UNIVERSITY OF CALIFORNIA
RIVERSIDE

The Pro-Inflammatory but not Protective Effects of Protease-Activated-Receptor-2 In the Airways are Abolished in Beta-Arrestin-2 Mice in OVA, Cockroach Frass and *Alternaria*-Induced Models of Allergic Asthma

A Dissertation submitted in partial satisfaction
of the requirements for the degree of

Doctor of Philosophy

in

Biomedical Sciences

by

Heddie Lynn Nichols

December 2012

Dissertation Committee:
Dr. Kathryn DeFea, Chairperson
Dr. Emma Wilson
Dr. Iryna Ethell

The Dissertation of Heddie Lynn Nichols is approved:

Committee Chairperson

University of California, Riverside

Acknowledgements

The experiments in this dissertation, as well as the reagents, costs and time they required were made possible with grant funding obtained by my principal investigator and mentor Dr. Kathryn DeFea, for whom I have the highest regard. My admiration arises in-part from her creative capacity to apply basic science techniques to research questions and provide novel answers. I'm sure we'd all be in better off if we were creative problem solvers and used wisdom in resource consumption as she has. I'm ever-more appreciative of her creativity and brilliant mind as she was simultaneously engaged in writing and submitting grant proposals, publishing articles in peer-reviewed journals, maintaining a successful graduate research lab and budget while teaching and coordinating in the UC Riverside Thomas Haider Medical School; and still being an incredibly nice person. I am grateful for the time spent alongside and under her supervision and to her for opening her lab and her remarkable mental faculties to me as a graduate student.

Dr. Emma Wilson and Dr. Iryna Ethell are members of my dissertation committee and have provided insightful comments on my research and publications. I appreciate their encouragement and generous support, including reagents and use of the BD FACS Canto II in the Wilson Lab. I am grateful to the faculty, staff and administration in the Biomedical Sciences Department at UC Riverside. The individual attention and professional insight from Dean Byus, Dr. Monica Carson, Dr. Ameae Walker and Kathy Redd enhanced and shaped years of positive experiences into my graduate career.

I appreciate the scientific understanding of Dr. Maria Zoudilova whose research and publications provided a springboard for my investigations into a mouse model of airway inflammation. Mike Lau provided expertise on tissue sectioning and we miss his singing during experiments. Current lab members including: Dr. Jungah Min, Dr. Alice Lin, and Kasturi Pal are extraordinary scientists, wonderful lab mates, and I am grateful for their friendship.

I am especially thankful for my parents Norma Jean and Wade Richards. My mother is first to congratulate me and celebrate my achievements and the scientific articles reported by my father, surely promoted my own academic endeavors. My siblings Ezra, Sam, Edie, their spouses and children have expressed their encouragement and support since the beginning of my graduate program in 2007. Importantly, I thank my ever-attentive husband Ryan for his patience with my unpredictable timetable, his flexibility in adapting to my schedule and delivering dinner to me during late night experiments. His light-hearted laughter and kindness diminished the magnitude of obstacles I've encountered in my multi-faceted life.

For the great men and women in my life who either made my life sublime, helped me take heart again; or both.

ABSTRACT OF THE DISSERTATION

The Pro-Inflammatory but not Protective Effects of Protease-Activated-Receptor-2 In the Airways are Abolished in Beta-Arrestin-2 Mice in OVA, Cockroach Frass and *Alternaria*-Induced Models of Allergic Asthma

by

Heddie Lynn Nichols

Doctor of Philosophy, Graduate Program in Biomedical Sciences
University of California, Riverside, December 2012
Dr. Kathryn A. DeFea, Chairperson

Proteinase Activated Receptor-2 (PAR_2), a G Protein Coupled Receptor (GPCR) activated by serine-like proteases, is reported to have both protective and pro-inflammatory effects in the airway. Given these dual and apparently opposing actions, both inhibitors and activators of PAR_2 have been proposed as therapeutics for asthma. PAR_2 can signal through two independent pathways: a G-protein-dependent and a beta-arrestin-2-dependent/G-protein-independent one. The beta-arrestin-dependent pathway promotes leukocyte migration, while bronchiolar smooth muscle relaxation requires G-protein signaling intermediates. These studies address the hypothesis that inflammatory responses to PAR_2 activation are mediated by beta-arrestins, while prostaglandin production and smooth muscle relaxation are not. Our initial studies focused on a mouse ovalbumin model for PAR_2-modulated airway inflammation to focus specifically on PAR_2 effects. During the course of our studies, two models of PAR_2 dependent airway inflammation were introduced. They are: Alternaria alternata, a fungus that commonly grows in homes and on plant; and Cockroach Frass from Blatella germanica a common household pest. These models induce a more robust inflammatory response and are more physiologically relevant as they used common household allergens. As determined by

flow cytometry, cytospin and immunohistochemistry, PAR_2-induced overall lung inflammation, mucus production, airway responsiveness and recruitment of eosinophils and CD4+-lymphocytes to the Broncho Alveolar Lavage Fluid (BALF) were abolished in beta-arrestin-$2^{-/-}$, compared with wild type mice. These results were exacerbated in our AltA and BG models. In contrast, PAR_2 promoted equivalent bronchial epithelium-dependent tracheal smooth muscle relaxation and production of PGE2 in both wild type and beta-arrestin-$2^{-/-}$ mice. Our data suggest that the PAR_2-enhanced inflammatory process is beta-arrestin-2-dependent, while the 'protective' anti-constrictor effect of bronchial epithelial PAR_2 is beta-arrestin-independent.

TABLE OF CONTENTS

TABLE OF CONTENTS (continued)

List of Figures

Chapter 1: Protease Activated Receptor -2 mediated signaling via β-arrestins

Chapter 2: β-arrestin-2 mediates the pro-inflammatory effects of Proteinase-Activated-Receptor-2 in the airway

Chapter 3: PAR_2-induced signaling through β-arrestin-2 mediates BG and AltA-induced airway inflammation

List of Tables:

Chapter 1

Chapter 2

List Of Cartoons

Chapter 1

This page intentionally left blank

Chapter 1

Part A: Background

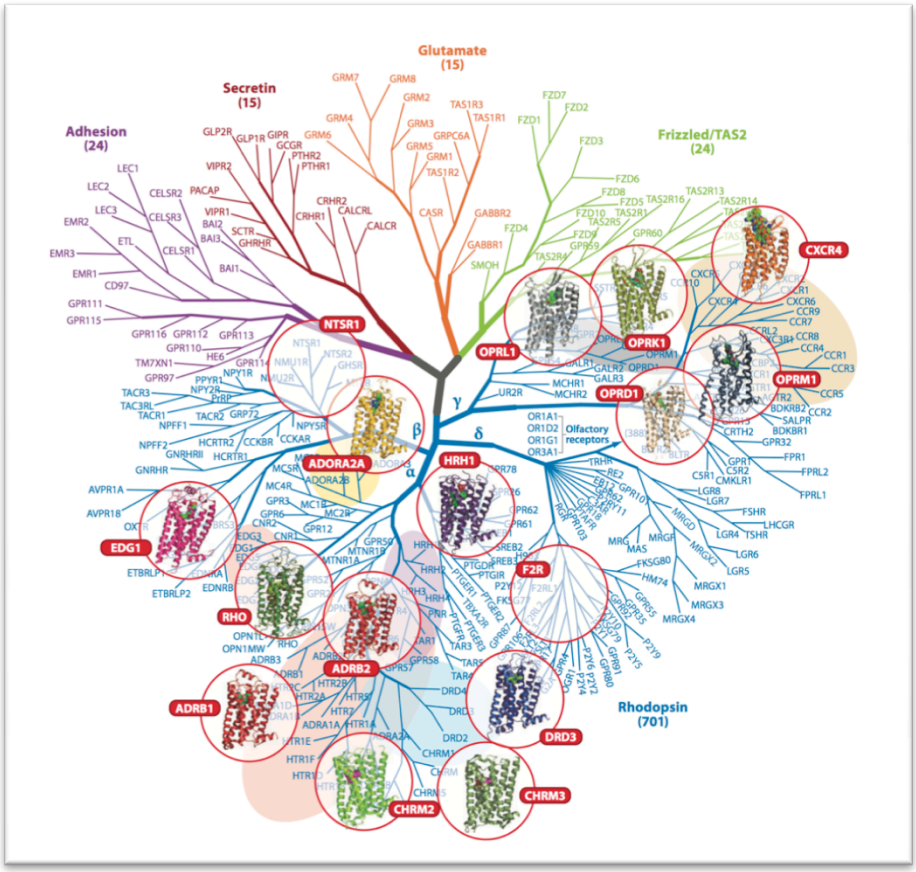
β-Arrestin-Dependent Modulation of G Protein Coupled Receptor (GPCR) Signal Transduction: Implications in Pathophysiology and Putative Therapeutics

Part A: Background

Section 1.1

GPCRs

G-protein coupled receptors (GPCRs) are the largest family of cell surface receptors, responsible for translation of numerous extracellular stimuli into intracellular signals. They are also called Seven Transmembrane Receptors (7TMRs) because of their unique morphology that spans the cells membrane seven times (1). For our discussion, I will refer to them only as GPCRs. Based on amino acid sequence, the 800 human GPCRs are grouped into five super families, namely: *Rhodopsin, Secretin, Adhesion, Glutamate* and *Frizzled/TAS2,* with a majority being in the *Rhodopsin* family (Cartoon 1.1) (2). GPCRs control a wide variety of cellular processes including: vision, smell, and taste to neurological, cardiovascular, endocrine, and reproductive and, as a result, they are currently a major target for therapeutic intervention. The super family of GPCRs is very diverse in terms of activation, however structural coverage of the GPCR super family has exponentially increased from two known structures in 2007, to 16 crystal structures in 2012. As the structural diversity within the super family has been explored, new insight has been shed on variations in the extracellular domains, and other topologies that alter physical properties of the ligand binding pockets. For greater insight on crystal structures on the GPCR super family, please see reference (2).

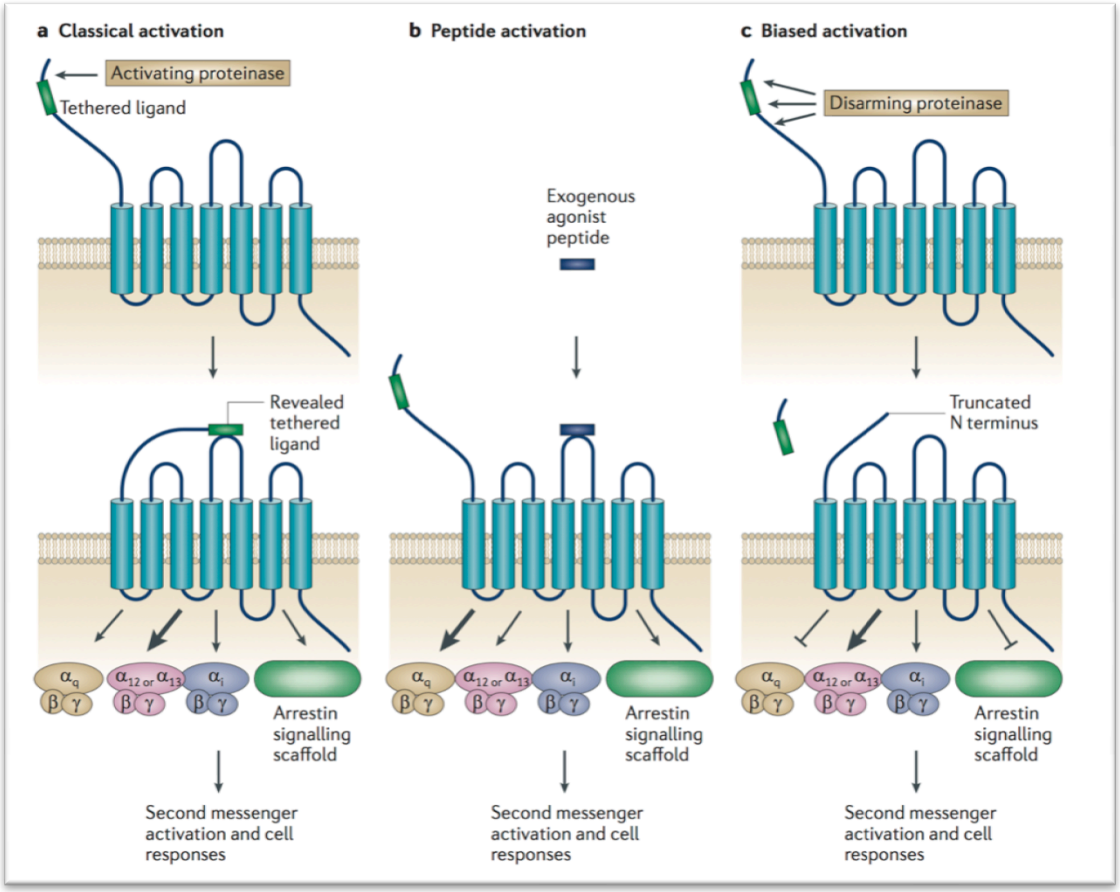


Cartoon 1.1 Dendrogram of the human G protein-coupled receptor (GPCR) super family with the crystal structures solved by September 2012. According to this notation human GPCRs include the *Rhodopsin* family (Class A GPCRs), the *Secretin* and *Adhesion* families (Class B GPCRs), the *Glutamate* family (Class C GPCRs), and the *Frizzled/TAS2* family. The *Rhodopsin* family is divided into subgroups: the α-group, the β-group, the γ-group, and the δ-group, as labeled GPCRs can be further provisionally divided into clusters (e.g., aminergic), subfamilies (e.g., adrenergic or opioid), and individual GPCR subtypes (e.g., dopamine receptor subtypes D1-D5). Olfactory receptors comprise the largest distinct cluster of 388 receptors (only 4 subtypes are shown here) in the s-group of the *Rhodopsin* family of GPCRs. Courtesy of Katritch et al. 2012, Annul Rev. Pharmacol Toxicol.

