$2^{-/-}$ mice, cofilin dephosphorylation by both measurements (decreased MFL and decreased numbers of p-cofilin positive cells) was diminished (Fig. 11E, F). No differences in total cofilin levels were observed in BALF cells from each treatment group. Thus, these allergens promote robust β-arrestin-dependent dephosphorylation of cofilin *in vivo*.

Alternaria-stimulated prostaglandin-dependent bronchorelaxation. In our work with an ovalbumin model of asthma, we also observed that inflammatory cell infiltration into the lung was dependent on β-arrestin-2. We also observed that the epithelium-dependent-prostanoid-mediated bronchodilation caused by PAR_2 activation was β-arrestin-independent (12). We thus evaluated the ability of the *Alternaria*-derived serine protease to cause bronchodilation. As shown in Figure 12, the partially purified *Alternaria* protease caused a bronchorelaxation that was blocked by the cyclooxygenase inhibitor, indomethacin. Thus, the *Alternaria* enzyme was able to activate not only the influx of inflammatory cells into the lung but also the counter-regulatory 'protective' bronchodilation response due to the epithelial cell-generated production of cyclooxygenase-produced agonists (presumably, prostaglandins).

Section 2.5

Discussion

The main finding of our study was that the asthma allergen from *Alternaria alternata* contains a single trypsin-like serine protease, AASP, that can activate PAR_2, to promote airway inflammation via a β-arrestin-dependent mechanism. This PAR_2-activating protease produced by *Alternaria alternata* has elements in common with PAR_2-activating enzymes present in other allergens like those from the cockroach and dust-mite (2). *Alternaria* allergens are of particular interest because they are highly correlated with early onset allergic asthma and increased asthma-associated mortality. We had previously demonstrated that *Alternaria* extracts promote canonical PAR_2/Gαq-protein signaling in cultured cells and allergic airway inflammation *in vivo*. The sensitivity of airway inflammation to serine protease inhibitors suggested that *Alternaria* allergen extracts contain serine proteases that activate PAR_2, leading to signaling events that promote the inflammation observed. Here we demonstrate that the filtrates and the isolated protease can induce both G-protein associated signaling events (e.g. Ca^{2+} mobilization) and β-arrestin-dependent ones (recruitment of β-arrestins to PAR_2, activation of cofilin and cell migration) in cultured cells and primary leukocytes. Whether T-cells directly encounter *Alternaria* proteases, or whether the PAR_2 response in vivo is a result of other serine proteases and chemokines released in response to PAR_2 is still unclear. However, both *Alternaria* and 2fAP-mediated PAR_2 activation promote barrier

damage in cultured airway epithelial cells (Fig. S2), raising the possibility that some of the apically acting proteases might gain access to the serosa.

We have shown previously that β-arrestin-dependent cofilin activation is essential for PAR_2-stimulated migration of many cell types, including primary leukocytes (35), and our studies suggest that PAR_2-stimulated β-arrestin-dependent cell migration is likely 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 observation here that the cells recruited to the BALF by administration of *Alternaria* have more active (dephosphorylated) cofilin, as compared to 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 PAR_2/β-arrestin signaling axis is associated with the inflammatory process induced by *Alternaria* in vivo. These studies are the first to demonstrate household allergen induced activation of β-arrestin-dependent signaling downstream of PAR_2, and the first to show an in vivo correlation of β-arrestin-dependent cofilin activation and leukocyte migration with lung inflammation. We have also demonstrated that airway inflammation induced by *Alternaria* is abolished in the absence of either PAR_2 or β-arrestin-2, implicating this signaling axis in the asthma-inducing effects of *Alternaria* exposure.

While many of the parameters of lung inflammation were similar in the *Alternaria*-asthma model we used here and the ovalbumin asthma model used in previous studies,

there were some notable differences. First, we see a striking increase in neutrophil infiltration in this asthma model compared with previously published studies using an ovalbumin model of lung inflammation in which PAR_2 activating peptides were added during sensitization. The neutrophilia may explain the dramatic increase in width of the basement membrane seen in the allergen-treated wild-type mice as they release preformed mediators such as metalloproteases, elastase, lactoferrin, and myeloperoxidase, all of which correlate with decreased pulmonary function (37). While airway eosinophilia often characterizes allergic asthma, it is widely recognized that neutrophils are found in patients with acute severe asthma (38,39). Interestingly, neutrophil infiltration was slightly less dependent upon PAR_2/β-arrestin-2 signaling and trypsin-like protease activity than were eosinophils and leukocytes, suggesting a more complex signaling network at play, perhaps involving direct activation of immune cells by PAR_2 agonists as well as release of chemokines that then act to recruit additional cells. Furthermore, treatment of mice with the purified AASP revealed an interesting feature of the *Alternaria*-induced asthma. While AASP induced eosinophil and lymphocyte recruitment to the same extent as the *Alternaria* extract, neutrophil recruitment was only marginally increased, suggesting that recruitment of different cell types may be mediated by different components of the *Alternaria* allergen. Taken together with our previous evidence that IgE production is not dependent upon the β-arrestin pathway but that PAR_2 contributes to the process, these data suggest that AASP may mediate the localized PAR_2

exacerbation of asthma in the airways via PAR_2/β-arrestin signaling, while the systemic responses may be mediated by other components.

Lung histology also demonstrated a marked airway remodeling in response to *Alternaria* treatment as evidenced by pseudo-thickening of the epithelium. This remodeling was reduced or absent in $PAR_2^{-/-}$ and β-arrestin-$2^{-/-}$ mice. Changes to the basement membrane of the epithelial tissue can result from deposition of extracellular matrix components as a result of communication between structural cells and immune cells. Thus, the recruitment of leukocytes, which was dependent upon both PAR_2 and β-arrestin-2, may be essential for epithelial thickening.

Although we and others have demonstrated a major role for PAR_2 in the progression of airway inflammation, PAR_2 activation has also been reported to orchestrate protective effects in the airway by promoting prostaglandin E2 (PGE2)-mediated bronchiolar smooth muscle relaxation leading to broncho-dilation (21). We observed a similar indomethacin-sensitive broncho-relaxing action of the AASP, indicating the 'dual' effect of PAR_2 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' (21,40). Thus, chronically, the overall effect of PAR_2 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 PAR_2 on the airway epithelium to enhance bronchorelaxation represents an intrinsic 'protective' action of PAR_2 stimulation (40). These seemingly biphasic effects with

respect to PAR_2 signaling in the airway have led to the current interest in developing both PAR_2-signaling biased agonists and antagonists as putative therapeutic agents for asthma (41). Because our previous studies suggest that the 'protective effects' of PAR_2 activation are independent of β-arrestin signaling we suggest that it will be of therapeutic value for asthma treatment to develop biased PAR_2 antagonists that target the β-arrestin-dependent PAR_2 signaling pathway, but which do not affect the β-arrestin-independent PAR_2 signaling pathways that may be of benefit in the setting of asthma induced by PAR_2-activating household allergens such as *Alternaria alternata*.

Section 2.6

Figures and Tables

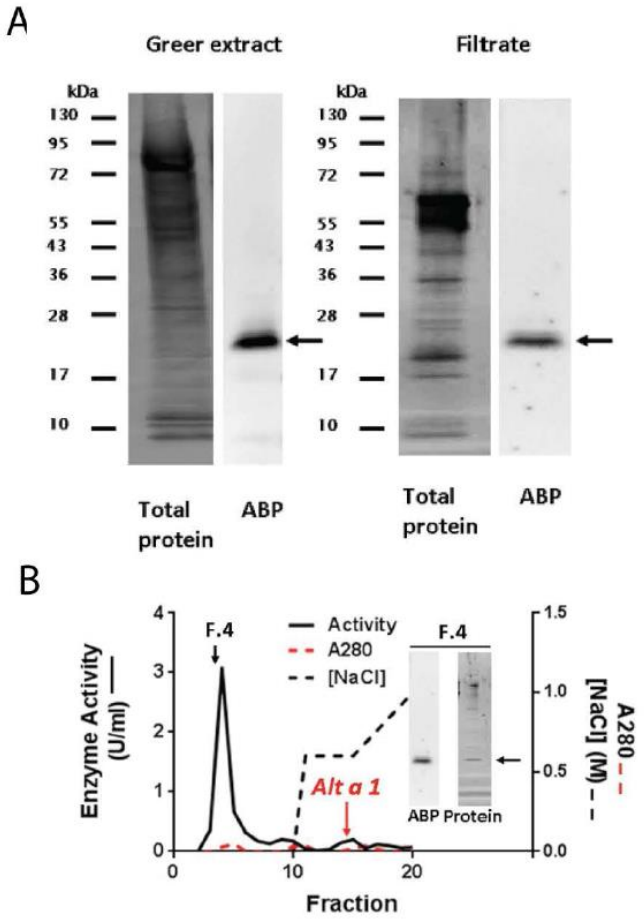

Figure 2.1. Identification of a single PAR_2-activating protease in *Alternaria* filtrates. A Activity based probe (ABP) labeling and protein detection. Either crude extract or culture filtrate from *Alternaria Alternata* were labeled with the biotinylated ABP as outlined in Methods. Proteins were then resolved by gel electrophoresis and either visualized directly in the gel using the SYPRO Ruby protein stain (left) or after western blot transfer and detection of biotinylated enzymes (ABP: arrows) using HRP-coupled avidin (right). **B.** Chromatographic separation of ABP-labeled enzyme from Alt a 1 antigen. Filtrate protein was applied to the High Q anion exchange column and non-adsorbed fractions (fractions 1-10) and those eluted with a two-step NaCl gradient (dashed black line) were collected. Fractions were monitored for enzyme activity (U/ml: left-hand Y-axis, solid black lines) and ultraviolet absorption/protein (A280: red lines, right-hand ordinate). An ELISA assay was used to detect the elution of the Alt A 1 allergen. Protein in fraction 4 was labeled with the ABP and then analyzed by gel electrophoresis followed by detection of the ABP-labeled enzyme (inset: left gel) and protein (inset: right gel) as outlined for panel A (Courtesy of Danny Polley and Dr. Morley Hollenberg, University of Calgary).

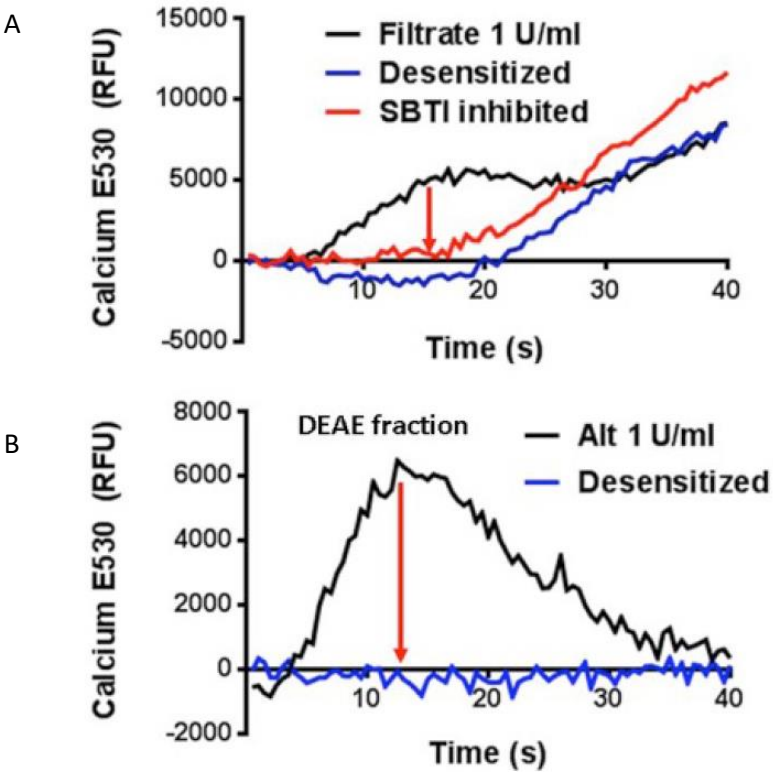

Figure 2.2. Calcium signaling by filtrate and purified enzyme. KNRK cells transfected with PAR_2 were loaded with Fluo-4 and treated with either *Alternaria* filtrate **(A)** or the enzyme containing fraction F4 **(B)** from Figure 1B. The increase in fluorescence emission at 540nm is proportional to the Ca2+ released and is shown as a function of time (Courtesy of Danny Polley and Dr. Morley Hollenberg, University of Alberta, Canada).

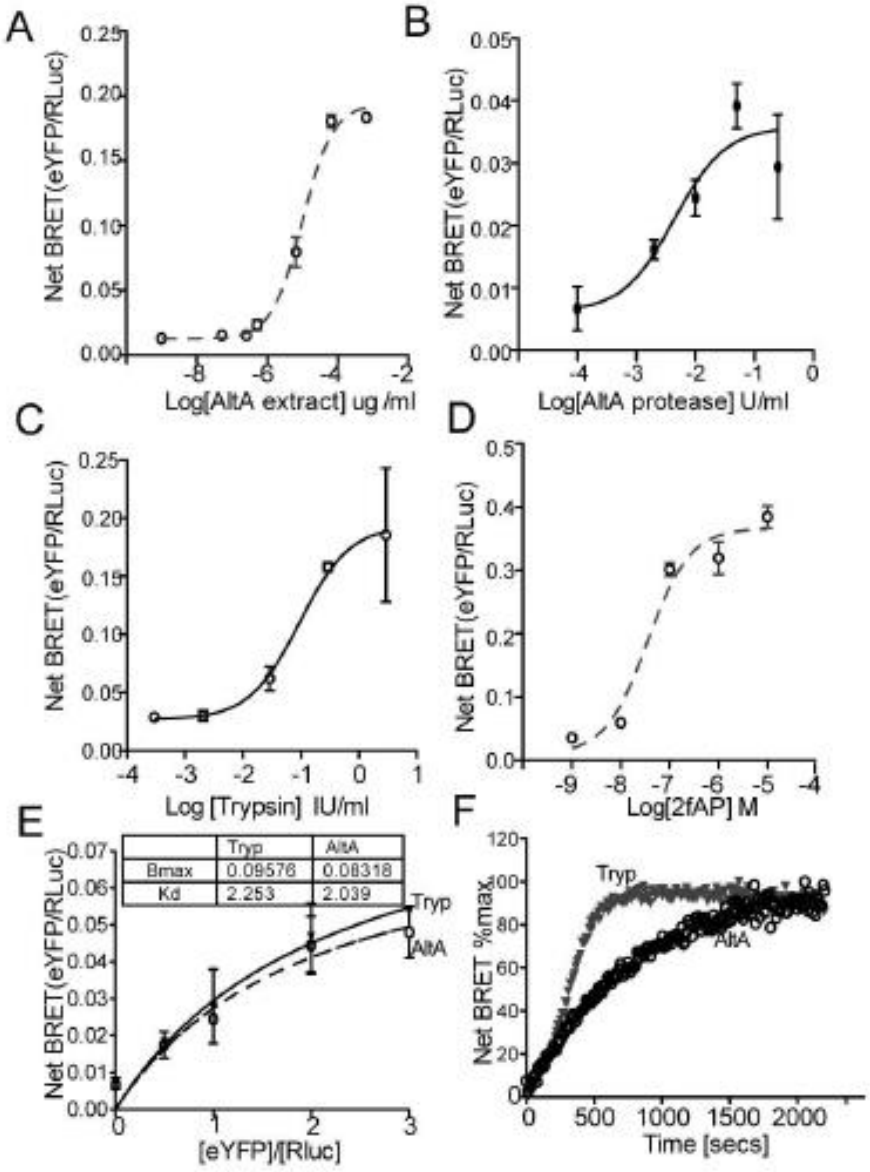
A
Net BRET(eYFP/RLuc)
Log[AltA extract] ug /ml
B
Net BRET(eYFP/RLuc)
Log[AltA protease] U/ml
C
Net BRET(eYFP/RLuc)
Log [Trypsin] IU/ml
D
Net BRET(eYFP/RLuc)
Log[2fAP] M
E
Net BRET(eYFP/RLuc)
[eYFP]/[Rluc]
Tryp
AltA
Bmax 0.09576 0.08318
Kd 2.253 2.039
F
Net BRET %max
Time [secs]
Tryp
AltA

Figure 2.3. Alternaria promotes recruitment of β-arrestins to PAR_2. A-D. Cells were transfected with luciferase-β-arrestin-2 and PAR_2-YFP and Bioluminescence Resonance Energy Transfer (BRET) was measured upon addition of increasing concentrations of Alternaria extract (A) partially purified Alternaria enzyme (B), trypsin (C) or 2fAP (D). Values are all shown as net-BRET (the ratio of emission in the YFP and the luciferase channel (e510/e435) in the presence of PAR_2-YFP and luc-β-arrestin minus the same ratio in cells transfected with luc-arrestin alone). **E.** BRET50 and $BRET_{max}$ values were determined for recruitment of β-arrestin-2 to PAR_2, in response to 65mg/ml Alternaria extractor 2.5u/ml of trypsin, by holding donor concentrations constant (β-arrestin-Luc) and increasing the concentration of acceptor (PAR_2-YFP). Insets show mean values for $BRET_{max}$ (maximal signal observed) and Bret50 (ratio at which ½ maximal Bret signal is observed. **F.** The time to reach maximal BRET signal was determined in response to Alternaria extract or trypsin (Courtesy of Kasturi Pal).

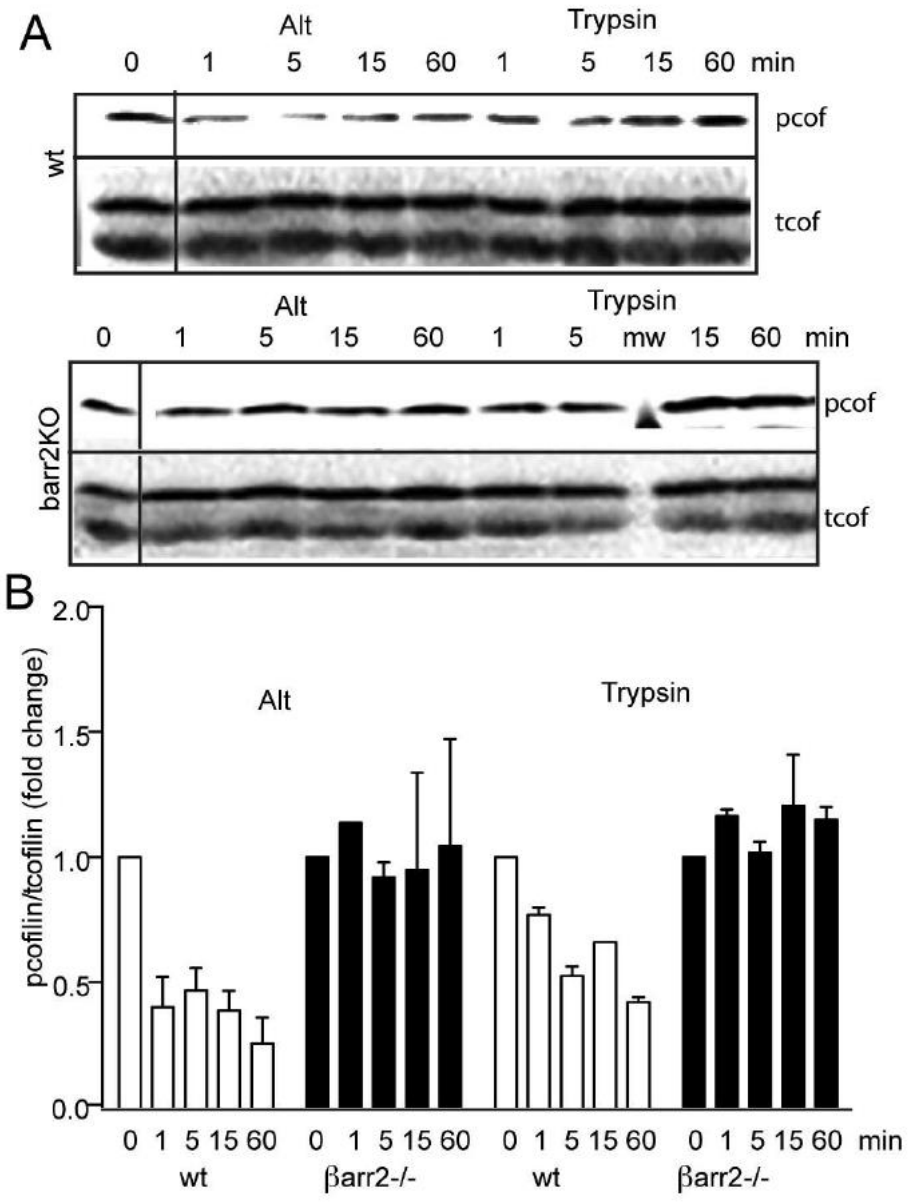

Figure 2.4. ***Alternaria*** **promotes β-arrestin-dependent activation of cofilin.** Cells (~80% lymphocytes) were prepared from spleens of wt and β-arrestin-2$^{-/-}$ mice, allowed to adhere for 12 hours and treated with or without 65μg/ml of *Alternaria* or 2U/ml trypsin. Lysates were analyzed by SDS-PAGE followed by western blotting with anti-p-cofilin and anti-t-cofilin. **A.** Representative western blot. **B.** Quantification of normalized phospho-cofilin levels, expressed as fold change from untreated cells. Values represent mean ± SEM. n=3. *significant difference between saline and Alternaria treated or between bracketed groups (p<.01). (Experiments done concurrently with Kasturi Pal).