Section 1.2

Structure, function and pathophysiology of protease activated receptors

Within the *Rhodopsin* super family exists the protease activated receptor subgroup. In this subgroup, there are four PAR family members (PAR_1, PAR_2, PAR_3, PAR_4). In contrast to the GPCR super family, they are not activated in vivo by a soluble ligand (3). Instead, they are activated by proteolytic cleavage of the N-terminus that reveals a tethered ligand that binds to an extracellular domain and induces a conformational change in to recruit heterotrimeric G-protein coupling and activation. They can also be activated by peptidomimetics corresponding to the tethered ligand amino acid sequence (Cartoon 1.2) (4). PAR_1 is activated by multiple trypsin serine proteases, including thrombin, kallikreins and interestingly, proatherocytin from snake venom (3). In addition to being proteolytically activated by trypsin, PAR_2 is also activated by mast cell tryptase, neutrophil elastase and the allergens isolated from German cockroach frass and fungal filtrate *Alternaria alternata*. PAR_3 is primarily targeted by thrombin and PAR_4 responds to trypsin and thrombin, the complement pathway and plasmin to name a few. For further insight on the pathophysiologies these receptors are involved, please see Table 1.1 (5). Once activated, receptors also engage G-protein receptor kinases (GRKs) and β-arrestins known for their role to uncouple GPCRs from their cognate heterotrimeric G-proteins and facilitate clathrin-mediated endocytosis. A recent paradigm shift in the field is the discovery that GPCRs can also signal independent of G-protein coupling through a family of proteins known as β-arrestins which serve as scaffolds to localize activation and inhibition of signaling molecules.



Cartoon 1.2 Mechanism of proteinase-activated receptor activation. The figure depicts the activation of proteinase-activated receptors (PARs) by proteolytic cleavage and the revealing of the tethered ligand to stimulate signaling (part a), the activation of PARs by exogenous application of synthetic agonist peptides in the absence of proteolytic unmasking of the tethered ligand (part b), and the disarming of signaling through PARs by proteolytic cleavage downstream of the receptor activating site to truncate the tethered ligand and make it unavailable for activating proteinase (part c). Such processing of PARs can, in addition to disarming some signaling pathways, simultaneously activate other signaling pathways. Disarmed receptors may sometimes be retained on the cell surface and be valuable for activation by synthetic agonist peptides. Courtesy of Ramachandran et al., 2012 Nature Drug Discovery.

Section 1.3

PAR_2 proinflammatory responses mediated by exogenous and endogenous proteases

Protease Activated Receptor-2 is a GPCR expressed on a plethora of tissues and plays a role in many pathologies (Table 1.1). There are many potential physiological roles, with corresponding animal models, that investigate PAR_2 including: endothelial cell function, intestinal function, skin pigmentation, tumour cell growth and metastasis and allergic asthma(4). We chose to emphasize the latter because of examination of a long-standing controversial role behind PAR_2 in the airways, the widespread expression of PAR_2 airway tissues, diverse inflammatory cells potentially recruited with PAR_2 activation and numerous proteases that gain access to the airways during allergic events (Table 1.2, 1.3) (6). Further a key paper illustrated that over-expression of PAR_2 increased the total number of cells and eosinophils in the airways over wt mice and was abolished in absence of the receptor (7). An example of inflammatory cell type recruited to the lung is the mast cell. In allergic events, they release histamine, tryptase chymase and cathepsin-G in response to IgE binding. These proteases are found in abundance in the airways and are involved in matrix destruction and remodeling, hydrolyzing chemokines and cytokines as well as inactivating allergens and neuropeptides, and are known to specifically activate PARs (8). Trypsin, tryptase and human airway trypsin have all been reported in the airways and promote allergic inflammation, airway hyperresponsivenss and tissue remodeling (9);(10) and activate PAR_2. There are also polymorphonuclear cells that migrate to the airways under inflammatory conditions, each with a unique cocktail of proteinases that exacerbate the inflammatory responses.

Potential role	*Comment*	*Reviewed by*
CNS neuronal and astrocyte function	Upregulation of PARs observed in the setting of CNS inflammation	Noorbakhsh *et al.*, 2003
Endothelial cell function: (PARs 1, 2 and 4)	Regulate release of NO, von Willebrand factor; increase neutrophil adherence; promote cell migration	Coughlin, 2005
Intestinal function: (PARs 1, 2 and 4)	Regulation of motility (GI smooth muscle) and secretion (GI epithelial cell)	Vergnolle, 2005a
Myenteric neuron function	Also affects GI motility and inflammatory response	Vergnolle, 2003
Vascular smooth muscle function	Activation of contractility; angiogenesis?	Coughlin, 2005
Renal vascular function	Regulation of flow and afferent arteriolar function	Vesey *et al.*, 2007
Skin pigmentation	Proteinase inhibition affects skin pigmentation. Involvement of PAR_2 in ethnic skin color phenotypes	Seiberg, 2001
Hyperalgesia and analgesia	PAR activation can increase (PAR_2 mediated) or decrease (PAR_1 and PAR_4 mediated) pain sensation	Vergnolle, 2005b
Platelet activation, haemostasis: Thrombin-activated receptors (PARs 1, 3, 4)	Regulate both secretion and aggregation; PARs 1 and 4 can play opposing roles possibly due to differential coupling to g-protiens	Coughlin, 2005
Response to joint injury	Use of PAR_2 null mice and PAR antagonists suggests key role for PAR_2 in arthritis	McIntosh *et al.*, 2007
Tumour cell growth and metastasis	Both PARs 1 and 2 may play roles, activated by tumor-derived serine proteinases and matrix metalloproteinases (e.g. MMP-1)	Ruf *et al.*, 2006; Soreide *et al.*, 2006

Abbreviations: CNS, central nervous system; PAR, proteinase-activated receptor

Table 1.1: Potential Physiological Roles for PARs. Courtesy of Ramachandran et al., 2008. BJP.

Section 1.4

Allergens with PAR_2 specificity

Besides the endogenous proteinases released from invading leukocytes, two exogenous proteases gain access to the airways that specifically activate PAR_2. They are cockroach frass from *Blatella germanica* (11)and fungal filtrate from *Alternaria alternata* (12). Because both BG and AltA specifically activate PAR_2, we further investigated their effects in β-arrestin-$2^{-/-}$ mice. These findings will be more discussed in detail in chapter 3. Cockroach frass (or feces), obtained from the German cockroach (*Blattella germanica*) (BG) contain serine proteases shown to specifically activate airway epithelial cells in a PAR_2-dependent fashion (11). The frass contains serine proteases that likely originate in the gastrointestinal track of the cockroach and aid in digestion. Per a 10 is one of the specific allergens found in the frass that has trypsin-like serine protease activity, and thus activates PAR_2 in the airways. Intranasal challenge of the allergen in wt mice results in an increase in number of eosinophils found in the airways, hyperresponsiveness and the increased presence of cockroach-specific IgG1 and total IgE as compared with saline controls. As predicted, $PAR_2^{-/-}$ mice challenged with cockroach frass mice show decreased numbers of total cells and eosinophils in the BALF and reduced airway hyperresponsiveness compared with wt mice challenged with cockroach frass. While the levels of cockroach-specific IgG1 were increased in challenged $PAR_2^{-/-}$ mice, they were reduced in comparison with BG challenged wt mice suggesting that PAR_2 is required for efficient mucosal sensitization to BG, though a lower degree of sensitization can develop in absence of PAR_2.

Alternaria (AltA) is a common fungal mold associated with allergic disease in arid regions of the US Midwest during the spring and summer months and identified as a risk factor for asthma attacks in children and young adults (12, 13) Alt a I is the major allergen found in fungal filtrates localized exclusively in the cell wall of *Alternaria* species spores and may be involved in spore germination, which may explain how it proteolytically activates PAR_2. As the spores are distributed in an airborne fashion and inhaled, they gain access to the airways and induce an IgE antibody response. Alt a 1 has been crystallized and the structure consists of a β-barrel that dimerizes through a disulfide bond and hydrophobic and polar interactions, exposing residues that were reported to be IgE antibody-binding epitopes (13). Intranasal challenge of the allergen in wt mice results in PAR_2-dependent cellular airway inflammation of and heat-inactivation of the allergen abolishes the recruitment (12).

Section 1.5

PAR_2 Protective Responses

A poignant paper that illustrates the protective role of PAR_2, used immunohistochemical analysis to show that PAR_2 co-localized with trypsin in the airway epithelium. PAR_2 peptides that mimic the tethered ligand were applied to airway smooth muscle preparations from mouse, rat, guinea-pig and human and caused relaxation by cyclooxygenase released and these results were confirmed by others (14, 15). In vivo models of LPS-induced airway hyperreactivity also illustrated PAR_2's protective role, as administration of the synthetic PAR_2 agonist activated COX-1 and COX-2 activity and release of [PGE2] that fine-tune the counter-inflammatory response of airway smooth

muscle relaxation. It is also well-known, the PAR_2 activation produces protective relaxation responses in isolated tracheal and bronchial segments obtained from lab animals (16). These responses were dependent on release of cyclooxygenase (COX)-derived relaxant prostanoids form the airway epithelia (15, 17, 18). As these studies were all in vivo, they warrant further investigation into the molecular mechanisms in which PAR_2 might favor the COX/[PGE2] protective response.

In short, the controversy refers to seemingly opposite and opposing responses, or, protective (smooth muscle relaxation) and proinflammatory (proteases from infiltrating leukocytes and/or airborne allergens) responses with exposure to PAR_2 activating peptide. Cartoon 1.3 humorously displays the back-and-forth arguments regarding the role of PAR_2 in the airways and how it might signal both ways (19). See Cartoon 1.4 for summary of the signaling that may explain the duplicitous nature of PAR_2. The proinflammatory effects downstream of PAR_2 activation may be mediated by β-arrestin recruitment and scaffold of actin machinery. The protective effects of Par_2 are likely a results of PGE2 produced in G-protein dependent fashion. The DeFea lab has shown that these two signaling pathways are completely independent of each other and, using a deductive experimental approach, we sought to understand how a single receptor might be responsible for both pro- and anti- inflammatory events in the airways and previous research in the DeFea Lab on G-protein dependent and β-arrestin-dependent pathways downstream of PAR_2 activation served as a starting hypothesis.

Sources and actions of PAR-activating proteinases in the lung.

Proteinase	Source	PAR	Other actions
Thrombin	Blood	PAR_1,PAR_3,PAR_4	Non-PAR receptors exist
Trypsin	Epithelium	PAR_1, PAR_2, PAR_4	Non-specific protease
Mast cell tryptase	Mast cell, basophil	PAR_2	Cleaves several peptides
Factor Xa	Blood	PAR_1,PAR_2	Non-PAR receptors exist
Factor VIIa	Blood	PAR_2	Non-PAR receptors might exist
Neutrophil proteainase-3	Neutrophils	PAR_2	Non-specific elastase
HAT	Epithelium	PAR_2	Non-PAR actions reported
Der P1, P3, P9	Dust mite allergen	PAR_2	Non-PAR actions reported
Cathepsin G	Monocytes, neutrophils	PAR_4	?
Matriptase (MT-SP1)	Epithelium	PAR_2	?

HAT, human airway trypsin-like protease.

Table 1.2 (Top) Sources and actions of PAR-activating proteinases in the lung. Courtesy of Moffatt et al., 2004, Curr Opinion in Pharmacology.

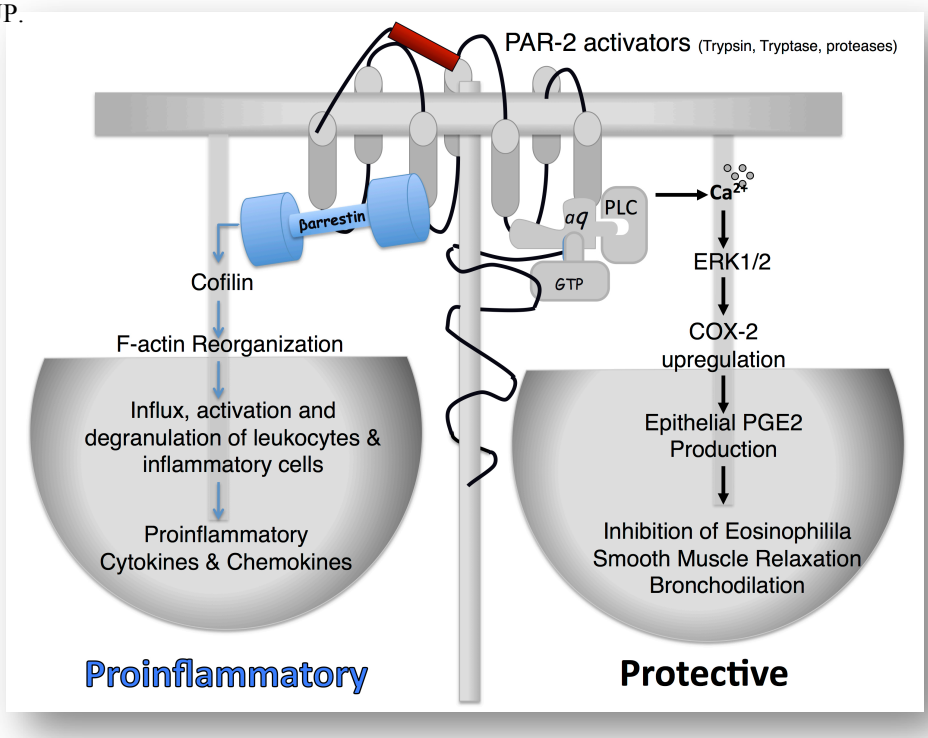
Cartoon 1.3 (Bottom) Is it pro- or anti- inflammatory? The duplicitous nature of PAR_2 in airway inflammation combined with the back-and-forth arguments depicted in the cartoon above have left a 'big dark hole' to possibly be filled by β-arrestin scaffolds of actin migration machinery. Courtesy of Scott Stantis. 2011, Chicago Tribune.

Summary of in vitro findings in pulmonary cells and tissues[a].

Cell or tissue	Species	PAR	Effect
Airway	Mouse	PAR_1	Relaxation/contraction
	Mouse	PAR_4	Relaxation/contraction
	Human	PAR_1	Contraction
	Guinea-pig	PAR_1	Contraction
	Mouse	PAR_2	Relaxation
	Human	PAR_2	Relaxation
	Guinea-pig	PAR_2	Hyperresponsiveness
	Human	PAR_2	Contraction
Airway myocyte	Human	PAR_2	Proliferation
Fibroblast	Mouse	PAR_1	Proliferation
	Human	PAR_1	Procollagen secretion
	Human	PAR_1	Myfibroblast differentiation
	Rat	PAR_1	CTGF secretion
	Human	PAR_1	CTGF secretion
	Human	PAR_2	Proliferation
	Human	PAR_1	IL-8 secretion
Arteries	Human	PAR_1	Relaxation via NO
	Pig	PAR_1	Relaxation via NO
	Pig	PAR_2	Relaxation via NO
	Rat	PAR_2	Relaxation via NO/contraction
Epithelial cell	Human	PAR_1	PDGF secretion
	Human	PAR_1	IL-6, IL-8, PGE_2 secretion
	Human	PAR_2	GMCSF, eotaxin secretion
	Human	PAR_2	MMP-9 secretion
	Human	PAR_2	Decreased ion transport
Neutrophil	Human	PAR_2	CD11b expression (activation)
Eosinophil	Human	PAR_2	Degranulation
Dendritic cell	Mouse	PAR_2	Differentiation from precursor

Table 1.3 (Above) Summary of in vitro findings in pulmonary cells and tissues. Courtesy of Moffatt et al., 2004, Curr Opinion in Pharmacology.

Cartoon 1.4 (Below) PAR_2-mediated airway inflammation is a balancing act. Both G-protein mediated responses and β-arrestin-dependent responses occur downstream of PAR_2. Evidence in the DeFea Lab suggests β-arrestin-2 serves as a molecular switch (shown above as a bar bell) to tip the scales, favoring a proinflammatory response. Adopted from a cartoon published by Peter J. Henry, (2006) EJP.



Section 1.6

Targeting GPCRs with putative therapeutics

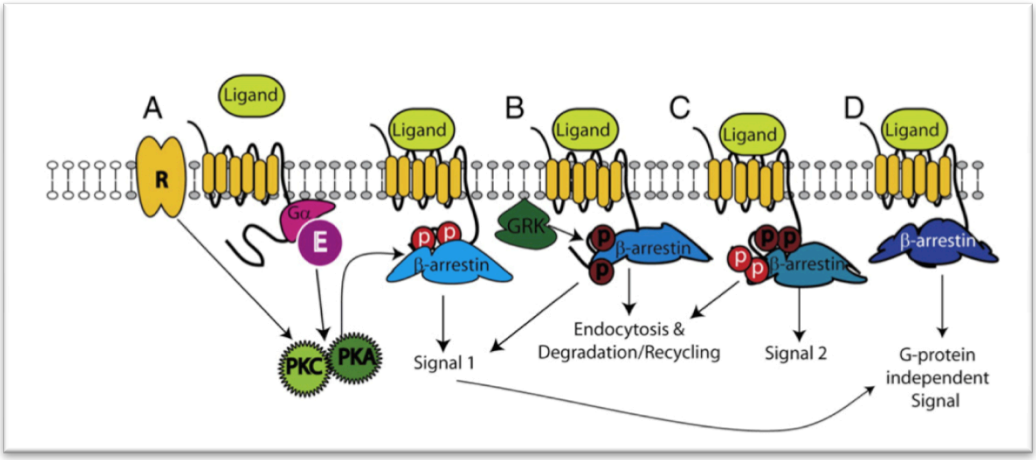
For a number of GPCRs, e.g. Angiotensin IIa Receptor, β_2-adrenergic receptor and parathyroid hormone receptor, and Parathyroid hormone receptor, biased agonists that trigger only the heterotrimeric G-protein pathway or the β-arrestin pathway have been developed and there is a growing interest in developing more biased agonists of GPCRs as therapeutics (Cartoon 1.2 C). Although β-agonists are bronchodilators of choice to alleviate acute bronchospasm, their chronic use is associated with loss of bronchoprotection, worsening of asthma control and asthma-related death. In murine models, the asthma phenotype is significantly promoted by chronic β-agonist treatment (20) or impaired in mice having genetic or pharmacologic ablation of β_2-ARs. Similarly, mice lacking β-arrestin-2 do not develop the asthma phenotype (21), placing β-arrestin-2 downstream of GPCRs in the pro-asthmatic signaling pathway. Thus, the seemingly paradoxical effects of PAR_2 and β_2-AR agonists on the asthma phenotype can likely be explained by the activation of dual signaling pathways; a pro-inflammatory β-arrestin-dependent signaling pathway in addition to the classical G protein-mediated bronchorelaxation pathway.

Section 1.7

An emerging paradigm for β-arrestins

β-arrestins were known for their classical role to desensitize, internalize and recycle GPCRs. A relatively new paradigm is emerging that highlights the ability of β-arrestins

to scaffold proteins in microdomains and spatially regulate activity (Cartoon 1.5) (22) . They play a requisite role in chemotaxis and reorganization of the actin cytoskeleton, downstream of multiple receptors (reviewed in refs (23); (24);(25). The DeFea Lab demonstrates the importance of PAR_2 induced β-arrestin-dependent scaffolds of the MAPK cascade and actin migration machinery. Here, I will focus on the identification of β-arrestin-dependent actin assembly scaffolds that serve to orchestrate actin cytoskeletal rearrangements evidenced by the observation that β-arrestins promote chemotaxis in both cultured cells and primary leukocytes. Currently, there are still multiple theories as to how β-arrestins contribute to actin reorganization and chemotaxis. While some studies strongly support the hypothesis that β-arrestins scaffold signaling molecules at the leading edge to enhance actin assembly, others suggest a more reciprocal role of the actin cytoskeleton in receptor internalization. The former hypothesis is supported by the identification of signaling scaffolds that enhance actin polymerization and membrane protrusion and by the fact that β-arrestin biased agonists can promote chemotaxis. The latter hypothesis is supported by evidence that many of the targets of β-arrestin-dependent scaffolding are cytoskeletal proteins involved in clathrin-mediated endocytosis. As tight spatio-temporal control over both actin assembly and receptor internalization is important for chemotaxis, both theories still appear to be valid.



Cartoon 1.5 Potential role of Phosphorylation in β-arrestin recruitment and signaling. Model depicting 4 potential scenarios in which GPCR phosphorylation affects β-arrestin recruitment and downstream signaling. In (A) second messenger kinases, e.g. PKC and PKA can be activated either by a heterologous receptor (R), in the absence of GPCR agonist, or downstream of ligand binding to a given GPCR. In either case, β-arrestins to the GPCR to generate signal 1. If this occurs in the absence of agonist binding, it is an example of G-protein independent signaling. In (B) GRK can phosphorylate a ligand bound GPCR to recruit β-arrestin, leading to receptor internalization or to the same signal observed in response to second messenger kinase phosphorylation. In (C), ligand binding to a GPCR results in GRK phosphorylation and activation of second messenger kinases that all phosphorylate the receptor. β-arrestin is recruited to the phosphorylated receptor but in this case generates a different signal (signal 2) that is dependent on phosphorylation by multiple kinases and upon heterotrimeric G-protein activation. This complex may also promote receptor internalization. In (D) ligand bound GPCR recruits β-arrestin independent of phosphorylation to generate a G-protein independent signal. Courtesy of K. DeFea (2011) Cellular Signaling

Section 1.8

β-arrestin dependent chemotaxis and disease:

β-arrestin-dependent actin machinery scaffolds and chemotaxis suggests their importance in mediating leukocyte responses to inflammation and tumor cell metastasis. Further, biased ligands can induce β-arrestin dependent chemotaxis in the absence of G-protein engagement (26, 27), (28). Inappropriate regulation of cytoskeleton can have pathological consequences, leading to tumor cell metastasis, uncontrolled inflammation and developmental defects. A prime example is the proposed role of β-arrestin-mediated chemotaxis in cancer progression and metastasis (29-31). Two studies have shown that β-arrestin-dependent signaling contributes to cancer cell migration: CXCR4-mediated p38 activation in HELA and HEK-293 cells (31) and PAR_2-activated ERK1/2 activation in MDA MB-231 breast cancer cells (29). Silencing of siRNA or MAPK (either p38MAPK or ERK1/2) reduced constitutive migration of tumor cells and β-arrestin levels and basal ERK1/2 activation were higher in metastatic than non- metastatic cell lines. More recent studies have suggested that LPA Receptor stimulates cell migration and cytoskeletal reorganization in MDA-MB 231 and this is reduced with expression of a mutant of RalGDS ($RalGDS^{616-768}$) that is deficient in β-arrestin binding (30). Another study showed that Prostaglandin E2 [PGE2] induces lung cancer cell migration via EP4/β-arrestin-1/c-Src signaling complex (32), suggesting β-arrestin can result in differential modulation of signaling by bringing target proteins into scaffolding complexes in response to extracellular signals.

Evidence that β-arrestins promote leukocyte chemotaxis and that allergic asthma is reduced in β-arrestin-2$^{-/-}$ mice suggests that they are also important mediators of inflammation (21, 33) or after small interfering RNA knockdown of β-arrestin-2. Studies have also demonstrated that β-arrestin-2 augments activation of MAPKs and cofilin in leukocytes (31, 34, 35). Over the years many chemokine receptors have been shown to require β-arrestins for chemotaxis and receptor desensitization/internalization. There have been only a few mechanistic studies on chemokine receptors to address the role β-arrestin-dependent scaffolding as opposed to internalization in chemotaxis. A recent study also proposed a role for a β-arrestin scaffold in CXCR4-mediated chemotaxis in WHIM syndrome (WS). WHIM syndrome is an immunodeficiency syndrome linked to heterozygous mutations of the CXCR4 resulting in a truncated receptor that lacks the last 15 residues of the C-tail. In the aforementioned study, it was shown that in leukocytes from WS patients the mutant receptor was recruited more slowly and to a lesser extent than wild type CXCR4, suggesting that defective β-arrestin signaling may contribute to WS (33).

Section 1.9

β-arrestins scaffold cell migration machinery

A major goal of my research was to define the molecular mechanisms of β-arrestin-dependent chemotaxis, and the regulation of cofilin. β-arrestins are multifunctional proteins that mediate receptor desensitization and internalization, and serve as signaling scaffolds. A role for β-arrestin scaffolds in signaling by PAR_2 and other receptors was first identified for the spatial regulation of ERK1/2 activity (29, 36,

37). They are now known to scaffold numerous other signaling molecules (28, 38-41), many of which are involved in actin reorganization and chemotaxis **(42-45).** An attractive hypothesis is that β-arrestins exert spatial control over actin assembly events at the leading edge to promote membrane protrusion and cell migration. A recent advance in this field was the discovery that β-arrestins are required for PAR_2-dependent activation of the actin filament severing protein, cofilin which binds to the sides of actin filaments, destabilizing them and promoting their severing. Filament severing has two functions: the reorganization of existing filaments and the creation of free actin barbed ends for monomer addition (46). Actin is a polar molecule with a barbed and pointed end containing; addition of actin monomers to a growing filament occurs at the barbed end. Although actin monomers spontaneously assemble into filaments very slowly, the generation of multiple small filaments with free barbed ends increases the rate of actin assembly dramatically. Thus, the presence of active cofilin within the leading edge of a migrating cell controls the availability of polymerization competent free barbed ends, which in turn is required for membrane protrusion and cell migration (28, 35). Spatial control over cofilin activity is essential, as either too much or too little actin filament-severing activity will inhibit efficient cell migration.

Section 1.10

Proteinase receptors and modulation of trafficking by β-arrestins to induce Chemotaxis in inflammatory cells by scaffolding actin migration machinery CIN and cofilin

My research in the DeFea Lab began in 2008 to study the protein binding domains

of CIN, cofilin and β-arrestins. That work would continue the investigations begun by Maria Zoudilova. The remaining background and Part B of Chapter 1 summarizes Maria's biochemical investigations into the relationship between the aforementioned protein scaffold which eventually led to chemotaxis in primary cell lines. At this point, I chose to continue her discoveries by evaluating β-arrestins in an in vivo model given the wealth of studies demonstrating a role for PAR_2 in mediating leukocyte infiltration during inflammation, primary leukocytes isolated from bone marrow of wild type, β-arrestin-$1^{-/-}$ mice and β-arrestin-$2^{-/-}$ mice and first demonstrated that the PAR_2/β-arrestin/cofilin pathway is relevant in these cells by examining cofilin phosphorylation levels and PAR_2 stimulated cell migration. My lab mate Alice Lin, took over the protein binding investigations. Continue reading for the background studies in the lab that led my thesis project.

Bone marrow preparations consisted of 55-60% neutrophils, 24-28% lymphocytes, 14-16% monocytes and less than 1% eosinophils; no significant differences were observed in the distribution of these cell types between wt and β-arrestin-1 or 2 knockout mice (Figure 1.1 A). PAR_2 also promotes β-arrestin-dependent cell migration in leukocytes, as it does in other cell lines (29, 35, 47), by seeding them onto Transwell filters and treating with or without the specific PAR_2 peptide agonist, 2-furoyl-LIGRL (2fAP) for 3 hours. Non-migratory cells were removed from the top of the membrane, and cells that had migrated to the filter underside were stained and counted. PAR_2 promoted an 8-fold increase in migration in wt leukocytes and this was reduced to 1.6-fold in the absence of β-arrestin-2 (Figure 1.1 B, C). This effect was not restricted to

bone marrow leukocytes, as PAR_2 also promoted an 8.5-fold increase in cell migration in wild type neutrophils and this was reduced to 2.2- and 2-fold in neutrophils from β-arrestin-$1^{-/-}$ and β- arrestin-$2^{-/-}$ mice, respectively (data not shown).