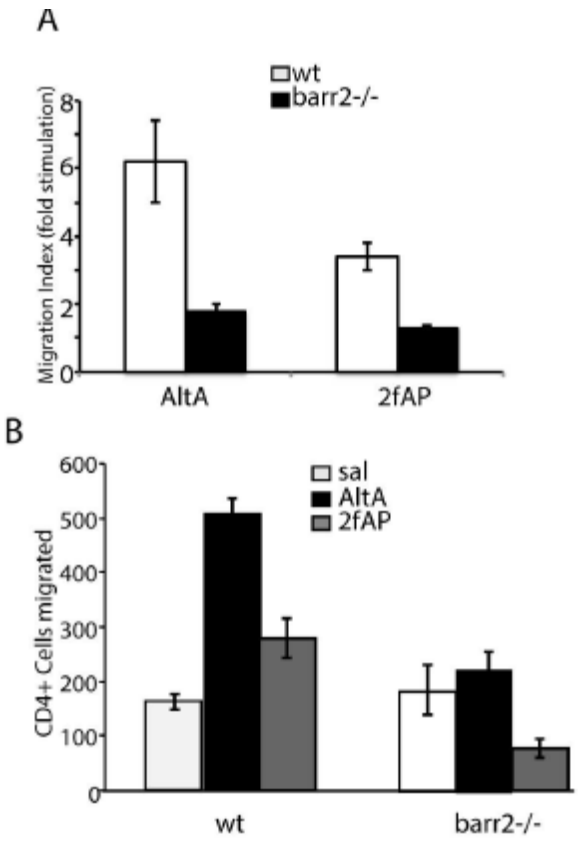

Figure 2.5. Alternaria promotes β-arrestin-dependent lymphocyte migration. Lymphocytes, isolated as described in Fig .4, were seeded onto Transwell filters and either *Alternaria*, PAR_2 agonist 2f-LIGRL-Orn-NH2 or saline was added to the underside. Cells were allowed to migrate for 1hour, after which non-migratory cells were removed from the upper side of the filter and cells on the underside were either stained with Crystal Violet and counted manually (A) or scraped into 0.5ml FACS buffer, incubated with antibody to CD4 (to identify Th2 cells) and analyzed by flow cytometry (cells/ml) (B). Values represent mean ± SEM. n=3. *significant difference between saline and Alternaria treated or between bracketed groups ($p<.01$). (Courtesy of Heddie Nichols).

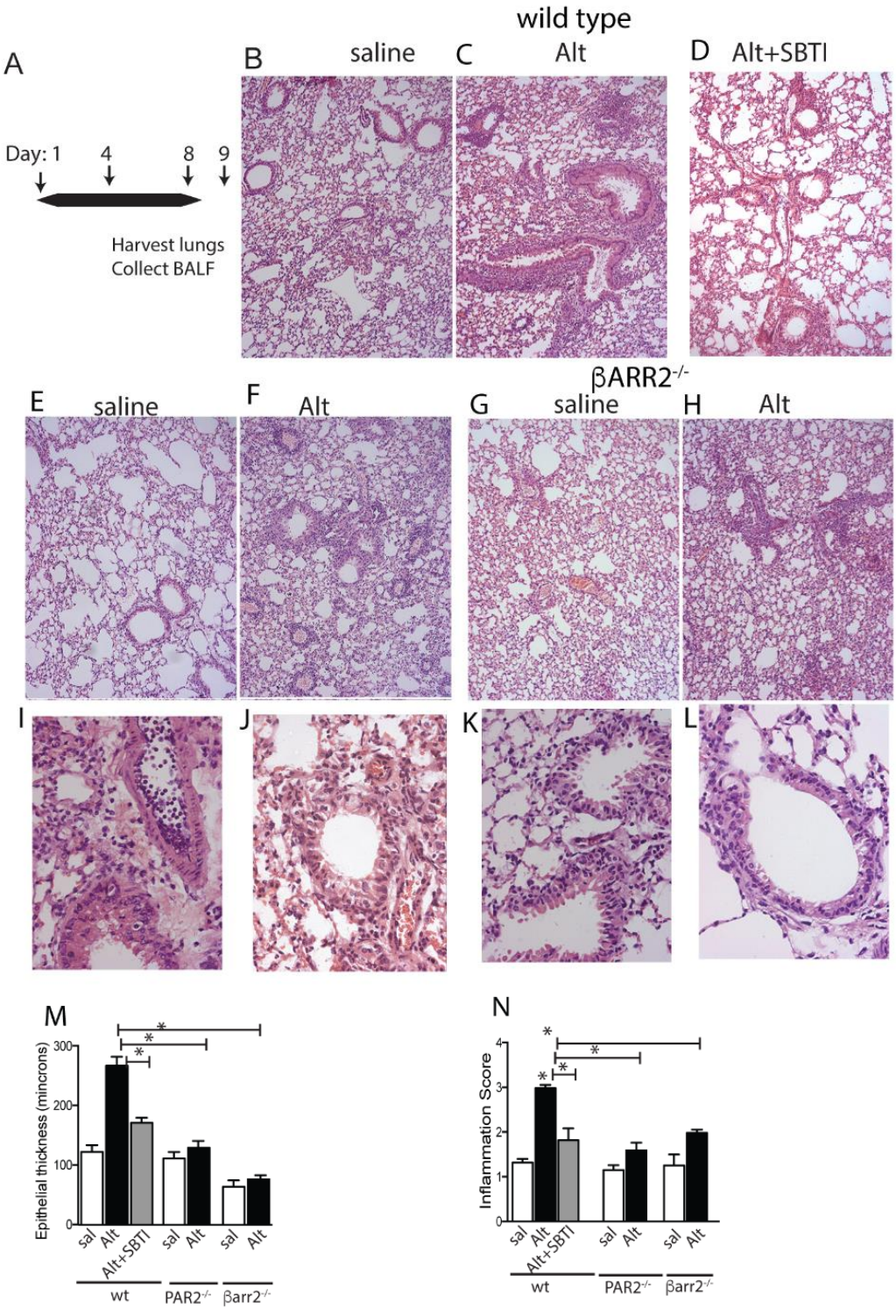
wild type
A
B
saline
C
Alt
D
Alt+SBTI
Day: 1
4
8
9
Harvest lungs
Collect BALF
βARR2-/-
E
saline
F
Alt
G
saline
H
Alt
I
J
K
L
M
Epithelial thickness (mincrons)
300
200
100
0
sal
Alt
Alt+SBTI
sal
Alt
sal
Alt
wt
PAR2-/-
βarr2-/-
N
Inflammation Score
4
3
2
1
0
sal
Alt
Alt+SBTI
sal
Alt
sal
Alt
wt
PAR2-/-
βarr2-/-

Figure 2.6. *Alternaria* induces PAR_2 and β-arrestin-2-dependent inflammation of the airway epithelium. A. Timeline of *Alternaria* exposure. Wild type (wt), PAR_2-/- or β-rrestin-2-/- mice were exposed to either saline solution (negative control) or *Alternaria* filtrates in saline 4 times over a period of 8 days, after which mice were euthanized and lungs and Bronchoalveolar lavage fluid (BALF) were collected for analysis. 4 mice per treatment group, repeated 3 times. **B-L.** Representative images of H and E stained lungs after saline treatment **(B, E, G)**, *Alternaria* treatment **(C, F, H)** or *Alternaria* + SBTI **(D)** of wt **(B-D)**, $PAR_2^{-/-}$ **(E, F)** or β-arrestin-$2^{-/-}$ mice **(G, H)**. Lower magnification (10X) are shown in B-G and higher magnification (40X) of lungs from *Alternaria*-treated mice are shown in I-L. **M, N.** Histological grading of slides. **M.** Basement membrane thickness was determined by measuring the distance from the basolateral surface of the epithelial cells to the smooth muscle layer. **N.** Inflammation score was determined by the density of infiltrating cells in the airway tissue (1=no invading cells, 2=scattered cells, 3=clusters of invading cells throughout, 4=invading cells comprise more than 70% of the field of vision). Significant differences between *Alternaria*- treated and saline controls or between bracketed Alternaria-treated groups are indicated by * (P≤.05) and ** (P≤.005).

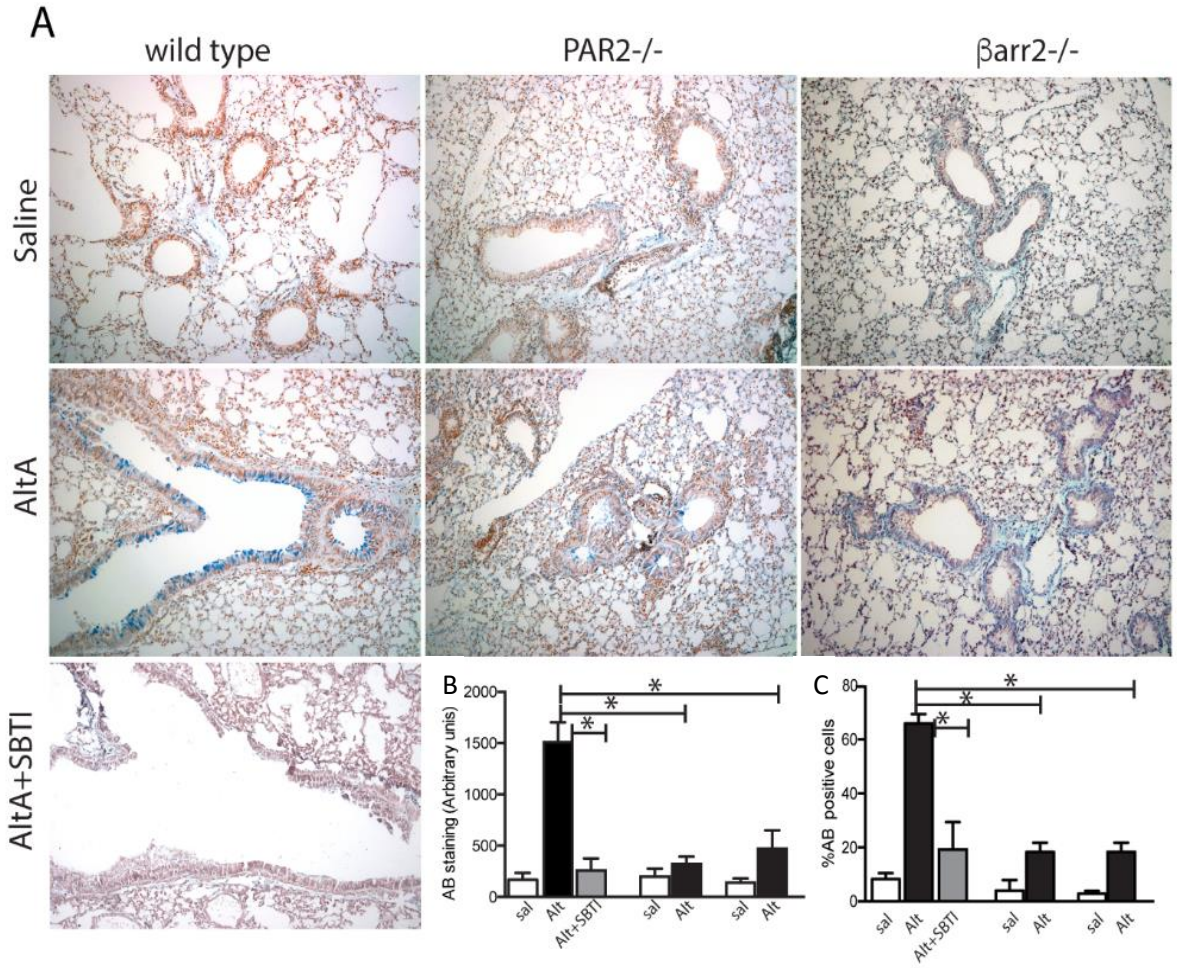

Figure 2.7. Mucus production and Goblet Cell hyperplasia require PAR_2 and β-arrestin-2. A. Representative images of paraffin-embedded lung sections from wt, $PAR_2^{-/-}$ and β-arrestin-$2^{-/-}$ mice, treated as described in Figure 1A, stained with Alcian Blue (AB) to identify acidic mucins. (10X mag). **B.** Quantification of mucin staining, as determined by integrated intensity of AB staining. **C.** Quantification of the percentage of AB positive (goblet) cells per 10mm section of epithelium. Significant differences between Alternaria- treated and saline controls or between bracketed Alternaria-treated groups are indicated by * (P≤.01) and ** (P≤.001).

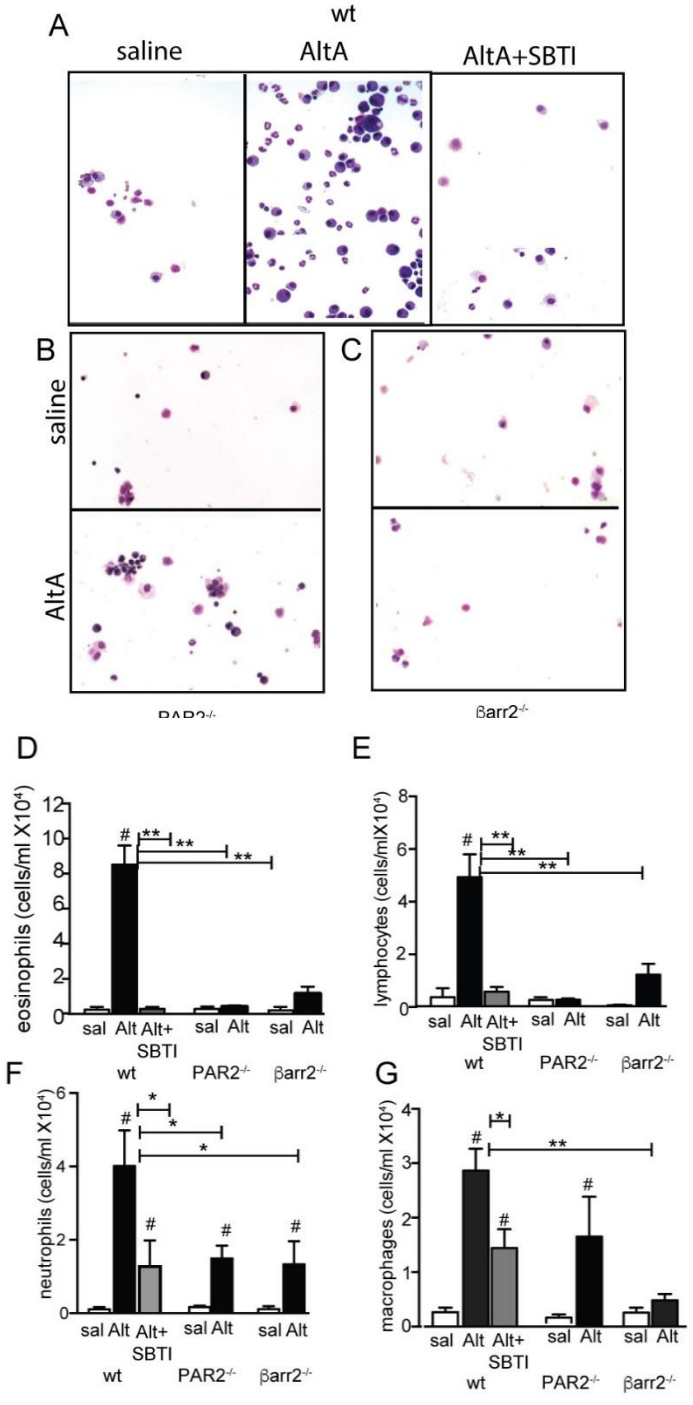
A
wt
saline
AltA
AltA+SBTI
B
C
saline
AltA
βarr2-/-
D
E
eosinophils (cells/ml X10⁴)
lymphocytes (cells/mlX10⁴)
sal Alt Alt+ SBTI sal Alt sal Alt
wt PAR2-/- βarr2-/-
F
G
neutrophils (cells/ml X10⁴)
macrophages (cells/ml X10⁴)

Figure 2.8. *Alternaria* increases the number of leukocytes in the BALF by a PAR_2 and β-arrestin-2-dependent mechanism. A-C. Representative GIEMSA stained cytospins from BALF of wt (A), $PAR_2^{-/-}$ (B) and β-arrestin-$2^{-/-}$ (C) mice. **D-G.** Differential counts of eosinophils (D), neutrophils (E), lymphocytes (F) and macrophages (G) in BALF cytospins. N=8, 4 slides/treatment, 2 fields of vision per slide. #Significant differences between *Alternaria*-treated and saline controls, Siginificant difference between bracketed *Alternaria*-treated groups are indicated by * (P≤.05) and ** (P≤.005).

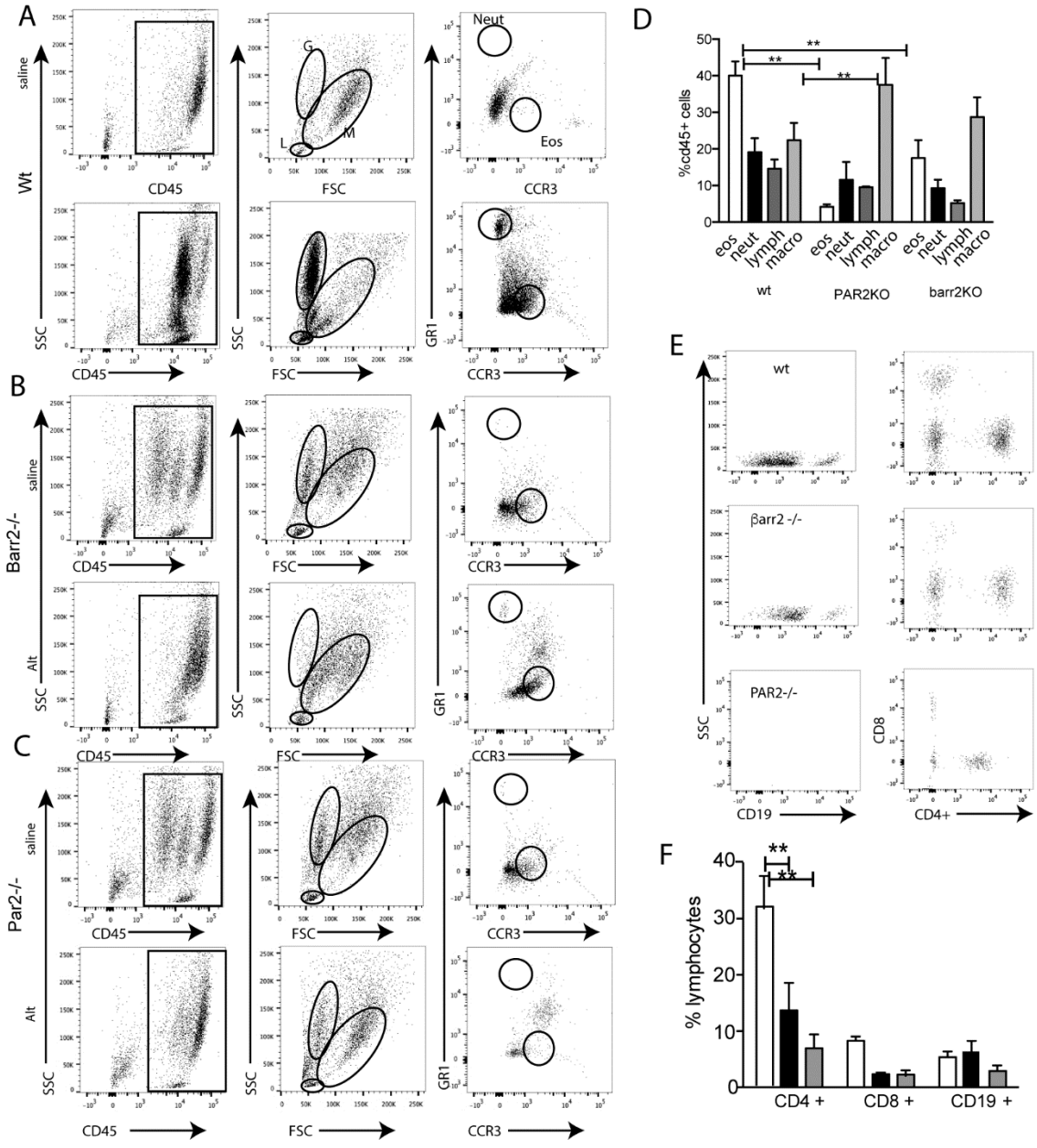
A
saline
Wt
Alt
SSC
CD45
FSC
G
L
M
Neut
Eos
CCR3
GR1
B
Barr2-/-
C
Par2-/-
D
%cd45+ cells
eos
neut
lymph
macro
wt
PAR2KO
barr2KO
**
E
wt
βarr2 -/-
PAR2-/-
CD19
CD4+
CD8
F
% lymphocytes
CD4 +
CD8 +
CD19 +

Figure 2.9. Alternaria treatment markedly increases the percentage of eosinophils and CD4+ lymphocytes in the BALF by a PAR_2/β-arrestin-2-dependent mechanism. A-C. Representative histograms showing the gating strategy for identification of BALF cell types in wt **(A)**, $PAR_2^{-/-}$ **(B)** and b-arrestin-$2^{-/-}$ **(C)** mice. **D.** Percentage of CD45+ cells that are scored as eosinophils (high SSC, med FSC, CCR3+), neutrophils (high SSC, high FSC, GR1+), lymphocytes (low SSC, med FSC, CD3+) or macrophages (high SSC/high FSC) by flow cytometric analysis of BALF. **E.** Percentage of CD45+ cells that are CD4+ (Th2), CD8+(NK) or CD19+ (B-cells). **F.** Flow-cytometric determination of the percentage of lymphocyte populations that were CD4+, CD8+, or CD19+. (Experiments done concurrently with Heddie Nichols)