Cofilin activation is controlled by opposing actions of LIMKs (which inactivate it by phosphorylation on Ser3) and cofilin-specific phosphatases (chronophin (CIN) and slingshot) that activate it (46, 48, 49), and by intracellular pH and PIP_2 levels (46). PAR_2 promotes rapid cofilin dephosphorylation that is decreased in the absence of β-arrestins or by expression of a dominant negative CIN mutant (35). PAR_2 activation results in recruitment of CIN and cofilin to β-arrestins into a scaffolding complex to promote localized generation of free barbed ends and membrane protrusion. We have previously shown: 1) Cofilin activation via PAR_2, was dependent on β-arrestin-2; 2) Dephosphorylation of cofilin involved both facilitation of CIN and inhibition of LIMK; and 3) β-arrestins and CIN are required for PAR_2-stimulated barbed end generation and subsequent membrane protrusion. PAR_2 stimulated cofilin activation is abolished by siRNA depletion of β-arrestins, expression of a dominant negative β-arrestin mutant, or genetic deletion of β-arrestins (28).

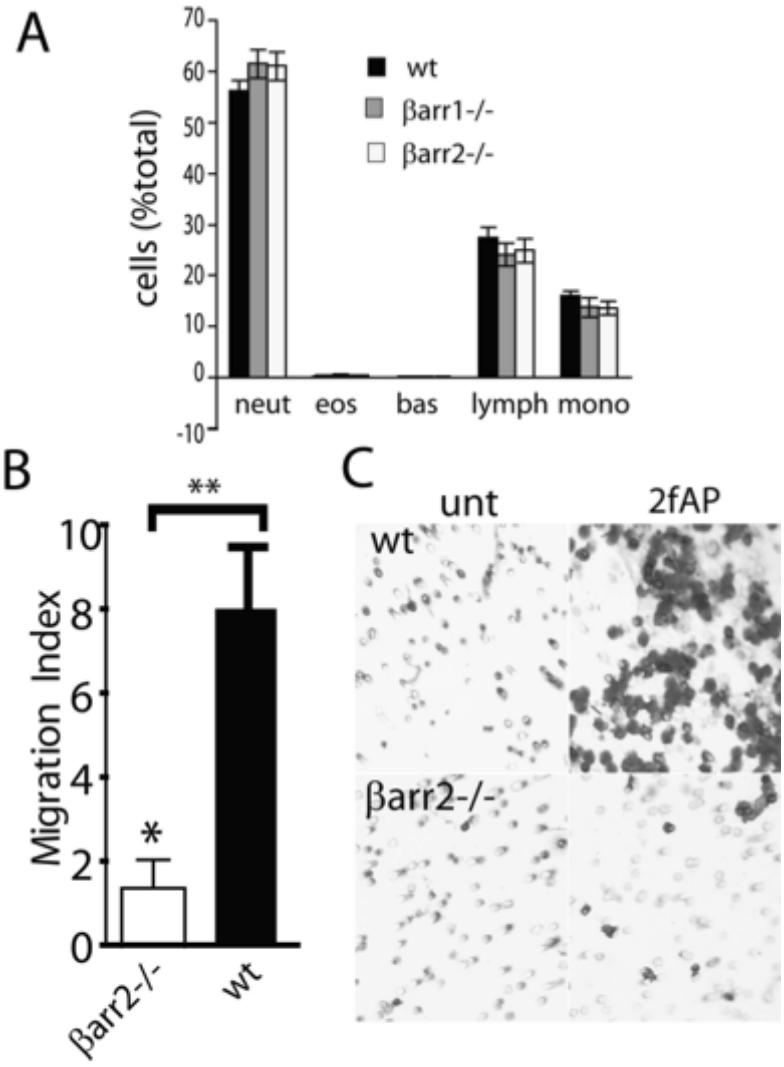


Figure 1.1 β-arrestins are required for PAR_2 stimulated primary leukocyte migration. A. Percentages of lymphocyte, neutrophil, eosinophil, monocyte precursors and mature cells were determined from Cytospin preparations of bone marrow leukocytes from wild type (wt), β-arrestin-$1^{-/-}$ and β-arrestin-$2^{-/-}$ mice. **B.** Graph showing fold increase (over PBS-treated controls) in migration of leukocytes from wt and β-arrestin-$2^{-/-}$ mice treated with 2fAP (mean +/- SEM, n=3). Statistically significant increase in response to 2fAP versus PBS. **C.** Image of migrated cells on underside of filter. Courtesy of Zoudilova et al., 2010. JBC.

Section 1.11

References

1. Reiter E, Ahn S, Shukla AK, & Lefkowitz RJ (2012) Molecular mechanism of beta-arrestin-biased agonism at seven-transmembrane receptors. (Translated from eng) *Annu Rev Pharmacol Toxicol* 52:179-197 (in eng).
2. Katritch V, Cherezov V, & Stevens RC (2012) Diversity and modularity of G protein-coupled receptor structures. (Translated from eng) *Trends Pharmacol Sci* 33(1):17-27 (in eng).
3. Adams MN*, et al.* (2011) Structure, function and pathophysiology of protease activated receptors. (Translated from eng) *Pharmacol Ther* 130(3):248-282 (in eng).
4. Ramachandran R, Noorbakhsh F, DeFea K, & Hollenberg MD (2012) Targeting proteinase-activated receptors: therapeutic potential and challenges. *Nat Rev Drug Discov* 11(1):69-86.
5. Ramachandran R & Hollenberg MD (2008) Proteinases and signalling: pathophysiological and therapeutic implications via PARs and more. (Translated from eng) *Br J Pharmacol* 153 Suppl 1:S263-282 (in eng).
6. Moffatt JD, Page CP, & Laurent GJ (2004) Shooting for PARs in lung diseases. (Translated from eng) *Curr Opin Pharmacol* 4(3):221-229 (in eng).
7. Schmidlin F*, et al.* (2002) Protease-activated receptor 2 mediates eosinophil infiltration and hyperreactivity in allergic inflammation of the airway. (Translated from eng) *J Immunol* 169(9):5315-5321 (in eng).
8. Reed CE & Kita H (2004) The role of protease activation of inflammation in allergic respiratory diseases. (Translated from eng) *J Allergy Clin Immunol* 114(5):997-1008; quiz 1009 (in eng).
9. Cairns JA (1998) Mast cell tryptase and its role in tissue remodelling. (Translated from eng) *Clin Exp Allergy* 28(12):1460-1463 (in eng).
10. Berger P*, et al.* (1999) Mast cell tryptase as a mediator of hyperresponsiveness in human isolated bronchi. (Translated from eng) *Clin Exp Allergy* 29(6):804-812 (in eng).
11. Arizmendi NG*, et al.* (2011) Mucosal allergic sensitization to cockroach allergens is dependent on proteinase activity and proteinase-activated receptor-2 activation. (Translated from eng) *J Immunol* 186(5):3164-3172 (in eng).
12. Boitano S*, et al.* (2011) Alternaria alternata serine proteases induce lung inflammation and airway epithelial cell activation via PAR2. (Translated from eng) *Am J Physiol Lung Cell Mol Physiol* 300(4):L605-614 (in eng).
13. Chruszcz M*, et al.* (2012) Alternaria alternata allergen Alt a 1: a unique beta-barrel protein dimer found exclusively in fungi. (Translated from eng) *J Allergy Clin Immunol* 130(1):241-247 e249 (in eng).
14. Lan RS, Stewart GA, & Henry PJ (2000) Modulation of airway smooth muscle tone by protease activated receptor-1,-2,-3 and -4 in trachea isolated from

influenza A virus-infected mice. (Translated from eng) *Br J Pharmacol* 129(1):63-70 (in eng).
15. Chow JM, Moffatt JD, & Cocks TM (2000) Effect of protease-activated receptor (PAR)-1, -2 and -4-activating peptides, thrombin and trypsin in rat isolated airways. (Translated from eng) *Br J Pharmacol* 131(8):1584-1591 (in eng).
16. De Campo BA & Henry PJ (2006) Protease-activated receptors (PARs) are partly pro-inflammatory and partly anti-inflammatory: will PAR agonists or antagonists participate in future drug therapies? (Translated from eng) *Curr Drug Targets* 7(5):629-637 (in eng).
17. Cocks TM*, et al.* (1999) A protective role for protease-activated receptors in the airways. (Translated from eng) *Nature* 398(6723):156-160 (in eng).
18. Kawabata A*, et al.* (2004) Distinct roles for protease-activated receptors 1 and 2 in vasomotor modulation in rat superior mesenteric artery. (Translated from eng) *Cardiovasc Res* 61(4):683-692 (in eng).
19. Stantis S (2011) Prickly City Comic. *Chicago Tribune*.
20. Lin R*, et al.* (2012) Chronic treatment in vivo with beta-adrenoceptor agonists induces dysfunction of airway beta(2) -adrenoceptors and exacerbates lung inflammation in mice. (Translated from eng) *Br J Pharmacol* 165(7):2365-2377 (in eng).
21. Walker JK*, et al.* (2003) Beta-arrestin-2 regulates the development of allergic asthma. (Translated from eng) *J Clin Invest* 112(4):566-574 (in eng).
22. DeFea KA (2011) Beta-arrestins as regulators of signal termination and transduction: how do they determine what to scaffold? (Translated from eng) *Cell Signal* 23(4):621-629 (in eng).
23. Cotton M & Claing A (2009) G protein-coupled receptors stimulation and the control of cell migration. (Translated from eng) *Cell Signal* 21(7):1045-1053 (in eng).
24. Defea K, Schmidlin F, Dery O, Grady EF, & Bunnett NW (2000) Mechanisms of initiation and termination of signalling by neuropeptide receptors: a comparison with the proteinase-activated receptors. (Translated from eng) *Biochem Soc Trans* 28(4):419-426 (in eng).
25. DeWire SM, Ahn S, Lefkowitz RJ, & Shenoy SK (2007) Beta-arrestins and cell signaling. (Translated from eng) *Annu Rev Physiol* 69:483-510 (in eng).
26. Hunton DL*, et al.* (2005) Beta-arrestin 2-dependent angiotensin II type 1A receptor-mediated pathway of chemotaxis. (Translated from eng) *Mol Pharmacol* 67(4):1229-1236 (in eng).
27. Xiao K*, et al.* (2010) Global phosphorylation analysis of beta-arrestin-mediated signaling downstream of a seven transmembrane receptor (7TMR). (Translated from eng) *Proc Natl Acad Sci U S A* 107(34):15299-15304 (in eng).
28. Zoudilova M*, et al.* (2010) beta-Arrestins scaffold cofilin with chronophin to direct localized actin filament severing and membrane protrusions downstream of protease-activated receptor-2. (Translated from eng) *J Biol Chem* 285(19):14318-14329 (in eng).

29. Ge L, Shenoy SK, Lefkowitz RJ, & DeFea K (2004) Constitutive protease-activated receptor-2-mediated migration of MDA MB-231 breast cancer cells requires both beta-arrestin-1 and -2. (Translated from eng) *J Biol Chem* 279(53):55419-55424 (in eng).
30. Li JP & Vlodavsky I (2009) Heparin, heparan sulfate and heparanase in inflammatory reactions. (Translated from eng) *Thromb Haemost* 102(5):823-828 (in eng).
31. Sun Y, Cheng Z, Ma L, & Pei G (2002) Beta-arrestin2 is critically involved in CXCR4-mediated chemotaxis, and this is mediated by its enhancement of p38 MAPK activation. (Translated from eng) *J Biol Chem* 277(51):49212-49219 (in eng).
32. Kim JI, Lakshmikanthan V, Frilot N, & Daaka Y (2010) Prostaglandin E2 promotes lung cancer cell migration via EP4-betaArrestin1-c-Src signalsome. (Translated from eng) *Mol Cancer Res* 8(4):569-577 (in eng).
33. Lagane B*, et al.* (2008) CXCR4 dimerization and beta-arrestin-mediated signaling account for the enhanced chemotaxis to CXCL12 in WHIM syndrome. (Translated from eng) *Blood* 112(1):34-44 (in eng).
34. Fong AM*, et al.* (2002) Defective lymphocyte chemotaxis in beta-arrestin2- and GRK6-deficient mice. (Translated from eng) *Proc Natl Acad Sci U S A* 99(11):7478-7483 (in eng).
35. Zoudilova M*, et al.* (2007) Beta-arrestin-dependent regulation of the cofilin pathway downstream of protease-activated receptor-2. (Translated from eng) *J Biol Chem* 282(28):20634-20646 (in eng).
36. DeFea KA*, et al.* (2000) beta-arrestin-dependent endocytosis of proteinase-activated receptor 2 is required for intracellular targeting of activated ERK1/2. (Translated from eng) *J Cell Biol* 148(6):1267-1281 (in eng).
37. Canto I Fau - Soh UJK*, et al.* (Allosteric modulation of protease-activated receptor signaling)
38. Zhang P, He X, Tan J, Zhou X, & Zou L (2011) beta-arrestin2 mediates beta-2 adrenergic receptor signaling inducing prostate cancer cell progression. (Translated from eng) *Oncol Rep* 26(6):1471-1477 (in eng).
39. Sun J & Lin X (2008) Beta-arrestin 2 is required for lysophosphatidic acid-induced NF-kappaB activation. (Translated from eng) *Proc Natl Acad Sci U S A* 105(44):17085-17090 (in eng).
40. Schreiber G, Golan M, & Avissar S (2009) Beta-arrestin signaling complex as a target for antidepressants and as a depression marker. (Translated from eng) *Drug News Perspect* 22(8):467-480 (in eng).
41. Mo W*, et al.* (2008) Nuclear beta-arrestin1 functions as a scaffold for the dephosphorylation of STAT1 and moderates the antiviral activity of IFN-gamma. (Translated from eng) *Mol Cell* 31(5):695-707 (in eng).
42. Scott MG*, et al.* (2006) Cooperative regulation of extracellular signal-regulated kinase activation and cell shape change by filamin A and beta-arrestins. (Translated from eng) *Mol Cell Biol* 26(9):3432-3445 (in eng).

43. Penela P, Ribas C, Aymerich I, & Mayor F, Jr. (2009) New roles of G protein-coupled receptor kinase 2 (GRK2) in cell migration. (Translated from eng) *Cell Adh Migr* 3(1):19-23 (in eng).
44. Miller WE*, et al.* (2001) Identification of a motif in the carboxyl terminus of beta -arrestin2 responsible for activation of JNK3. (Translated from eng) *J Biol Chem* 276(30):27770-27777 (in eng).
45. Ge L, Ly Y, Hollenberg M, & DeFea K (2003) A beta-arrestin-dependent scaffold is associated with prolonged MAPK activation in pseudopodia during protease-activated receptor-2-induced chemotaxis. (Translated from eng) *J Biol Chem* 278(36):34418-34426 (in eng).
46. Huang TY, DerMardirossian C, & Bokoch GM (2006) Cofilin phosphatases and regulation of actin dynamics. (Translated from eng) *Curr Opin Cell Biol* 18(1):26-31 (in eng).
47. Nichols HL*, et al.* (2012) beta-Arrestin-2 mediates the proinflammatory effects of proteinase-activated receptor-2 in the airway. (Translated from eng) *Proc Natl Acad Sci U S A* 109(41):16660-16665 (in eng).
48. Wang Y, Shibasaki F, & Mizuno K (2005) Calcium signal-induced cofilin dephosphorylation is mediated by Slingshot via calcineurin. (Translated from eng) *J Biol Chem* 280(13):12683-12689 (in eng).
49. Soosairajah J*, et al.* (2005) Interplay between components of a novel LIM kinase-slingshot phosphatase complex regulates cofilin. (Translated from eng) *EMBO J* 24(3):473-486 (in eng).

Chapter 1: Introduction

Molecular Mechanisms Linking Receptor Activation to Actin Reorganization: PAR_2 Signaling via β-arrestins

Part B: Introduction

Section 1.12

My thesis work investigates the important role *in vivo* that PAR_2 plays in the recruitment of leukocytes to the sites of inflammation, as this is impaired in $PAR_2^{-/-}$ mice and enhanced by administration of PAR_2 agonists (3-8). Recently, proteases found in cockroach frass from *Blatella germanica* and fungal isolates of *Alternaria alternata* have also been shown to recruit leukocytes in a PAR_2-dependent fashion (43; 44). These studies are the first to link β-arrestin-dependent scaffolding of actin assembly proteins to PAR_2 stimulated chemotaxis under physiological conditions. The findings from our mouse models of PAR_2 exacerbated ova-induced airway and BG- or AltA- PAR_2-dependent allergen inflammation will be presented in further detail in Chapters 2 and 3, respectively, of this dissertation.

Section 1.13

Experiments which served as a springboard for the PAR_2-dependent airway inflammation model.

PAR_2 promotes the formation of a β-arrestin/cofilin/CIN association in primary leukocytes and cultured cells. In PAR_2 stimulated cells, cofilin dephosphorylation was abolished by expression of dominant negative CIN, suggesting that β-arrestins scaffold CIN with cofilin to promote its dephosphorylation and activation, which is ultimately necessary for migration. Former DeFea Lab member Maria Zoudilova used size exclusion chromatography (SEC) coupled with co-immunoprecipitations to investigate whether β-arrestins form an oligomeric complex with CIN and cofilin. Maria skillfully

demonstrated that isolated bone marrow leukocytes treated with 2fAP for 5 min, then lysed and fractionated by SEC on Sephacryl S300 to identify higher molecular weight fractions; contained the putative protein complex. Co-elution and thus association of the proteins was then confirmed by co-immunoprecipitation from these same fractions. In response to PAR_2 activation wild type mice, endogenous β-arrestin-1 and 2, cofilin and CIN co-eluted in fractions corresponding to a Stokes radius of approximately 4.85-5.3nm (Figure 1.2-1 A, B), which is distinctly larger than those reported for either cofilin or β-arrestins alone (30;31). To better illustrate co-elution, for each protein, the fraction of total protein present in each elution was calculated and graphed as a function of the Stoke's radius (Figure 1.2-1 C, D). To prove that co-elution of these proteins reflected their presence in a complex, the fractions corresponding to a 4.5-6nm Stoke's radius were immunoprecipitated with either with anti-β-arrestin-1/2 or IgG, and immune complexes analyzed by SDS-PAGE followed by western blotting with anti-CIN, anti-cofilin and anti-β-arrestin-1/2 (Figure 1.2 -1 E) (50).

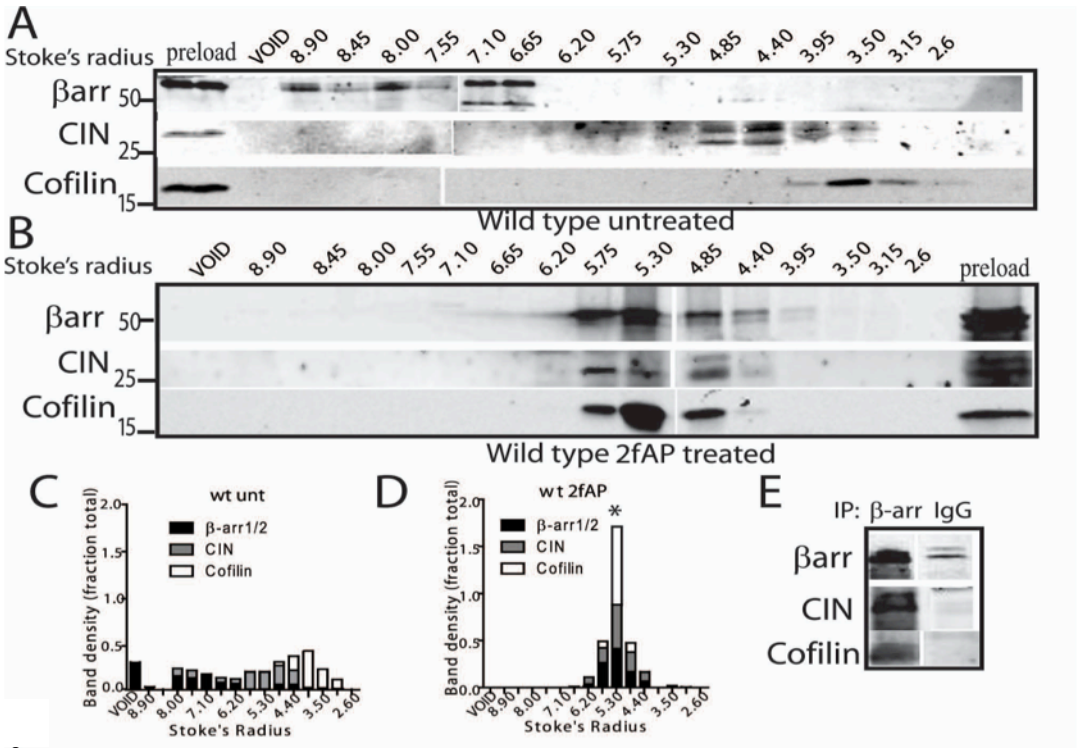


Figure 1.2-1 β-arrestins are required to scaffold CIN with cofilin in primary leukocytes. A, B. Leukocytes from wild type mice were treated with or without 2fAP and analyzed by size exclusion chromatography (SEC). Protein from every other 7ml fraction within the included volume was analyzed by SDS-PAGE followed by western blotting with antibodies to β-arrestins, CIN and cofilin. For wt cells, both untreated (A) and 2fAP treated (B) samples are shown. **C.,D.** Stacked bar graphs showing distribution of each protein over the column as a function of Stoke's Radius are shown for each treatment group. (Band intensity in each elution was divided by the sum of band intensities in all fractions). **E.** Fractions from 2fAP-treated wild type mice (B), in which cofilin/CIN and β-arrestins co-eluted, were pooled and immunoprecipitated with anti-β-arrestin-1/2 or IgG (negative control), followed by western analysis with anti-β-arrestin1/2, CIN and cofilin. Zoudilova et al., 2010. JBC.

The gel filtration experiment was then repeated in leukocytes from β-arrestin-$1^{-/-}$ and β-arrestin-$2^{-/-}$ mice (Figure 1.2-2 F-I). PAR_2 -stimulated co-elution of cofilin with CIN was only severely impaired in β-arrestin-$2^{-/-}$ leukocytes and some co-elution of CIN and cofilin with β-arrestin-2 was still observed in β-arrestin-1-/- leukocytes. These data suggest a major role for β-arrestin-2 in mediating cofilin/CIN association. Consistent with this hypothesis, PAR_2 promoted co-immunoprecipitation of CIN with cofilin in total leukocyte lysates from wild type mice and this was reduced by ~60% in β-arrestin-$2^{-/-}$ leukocytes (Figure 1.2-2 J). These data suggest that β-arrestins are required for PAR_2 stimulated primary leukocyte chemotaxis and recruit cofilin into a scaffolding complex with CIN in these cells (50)..

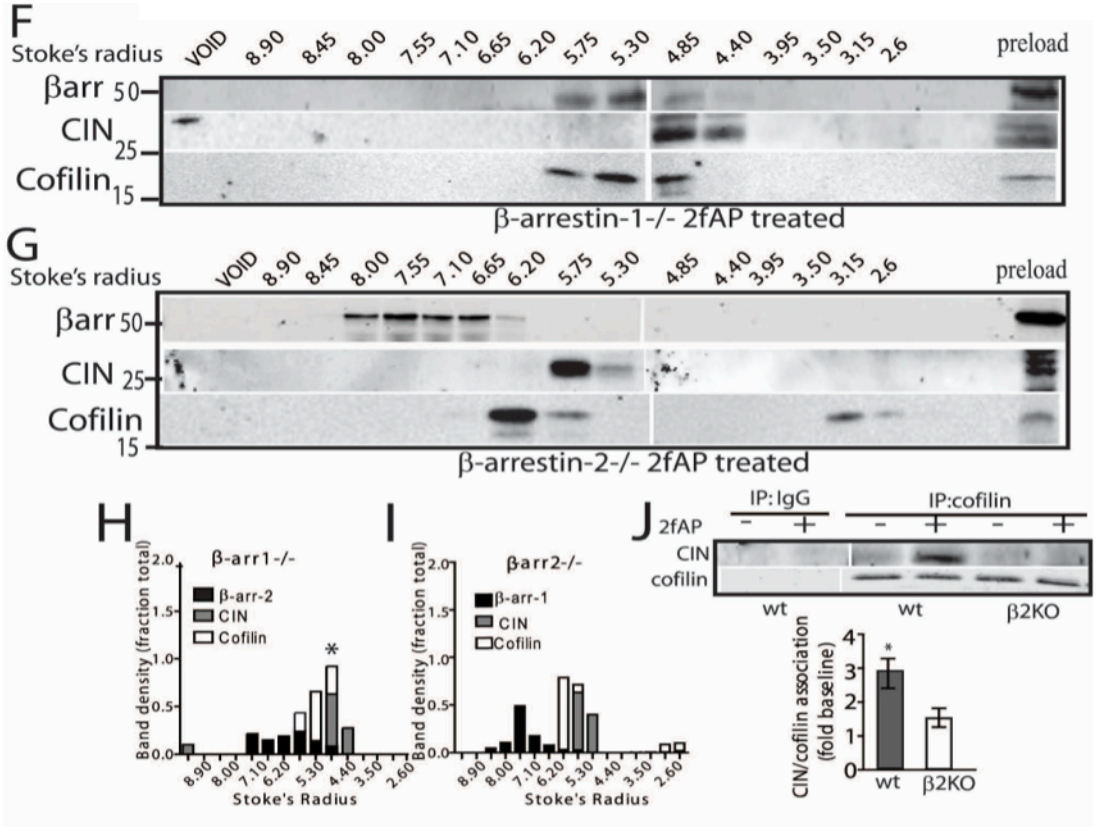


Figure 1.2-2 SEC was repeated on 2fAP-treated leukocytes from β-arrestin-1$^{-/-}$ (**F**) and β-arrestin-2$^{-/-}$ (**G**) knockout mice. **H, I.** Stacked bar graphs of protein distribution as describe for C, D are shown. **J.** Total lysates from wt or β-arrestin-2$^{-/-}$ bone marrow leukocytes, treated with or without 2fAP, were immunoprecipitated with anti-cofilin or IgG, followed by western analysis with anti-cofilin and anti-CIN. Integrated intensities of immunoprecipitated CIN and cofilin were calculated. CIN was normalized to the amount of cofilin present in each lane and the fold increase in CIN/cofilin co-immunoprecipitated after PAR-2 stimulation, compared with untreated controls was determined. *indicates a statistically significant increase in PAR-2 stimulated co-immunoprecipitation, p=.025. Co-immunoprecipitation of cofilin with CIN was reduced by 60% in the absence of β-arrestin-2 (p=.04), n=4. Zoudilova et al., 2010. JBC.

These findings were confirmed in MDA MB-468 cells, the breast cancer cell line in which PAR_2 stimulated, β-arrestin-dependent cofilin activation was originally demonstrated. MDA MB-468 cells were treated with or without 2fAP and fractionated by SEC as described for leukocytes. Once again β-arrestins co-eluted with CIN and cofilin in fractions corresponding to a Stoke's radius of 5.3-6.2 after 2fAP-treatment (Figure 1.3 A). That these proteins exist in an oligomeric complex was again confirmed by the fact that cofilin and β-arrestins could be co-immunoprecipitated with CIN from the fractions in which they co-eluted (Figure 1.3 D). The slight variation in size of eluted complexes between cell types may reflect differences in the presence of other proteins, in each complex, differences between human and mouse complexes, or cell-type specific differences in complex composition. Other studies on β-arrestin/src and β-arrestin/ERK complexes have demonstrated a similar disparity in complex size between species and cell type (10;33). In untreated cells this complex was not detected. In fact, the majority of CIN eluted in early fractions and in the void volume, consistent with its aggregation into very large complexes; a similar phenomenon was observed with src in prior studies (33). Furthermore, β-arrestins and cofilin were co-immunoprecipitated with CIN from total cell lysates and this co-precipitation was significantly increased upon PAR_2 activation (Figure 1.3 E, F). Thus, PAR_2 promotes the formation of a scaffolding complex containing β-arrestin-1 and 2, CIN and cofilin in multiple motile cell types (50).