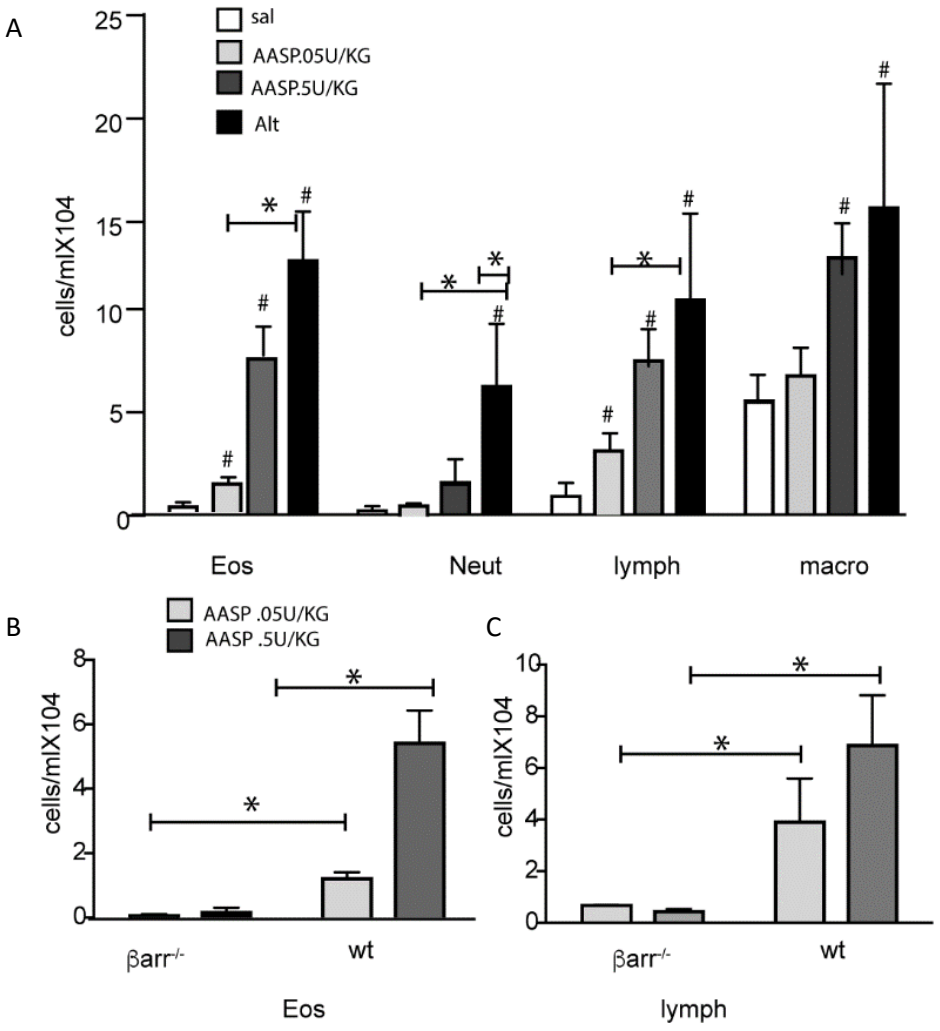

Figure 2.10. AASP promotes β-arrestin-2-dependent recruitment of eosinophils and lymphocytes to the airways. AASP (.05U/kg or .5U/kg) was administered intranasally to wild type and β-arrestin-$2^{-/-}$ mice as described for *Alternaria* extract in Figure 8. Negative controls (saline) and positive controls (*Alternaria*) were also included. BALF was collected and differential counts performed as described in Figure 9. **A.** Quantification of cell types in wild type mice. **B-C.** Comparison of eosinophils and lymphocytes in *Alternaria*-treated wt compared with β-arrestin-$2^{-/-}$ mice.

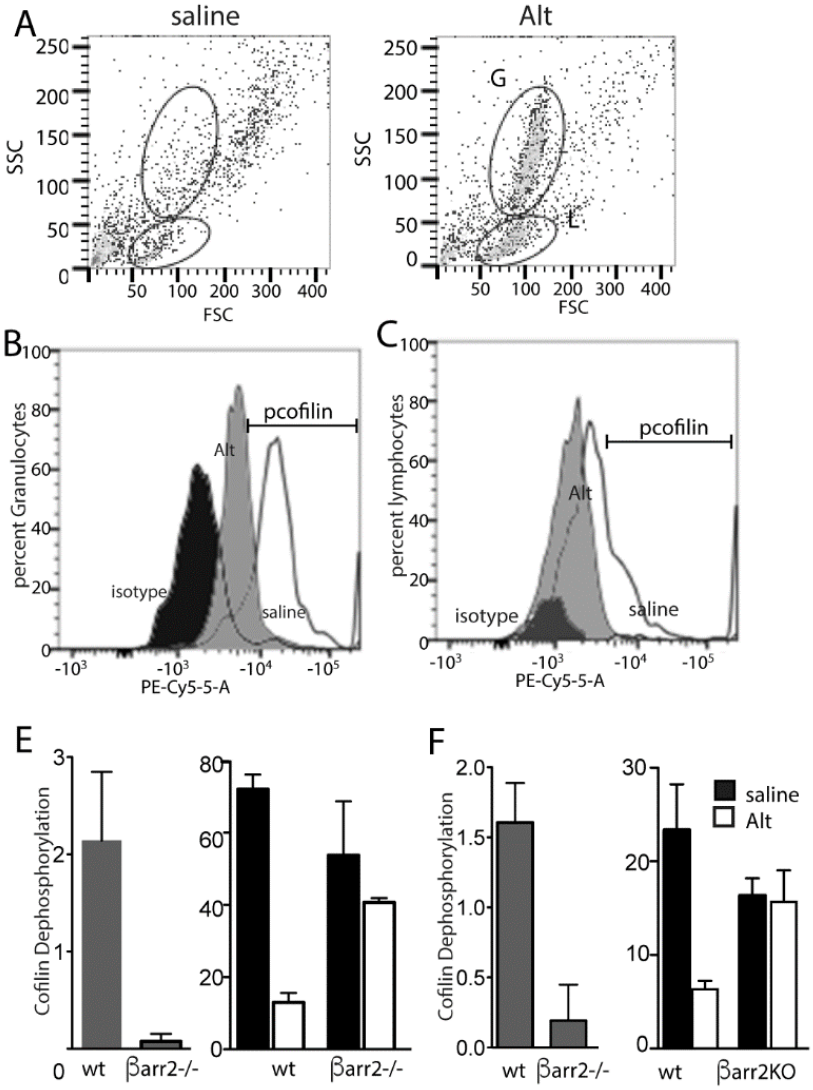

Figure 2.11. Cofilin dephosphorylation in granulocytes and lymphocytes is increased after Alternaria treatment. BALF cells, isolated after treatment of wt and β-arrestin-2$^{-/-}$ as described in Figure 1A, were incubated with antibody to CD45 and phospho-cofilin and analyzed by flow cytometry. **A.** Representative dot plot of CD45+ cells analyzed for forward and side scatter (FSC/SSC). Granulocytes (G) and lymphocyte (L) populations are indicated. **B, C.** Histogram analysis of phospho-cofilin fluorescence in the granulocyte (B) and lymphocyte (C) population after treatment with Alternaria (grey) or saline (white). Isotype saline-treated controls (white) are included to show background fluorescence levels. The positive phospho-cofilin gate was set at fluorescence levels above 5X10^3 and is indicated by the bracket. **D.** Bar graphs showing average mean fluorescence levels (MFL), after subtraction of background fluorescence (isotype control), for saline and Alternaria-treated granulocytes (left) and lymphocytes (right) ±SEM. The percentage of cells falling into the positive phospho-cofilin gate was graphed for granulocytes (**E**) and lymphocytes (**F**).

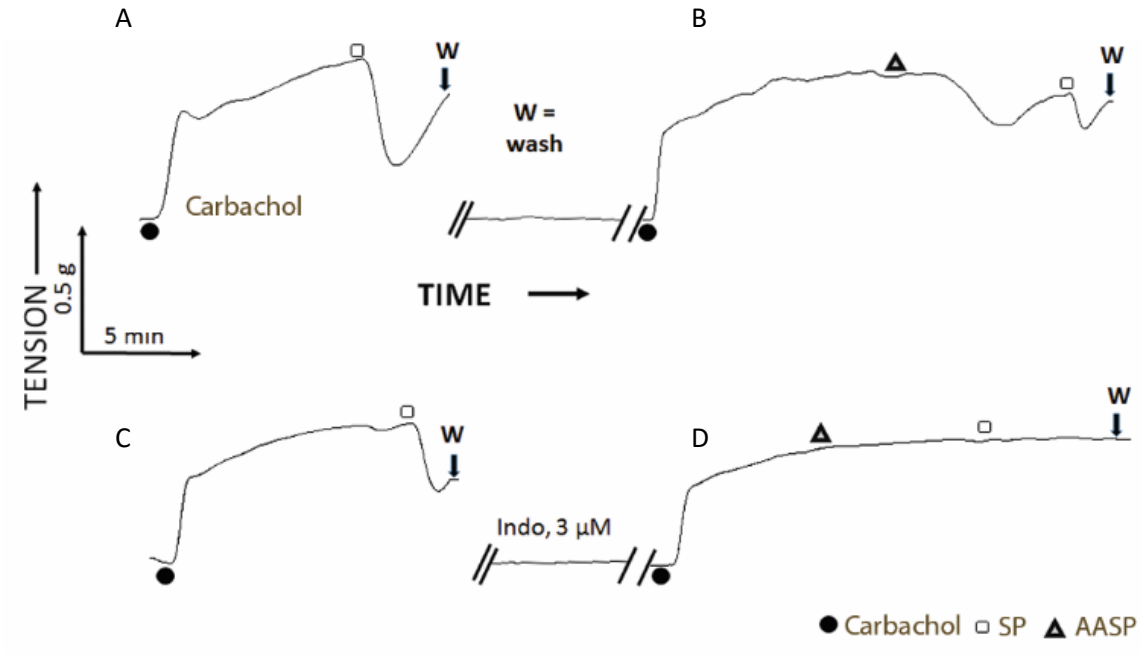

Figure 2.12. *Alternaria* protease-mediated bronchorelaxation. Bronchorelaxation in response to the *Alternaria* protease was measured in second-order mouse bronchiolar ring segments as outlined previously (Nichols et al, 2012). Rings were first contracted with 1 µM carbachol and preparations either untreated **(A, C)** or treated with 3 µM indomethacin **(D)** were then exposed to substance P (SP: 20 nM) to induce a prostanoid-generated indomethacin-blocked epithelium-dependent relaxation. The relaxant response to 0.6 U/ml *Alternaria* enzyme (AASP) activity is shown in tracing **B and D**, in comparison with the relaxation caused in the same preparation by substance P. (Courtesy of Dr. Morley Hollenberg, University of Calgary).

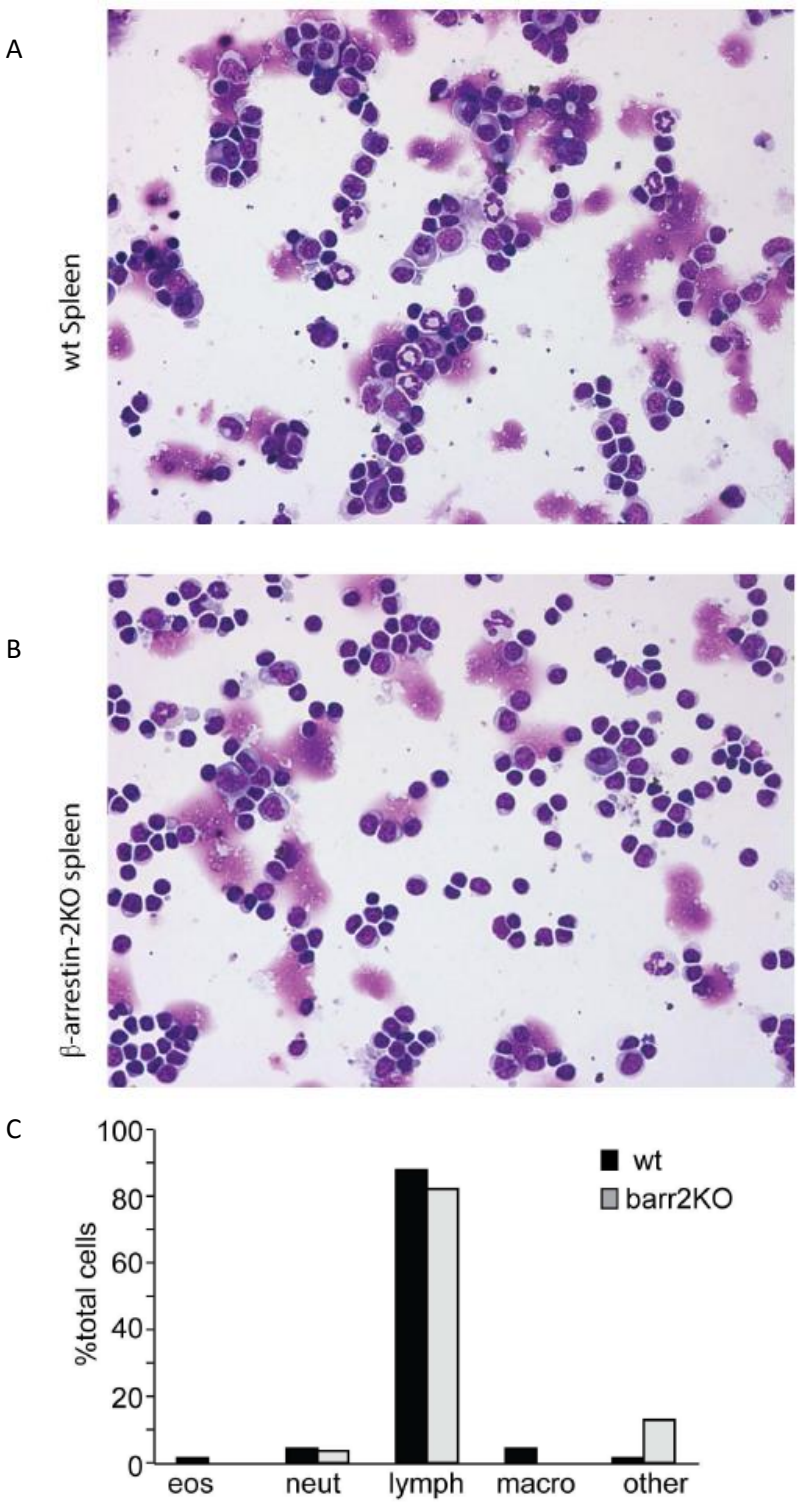

Figure 2.S1. Representative cytospin images of spleen preparations. A-B. Images illustrate no difference in basal numbers of lymphocytes. **C.** Bar graph of lymphocytes, determined from a differential cell count of cytospins.

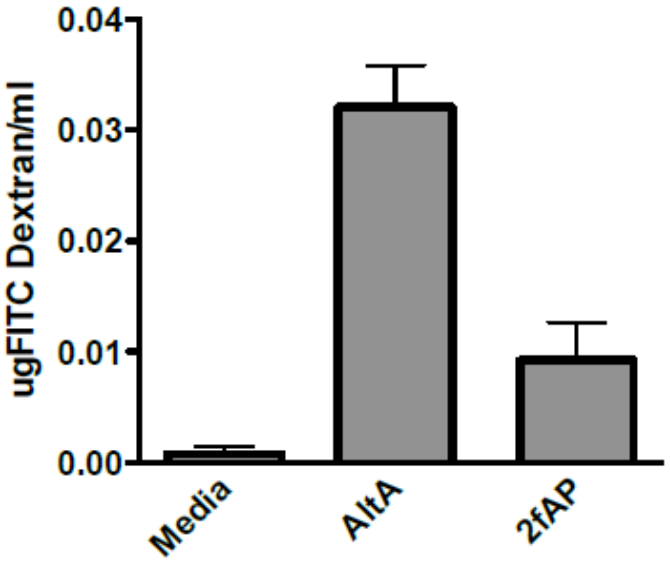

Figure 2.S2. *Alternaria* and 2fAP-mediated PAR_2 activation promote barrier damage in cultured airway epithelial cells. FITC-dextran (4 kDa) was added to the apical side of BEAS-2B cell monolayers, grown on a collagen-coated permeable support. After administration of media alone, 2fAP, the PAR_2 agonist, Alternaria, or cockroach allergens, the presence of FITC-dextran was quantified on the basolateral side by absorbance at 488 nm (Courtesy of Heddie Nichols).

Table 2.1. Biochemical Characteristics of *Alternaria* Serine Protease. Partially purified *Alternaria* protease (Alt) and purified trypsin activities were with 3 substrates: QAR (trypsin specific), FVR (thrombin specific) and GGR (urokinase specific) and Km values were calculated (left panel). Sensitivity to inhibition by SBTI (trypsin specific inhibitor) and TLCK (chymotrypsin specific inhibitor) was determined and Ki values calculated (right panel). Mean ± SEM Km and Ki values are shown, n=3 (Courtesy of Dr. Morley Hollenberg and Danny Polley, University of Calgary)

	Substrate Km (μM)			Inhibitor Ki	
	QAR (Trypsin)	FVR (Thrombin)	GGR (Urokinase)	SBTI (pM)	TLCK (nM)
Alt	16 ± 2	366 ± 57	98 ± 31	18 ± 4	12879 ± 842
Trypsin	5 ± 0.6	24 ± 5	1054 ± 211	4 ± 0.4	2 ± 0.2

Table 2.2. Amino Acid Sequence of *Alternaria* Enzyme

(Courtesy of Dr. Morley Hollenberg, University of Calgary)

gi|3333333 – Alt:

MRFQSIIAIALPALVLAAPTPQDPDYEFPEDAPADDIVGGTTASAGEFPFIVSL
QRSGSHFCGGSLLDSTTVITAAHCSVSSVIGSVSGLRVRAGSLNKSSGGTL
VGVSSVTVHPSYRSSGQDFDVAIWKLSTAVPTSSTIGYATLPASGSDPAAGS
TATVAGWGALTEGGSSPSTLYKVSVPIVSRTECRSSYGTSAITNNMFCAGYT
TGGRDSCQGDSGGPIVNSAKTLIGLVSWGNGCAQPNFPGVYARTAALLSFI
NSV

99% confidence 95% confidence

Table 2.3. Levels of IL-13 and IL-6 in BALF supernatants (in pg/ml) after treatment of wt and β-arrestin-$2^{-/-}$ mice with Alternaria were determined by ELISA. Significant differences were calculated by 1-way ANOVA with TUKEY post-tests. (Courtesy of Heddie Nichols)

	Wt		**β-arrestin-$2^{-/-}$**	
	HBSS	*Alternaria*	HBSS	*Alternaria*
IL-13	2.1±0.01	4.2±0.2*	2.1±.03	3.3±0.2*+
	* p=.01, difference compared to saline;			
	+ p=.05, difference compared to wt			
IL-6	7.3±.27	10.2±0.3*	8.0±0.3	8.9±0.6
	* p=.01, difference compared to saline			

Section 2.7

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Chapter 3:

Protease-activated receptor-2 Antagonist C391 Blocks *Alternaria* Allergen induced β-arrestin signaling and airway inflammation

Section 3.1

Abstract

As discussed in the previous chapters, protease-activated-receptor-2 promotes independent signaling pathways in the lung – one protective activated through G-protein-dependent signaling, and the other being β-arrestin-dependent promotion of pro-inflammatory responses. Recently, our collaborators have developed a number of peptidomimetic PAR_2 agonists, one of which is C391, although a weak agonist, previously shown to antagonize PAR_2 signaling, as determined by Ca2+ mobilization and inhibit protease-induced, mast cell mediated hyperalgesia *in vivo*. However, the IC50 for Ca2+ inhibition in these studies was very high (micromolar range) and whether it preferentially inhibits the β-arrestin pathway was not investigated. As PAR_2 is known to promote both protective and proinflammatory effects and we have shown in our previous studies that the *Alternaria* protease AASP is capable of promoting the protective smooth muscle relaxation effects in isolated bronchioles, an ideal asthma therapeutic might be a β-arrestin biased PAR_2 antagonist. In this chapter, I describe the ability of the PAR_2 antagonist C391 to block PAR_2 signaling in cultured cells. C391 blocks PAR_2 signaling including antagonizing G-protein-dependent signaling, but it affects β-arrestin-2 recruitment at lower concentrations where it does not affect Calcium mobilization, thus suggesting that it is acting as a biased antagonist. Furthermore, I demonstrate that C391 abrogates *Alternaria*-induced inflammatory responses in the

lung including bronchial epithelial thickening, mucus production, and leukocyte recruitment. Thus, my work demonstrates that C391 blocks *Alternaria*-induced inflammation and suggests that this is a good candidate for studies of PAR_2 biased antagonism.

Section 3.2

Introduction

With the discovery of the first biased GPCR agonist, there has been a growing interest in developing drugs that target either the G-protein or β-arrestin signaling arm of these receptors for therapeutics. A number of such compounds have been developed and are in clinical trials or are currently available for treatment of diseases such as MK-6892 for hyperlipidemia, valproate for schizophrenia and PTH(1-34) for osteoporosis (1–3). There has been a wealth of pharmacological characterization of agonist "bias," but the simplest view is that an agonist exhibits bias toward one pathway when receptor binding results in activation of one pathway at a significantly lower concentration than that needed to activate the other. Development of biased antagonists that inhibit one pathway at a concentration where the other pathway is unaffected has not yet been reported for any GPCR. Because of the therapeutic potential of targeting a specific pathway of PAR_2 activation, recent efforts from computational modeling based on a known agonist and antagonist, GB110 and GB88 respectively, have revealed a diverse panel of putative PAR_2-binding agonists and antagonists that may function in a biased fashion (4). In light of studies on PAR_2 described in the previous chapter, the advantages of a biased antagonist are clear: inhibiting the pro-inflammatory β-arrestin pathway

while preserving the protective effects has great potential as a strategy for treating asthma.

Targeting PAR_2 has proven difficult for many reasons, including lack of structural information on the receptor binding pocket and the fact that the endogenous ligand is physically attached to the receptor (the tethered ligand unveiled by proteolytic cleavage). However, several strategies have been utilized with partial success. A number of tethered ligand peptidomimetics, modified to increase affinity and stability or lipidated to increase membrane association, have been developed that can activate G-protein signaling by PAR_2 with an EC50 in the low nanomolar range (5). There have also been a number of PAR_2 antagonists developed, but none have been tested for potential bias toward the β-arrestin signaling pathway and are relatively low potency (6). A novel small molecule PAR_2 antagonist, N^1-3-methylbutyryl-N^4-6-aminohexanoyl-piperazine (ENMD-1068) reduced joint inflammation *in vivo* and reduced PAR_2-mediated Ca^{2+} signaling *in vitro* (7). However, this antagonist was of too low a potency to be used in the clinic. Pepducins, which are membrane permeant antagonists that interact with the cytosolic G-protein binding region of PAR_2 have been developed and one such compound, P2pal-18S pepducin, was shown to have a mild inhibitory effect on trypsin and tryptase-induced neutrophil migration *in vitro*, and decreased paw edema and *in vivo* (8). Pepducins act primarily by interfering with, or enhancing, G-protein/GPCR interactions; however, their effects on β-arrestin recruitment are not well defined. For the beta-2-adrenergic receptor, a Gαs biased pepducin that does not promote β-arrestin

recruitment has been proposed as an alternative to existing beta-agonists for the treatment of some diseases. Both the piperazine and pepducin antagonists target a more highly conserved portion of the receptor, making specificity more difficult to attain. For this reason, PAR_2 peptidomimetics have an advantage over small molecules both as agonists and antagonists of PAR_2.

Recent work by our collaborators has identified a number of high affinity, stable PAR_2 agonists and, pertinent to my work, a PAR_2 antagonist, C391. C391 is a modification of the agonist used in earlier studies by our laboratory, 2-f-LIGRLO-NH2 (2fAP) in which the L-I-G residues were substituted with an azabicycloalkane scaffold and the Ornithine was substituted with a Tyrosine. This compound acts as a weak agonist of PAR_2-stimulated Ca^{2+} mobilization at very high concentrations (~20 μM) and antagonized 2fAP- and trypsin-induced Ca2+ signaling and MAPK activation at 1-10 μM. Using a model of 48/80-induced thermal hyperalgesia (which is thought to be mediated by mast cell tryptase), injection of 25-75 μg of C391 inhibited the withdrawal response, suggesting this may be effective in treating pain.

The ability of C391 to antagonize PAR_2-induced pain *in vivo* despite the very high concentrations needed to antagonize G-protein signaling *in vitro* suggested that it may be preferentially acting through a β-arrestin-dependent pathway. A limiting factor in the characterization of many of these PAR antagonists is that no studies have investigated the β-arrestin signaling pathway. To this end, I examined whether C391 blocks Alternaria-induced inflammation, which we have shown to be mediated through

PAR_2/β-arrestin signaling, and whether it can inhibit β-arrestin recruitment at a lower concentration than it inhibits Ca2+ mobilization *in vitro*.

Section 3.3

Methods and Materials

C391: Compound C391 is the same used as previously described (9). The product was purified by HPLC with a reverse-phase column and confirmed by NMR and high-resolution mass spectrometry. C391 is a ligand designed to target PAR_2 and derived from the strong PAR_2 activating peptide, 2f-AP (Fig. 1A,B). C391 contains moieties with specific functions including a 2-furoyl group that protects it from endogenous proteases, azabicycloalkane that provides a scaffold with an isobutyrl side chain, a lysline-like side chain, and C-terminal Leu and Tyr.

Mouse inflammation models and measurement of airway inflammation: C57BL/6 mice that were 2 – 3 months old with an average weight of 20 grams were used in this experiment. Treatments were administered as done following the same schedule published previously (10). Briefly, *Alternaria* was administered intranasally in 25 ul per mouse at 0.1 ug / ul at Day 1, Day 4, and Day 8 with or without indicated amounts of C391 with surgical procedures and tissue collection done on Day 9. Mice were anesthetized and euthanized with ketamine:xylazine according to the approved IACUC protocol. Bronchoalveolar lavage fluid (BALF) collection and differential cell counts:

Mice were euthanized, and tracheotomy was performed. Polyethylene tubing with I.D. of 0.86 mm was connected to small 3-way stopcock by way of a 20G needle. After the tubing was inserted into the opening in the trachea, 1x PBS was pumped into the lung and collected with syringes attached to the other ends of the stopcock. Cells were collected, and cytospins were prepared using the Shandon Cytospin 3. The Hema 3 stain kit was used to obtain staining comparable to that of the Wright-Giemsa stain.

Hematoxylin and Eosin stain (H&E): The standard protocol for Hematoxylin and Eosin staining was used. Briefly, rehydrated lung tissue sections were stained in Hematoxylin for 8 mins, rinsed with tap water, counterstained with Eosin Y for 2 – 3 mins, and dehydrated for mounting.

Alcian blue stain: Alcian blue pH 2.5 staining along with counterstaining with Safranin O from Scytek Laboratories were done according to the manufacturer's protocol. Briefly, rehydrated lung tissue sections were first dipped in 3% acetic acid for 3 mins, then Alcian blue stained for 30 mins, rinsed with water, counterstained with Safranin O for 5 mins, and dehydrated for mounting.

Calcium signaling assay: The 16HBE14o- cell line used is an immortalized human bronchial epithelial cell line. This protocol was done as previously described (9). Briefly, the cells were loaded with 5 µM of Fura 2-AM for 45 mins. Fluorescence was recorded on the Olympus 1X70 microscope. Intracellular calcium concentrations, [Ca2+]i, was calculated by ratiometric analysis (11). All cells were traced within the field of view, and measurements were taken at least every second for all experiments. [Ca2+]i over time is

depicted in a line drawing as a mean [Ca2+]i of all cells within the field of view. Changes in Ca2+ response are graphed as a percentage of cells that increased [Ca2+]i over 150 nM. Changes in [Ca2+]i are shown as color maps over time in response to *A. alternata* filtrate. Cells were treated with *Alternaria* or first exposed to C391 and then a combination of *Alternaria* and C391. With C391, there was a preincubation period of 2 mins with continual treatment with C391 during *Alternaria* exposure.

Bioluminescence Resonance Energy Transfer (BRET): BRET analysis was done as previously described with some modifications (12). Briefly, HEK293 cells were seeded on 96-well plates coated with 0.01% poly-l-lysine with Trans-It X2 transfection reagent (Mirus). YFP-tagged PAR_2 and β-arr-2-R-luciferase were co-expressed in HEK293 cells, single transfection with YFP-PAR_2 alone was done for positive control, or βarr2-R-Luc alone was expressed for negative control to determine background readings. 24 hours post-transfection, cells were treated with appropriate concentrations of either 2fAP agonist, *Alternaria*, or *Alternaria* with antagonist C391 along with h-coelenterazine substrate. For cells treated with C391, cells were also preincubated with the peptide for 5 minutes prior to addition of *Alternaria*. BRET signals were calculated as the ratio of emission of eYFP compared to that of RLuc readings obtained with the TRISTAR LB941 plate reader (Berthold Technologies) with detection of light at 460 – 500 nm for RLuc and 510 – 550 nm for YFP. For dose curves, readings were taken 15 mins after treatment. For kinetics measurements, readings were taken immediately after agonist and h-coelenterazine addition and monitored for 20 mins.

Transwell Migration Assays: 24-well plates with transwell filters containing 5 um pores (Corning Costar #3412) were used. Spleen lymphocytes were collected from wild-type C57BL/6 mice and seeded on top of the transwell filters. Seeded cells were either unchallenged or preincubated with C391 for at least 5 minutes prior to challenge. Challenge media containing either RPMI only, *Alternaria* (at a final concentration of 0.1 mg / ml), or *Alternaria* with C391 was placed in the well on the basolateral side of the filters. Cells were incubated with challenge media for 90 minutes. Cells that had passed through the filter were collected from the basolateral side and counted manually.

Section 3.4

Results

C391 exhibits biased inhibition of β-arrestin-dependent signaling over inhibition of calcium signaling. In Chapter 2, I showed that *Alternaria* and AASP induced PAR_2-dependent calcium mobilization in cultured HEK293 cells, and other studies have shown *Alternaria* induces Ca2+ mobilization in human bronchoepithelial cells (10). While the IC50 for Ca2+ inhibition was in the micromolar range, suggesting it is not a potent inhibitor of G-protein signaling, injection of C391 into the mouse hindpaw inhibited thermal hyperalgesia. Since our previous studies have demonstrated that inflammatory effects of PAR_2, such as leukocyte infiltration, are dependent upon β-arrestin signaling and not G-protein signaling, we hypothesized that C391 might inhibit β-arrestin signaling

pathways preferentially over the G-protein pathway, thus explaining the positive effects *in vivo* in the face of what appears to be a non-physiological IC50. We thus compared the ability of C391 to inhibit Ca2+ mobilization and β-arrestin recruitment to PAR_2 in cultured cells. To monitor β-arrestin recruitment, cells were transfected with Luc-β-arrestin-2 and PAR_2-YFP and treated with 100 nM 2fAP to activate PAR_2 in the presence of increasing concentrations of C391 (0-10 μM). BRET was measured as the ratio of eYFP (530) to eLuciferase (488) and background values (eYFP/eLUC) in the presence of Luciferase-β-arrestin-2 alone were subtracted to give net BRET. In the absence of 2fAP, no increase in net BRET is observed, as has been reported in our previous studies and is shown in Chapter 2. 2fAP-stimulated net BRET values were decreased in a dose dependent fashion by addition of C391, with an IC50 of 7.58 nM (Fig. 2). Thus, C391 antagonizes β-arrestin signaling induced by the peptidomimetic compound C391 with an IC50 in the nanomolar range.

I next examined whether C391 also antagonizes β-arrestin signaling by *Alternaria*. Cells, transfected with Luc-β-arrestin-2 and PAR_2-YFP, were treated with 600 μg/mg *Alternaria* extract (as described in Chapter 2) in the presence of increasing doses of C391. Once again, C391 inhibited β-arrestin-dependent PAR_2 signaling in a dose-dependent fashion with an IC50 of 12.5 nM (Figure 3A). To analyze the antagonist ability of C391 on *Alternaria*-induced G-protein signaling, we used the calcium mobilization assay. Cells were treated with *Alternaria* only or *Alternaria* with 3 μM C391. Whereas *Alternaria* stimulated increased calcium signaling over time (Fig. 3B-E), incubation with 3

μM C391 effectively inhibited calcium signaling (Fig. 3F-I). No inhibition of Ca2+ mobilization was observed below 3 μM. Thus, C391 blocks *Alternaria*-induced βarr2 signaling at a lower concentration where it does not affect calcium signaling.

C391 inhibits Alternaria-induced cellular inflammation, mucus production, and goblet cell hyperplasia in the lung. A major hallmark of asthmatic inflammation can be observed in airway histological changes. Marked accumulation of leukocytes around the vasculature and infiltrating the airway epithelium as well as thickening of the epithelial basement membrane are associated with the asthmatic lung. Accompanying these changes, mucus producing goblet cells increase in number and the amount of mucus lining the apical surface of the epithelial cells increases, eventually forming a mucus plug that can impede airflow. To examine these features, C57BL/6 mice were treated with the same schedule as that used in a previously published study (13), with saline or *Alternaria* administered intranasally on days 1, 5, and 8 after which lungs were lavaged, then used for histology. In the current study, the addition of C391 was simultaneously administered with *Alternaria* filtrates. Histological analysis of H&E stained airway sections reveals marked increase in Alternaria-induced epithelial thickening and inflammation (Fig. 4A, B) that was reduced by C391 (Fig. 4C, D). Quantification of epithelial thickening and inflammation score, as determined by rings of leukocytes around the blood vessels, indicated 2-fold increases induced by Alternaria that were reduced by 2.5 nmol C391, whereas 0.25 nmol C391 reduced inflammation score but not epithelial thickening (Fig. 4E, F).

To examine mucus production and goblet cell hyperplasia, we stained lung tissue sections from mice treated as in Figure 2B, with Alcian blue dye, which binds acidic mucins (Fig. 5A-D). *Alternaria* increased the total amount of mucin approximately 7-fold, as evidenced by the total intensity of Alcian blue and the number of Alcian blue-positive goblet cells per mm within the bronchioles by approximately 4-fold, both of which are diminished by C391 (Fig. 5E, F). Together, these data demonstrate that the cellular inflammation and production of mucus plugs associated with *Alternaria*-induced asthma can be ameliorated with intranasal administration of the PAR_2 antagonist, C391.

Allergic asthma is associated with infiltration of leukocytes, particularly eosinophils and CD4+ lymphocytes to the lungs, which can be recovered in the bronchoalveolar lavage fluid (BALF). With *Alternaria*-induced asthma, we observe marked eosinophil, lymphocyte, macrophage, as well as neutrophil recruitment (Fig. A-B, E). Recruitment of eosinophils, lymphocytes, and macrophages were reduced with administration of 0.25 nmoles and 2.5 nmoles of C391; however, neutrophil recruitment was reduced with 2.5 nmoles of C391, but not 0.25 nmoles of C391 (Fig. 6C-E).

Since β-arrestin-dependent PAR_2 signaling results in cofilin activation, actin reorganization leading to cell migration, a crucial step in airway inflammation, we examined the ability of C391 to inhibit *Alternaria*-induced lymphocyte migration, using a transwell filter assay. Naive lymphocytes were seeded on the top of transwell filters containing 5 µm pores and treated with either 2fAP or *Alternaria* in the absence or presence of C391, with a preincubation step and addition to the lower chamber. C391

alone did not increase in cell migration (data not shown). However, *Alternaria* increased cell migration by approximately 4-fold, and this was inhbited by C391 (Fig. 7).

Section 3.5

Discussion

While I previously demonstrated in Chapter 2 that a single *Alternaria alternata* serine protease induced inflammatory responses by activating PAR_2 signaling via β-arrestin-2, here in this chapter, I demonstrate that C391 was able to reduce *Alternaria*-induced inflammatory responses including bronchoepithelial thickening, goblet cell hyperplasia, and leukocyte infiltration. Recruitment of eosinophils, lymphocytes, and macrophages was reduced with both 2.5 nmoles and 0.25 nmoles of C391, but neutrophil recruitment was reduced with 2.5 nmoles of C391 but not 0.25 nmoles of C391. C391 was also able to inhibit *Alternaria*-induced migration of spleen cells, which was previously shown to be β-arrestin-dependent. These results are consistent with our previous findings regarding the role for PAR_2-β-arrestin-2 signaling in regulating pro-inflammatory responses, which demonstrated that neutrophil recruitment was less dependent on the protease component of *Alternaria* and less dependent upon the PAR_2-β-arrestin signaing axis. If, as our data suggest, C391 is a β-arrestin biased agonist, it may preferentially inhibit the T_H2 component of the inflammatory response and have a lesser effect on neutrophilia. Together, these data also suggest the involvement of a

separate PAR_2-independent mechanism involved in neutrophil infiltration. Preliminary data from our collaborators examining the effects of C391 on isolated bronchioles suggests that doses up to 50 nM C391 do not inhibit 2fAP-mediated smooth muscle relaxation. Thus, C391 may preserve the protective effects of PAR_2 *in vivo*, something that needs to be addressed in future studies.

Because C391 has previously been shown to outcompete other PAR_2 agonists *in vitro* and blocked hyperalgesia when injected into mice hindpaws *in vivo*, C391 was proposed to work as an orthosteric antagonist directly blocking the proteolytically exposed tethered ligand. However, the concentration of C391 that blocks 2fAP-induced β-arrestin recruitment is lower than that previously shown to compete off 2fAP in a PAR_2 competitive binding assay. C391 does not prevent trypsin cleavage of PAR_2, but it can inhibit trypsin induced Ca2+ mobilization. The biased effect observed at sub-micromolar concentrations of C391 may reflect an allosteric effect on PAR_2 activation. While further characterization is needed to fully understand the mechanism by which C391 exhibits biased antagonism, these results suggest that it may be an effective method of reducing *Alternaria*-induced inflammatory responses *in vivo*.

Section 3.6

Figures

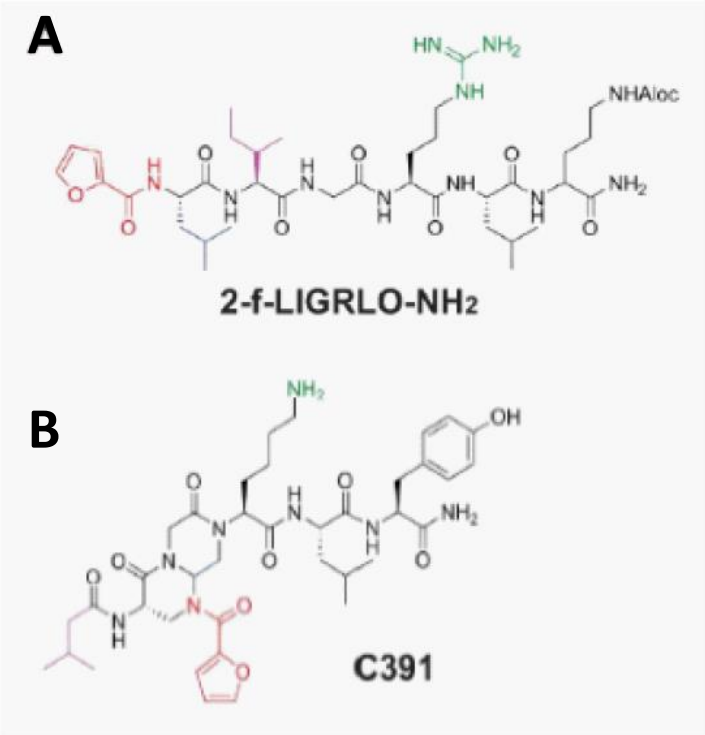

Figure 3.1. Structure of C391 derived from 2fAP. A. Structure of PAR_2 activating peptidomimetic ligand 2-furoyl-LIGRLO-NH2 as previously described (9). **B.** Structure of C391 antagonist is shown here as previously described (9).

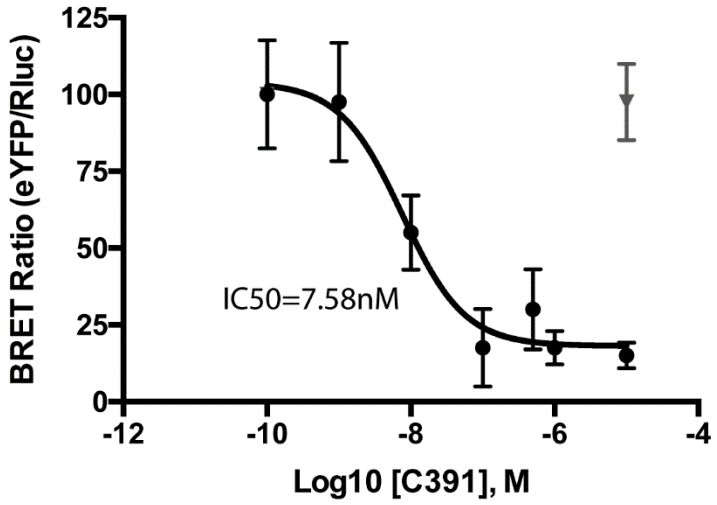

Figure 3.2. β-arrestin biased antagonism of 2-furoyl-LIGRL induced PAR_2 signaling by C391. Cells were transfected with R-luciferase-β-arrestin-2 and PAR_2-YFP, and bioluminescence resonance energy transfer (BRET) assay was performed upon the addition of *Alternaria* with C391. $BRET_{max}$ values (max signal observed) were determined for recruitment of βarr2 to PAR_2, and the effect of C391 was measured as a percentage of $BRET_{max}$. Positive control was shown for trypsin (triangle). Data points are plotted as ± SEM and shown with a non-linear regression curve. Data points are plotted as ± SEM and shown with a non-linear regression curve. The IC50 value was calculated using Graphpad Prism software (Courtesy of Monica Ming).