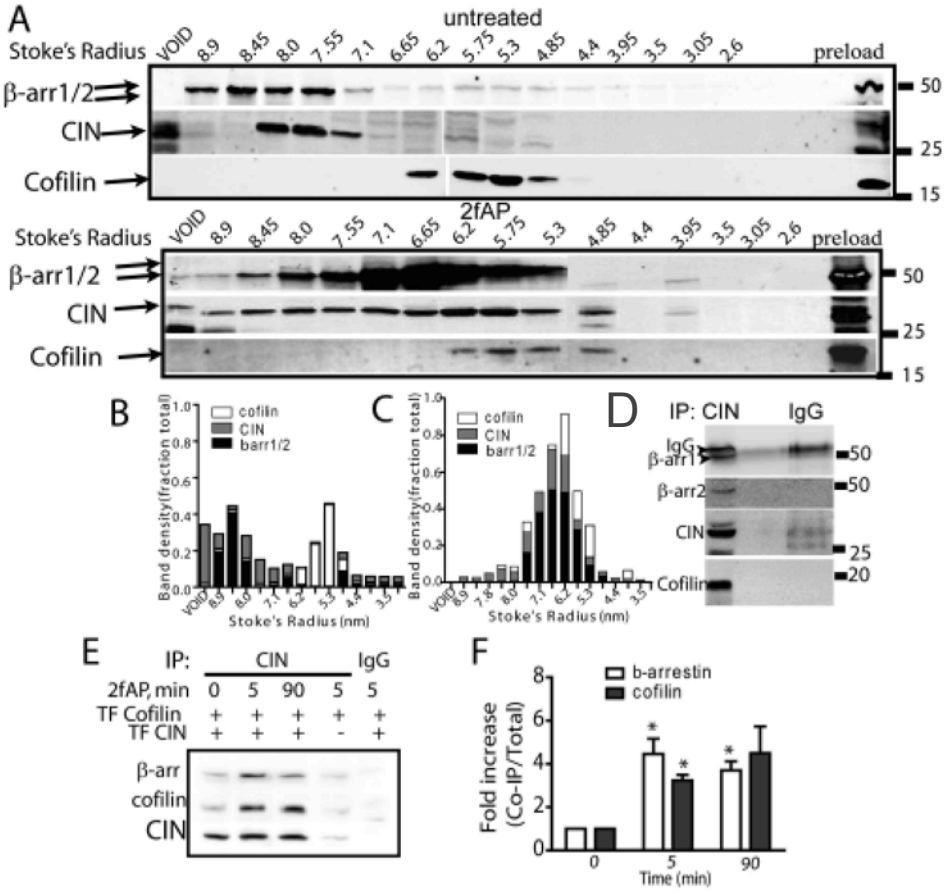


Figure 1.3. Formation of a scaffolding complex containing β-arrestins, CIN and cofilin in breast cancer cells. A. MDA MB-468 cells were transfected with myc-CIN and treated with or without 2fAP for 5 minutes. Cell lysates were fractionated by SEC followed by western blotting with antibodies to β-arrestins, cofilin and myc (to visualize CIN) as described in Fig. 2. **B-C.** Graphs showing distribution of each protein over the column as a function of Stoke's radius are shown for untreated (**B**) and 2fAP-treated (**C**) samples. **D.** Co-immunoprecipitation of CIN (using anti-myc) with β-arrestins and cofilin was performed on pooled samples from 2fAP-treated cells in which proteins co-eluted. **E.** Co-immunoprecipitation of GFP-cofilin and endogenous β-arrestins with myc-CIN in MDA MB-468 cells, treated with or without 2fAP for 0-90 minutes. Lanes 4 and 5 are negative controls: anti-myc and anti-IgG IP from cells transfected with GFP-cofilin alone. **F.** Graph depicting fold increase in the amount of β-arrestin and cofilin associated with CIN upon PAR-2 activation. *indicates a statistically significant increase in PAR_2 stimulated co-immunoprecipitation, p=.01, n=3. Zoudilova et al., 2010. JBC.

Section 1.14

β-arrestins and CIN activity are required for the formation of membrane protrusions downstream of PAR_2

The studies described thus far suggest that β-arrestins are required to bring CIN in contact with its substrate so that it can dephosphorylate and activate cofilin. As cofilin activity is required for the formation of a leading edge during the initial steps of chemotaxis, we predicted that protrusion formation required both β-arrestins and CIN. Examination of cell morphology in response to directed activation of PAR_2 in embryonic fibroblasts from wild type (MEFwt) and β-arrestin1/2$^{-/-}$ mice (MEFβarrDKO), or from MEFβarrDKO stably expressing β-arrestin-1 (DKO+βarr1) or β-arrestin-2 (DKO+βarr2). Mouse embryonic fibroblasts from wild type mice (MEFwt), β-arrestin-1/2$^{-/-}$ mice (MEFβarrDKO), and MEFβarrDKO cells stably transfected with physiological levels of either β-arrestin-1 or β-arrestin-2 were from Dr. Robert Lefkowitz and have been described previously (14;28;29). Cells were monitored by time-lapse live video microscopy prior to, and after for localized PAR_2 activation. Within 1-2 minutes, MEFwt exhibited rapid formation of membrane protrusions towards the agonist, while MEFβarrDKO remained unchanged. DKO+βarr1 and DKO+βarr2 cells constitutively formed small, random protrusions, but no directional protrusions were formed in response to 2fAP (Figure 1.4 A) (50).

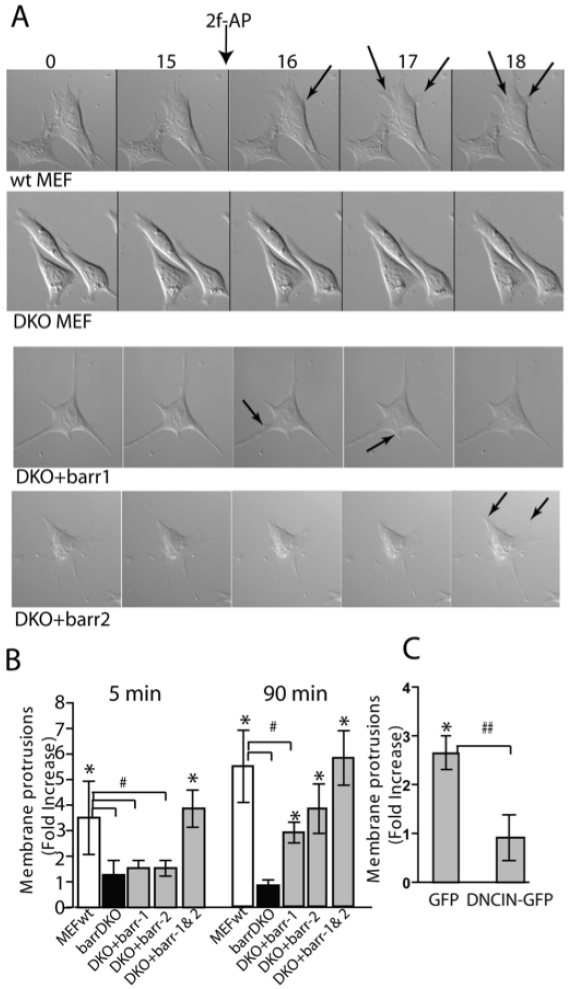


Figure 1.4. β-arrestins and CIN are required for initial membrane protrusion formation. A. Time lapse images of MEFwt, MEFβarrDKO, DKO+βarr-1 and DKO+βarr2 were observed from 15 minutes before to 2 minutes after addition of an agar cube containing the PAR_2 agonist 2fAP, to the upper right corner of the dish. Arrows indicate membrane protrusions. **B.** Protrusion formation in response to PAR_2 activation (mean protrusion number ± SEM) was quantified using a modified Transwell filter assay in MEFwt and MEFβarrDKO treated with or without 2fAP for 5 and 90 minutes. Rescue of protrusion formation by β-arrestin-rescue of protrusion formation determined in MEFDKO transfected with β-arrestin-1 (DKO+βarr1), β-arrestin-2 (DKO+βarr2), or with both β-arrestins (DKO+βarr1&2). **C.** Protrusion formation was determined in MDA MB-468 cells, transfected with GFP-tagged dominant negative CIN (DN-CIN) or GFP alone (negative control) and treated with or without 2fAP for 90 minutes. Data are expressed as a fold change in the number of protrusions in agonist compared with untreated controls *Statistically significant increase in protrusions ($p<.01$). Statistically significant difference in agonist-induced membrane protrusion between bracketed groups is indicated by # ($p<.01$) or ## ($p<.04$), n=3. Zoudilova et al., 2010. JBC.

Section 1.15

PAR_2 promotes rapid and stable membrane protrusions and both β-arrestins-1/2, and CIN activity are required.

Further, using a pseudopodia assay to quantify membrane protrusions in MEFwt compared with MEFβarrDKO (1;34), we observed that PAR_2 activation increased rapid membrane protrusions (observed after 5 minutes of agonist addition) and stable protrusions (observed after 90 minutes of agonist) compared to vehicle-treated controls. In MEFβarrDKO, formation of both rapid and stable protrusions was abolished, and this was rescued by transfection of both β-arrestins-1 and 2 (Figure 1.4 A). In support of previous observations that both β-arrestins are required for PAR_2 stimulated membrane protrusion and cell migration (1), Restoring either β-arrestin-1 or β-arrestin-2 into MEFβarrDKO partially rescued stable protrusion formation to 65-70% of that observed in MEFwt but did not rescue rapid membrane protrusion formation.

Our working hypothesis is that β-arrestins sequester cofilin with its upstream activator, CIN, to promote its localized activity, which is known to be essential for both migration and membrane protrusion formation (23;24). To determine whether CIN was also required for PAR_2 stimulated membrane protrusions, cells were transfected with GFP (negative control) or GFP-tagged dominant negative CIN (DNCIN). DNCIN is an inactive point mutant that specifically blocks endogenous CIN activity and was previously shown to inhibit PAR_2 stimulated cofilin dephosphorylation (14;26). Transfection of dominant negative CIN abolished membrane protrusion formation

(Figure 1.4 C). We conclude that both β-arrestins-1 and 2, and CIN activity, are required for directional membrane protrusion in response to PAR_2 activation (50).

Section 1.16

Actin monomer incorporation into membrane protrusions

PAR_2 induced generation of free barbed ends in lamellipodia is reduced in the absence of β-arrestins. Cofilin-induced actin filament severing contributes to chemotaxis by providing free barbed ends at the leading edge for polymerization (24;35). Barbed end formation can be visualized by determining incorporation of fluorescently labeled actin monomers into cells by confocal microscopy. The fluorescence intensity can be quantified over a defined distance to determine the level of actin monomer incorporation at the leading edge (35-39). Upon treatment of MEFwt with 2fAP for 1 minute, actin monomers were incorporated into membrane protrusions in MEFwt, within 0.5-2μ from the cell edge, but no significant monomer incorporation into MEFβarrDKO was observed (Figure 1.5A, B, D, E). PAR_2 induced generation of free barbed ends at the leading edge could be rescued by transfection of both β-arrestins (Figure 1.5 C-E). Transfection of either β-arrestin-1 or β-arrestin-2 alone marginally rescued actin monomer incorporation at the cell edge although we did not observe as distinct membrane protrusions as with transfection of both β-arrestins. Actin monomer incorporation was also decreased in cells expressing DN-CIN, compared with mock-transfected controls (Figure 1.5 F-I). We conclude that PAR_2 -induced generation of new barbed ends and subsequent membrane protrusion requires both β-arrestins-1 and 2, and the activity of CIN(50).