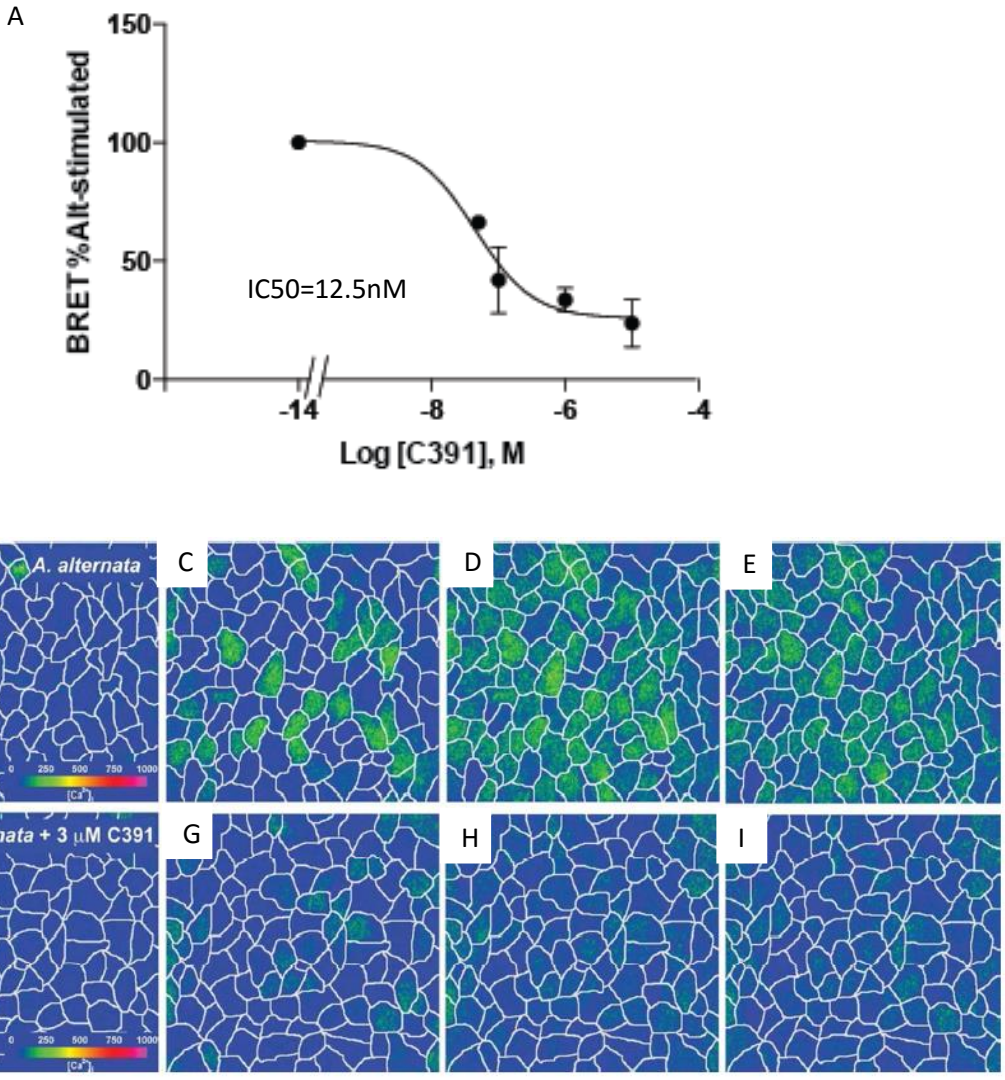
A
150
100
50
0
BRET %Alt-stimulated
IC50=12.5nM
-14
-8
-6
-4
Log [C391], M
B
A. alternata
C
D
E
F
A. alternata + 3 μM C391
G
H
I

Figure 3.3. β-arrestin biased antagonism of *Alternaria*-induced PAR_2 signaling by C391. A. Cells were transfected with R-luciferase-β-arrestin-2 and PAR_2-YFP, and bioluminescence resonance energy transfer (BRET) assay was performed upon the addition of *Alternaria* with C391. $BRET_{max}$ values (max signal observed) were determined for recruitment of Barr2 to PAR_2, and the effect of C391 was measured as a percentage of $BRET_{max}$. **B - E**. No inhibition of Ca2+ mobilization was observed below 3uM. Changes in [Ca2+]i are shown as color maps over time in response to *Alternaria*. Individual panels are from 20 sec prior to *Alternaria* addition and at 60 sec intervals. White lines approximate cell borders, and color key for [Ca2+]i is displayed in lower right of first panel. **F - I**. The same experiment as (**B - E**) excepting a 2 min pre-incubation with 3 µM C391 and continued 3 µM C391 treatment during *Alternaria* exposure. C391 blocks *Alternaria*-induced Ca2+ changes in 16HBE14o- cells. (Courtesy of Monica Ming and Scott Boitano)

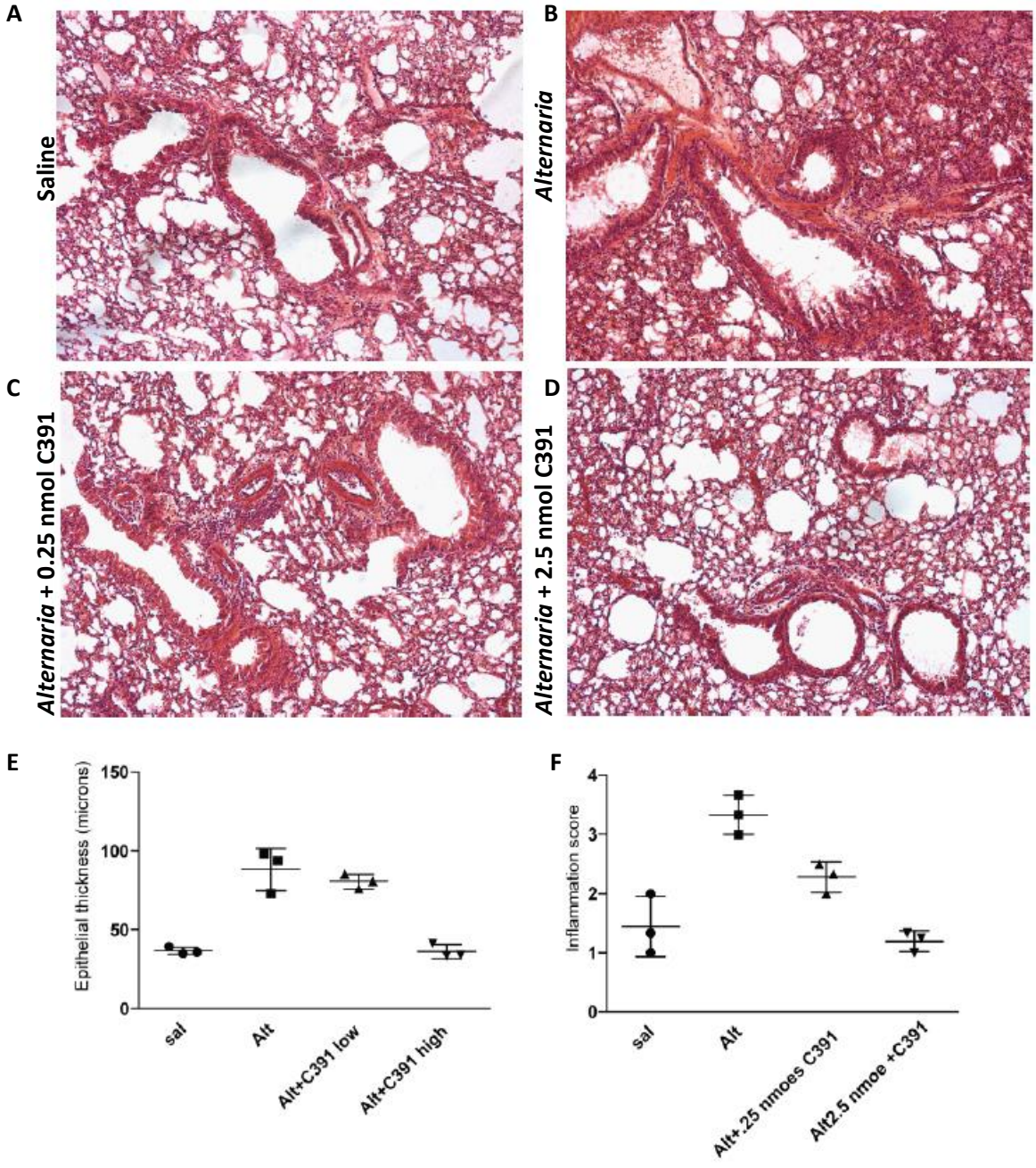
A
Saline
B
Alternaria
C
Alternaria + 0.25 nmol C391
D
Alternaria + 2.5 nmol C391
E
Epithelial thickness (microns)
150
100
50
0
sal
Alt
Alt+C391 low
Alt+C391 high
F
Inflammation score
4
3
2
1
0
sal
Alt
Alt+.25 nmoes C391
Alt2.5 nmoe +C391

Figure 3.4. C391 limits *Alternaria*-induced bronchiolar thickness. Lung tissue from mice of either Saline-treated (**A**), *Alternaria*-treated (**B**), *Alternaria* treatment with 0.25 nmol C391 (**C**), or *Alternaria* treatment with 2.5 nmol C391 (**D**) were stained with H&E. Histological grading of slides. **E.** Epithelial thickness was determined by measuring the distance from the basolateral surface of the epithelial cells to the smooth muscle layer. **F.** Inflammation score was determined by the density of infiltrating cells in the airway tissue (1=no invading cells, 2=scattered cells, 3=clusters of invading cells throughout, 4=invading cells comprise more than 70% of the field of vision). Significant differences between *Alternaria*- treated and saline controls or C391 and *Alternaria*-treated groups are indicated by * ($P \leq .05$) and ** ($P \leq .005$).

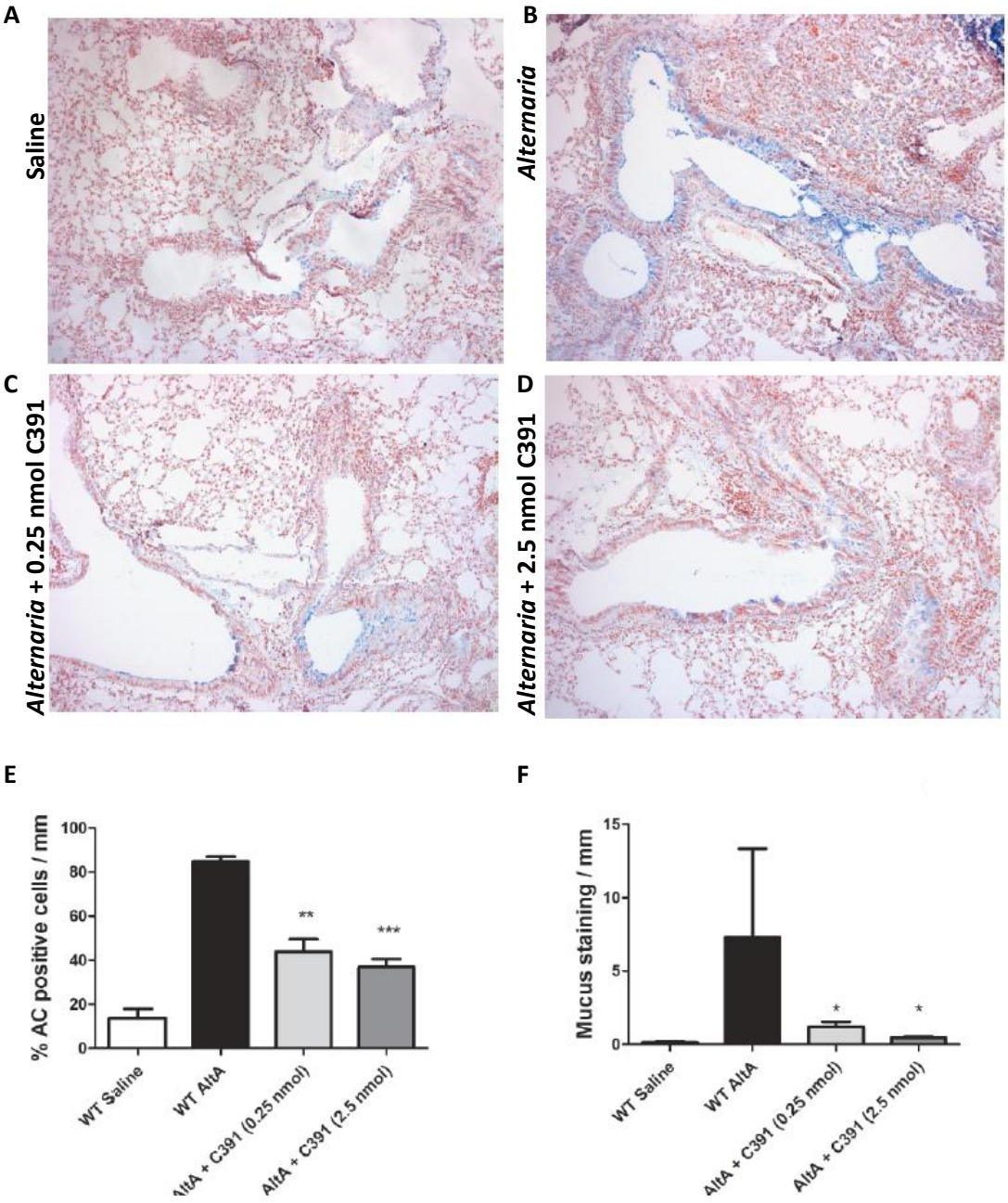

Figure 3.5. C391 inhibits goblet cell hyperplasia in the lung. A. Lung tissue from mice of either Saline-treated (**A**), *Alternaria*-treated (**B**), *Alternaria* treatment with 0.25 nmol C391 (**C**), or *Alternaria* treatment with 2.5 nmol C391 (**D**) were stained with Alcian blue. The number of Alcian blue-positive goblet cells (**E**) and amount of mucin per mm of bronchiole (**F**) were counted. Significant differences between *Alternaria*-treated and saline controls or C391 and Alternaria-treated groups are indicated by * (P≤.05), ** (P≤.005), *** (P≤.001).

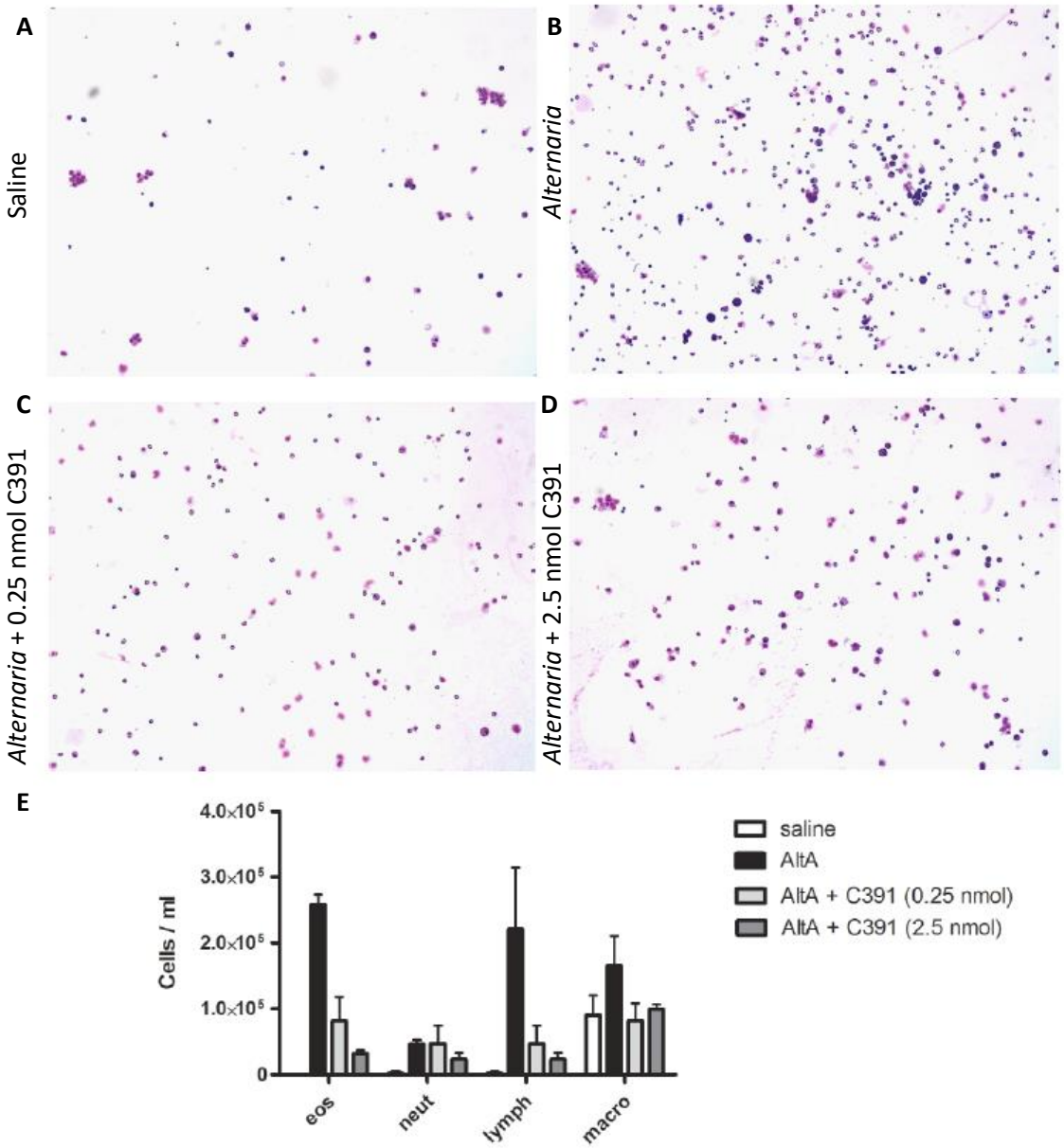

Figure 3.6. C391 inhibits the *Alternaria*-induced infiltration of eosinophils and macrophages in the lung. A. Bronchoalveolar lavage fluid was collected from mice that were either Saline-treated (**A**), *Alternaria*-treated (**B**), *Alternaria* with 0.25 nmol C391 (**C**), or *Alternaria* with 2.5 nmol C391 (**D**), and cytospins were collected and stained with Hema 3. **E.** Differential cell counts of cytospins were acquired. Significant differences between *Alternaria*- treated and saline controls or C391 and *Alternaria*-treated groups are indicated by * (P≤.05) and ** (P≤.005),

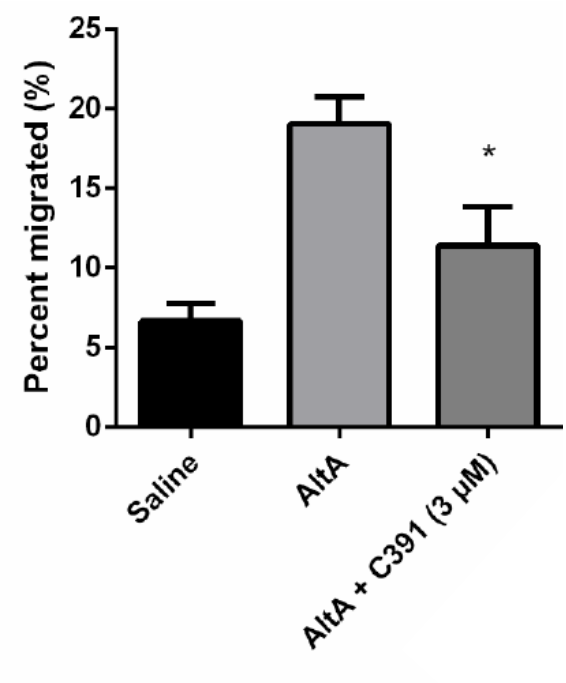

Figure 3.7. C391 blocks *Alternaria*-induced leukocyte migration in a dose-dependent manner. Primary cells collected from spleen of wild-type mice were challenged on the basolateral side of the transwell filter with saline, *Alternaria*, or *Alternaria* with C391. Cells designated for C391 treatment were also pretreated with C391 after they were seeded on the top side of the transwell filter 5 minutes before basolateral challenge. Significant differences are indicated by * ($P \leq .05$). (Experiments done concurrently with Marisol Arellano.)

Section 3.7

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Chapter 4:

Conclusions and Perspectives

Section 4.1

The role of PAR_2 signaling via β-arrestin-2 in allergic asthma

PAR_2 activation in the airways can have paradoxical roles being bronchoprotective or pro-inflammatory. Our lab recently showed in the ovalbumin (OVA)-induced model of airway inflammation with exacerbation by a PAR_2 agonist that the pro-inflammatory responses of PAR_2 activation in the lung are mediated by β-arrestin-2 (βarr2), demonstrating a distinct role for β-arrestin-2 in the airway separate from G-protein-dependent bronchoprotective effects. To understand the role of PAR_2-induced β-arrestin-2 signaling in a more physiologically relevant model, we focused on allergens from *Alternaria* alternata, one of the most common environmental allergens because evidence showed that they *contain serine proteases able to* activate PAR_2-dependent calcium signaling and airway inflammation. Based on our previous studies that demonstrated the role of β-arrestin-2 in regulating pro-inflammatory effects, I hypothesized that activation of the PAR_2/β-arrestin signaling axis as a direct consequence of PAR_2 activation by proteases found in allergens plays a key role in regulating *Alternaria*-induced lung inflammation.

In this investigation, we have shown that *Alternata*-induced airway inflammation requires both PAR_2 and βarr2 as demonstrated by the reduced recruitment of immune cells into the lung, mucus production, and thickening of the lung epithelium in PAR_2-knockout ($PAR_2^{-/-}$) or β-arrestin-$2^{-/-}$ mice compared to normal, wild-type mice.

Furthermore, I demonstrate that the majority of these acute inflammatory responses are promoted by the purified AASP and blocked by Soybean Trypsin Inhibitor. *Alternaria* allergens and its purified protease promote recruitment of β-arrestins to PAR_2 and trigger downstream signaling including cofilin activation and cell migration. Yet *Alternaria* protease-induced bronchorelaxation relied on COX production, showing that *Alternaria* is also able to activate G-protein-dependent protective effects. Thus, we have demonstrated a key role for PAR_2 signaling via β-arrestin-2 in regulating multiple aspects of the allergic inflammatory response stimulated by a single serine protease from *Alternaria*, while *Alternaria* is also able to induce protective effects, consistent with its effects on PAR_2-dependent activation of G-protein signaling.

This paradigm is seen in a wide variety of allergy-induced models of allergic asthma including those using cockroach and house dust mite allergens. Previously, *B. germanica* was shown to activate PAR_2-dependent calcium signaling; *B. germanica*-dependent airway inflammation including recruitment of eosinophils was abolished in $PAR_2^{-/-}$ mice and by addition of trypsin inhibitors(1,2). Airway inflammation was dependent upon serine protease activity, and analysis of extracts from *B. germanica* revealed a single serine protease activity (2,3). *B. germanica* induced production of G-CSF and CCL20 / MIP3A was inhibited by a protease inhibitor and also $PAR_2^{-/-}$ mice (4). *B. germanica*-induced activation of calcium signaling was also inhibited by prior sensitization to PAR_2-activating proteases and a trypsin inhibitor (5).

Furthermore, in our studies, we have demonstrated that *B. germanica* promotes lung inflammation including bronchoepithelial thickening, goblet cell hyperplasia, and immune cell recruitment that is dependent on β-arrestin-2 (See Appendix A Fig. 1, 2, and 3). *B. germanica* promoted β-arrestin-dependent cofilin activation in immune cells infiltrating the airway dependent on β-arrestin-2 (See Appendix A Fig. 4). *B. germanica* activates key readouts of PAR_2 signaling including recruitment of β-arrestin-2 (See Appendix A Fig. 4). Altogether, our results show that *B. germanica* promotes airway inflammation through β-arrestin-2-dependent activation of PAR_2 signaling (See Appendix A).

We also found that *B. germanica*-induced recruitment of immune cells requires hematopoietic PAR_2. Similar to what we previously demonstrated in our OVA+2fAP asthma model with adoptive transfer of bone marrow from $PAR_2^{-/-}$GFP (PAR_2 knockout) into wild type mice resulted in a significant reduction in leukocyte infiltration into the airway, we compared *B. germanica* challenged mice with adoptive transfer of WT/GFP with $PAR_2^{-/-}$GFP bone marrow cells. Our results show that GFP-positive cells in the lung were recruited by 20-fold, and flow cytometric analysis confirmed that the recruitment of eosinophils, lymphocytes, neutrophils, and macrophages were recruited with adoptive transfer of WT/GFP cells, but in wt mice receiving $PAR_2^{-/-}$GFP bone marrow, we observed a loss of recruitment of eosinophils, lymphocytes, and macrophages but not neutrophils. This suggests that hematopoietic PAR_2 is necessary for recruitment of these immune cells except for neutrophils to the airway.

In other models of allergic asthma, PAR_2 has also been shown to play a significant role in regulating inflammatory responses. House dust mite-induced inflammation in the lung was shown to require PAR_2 (6). However, the role of β-arrestin-2 in regulating allergic responses in this model has not been addressed. To analyze the translational relevance of our models and contributions of β-arrestins to exacerbation of asthma symptoms in patients, we compared key elements of PAR_2-β-arrestin signaling in cells collected from asthmatics and non-asthmatics. Using immunohistochemistry and microscopic analyses of PBMCs collected from these patients, expression of β-arrestin-1 and β-arrestin-2 was observed in human PBMCs collected from both healthy and asthmatic patients (see Appendix A). We next analyzed cofilin dephosphorylation levels as readouts of activation of β-arrestin signaling, and we found that PAR_2 agonists were able to activate cofilin in both healthy and asthmatic PBMCs. These results suggest that PAR_2 activation via β-arrestins is a relevant mechanism to consider in clinical cases of asthma where allergens that contain PAR_2-activating proteases are involved. In another model relevant to clinical manifestations of asthma, an increasing number of studies have established the link between outdoor air pollution and asthma where diesel exhaust particles (DEP) and particulate matter (PM2.5 or PM10) have been shown to damage airway epithelial cells (7). Recently, DEP has been shown to promote PAR_2-dependent calcium signaling and TRPV4 sensitization that was reduced with PAR_2 siRNA in bronchial epithelial cells (8). This suggests that DEP could either act like an agonist of PAR_2 or that endogenous proteases such as the release of MMP-1 are released from

epithelial cells that could then activate PAR_2, but these studies need to be verified (8–10).

Section 4.2

C391 Antagonizes *Alternaria*-induced PAR_2 signaling via β-arrestin-2

The use of PAR_2 agonists and antagonists have been proposed for the treatment of asthma. We have demonstrated a role for PAR_2 signaling through β-arrestins in the pathogenesis of *Alternaria*-induced airway inflammation and allergic asthma. However, *Alternaria* can still promote protective responses such as bronchorelaxation, which is regulated via a β-arrestin-independent, G-protein mediated pathway, involving the release of PGE2 and COX1/2 (11). Thus, the ideal antagonist would exhibit bias toward the β-arrestin signaling pathway and would not block the protective responses at doses that inhibit inflammation. Recently, a novel ligand targeting PAR_2, C391, was described and shown to block PAR_2-induced Ca2+ mobilization and PAR_2-mediated pain in a thermo-analgesic mouse model (12). Based on its antagonistic properties, I hypothesized that it would inhibit *Alternaria*-induced airway inflammation by targeting β-arrestin-dependent PAR_2 signaling.

Our main finding is that C391 was not only able to antagonize *Alternaria*-induced calcium signaling in bronchial epithelial cells, suggesting that it can still block *Alternaria*-induced G-protein signaling, but that it is preferentially blocking *Alternaria*-induced β-

arrestin-2 recruitment at lower doses. This observation suggests that C391 may be acting as a biased antagonist that preferentially blocks β-arrestin recruitment at a lower dose. Thus, C391 is a good candidate for the study of biased antagonism.

In this investigation, we have also shown that C391 abrogates *Alternaria*-induced inflammatory responses including the reduction of bronchoepithelial thickening, goblet cell hyperplasia, and immune cell recruitment to the lung. Therefore, these results suggest that targeting PAR_2 activation via β-arrestin signaling may be a new avenue for therapeutic intervention. This study of C391 could pave the way for targeting β-arrestin-dependent PAR_2 signaling as a new avenue for therapeutic intervention.

One major problem for the treatment of obstructive lung disease is the decreased responsiveness of bronchodilators with repeated use over time, resulting in ineffective treatment and increased health risks and possible fatal attacks (13). Furthermore, ligands targeting PAR_2 have thus far been unattractive therapeutics because of the relatively high inhibitory concentrations needed to be effective *in vitro* and in pre-clinical trials. Our studies show that C391 may function effectively at nanomolar ranges that are ideal for further stages of analyses.

However, newer, enhanced ligands targeting PAR_2 have been developed. A linker modification by the addition of polyethylene glycol into the ligand more closely mimics the activation of PAR_2 and has been shown to be more potent (14). Our collaborators have successfully shown that pegylated aminothiol-modified ligands activated PAR_2 more effectively than previously known agonists in activating PAR_2-dependent Ca2+

mobilization and changes in transepithelial resistance in human bronchioepithelial cells (15). Similarly, the next evolution of C391 is modified with polyethylene-glycol (PEG) and is called C391-PEG, which potentially enhances antagonist activity. Their studies also demonstrate that lipidation of C391-PEG increases membrane anchoring and targeting to membrane-bound PAR_2, thus more effectively inhibiting PAR_2-induced calcium signaling *in vitro* at an EC50 of 100 nM (Boitano, 2016 personal communication). Another ligand, known as C562, also shows an ability to inhibit PAR_2-βarr2 recruitment *in vitro* that could also be tested in this model of allergic asthma (DeFea lab, unpublished results). Thus, further studies have been underway using novel ligands targeting PAR_2 biased signaling in translational studies of diseases with promising therapeutic potential (16–19).

Section 4.3

The Role of Endogenous Proteases in activating PAR_2-β-arrestin signaling

We previously demonstrated in our OVA+2fAP and *B. germanica* asthma models that adoptive transfer of bone marrow from $PAR_2^{-/-}$GFP (PAR_2 knockout) into wild type mice resulted in a significant reduction in leukocyte infiltration into the airway (11). Preliminary studies suggest that in the cockroach model, recruitment of eosinophils, lymphocytes and macrophages but not neutrophils was severely impaired in wild-type mice receiving $PAR^{2-/-}$ bone marrow (Fig. 3A). This suggests that hematopoietic PAR_2 is

necessary for recruitment of these immune cells to the airway. A major conundrum arising from these results is how PAR_2 agonists, which presumably encounter the apical surface of the epithelium, are gaining exposure to the circulating leukocytes.

Because endogenously released proteases can activate PAR_2-induced allergic responses in the lung, the contributions of these proteases to *Alternaria*-induced inflammation need to be considered. Mast cells have long been understood as a major contributor to allergy and asthma and can release mast cell tryptase that has been shown to activate PAR_2. Because mast cell degranulation and thus the release of tryptase may be regulated by PAR_2-βarr2 activation, mast cell degranulation is a topic of interest for this model. However, in our studies, we have observed low numbers of mast cells in mouse lung tissue in our *Alternaria* and cockroach models. Yet β-arrestin-2 seems to regulate mast cell degranulation, which could contribute to the release of mast cell tryptase and other chemical mediators (See Appendix B). Thus, the contribution of mast cell tryptase to PAR_2 signaling via β-arrestin-2 is another topic of interest in the field of asthma in different models such as a chronic model of OVA or allergic asthma over the span of months and appropriate assays.

Studies of contributions from endogenous proteases that can activate PAR_2 also need to consider other trypsin-like proteases more recently studied. The human airway trypsin-like protease (HAT) has been isolated from the lung of patients with chronic airway disease and asthma and to activate PAR_2 (20,21). Although no mouse homolog is known, if *Alternaria* cannot completely penetrate the lung to activate PAR_2-βarr2

signaling, then these studies suggest that host-derived proteases such as HAT may play a greater role than previously expected. Neutrophils are found to be recruited in our model of *Alternaria*-induced airway inflammation, and neutrophil elastase can be released during inflammation to regulate neutrophil extracellular traps (NETs) to neutralize pathogens (22). Neutrophil elastase can cleave and activate PAR_2, but previous studies in our lab have shown that it does not recruit β-arrestins (23). Although it is not likely to promote pro-inflammatory effects through β-arrestins, it is possible that it activates PAR_2 signaling through G12/13.

Section 4.4

Figures

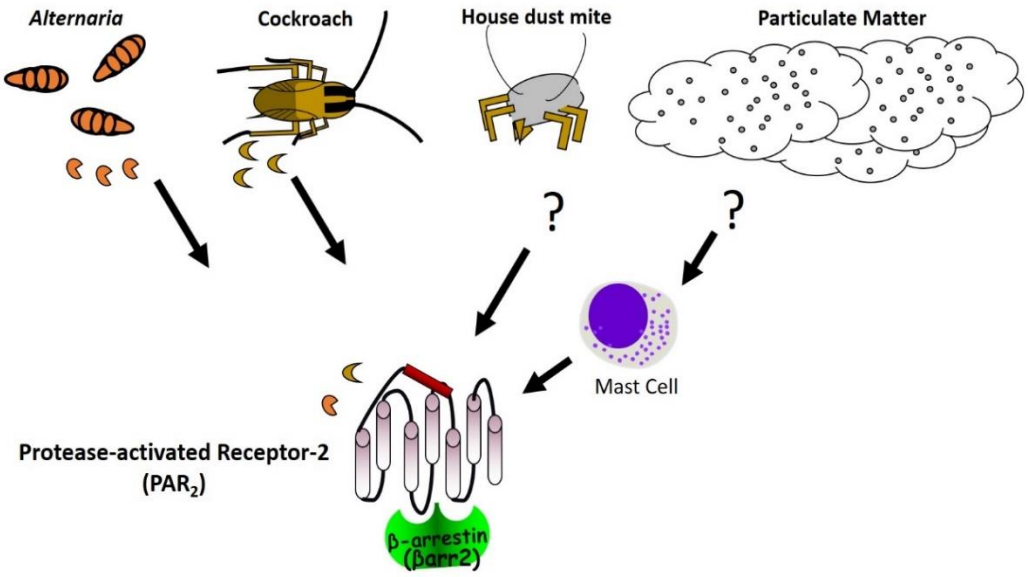

Figure 4.1. Model for the wide-spread role of PAR_2-β-arrestin-2 in allergic asthma. Proteases have been purified and identified from *Alternaria* and German cockroach allergens, and the requirement of PAR_2-β-arrestin signaling has been shown to regulate pro-inflammatory effects in these models. Although a role for PAR_2 has been shown with HDM-induced inflammation, the role of β-arrestin has not been addressed. Particulate matter found in air pollutants has a known history in association with increased prevalence of asthma, but the mechanisms and role of PAR_2-β-arrestin signaling have not been addressed. In this model, endogenous proteases may be released by key mediators such as mast cells in the allergic response where PAR_2-β-arrestin signaling may play a role.

Section 4.5

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Appendix A:

Methods Developed: Determining the Role of PAR_2-β-arrestin-2 signaling in a cockroach-induced model of allergic asthma and the translational relevance for clinical manifestations of asthma

Appendix A.1

Introduction

Cockroach frass can be found throughout the environment, and exposure to cockroach allergens is associated with increased incidence of asthmatic attacks. In recent studies, *Blattella germanica* (*B. germanica*) was shown to induce recruitment of immune cells that was dependent on PAR_2 and that a serine protease was identified in *B. germanica* allergens. Since PAR_2 signaling via βarr2 mediates pro-inflammatory responses in the lung challenged with *Alternaria* serine proteases, we hypothesized that βarr2 also plays a role in regulating *B. germanica*-induced allergic responses. While developing and applying the same techniques as discussed in Chapter 1 including histological analyses, I found that *B. germanica* promotes bronchoepithelial hyperplasia and goblet cell hyperplasia in wild-type mice but not $\beta arr2^{-/-}$ mice. Differential cell counts and flow cytometric analyses revealed that *B. germanica* promotes immune cell infiltration into the lung, but these effects were lost with $\beta arr2^{-/-}$ mice. Furthermore, I developed and applied the "phosphor-flow" cytometric assay, described in Chapter 1, and found that *B. germanica* promoted cofilin activation in infiltrating immune cells in the lung – the first to show another in vivo correlation between PAR_2-βarr2 signaling and lung inflammation. *B. germanica* also promoted β-arrestin recruitment to PAR_2, cofilin activation dependent on βarr2, and cell migration, suggesting that *B. germanica* activates PAR_2 signaling via β-arrestins. Altogether, our findings suggest that PAR_2

signaling via βarr2 plays an important role in regulating allergic responses induced by allergens that contain serine proteases.

To determine the translational relevance of our findings in mouse models to clinical manifestations of allergic asthma, I examined the expression of β-arrestins and responsivity of human PBMCs to PAR_2 agonist isolated from normal and asthmatic patients by developing techniques in immunohistochemistry and Western Blot analyses. Analyses of PBMCs revealed that βarr2 was expressed in both asthmatic and non-asthmatic PBMCs, and PBMCs isolated from asthmatic patients were more sensitive to the PAR_2 agonist, thus suggesting that PAR_2 signaling via βarr2 plays a key role in clinical manifestations of asthma.

Appendix A.2

Figures

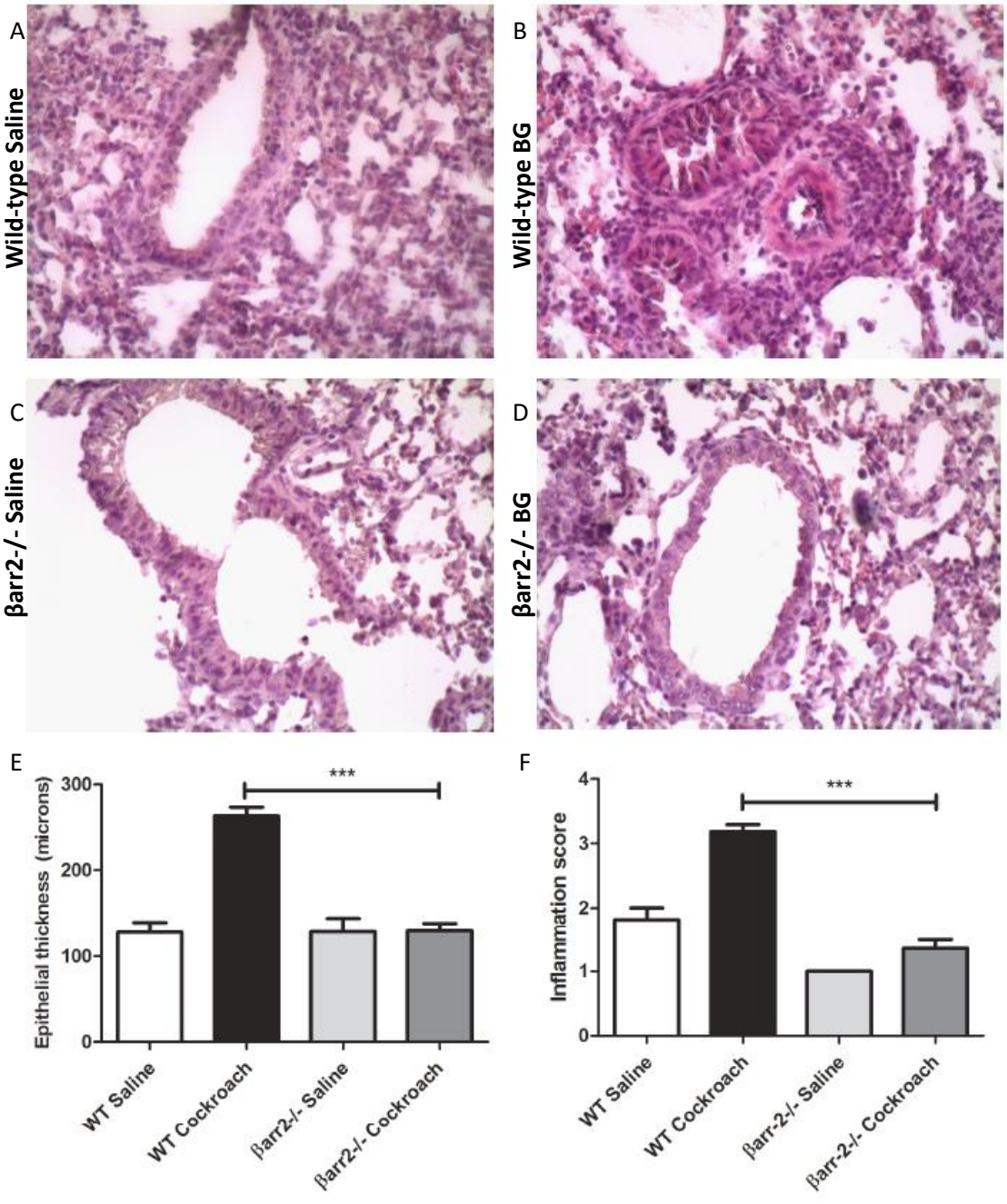
A
Wild-type Saline
B
Wild-type BG
C
βarr2-/- Saline
D
βarr2-/- BG
E
Epithelial thickness (microns)
300
200
100
0
***
WT Saline
WT Cockroach
βarr2-/- Saline
βarr2-/- Cockroach
F
Inflammation score
4
3
2
1
0
***
WT Saline
WT Cockroach
βarr-2-/- Saline
βarr-2-/- Cockroach

Figure A.1. *B. germanica* induces β-arrestin-2 dependent inflammation in the airway. H&E stained lung sections are shown from wild-type **(A, B)** and β-arrestin-$2^{-/-}$ mice **(C, D)** treated with either saline **(A, C)** or *B. germanica* **(B, D)**. Histological grading of slides. **E.** Epithelial thickness was determined by measuring the distance from the basolateral surface of the epithelial cells to the smooth muscle layer. **F.** Inflammation score was determined by the density of infiltrating cells in the airway tissue (1=no invading cells, 2=scattered cells, 3=clusters of invading cells throughout, 4=invading cells comprise more than 70% of the field of vision). Significant differences between bracketed *B. germanica*-treated groups are indicated by *** (P≤.001).