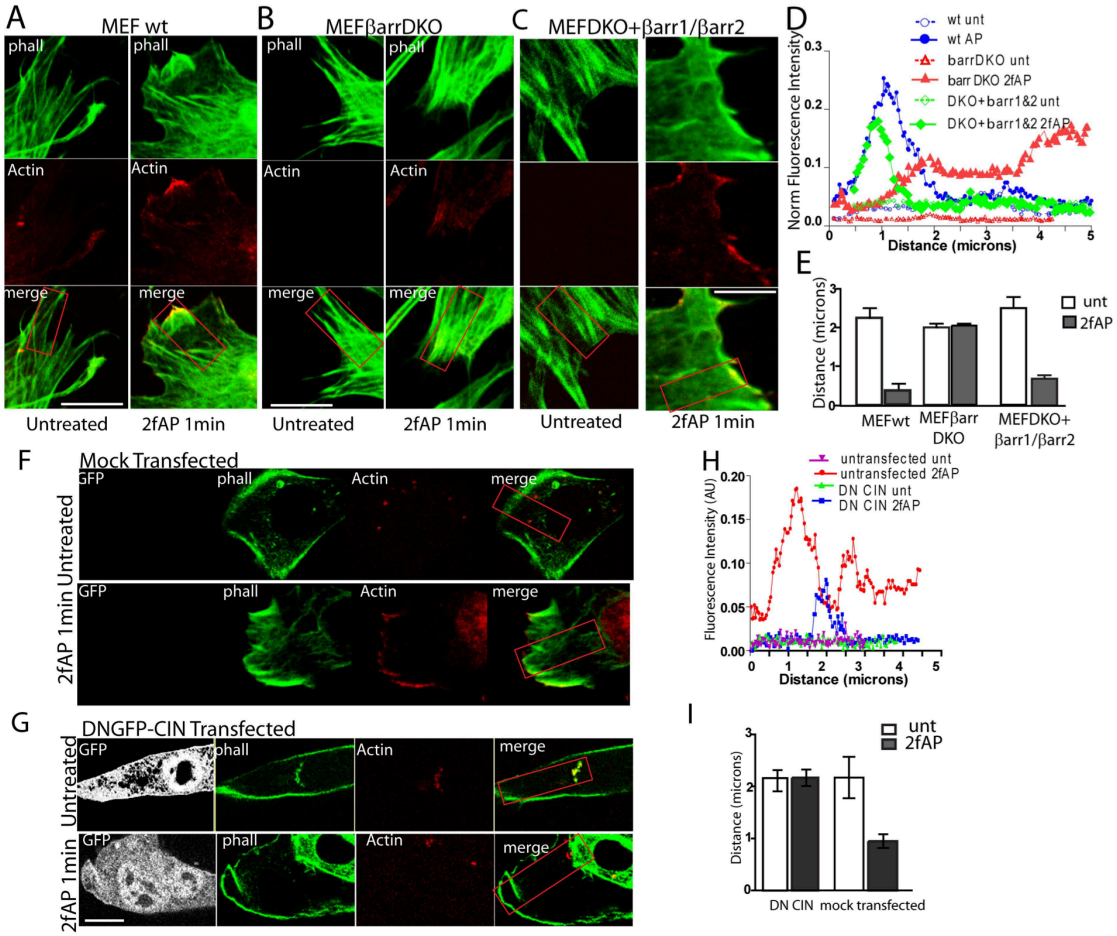


Figure 1.5. Formation of free barbed ends at the cell edge requires β-arrestins. A-C. Images (100X magnification) of MEFwt **(A)**, MEFβarrDKO **(B)** and DKO+βarr-1&2 **(C),** treated with or without 2fAP for 1 minute, in the presence of 300nM labeled actin monomers (red), fixed and cross-stained with phalloidin (phall, green). Scale bars=10μ. **D**. Representative traces of actin monomer incorporation (Fluorescence Intensity in arbitrary units (AU)), measured along the boxed regions, graphed as a function of distance from the cell edge are shown for MEFwt, MEFβarrDKO and DKO+βarr1&2. **E.** Mean distance (from the cell edge) at which peak rhodamine actin fluorescence was observed in MEFwt, MEFβarrDKO and DKO+βarr1&2. **F-G**. Rhodamine actin incorporation in MEFwt, either mock–transfected (F) or transfected with DNCIN (G) was performed as described above. **H.** Representative trace of actin monomer incorporation versus distance from the leading edge in mock-transfected and DN-CIN transfected cells. **I.** Mean distance from cell edge at which peak fluorescence was observed in mock- transfected and DN-CIN transfected cells. (For each group, n=12 cells from 3 separate experiments). Zoudilova et al. JBC. 2010

Section 1.17

β-arrestins facilitate co-localization of cofilin with chronophin (CIN) in membrane protrusions.

PAR_2 promotes redistribution of β-arrestins and cofilin to membrane protrusions (14) and β-arrestins are present in isolated pseudopodia (1) and might be required for localization of CIN with cofilin at the leading edge. To address this hypothesis, GFP-tagged-cofilin and Myc-tagged-CIN were expressed in MEFwt or MEFβarrDKO; cells were treated with 2fAP for 1 minute, then fixed and stained with phalloidin and anti-myc. Colocalization of cofilin and CIN was observed by confocal microscopy, and fluorescence intensity of each was again determined as a function of the distance from the cell edge. Untreated cells show cofilin was diffusely distributed throughout the cytoplasm, while CIN exhibited a more punctate cytosolic staining near the membrane (3-5μ from the cell edge). After treatment of MEFwt with 2fAP, we observed striking redistribution of a pool of cofilin to the membrane protrusions, where it colocalized with CIN (Figure 1. 6 A, B, E, F). No cofilin redistribution or colocalization with CIN was observed in MEFβarrDKO (Fig. 1.6 C, D, G, H). Quantification of fluorescence as a function of distance from the cell edge revealed that cofilin and CIN co-localized in peaked 0.75μ from the cell edge (Figure 1.6 I-M). Quantification of the fluorescence intensity reveals that PAR_2 promotes a 12-fold increase in both CIN and cofilin translocation to the leading edge (defined as the zone 0-2μ from the membrane, Figure 1.6 N) (50).

In contrast the mean fluorescence for both cofilin and CIN in both treated and untreated MEFβarrDKO was ~4μ from the cell edge, similar to what was observed in untreated MEFwt (Figure 1.6 M). The peak intensity of CIN and cofilin colocalization in MEFwt after PAR_2 activation overlapped with the peak intensity of actin monomer incorporation, consistent with a role for this scaffold in filament severing (Figure 1.6 O). These microscopic observations were corroborated with a previously described biochemical method for isolating membrane protrusions and cell bodies using (1, 2, 34). After inducing extension of protrusions through the 3μ pores of a Transwell filter either pseudopodia from the filter underside or cell bodies from the topside are collected, lysed and analyzed by western blot. Using this assay, we demonstrated the enrichment of both cofilin and CIN in membrane protrusions (Figure 1.6 P) (50).

Figure 1.6 (Facing Page). PAR_2 induced colocalization of cofilin and CIN in lamellipodia requires β-arrestins. MEFwt (**A, B**) and MEFβarrDKO (**C, D**) were transiently transfected with CIN and cofilin, either untreated (**A, C**) or treated with 2fAP for 1 minute (**B, D**), fixed, stained and imaged by confocal microscopy (40X magnification). Panels depict localization of cofilin (green), CIN (red), phalloidin (phal, blue) and a merge of all three (Scale bar=30μ). **E-H.** High magnification (63X) of membrane protrusions from cells in A-D. Lower panels in grayscale represent 5X zoomed images of cofilin and CIN in the indicated boxed regions of the protrusions, overlaid with tracings of the cell edge. **I-L.** Representative traces of fluorescence intensity calculated for CIN and cofilin, graphed on the y-axis in arbitrary units (AU), as a function of distance in microns from the cell edge. **M.** Redistribution of CIN and cofilin upon PAR-2 activation, as demonstrated by the decrease in mean distance from the cell edge at which peak fluorescence intensity for each was observed. **N**. Mean fluorescence intensity was determined for each protein at the leading edge (defined as 0-2μ from the cell edge). **O.** Overlay of PAR-2 stimulated CIN, cofilin and rhodamine actin fluorescence (from Figure 1) in PAR-2 activated MEFwt demonstrates overlap between barbed end formation, CIN and cofilin localization. **P.** Membrane protrusions were physically separated from cell bodies (see materials and methods), lysed and analyzed by western blotting with cofilin and CIN antibodies. Histone was included to demonstrate valid separation of the two fractions. A bar graph of the relative distribution of each protein is shown under the western blots. Zoudilova et al., 2010. JBC.

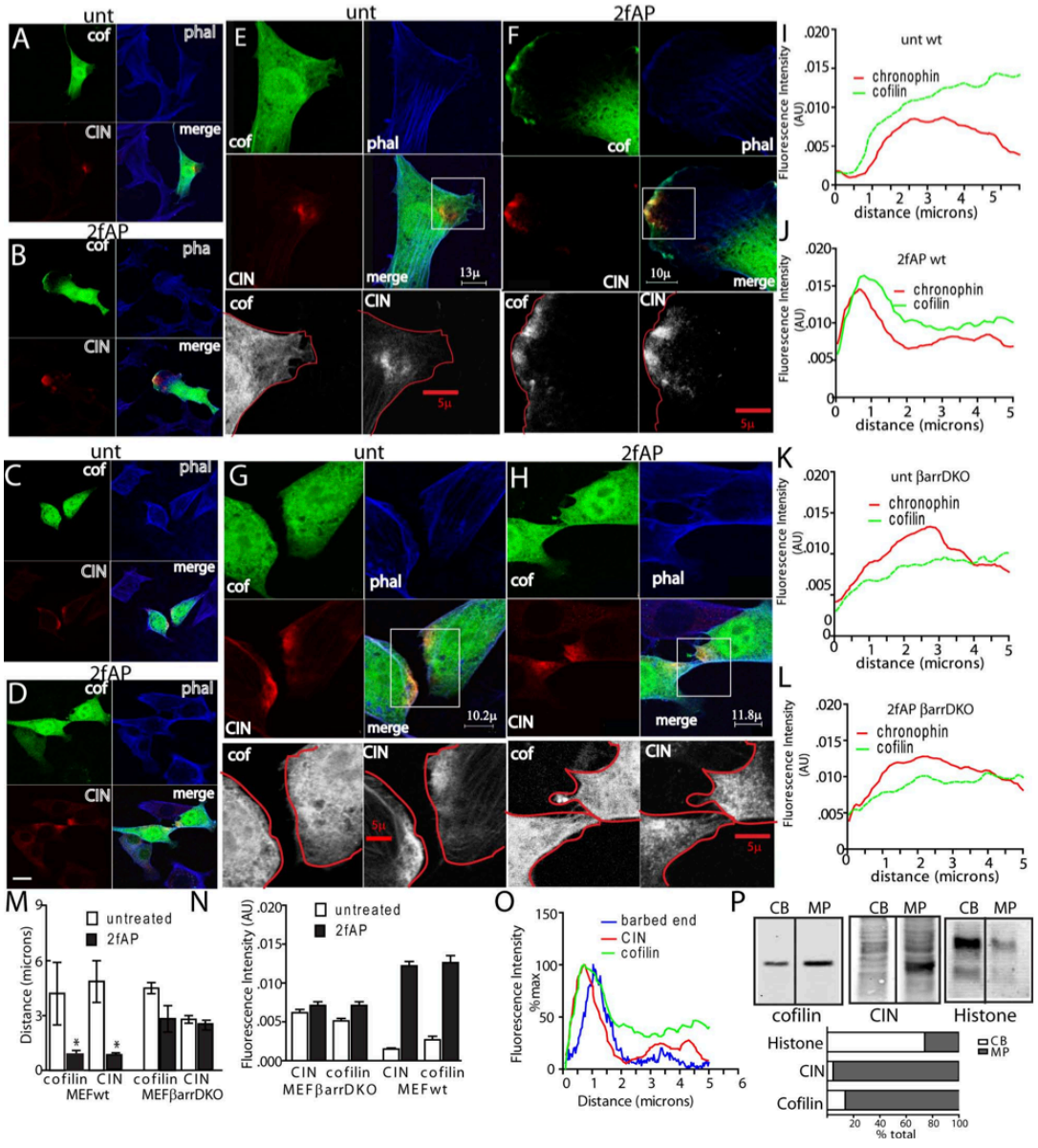

unt
2fAP
A
cof
phal
CIN
merge
B
pha
E
F
C
D
G
H
13μ
10μ
10.2μ
11.8μ
5μ
I
unt wt
J
2fAP wt
K
unt βarrDKO
L
2fAP βarrDKO
chronophin
cofilin
Fluorescence Intensity (AU)
distance (microns)
M
N
O
P
untreated
2fAP
Distance (microns)
cofilin
CIN
MEFwt
MEFβarrDKO
barbed end
% max
CB
MP
Histone
% total

Figure 1.6

Section 1.18

Linking receptor activation with actin reorganization and airway inflammation

A requirement for β-arrestins in chemotaxis has been reported for various receptors both in vivo and in vitro (13;16-18); we demonstrate the molecular mechanistic role for β-arrestins in PAR_2 stimulated migration in primary cells. Both leukocytes and neutrophils from β-arrestin knockout mice exhibited defects in PAR_2 stimulated chemotaxis, pointing to the possible importance of β-arrestins in PAR_2 -mediated inflammatory responses. These experiments provide a springboard into the in vivo models of OVA-, BG- and AltA-induced of airway inflammation discussed in the subsequent chapters. PAR_2 plays a significant role in airway inflammation and it has been reported to participate in the recruitment of lymphocytes, neutrophils and eosinophils to sites of inflammation a variety of disease models, including asthma and inflammatory bowel disease (3;4;7).

This work fills an informational gap in the understanding of how β-arrestins regulate actin assembly and cell migration and their role in PAR_2 stimulated chemotaxis, providing a novel mechanism for spatial regulation of cofilin. How β-arrestins regulate cell motility has been a topic of debate for some time. Some studies suggest that β-arrestins are essential for signal termination at the trailing edge, allowing for cell polarization in response to different chemotactic signals, while others suggest that they regulate actin binding proteins and other molecules involved in cell motility (13). These studies are the first to demonstrate a correlation between β-arrestin scaffolding of actin

assembly proteins and defective chemotaxis in primary cells, and to directly link CIN and β-arrestins to localized cofilin activity (50).

A major outcome of this study is the identification of β-arrestin-dependent scaffolding of cofilin and CIN in primary leukocytes, as well as β-arrestin-dependent, PAR_2 induced cell migration in these cells. The fact that phosphorylated (inactive) cofilin levels were elevated in the bone marrow leukocytes from either β-arrestin-$1^{-/-}$, β-arrestin-$2^{-/-}$ or PAR_2 $^{-/-}$ mice compared to wild type controls, is suggestive of a general role for the PAR_2 /β-arrestin/cofilin signaling axis in vivo (data not shown) β-arrestins may represent a novel means for spatially controlling cofilin activity to generate a localized pool of free barbed ends for other receptors besides PAR_2. However, the role of β-arrestins in cell signaling depends on the activating receptor; thus, this mechanism is unlikely to be shared by all receptors. For example, the role of cofilin in chemotaxis of tumor cells, lamellipodia formation, as well as its mechanism of activation, has been well characterized for EGF (23;35;40). EGF has been reported to activate and localize cofilin and filament severing, independent of cofilin dephosphorylation (41). Furthermore, EGF stimulates CIN localization to membrane protrusions, but while CIN is required for EGF-stimulated barbed end incorporation, it is not required for EGF-stimulated membrane protrusions in MTLn3 cells (C. DerMardirossian and G. Bokoch, personal communication). Studies by other labs on AT1-AR demonstrated a requirement for β-arrestin-2 in chemotaxis, but this requirement was not observed for EGF-stimulated chemotaxis (16). Thus, there are likely multiple mechanisms of spatially regulating

cofilin activity during chemotaxis and the role of β-arrestins and CIN in this process varies between receptors (50).