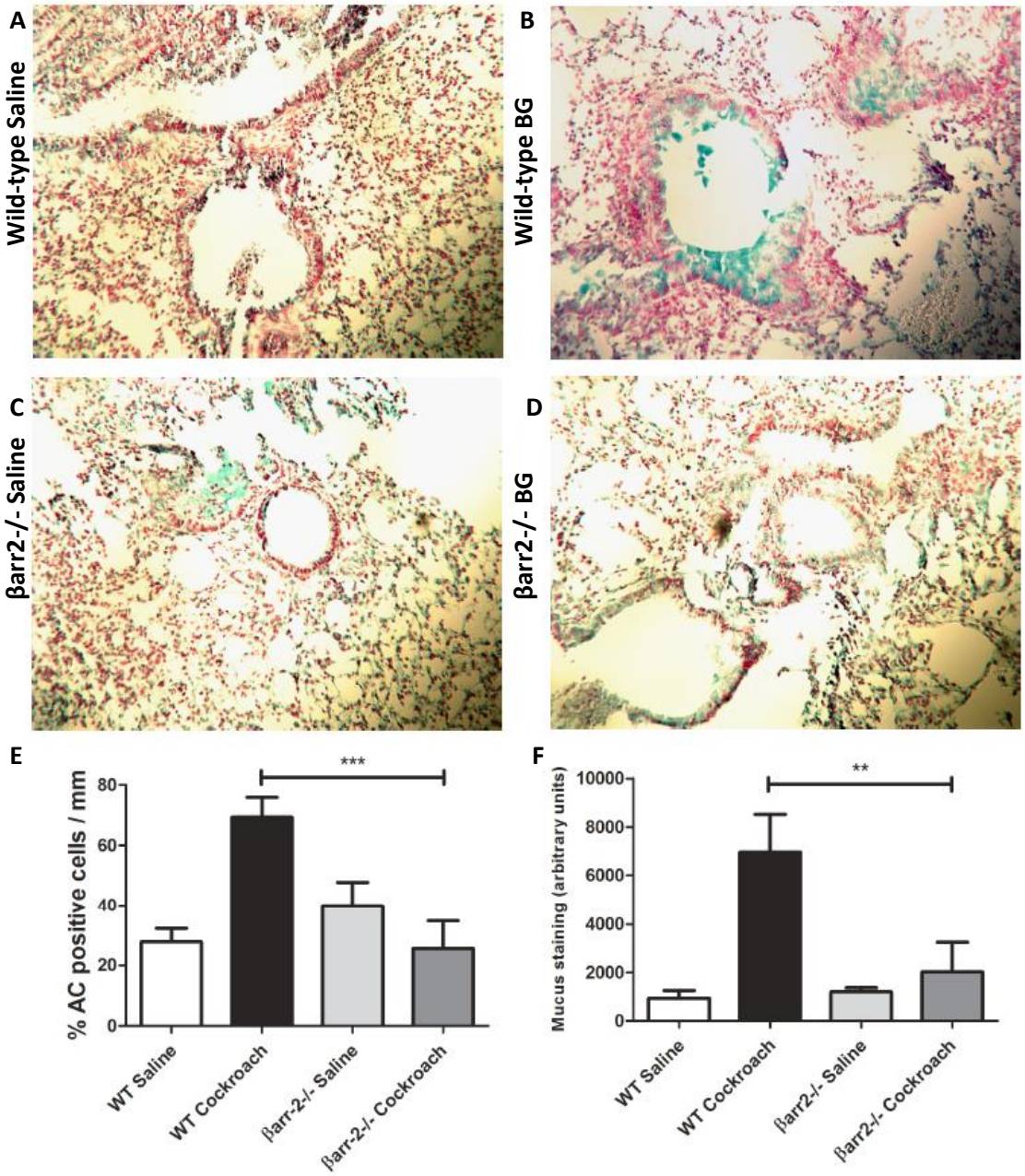
A
Wild-type Saline
B
Wild-type BG
C
βarr2-/- Saline
D
βarr2-/- BG
E
% AC positive cells / mm
80
60
40
20
0
***
WT Saline
WT Cockroach
βarr-2-/- Saline
βarr-2-/- Cockroach
F
Mucus staining (arbitrary units)
10000
8000
6000
4000
2000
0
**
WT Saline
WT Cockroach
βarr2-/- Saline
βarr2-/- Cockroach

Figure A.2. *B. germanica*-induced goblet cell hyperplasia require β-arrestin-2. Alcian blue stained lung section are shown from wild-type **(A, B)** and β-arrestin-$2^{-/-}$ mice **(C, D)** treated with either saline **(A, C)** or *B. germanica* **(B, D)**. The number of Alcian blue-positive goblet cells (**E**) and amount of mucin per mm of bronchiole (**F**) were counted. Significant differences between bracketed *B. germanica*-treated groups are indicated by ** (P≤.005) and *** (P≤.001).

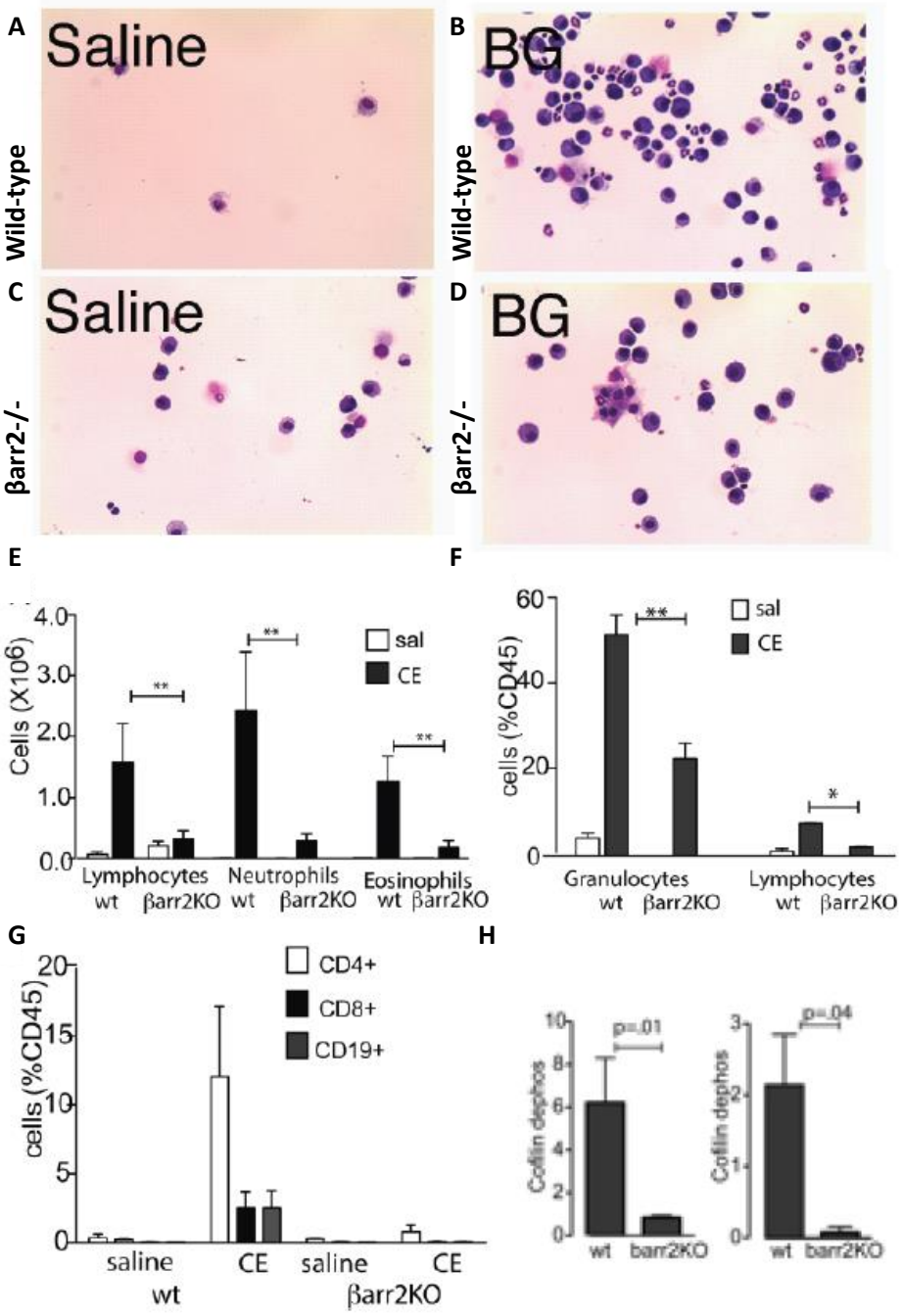
A
Saline
Wild-type
B
BG
Wild-type
C
Saline
βarr2-/-
D
BG
βarr2-/-
E
Cells (X10^6)
4.0
3.0
2.0
1.0
0.0
sal
CE
**
**
**
Lymphocytes
wt
βarr2KO
Neutrophils
wt
βarr2KO
Eosinophils
wt
βarr2KO
F
cells (%CD45)
60
40
20
0
sal
CE
**
*
Granulocytes
wt
βarr2KO
Lymphocytes
wt
βarr2KO
G
cells (%CD45)
20
15
10
5
0
CD4+
CD8+
CD19+
saline
CE
saline
CE
wt
βarr2KO
H
Coffilin dephos
10
8
6
4
2
0
p=.01
wt
barr2KO
Coffilin dephos
3
2
1
0
p=.04
wt
barr2KO

Figure A.3. *B. germanica* increases number of leukocytes in BALF that is dependent on β-arrestin-2. Total cells in BALF from wild-type **(A, B)** and β-arrestin-$2^{-/-}$ mice **(C, D)** treated with saline **(A, C)** or *B. germanica* **(B, D)** were counted as described. Differential cell counts were obtained from Hema 3 stained cytospins. **Flow cytometric analyses of *B. germanica*-induced recruitment of immune cells and cofilin activation dependent on β-arrestin-2. E)** B. germanica increases lymphocytes, neutrophils, and eosinophils that were lost with βarr$2^{-/-}$. **F)** *B. germanica* increases numbers of granulocytes and lymphocytes. Granulocytes were gated as high SSC and low FSC. Lymphocytes were gated as low SSC and low FSC. Percentage of CD45+ cells by flow cytometric analysis. **G)** Flow cytometric analyses confirmed *B. germanica*-induced CD4+ and CD8+ T cells and CD19+ B cells in BALF and revealed cofilin dephosphorylation in granulocytes and lymphocytes were dependent on β-arrestin-2H) "Phosphor-flow" assay was done to measure phosphor-cofilin levels minus isotype background levels for wild-type and β-arrestin-$2^{-/-}$ mice treated with *B. germanica*. Significant differences between bracketed *B. germanica*-treated groups are indicated by ** (P≤.005) and *** (P≤.001) (Experiments done concurrently with Heddie Nichols and courtesy of Heddie Nichols – Heddie Nichols' Dissertation 2012).

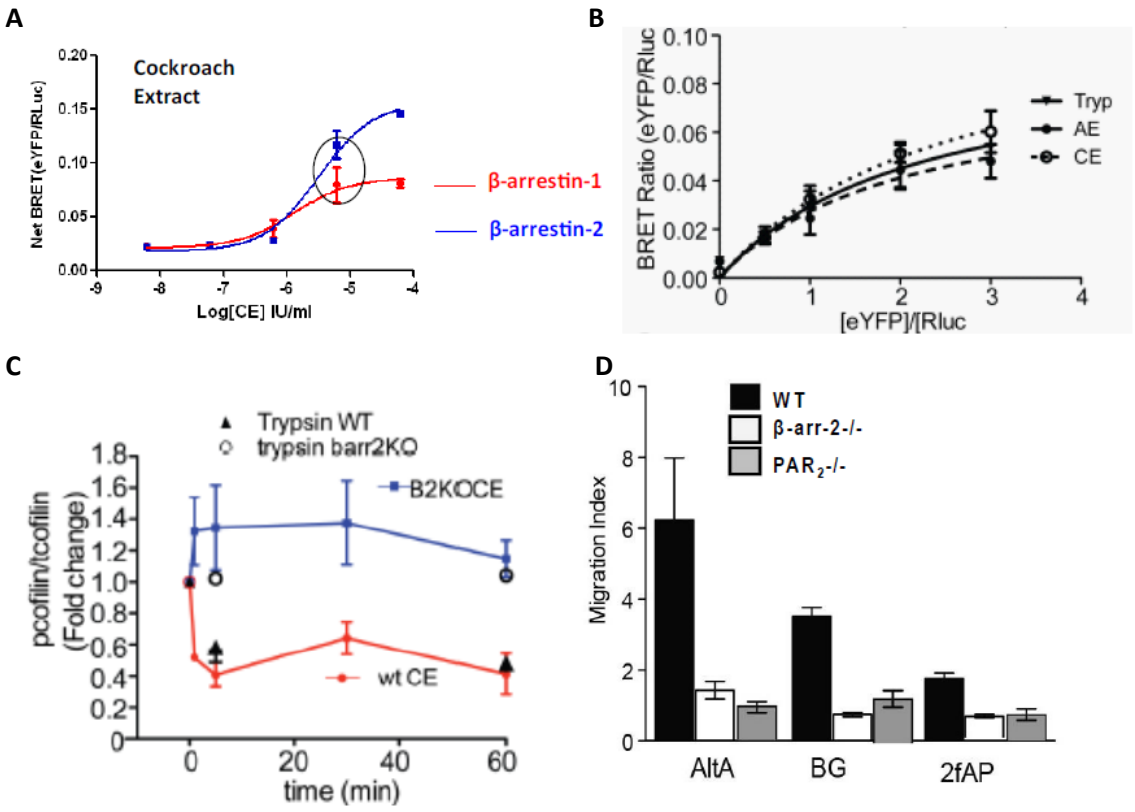

Figure A.4. ***B. germanica*** **promotes PAR_2 activation via β-arrestin-1 and β-arrestin-2.** Bioluminescence-resonance-energy transfer (BRET) analysis showing *B. germanica*-induced recruitment of β-arrestin-2 to PAR_2 with dose response profiles **(A)** and kinetics **(B)** similar to trypsin. **C)** Western Blot analysis of phospho-cofilin over total-cofilin levels in wild-type or β-arrestin-$2^{-/-}$ cells treated with *B. germanica* or trypsin positive control. **D)** Transwell migration assay was also performed with wild-type, β-arrestin-$2^{-/-}$, or $PAR_2^{-/-}$ cells treated with *B. germanica* (Courtesy of Kasturi Pal – Kasturi Pal's Dissertation 2013 and Heddie Nichols' Dissertation 2012).

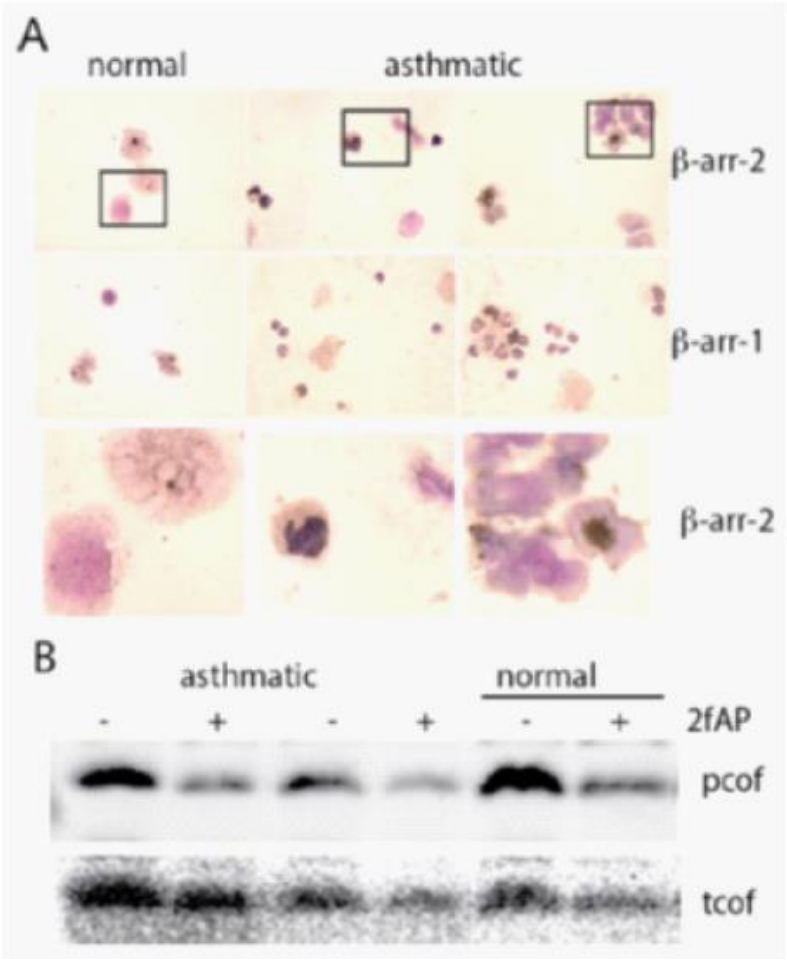

Figure A.5. Expression of β-arrestins and PAR_2 activation in human immune cells. A) Cytospins of peripheral blood mononuclear cells from a non-asthmatic and two asthmatic donors were stained with Hema 3 and antibodies to β-arrestin-1 or 2 followed by HRP-conjugated secondary antibodies and incubated with the HRP substrate, DAB, for colorimetric detection. Bottom row shows images taken at higher magnification of boxed regions showing vesicular staining of β-arrestin-2 visible in asthmatic samples. **B)** PBMCs from the same donors were incubated with or without 2fAP for 5 minutes, lysed, and analyzed by SDS-PAGE followed by Western Blot analyses using antibodies α-phospho-cofilin and α-total-cofilin.

Appendix B:

Methods Developed: Toluidine blue staining used to determine contributions from mast cells to PAR_2 activation in a model of *Alternaria*-induced allergic asthma

Appendix B.1

Abstract

Our lab has previously shown that adoptive transfer of bone marrow from $PAR_2^{-/-}$ GFP (PAR_2 knockout) into wild type mice resulted in a significant reduction in leukocyte infiltration into the airway in mouse models of allergic asthma, thus showing that PAR_2 expressed on leukocytes is required for recruitment. A major conundrum arising from these results is how PAR_2 agonists, which presumably encounter the apical surface of the epithelium, are gaining exposure to the circulating leukocytes. However, it is possible that other PAR_2-activating proteases are produced endogenously in the lung and released from the epithelium and surrounding cells that then contribute to the activation of PAR_2 and recruitment of leukocytes. Mast cells have long been understood as a major contributor to allergy and asthma and can release mast cell tryptase that has been shown to activate PAR_2. Here, I describe a histological staining technique using toluidine blue to analyze the numbers of mast cells and dispersion of mast cell granules in the mouse model of *Alternaria*-induced allergic asthma described throughout this Dissertation. Mast cells were detected in the lung of saline and *Alternaria*-challenged mice. *Alternaria*-treated wild-type mice displayed increased dispersion of granules that was lost in β-arrestin-$2^{-/-}$ mice. Thus, these results suggest that β-arrestin-2 regulates the dispersion of mast cell granules within lung tissue. My work demonstrates that toluidine blue staining

to visualize and analyze mast cells in the lung in a model of *Alternaria*-induced allergic asthma. This method may be applicable to other models of allergic asthma as well.

Appendix B.2

Introduction

We previously demonstrated that adoptive transfer of GFP-tagged hematopoietic cells resulted in infiltration into the lung of wild-type mice with OVA + 2fAP or *B. germanica* treatment while adoptive transfer of bone marrow from $PAR_2^{-/-}$GFP (PAR_2 knockout) resulted in a significant reduction in leukocyte infiltration into the airway (6). This suggests that hematopoietic PAR_2 is necessary for recruitment of immune cells to the airway. A major conundrum arising from these results is how PAR_2 agonists, which presumably encounter the apical surface of the epithelium, are gaining exposure to the circulating leukocytes. However, it is likely that other PAR_2-activating proteases are released in response from the epithelium and surrounding cells and contribute to the overall recruitment of leukocytes. Therefore, when considering the model of *Alternaria*-induced allergic asthma addressed in this Dissertation, the possibility that must be addressed is that other proteases can be released endogenously that can then activate PAR_2. This may explain the observed exacerbation of inflammatory responses. Host-derived proteases that can activate PAR_2 can come from a variety of sources within the lung including the epithelia that can produce a trypsin-like protease (HAT), mast cells that can produce mast cell tryptase, and in the case of inflammation, neutrophils that can produce neutrophil elastase.

Most widely studied of these in the context of allergy and asthma are mast cells that have long been understood as a major contributor. With sensitization to an allergen, dendritic cells take up the antigen and drive the development of Th2 cells, which then stimulate class switching of B cells to produce IgE. IgE can be produced at the site of the allergic challenge by Plasma B cells. Mast cells tightly bind IgE by FcεR and upon IgE binding to antigen act as key effector cells located in tissue that is our first line of defense including the skin and mucosa. Mast cells release granules after contact with antigens. They themselves can amplify the Th2 response by releasing IL-4 and IL-33 and by providing the co-stimulatory signal by expressing CD40 ligand binding CD40 on B cells. Of note, the ability of cells to migrate and arrive at these important loci is important including lymph nodes, the site of allergy, germinal centers containing B cells in the challenged tissue (Janeway's Immunobiology 8th Ed). Murine mast cells are found in the trachea and upper airways, and they release prostaglandins, acetylcholine, and 5-HT (serotonin) along with chemokines and cytokines. However, human mast cells are found in the trachea, upper airways, and in the rest of the lung, while these release prostaglandins and histamine along with chemokines and cytokines (7). Both contain mast cell tryptases, which has been shown to be expressed in the lung (8).

Clinically, mast cells are well-known for their involvement in allergic reactions. Mast cell numbers were found to be increased with mild and severe asthmatics whose endobronchial tissue tested positive for mast cell tryptase as well as in mouse models (1,9–11). Using immunohistochemical analyses of human biopsies from asthmatic lung

tissue, mast cell numbers were also found to be increased significantly (12). In human cases of allergic asthma, detection of mast cell tryptase was correlated with increased severity in allergic individuals including children (8,13). In a mouse model of chronic allergic inflammation using ovalbumin challenge, mast cells were found to enhance eosinophil recruitment (9). Mast cell numbers were also found to be increased in a chronic model of ovalbumin challenged mice (1).

Mast cell tryptase has been shown to activate PAR_2 similarly to well-known agonists of PAR_2 and is the most abundant protease found within mast cell granules (29–33). Mast cell tryptase activates PAR_2 as shown in fibroblasts transfected with PAR_2 and calcium mobilization assays (29). Two follow-up studies using desensitization assays with serine protease agonists in neuronal cell lines and HEK293T cells suggest that mast cell tryptase activates PAR_2 (31,32). Mast cell tryptase mMCP-6 cleaved the chromogenic substrate and showed that proteolytic cleavage was reduced when using a serine protease inhibitor leupeptin (33). However, it was also shown that it can cleave thrombin substrates - tosyl-Gly-Pro-Lys-*p*-nitroanilide and tosyl-Gly-Pro-Arg-*p*-nitroanilide even without the heparin glycosaminoglycan (19).

Several studies have also shown that tryptase can promote pro-inflammatory responses (20–24). A recent study has shown that mMCP-6 and MHC double-knockout mice resulted in abolished allergic responses to ovalbumin in the lung, but mMCP-6 knockout alone did not reduce mononuclear or Th2 cell infiltration. However, mMCP-6 knockout alone did result in a reduction of eosinophils recruited in the lung (25). A study

showed that injection of mMCP-6 into the peritoneal cavity caused recruitment of neutrophils as well as mast cell degranulation (26). Another study showed that injection of mast cell tryptase into the intestinal pleural cavity resulted in eosinophil recruitment dependent on PAR_2 that was blocked by PAR_2 inhibitors (27). Other mast cell proteases of interest include the tryptase mMCP-7 and the chymase mMCP-4.

PAR_2 and β-arrestins have been shown to play a role in the release of mast cell granules. In a model of collagen-induced arthritis, a PAR_2 antagonist, GB88, reduced degranulation that was measured by Alcian blue/Safranin O stained mast cells (34). Other studies have also shown that mast cell degranulation may be regulated by PAR_2 and βarr2 activation (35,36). Mast cell degranulation has also been analyzed by toluidine blue staining in several other studies published from a variety of labs (1–3). Degranulation can then lead to mast cell tryptase release that acts in a feedback loop in amplifying PAR_2 activation and further release of granules likely containing more tryptase. β-arrestin has been shown to play a role in regulating the release of these endogenous mediators in packaged granules. In a study of degranulation in polymorphonuclear cells comparing wild-type and beta-arrestin-1 mutants, β-arrestin was found to regulate the release of granules (37).

Since mast cell tryptase can activate PAR_2 and has been suggested to be involved with pro-inflammatory responses, then it is possible that βarr2 may regulate pro-inflammatory downstream effects, but this has largely been unexplored. Because mast cell degranulation and thus the release of tryptase may be regulated by PAR_2-βarr2

activation, mast cell degranulation needs to be analyzed further in this allergic model. Here, I demonstrate the histological staining technique using toluidine blue to analyze the numbers of mast cells and dispersion of mast cell granules in the mouse model of *Alternaria*-induced allergic asthma described throughout this Dissertation. I show the ability of toluidine blue staining to detect mast cells in murine lung either challenged with saline or challenged with *Alternaria* in wild-type or β-arrestin-2-KO mice.

Appendix B.3

Methods and Materials

Toluidine blue staining of mast cells: Lung sections were prepared as previously described in Chapter 2 and 3. Toluidine blue staining was used as a method of identifying mast cells with an established protocol. Toluidine blue binds strongly to heparin, and because mast cell tryptase is complexed with heparin, toluidine blue has been used at low levels from 0.1% to 1% to stain for mast cells in several published studies (1–3). Toluidine blue was diluted at 0.1% weight to volume in 0.5 N HCl. Tissue was stained for one hour in toluidine blue and briefly counterstained with eosin.

Appendix B.4

Results

Mast cells are present in the mouse lung

To address whether there are mast cells that are present in the lung of mice, we used a histological staining technique using toluidine blue to stain lung tissue sections. Analyses of lung tissue sections in our model of *Alternaria*-challenged mice have revealed the presence of toluidine blue-positive cells in the lung of our mice – both saline and *Alternaria* challenged either wild-type or β-arrestin-$2^{-/-}$ mice (Fig. 1). However, when the total number of mast cells were quantified in wild-type and β-arr-$2^{-/-}$ mice, there was no significant increase in mast cells with *Alternaria* surrounding bronchioles, large airways, and smooth muscle overall. However, in the large airway of $PAR_2^{-/-}$ mice treated with *Alternaria*, there was a significant increase of mast cells. Western blot analyses targeting the mast cell tryptase, mMCP-6, known to be expressed in the lung revealed that it was not detectable in the BAL fluid of mice (unpublished data).

β-arrestin-2 regulates dispersion of mast cell granules in the lung

Because previous studies have shown a role for β-arrestins in regulating degranulation, mast cell granularity was also analyzed. Toluidine-blue binds to heparin packaged in granules within mast cells and released extracellularly upon degranulation, and the percent granularity was quantified. Saline-treated mice contained cells showing

tight compaction of toluidine-blue stained granules. But with Alternaria challenge, there was a significant increase in dispersion of toluidine-blue stained granules around mast cells (Fig. 2A, B). However, with β-arrestin-$2^{-/-}$ and $PAR_2^{-/-}$, this increase in granule dispersion was lost (Fig. 2C - E).

Mast cells were detected in intestinal, tongue, and skin tissue

Previous studies have shown that mast cells were detectable by toluidine blue staining in intestinal, tongue, and skin tissue. To confirm the ability of this method to detect mast cells, these tissue were isolated and stained with the same protocol as that used for lung tissue. Toluidine blue-positive cells were detected with ovoid cellular morphology and distinct granular packets in tissue sections from the intestine (Fig. S1A). Toluidine blue-positive cells were also detected in tongue and skin tissue sections, but these cells were irregularly shaped (Fig. S1B, C). These cells staining positive for toluidine blue are consistent with previous publications that characterized mast cells.

Appendix B.5

Discussion

Here, I demonstrate the ability of toluidine blue staining to detect mast cells that were present in wild-type and β-arrestin-$2^{-/-}$ murine lung. There was no detectable

difference in the number of mast cells in the large airway between Wild-type or βarr2$^{-/-}$ mice with saline or *Alternaria* treatment, suggesting that mast cell numbers are not regulated by β-arrestin-2. The observed numbers were consistent with previously published studies that showed similar numbers of mast cells surrounding airways. Furthermore, a comparison between the numbers of mast cells found in our model compared to the model using a long-term treatment with ovalbumin over several months reveals that the mast cell counts observed in our *Alternaria* model are lower and suggest that contributions from mast cells are not as pronounced (1). Mast cells were also detected surrounding blood vessels and smooth muscle in our *Alternaria* model, and this suggests that they may still be contributing to modulating vasculature function and contractility within the lung.

Because toluidine-blue binds heparin, and mast cell tryptase is a major component of mast cells that is bound to heparin, mast cell tryptase could still be present and can be released by mast cell degranulation in the lung in low amounts. However, *Alternaria* induced greater dispersion of granules surrounding mast cells in wild-type mice, while this dispersion was lost with β-arrestin-2$^{-/-}$ mice, suggesting that β-arrestin-2 plays a role in regulating degranulation. Thus, β-arrestin-2 may fine-tune release of mast cell products but not contribute to the expansion or recruitment of mast cell populations. Altogether, my studies have shown that toluidine blue staining provides an effective means of visualizing and analyzing the contributions of mast cells

in an *Alternaria*-induced model of allergic asthma. This method can thus be applied to other models of allergic asthma.

Yet other trypsin-like proteases should also be considered that may be released in the lung that contribute to PAR_2 activation. Several other proteases have been analyzed in other models of airway inflammation. Since *Alternaria* allergens first contact the airway epithelium, which is also known to express human airway trypsin-like protease (HAT), its role has been studied. HAT has been isolated from the lung of patients with chronic airway disease and asthma and to activate PAR_2 (38,39). Although no mouse homolog is known, if *Alternaria* cannot completely penetrate the lung to activate PAR_2-βarr2 signaling, then these studies suggest that host-derived proteases such as HAT may play a greater role than previously expected.

Neutrophil elastase can cleave and activate PAR_2, but previous studies in our lab has shown that it does not recruit βarrestin-2 (40). However, the results suggest that it can also activate another arm of PAR_2 signaling possibly through G12/13. Neutrophils are found to be recruited in our model of *Alternaria*-induced airway inflammation, and neutrophil elastase can be released during inflammation to regulate neutrophil extracellular traps (NETs) to neutralize pathogens (41). In our model of allergen-induced asthma, then neutrophil elastase is not likely to contribute to the activation of PAR_2-βarr2 signaling but can contribute to the regulation of inflammatory responses.

Appendix B.6

Figures

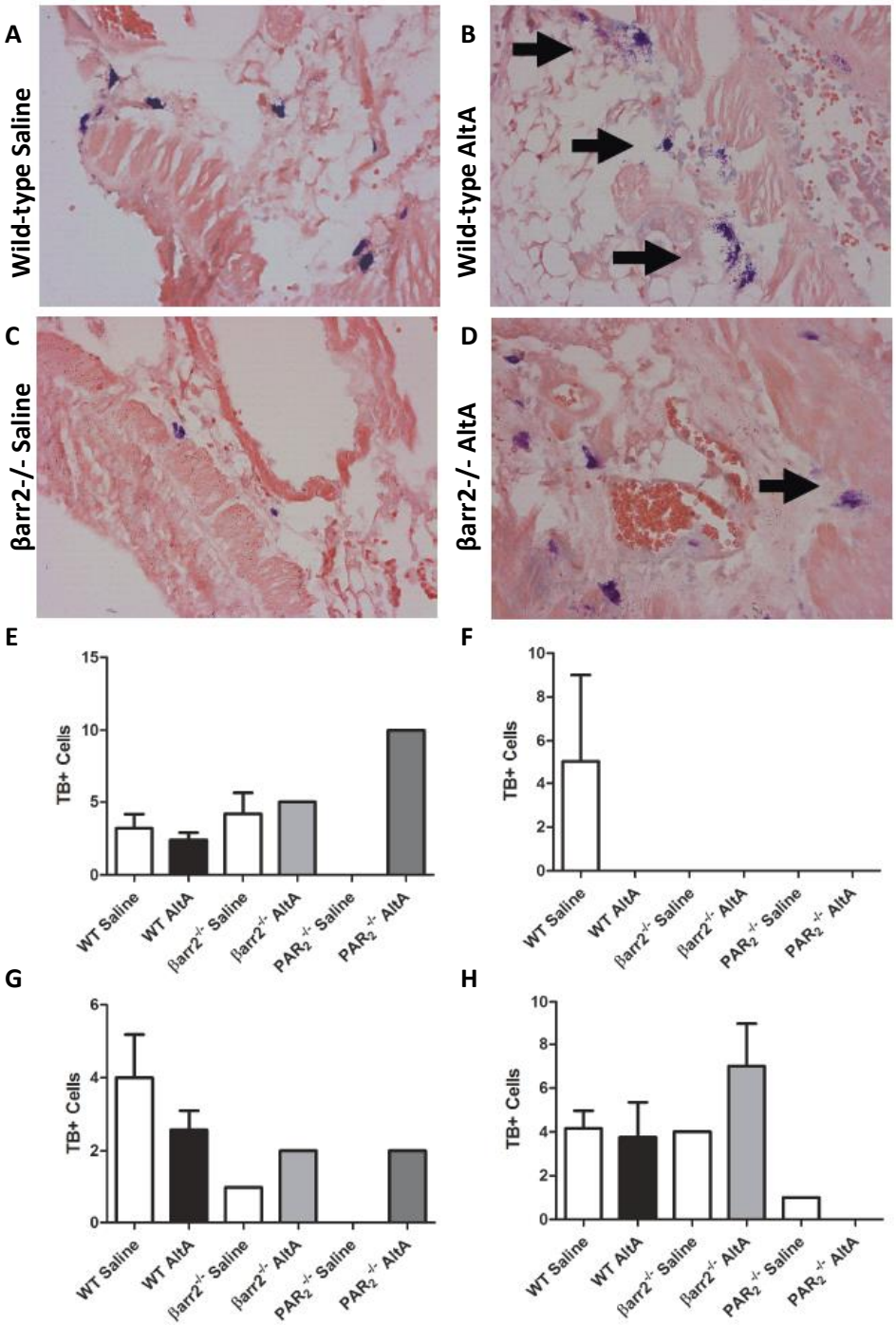
A
Wild-type Saline
B
Wild-type AltA
C
βarr2-/- Saline
D
βarr2-/- AltA
E
TB+ Cells
15
10
5
0
WT Saline
WT AltA
βarr2-/- Saline
βarr2-/- AltA
PAR2 -/- Saline
PAR2 -/- AltA
F
TB+ Cells
10
8
6
4
2
0
WT Saline
WT AltA
βarr2-/- Saline
βarr2-/- AltA
PAR2 -/- Saline
PAR2 -/- AltA
G
TB+ Cells
6
4
2
0
WT Saline
WT AltA
βarr2-/- Saline
βarr2-/- AltA
PAR2 -/- Saline
PAR2 -/- AltA
H
TB+ Cells
10
8
6
4
2
0
WT Saline
WT AltA
βarr2-/- Saline
βarr2-/- AltA
PAR2 -/- Saline
PAR2 -/- AltA

Figure B.1. **Imaging and Quantification of Mast cells in the Lung**. Presence of toluidine-blue-positive (TB+) stained mast cells was quantified according to proximity to structures in the lung including bronchioles, large airway, alveoli, and smooth muscle. Lung sections were prepared as previously described in Chapter 2 and 3. Toluidine blue staining was used as a method of identifying mast cells with an established protocol. Tissue was stained for one hour in 0.1% toluidine blue and briefly counterstained with eosin. **(A - D)** Representative images of lung sections from mice treated with saline or *Alternaria*. Quantification of TB+ cells surrounding large airway **(E)**, bronchioles **(F)**, alveoli **(G)**, and smooth muscle **(H)**.

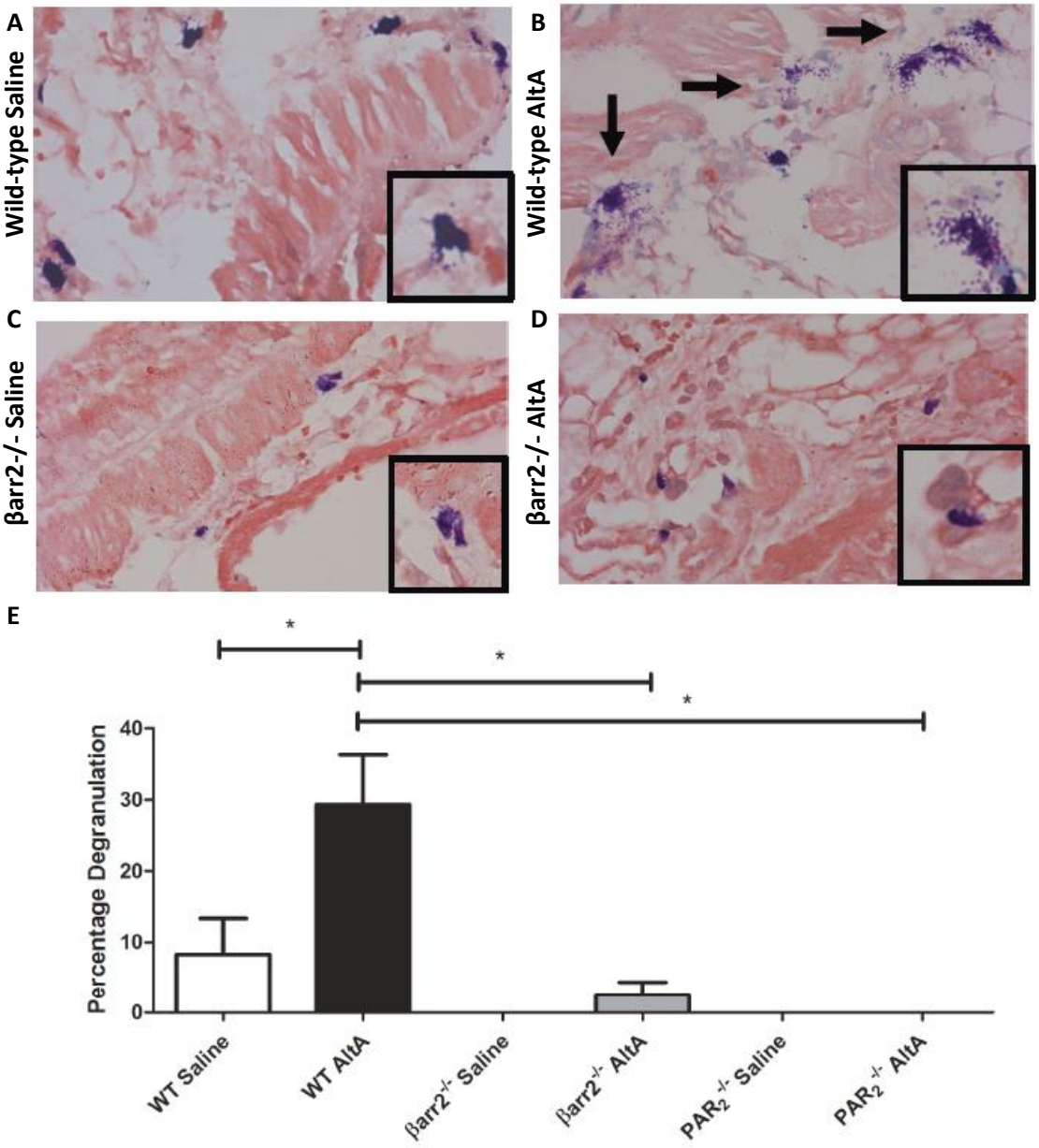
A
Wild-type Saline
B
Wild-type AltA
C
βarr2-/- Saline
D
βarr2-/- AltA
E
*
*
*
Percentage Degranulation
40
30
20
10
0
WT Saline
WT AltA
βarr2-/- Saline
βarr2-/- AltA
PAR2-/- Saline
PAR2-/- AltA

Figure B.2. Mast cell degranulation is regulated by β-arrestin-2. **(A - D)** Toluidine-blue (TB+) stained lung section counterstained with eosin. **A)** Wild-type saline-treated mouse lung section. **B)** Wild-type *Alternaria*-treated mouse lung section. **C)** β-arrestin-2-/- saline-treated mouse lung section. **D)** β-arrestin-2-/- *Alternaria*-treated mouse lung section. **E)** Quantification of the percentage of total TB+ cells in the image that showed dispersion of granules over 100 images analyzed. Insets show 40x magnification of TB+ cell.

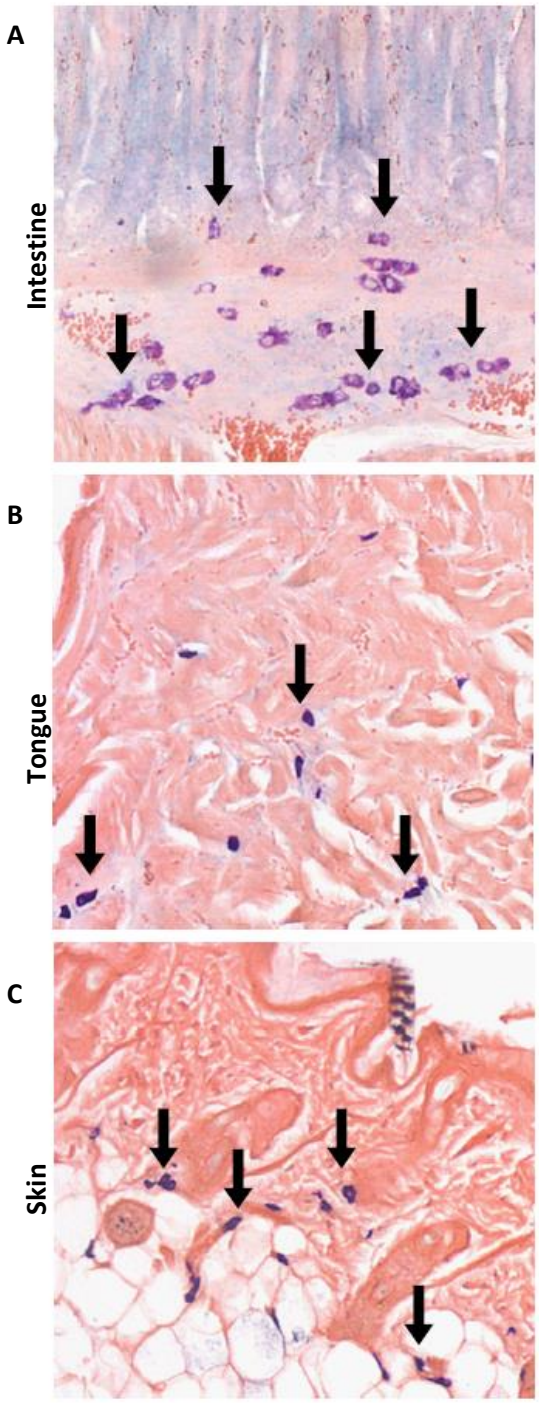

Figure S1. Mast cells were detected in intestine, tongue, and skin. Presence of toluidine-blue-positive (TB+) stained mast cells was detected using a previously established protocol. Tissue sections from unchallenged wild-type murine intestine **(A)**, tongue **(B)**, and skin **(C)** were stained for one hour in 0.1% toluidine blue and briefly counterstained with eosin.

Appendix B.7

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