As β-arrestins are pleiotropic proteins, they are likely to affect cell migration, actin cytoskeletal reorganization, and even cofilin activation on multiple levels. In addition to inhibition of LIMK activity (14), β-arrestins can also regulate Rho-GTPase activity, interact with actin-binding proteins and regulate MAPK activity at the leading edge (1;13;42), all of which are important pathways for cell migration. Still other actin assembly proteins can associate with β-arrestins (13). Future studies investigating the interplay between these other pathways and the cofilin pathway are essential to a complete understanding of the consequences of β-arrestin-dependent scaffolding in PAR_2 signaling, as well as other receptor signaling cascades (50).

Section 1.19

References

1. Ge, L., Ly, Y., Hollenberg, M., and DeFea, K. (2003) J.Biol.Chem. 278, 34418-34426
2. Ge, L., Shenoy, S. K., Lefkowitz, R. J., and DeFea, K. A. (2004) J Biol Chem. 279, 55419-55424
3. Ebeling, C., Forsythe, P., Ng, J., Gordon, J. R., Hollenberg, M., and Vliagoftis, H. (2005) J Allergy Clin Immunol 115, 623-630
4. Cenac, N., Coelho, A. M., Nguyen, C., Compton, S., Andrade-Gordon, P., MacNaughton, W. K., Wallace, J. L., Hollenberg, M. D., Bunnett, N. W., Garcia-Villar, R., Bueno, L., and Vergnolle, N. (2002) Am J Pathol 161, 1903-1915
5. Chin, A. C., Lee, W. Y., Nusrat, A., Vergnolle, N., and Parkos, C. A. (2008) J Immunol 181, 5702-5710
6. Hyun, E., Andrade-Gordon, P., Steinhoff, M., and Vergnolle, N. (2008) Gut 57, 1222-1229
7. Schmidlin, F., Amadesi, S., Dabbagh, K., Lewis, D. E., Knott, P., Bunnett, N. W., Gater, P. R., Geppetti, P., Bertrand, C., and Stevens, M. E. (2002) J Immunol. 169, 5315-5321
8. Ferrell, W. R., Lockhart, J. C., Kelso, E. B., Dunning, L., Plevin, R., Meek, S. E., Smith, A. J., Hunter, G. D., McLean, J. S., McGarry, F., Ramage, R., Jiang, L., Kanke, T., and Kawagoe, J. (2003) J Clin Invest 111, 35-41
9. Luttrell, L. M., Ferguson, S. S., Daaka, Y., Miller, W. E., Maudsley, S., Della Rocca, G. J., Lin, F., Kawakatsu, H., Owada, K., Luttrell, D. K., Caron, M. G., and Lefkowitz, R. J. (1999) Science 283, 655-661
10. DeFea, K. A., Zalevsky, J., Thoma, M. S., Dery, O., Mullins, R. D., and Bunnett, N. W. (2000) J.Cell Biol. 148, 1267-1281
11. Tohgo, A., Pierce, K. L., Choy, E. W., Lefkowitz, R. J., and Luttrell, L. M. (2002) J Biol Chem. 277, 9429-9436
12. Wang, P. and DeFea, K. (2006) Biochemistry 45, 9374-9385
13. DeFea, K. A. (2007) Annu.Rev Physiol 69, 535-560
14. Zoudilova, M., Kumar, P., Ge, L., Wang, P., Bokoch, G. M., and DeFea, K. A. (2007) J Biol Chem. 282, 20634-20646
15. DeWire, S. M., Ahn, S., Lefkowitz, R. J., and Shenoy, S. K. (2007) Annual Review of Physiology 69, 483-510
16. Hunton, D. L., Barnes, W. G., Kim, J., Ren, X. R., Violin, J. D., Reiter, E., Milligan, G., Patel, D. D., and Lefkowitz, R. J. (2005) Mol.Pharmacol 67, 1229-1236
17. Fong, A. M., Premont, R. T., Richardson, R. M., Yu, Y. R., Lefkowitz, R. J., and Patel, D. D. (2002) Proc.Natl.Acad.Sci.U.S.A 99, 7478-7483

18. Walker, J. K., Fong, A. M., Lawson, B. L., Savov, J. D., Patel, D. D., Schwartz, D. A., and Lefkowitz, R. J. (2003) J Clin.Invest 112, 566-574
19. Lagane, B., Chow, K. Y., Balabanian, K., Levoye, A., Harriague, J., Planchenault, T., Baleux, F., Gunera-Saad, N., Arenzana-Seisdedos, F., and Bachelerie, F. (2008) Blood 112, 34-44
20. Cooper, J. A. and Schafer, D. A. (2000) Curr.Opin.Cell Biol 12, 97-103
21. Delorme, V., Machacek, M., DerMardirossian, C., Anderson, K. L., Wittmann, T., Hanein, D., Waterman-Storer, C., Danuser, G., and Bokoch, G. M. (2007) Dev.Cell 13, 646-662
22. Ponti, A., Machacek, M., Gupton, S. L., Waterman-Storer, C. M., and Danuser, G. (2004) Science 305, 1782-1786
23. Mouneimne, G., DesMarais, V., Sidani, M., Scemes, E., Wang, W., Song, X., Eddy, R., and Condeelis, J. (2006) Curr.Biol 16, 2193-2205
24. Ghosh, M., Song, X., Mouneimne, G., Sidani, M., Lawrence, D. S., and Condeelis, J. S. (2004) Science 304, 743-746
25. Nagata-Ohashi, K., Ohta, Y., Goto, K., Chiba, S., Mori, R., Nishita, M., Ohashi, K., Kousaka, K., Iwamatsu, A., Niwa, R., Uemura, T., and Mizuno, K. (2004) J.Cell Biol. 165, 465-471
26. Gohla, A., Birkenfeld, J., and Bokoch, G. M. (2005) Nat Cell Biol 7, 21-29
27. Bamburg, J. R. (1999) Annual Review of Cell and Developmental Biology 15, 185-230
28. Kumar, P., Lau, C., Wang, P., Mathur, M., and DeFea, K. A. (2007) Am J Physiol Cell Physiol
29. Kohout, T. A., Lin, F. S., Perry, S. J., Conner, D. A., and Lefkowitz, R. J. (2001) Proc.Natl.Acad.Sci U.S.A 98, 1601-1606
30. Milano, S. K., Kim, Y. M., Stefano, F. P., Benovic, J. L., and Brenner, C. (2006) J Biol Chem. 281, 9812-9823
31. Matsuzaki, F., Matsumoto, S., Yahara, I., Yonezawa, N., Nishida, E., and Sakai, H. (1988) J Biol Chem. 263, 11564-11568
32. McGuire, J. J., Saifeddine, M., Triggle, C. R., Sun, K., and Hollenberg, M. D. (2004) J Pharmacol Exp Ther 309, 1124-1131
33. DeFea, K. A., Vaughn, Z. D., O'Bryan, E. M., Nishijima, D., Dery, O., and Bunnett, N. W. (2000) Proc.Natl.Acad.Sci.U.S.A 97, 11086-11091
34. Cho, S. Y. and Klemke, R. L. (2002) J Cell Biol 156, 725-736
35. Mouneimne, G., Soon, L., DesMarais, V., Sidani, M., Song, X., Yip, S. C., Ghosh, M., Eddy, R., Backer, J. M., and Condeelis, J. (2004) J.Cell Biol. 166, 697-708
36. Chan, A. Y., Raft, S., Bailly, M., Wyckoff, J. B., Segall, J. E., and Condeelis, J. S. (1998) J Cell Sci 111 (Pt 2), 199-211

37. Sidani, M., Wessels, D., Mouneimne, G., Ghosh, M., Goswami, S., Sarmiento, C., Wang, W., Kuhl, S., El Sibai, M., Backer, J. M., Eddy, R., Soll, D., and Condeelis, J. (2007) J Cell Biol 179, 777-791
38. Wang, W., Mouneimne, G., Sidani, M., Wyckoff, J., Chen, X., Makris, A., Goswami, S., Bresnick, A. R., and Condeelis, J. S. (2006) J.Cell Biol. 173, 395-404
39. Pollard, T. D. and Borisy, G. G. (2003) Cell 112, 453-465
40. Chan, A. Y., Bailly, M., Zebda, N., Segall, J. E., and Condeelis, J. S. (2000) J Cell Biol 148, 531-542
41. Song, X., Chen, X., Yamaguchi, H., Mouneimne, G., Condeelis, J. S., and Eddy, R. J. (2006) J Cell Sci 119, 2871-2881
42. Barnes, W. G., Reiter, E., Violin, J. D., Ren, X. R., Milligan, G., and Lefkowitz, R. J. (2005) J Biol Chem. 280, 8041-8050.
43. Arizmendi NG, et al. (2011) Mucosal allergic sensitization to cockroach allergens independent on proteinase activity and proteinase-activated receptor-2 activation. (Translated from eng) J Immunol 186(5):3164-3172 (in eng).
44. Boitano S, et al. (2011) Alternaria alternata serine proteases induce lung inflammation and airway epithelial cell activation via PAR2. (Translated from eng) Am J Physiol Lung Cell Mol Physiol 300(4):L605-614 (in eng).
45. Zoudilova M, et al. (2010). J Biol Chem 285(19):14318-14329.
46. Ramachandran R, Noorbakhsh F, DeFea K, & Hollenberg MD (2012). Nat Rev Drug Discov 11(1):69-86.
47. Moffatt JD, Jeffrey KL, & Cocks TM (2002). Am J Respir Cell Mol Biol 26(6):680 684.
48. Schmidlin F, et al. (2002). J Immunol 169(9):5315-5321 (in eng).
49. Ebeling C, et al. (2005). J Allergy Clin Immunol 115(3):623-630.
50. Zoudilova, M, et al (2010). J Biol Chem May 7;285(19):14318-29.

This page intentionally left blank

Chapter 2:

β-Arrestin-2 Mediates the Proinflammatory Effects of Proteinase-Activated Receptor-2 in the Airway

Section 2.1

Abstract

Proteinase Activated Receptor-2 (PAR_2), a G Protein Coupled Receptor (GPCR) activated by serine-proteinases, is reported to have both protective and pro-inflammatory effects in the airway. Given these opposing actions, both inhibitors and activators of PAR_2 have been proposed for treating asthma. PAR_2 signals through two independent pathways: a β-arrestin-dependent one that promotes leukocyte migration, and a G-protein/Ca^{2+} one that is required for prostaglandin E2 [PGE2] production and bronchiolar smooth muscle relaxation. We hypothesized that the pro-inflammatory responses to PAR_2 activation are mediated by β-arrestins, whereas, the protective effects are not. In a mouse ovalbumin model for PAR_2-modulated airway inflammation we observed decreased leukocyte recruitment, cytokine production and mucin production in β-arrestin-$2^{-/-}$ mice. In contrast, PAR_2-mediated PGE2 production, smooth muscle relaxation and baseline airway resistance (putative PAR_2 'protective' effects) were independent of β-arrestin-2. Flow cytometry and cytospins reveal that lung eosinophil and CD4-T-cell infiltration, and production of IL4, IL-6, IL-13, and TNFα, increased in wild type but not β-arrestin-$2^{-/-}$ mice. However, PAR_2 activation protects against airway hyperresponsiveness (AHR) by an unknown mechanism, possibly involving smooth muscle relaxation. Our data suggest that the PAR_2-enhanced inflammatory process is β-arrestin-2-dependent, while the 'protective' anti-constrictor effect of bronchial epithelial PAR_2 may be β-arrestin-independent.

Section 2.2

Materials and Methods

Animals. 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 IACUC at UC Riverside and Duke University. β-Arrestin2-/- mice in a C57BL/6 background were provided by Dr. 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 (Tokyo, Japan). Wild-type C57BL/6 (WT) mice were from Jackson Laboratories. All animals are bred in-house.

Sensitization and Challenge with Ovalbumin: Age matched male C57BL/6 β-arrestin-$1^{-/-}$ and β-arrestin-$2^{-/-}$ mice (2- 4 months old) were sensitized on days 1 and 6 with an intraperitoneal (IP) injection of OVA/alum (Sigma) (10μg OVA and 2mg $Al(OH)_3$ in .5mL saline) or saline alone. On days 12 and 14 were given an IN challenge of 25μL OVA (.2%w/v in saline) containing either PAR_2 activating peptide (2 furoyl- LIGRLO-$_{NH2}$ (2fAP) at 2.5 nanomoles) or PAR_2 control peptide (CP, 2-furoyl-OLRIGL-$_{NH2}$, 2.5 nanomoles), or given saline alone. Peptides were synthesized by GENEMED (Burlingame, CA). Control wt and β-arrestin$2^{-/-}$ mice received saline for IP injections and IN challenges. On day 15, mice were euthanized and BALF and lungs were collected. Lung digests and histology were performed as previously described (1) or analyzed for AHR as described below.

ELISA Immunoassays and Cytokine Bead arrays: OVA-IgE ELISA immunoassays and Prostaglandin AchR capture assays (MD BioProducts) were performed according to the manufacturer's instructions.

Bronchoalveolar Lavage Fluid (BALF) analysis: Cells from BALF samples were pelleted, washed and resuspended in FACS Buffer for flow cytometry. Total cell numbers were determined by hemocytometer counts. 100µl of lavage fluid was spun onto slides using Shandon Cytospin 3 as previously described (2). Cell differentials were determined by classifying 200 cells using standard morphological criteria. A minimum of 10 images were analyzed for each mouse; n=16 mice per treatment (4 mice per treatment group repeated 4 times). For flow cytometry, BALF cells were divided in two antibody staining sets and analyzed.

Lung Histology: Extracted lungs were either flash frozen in OCT or fixed and embedded in paraffin. 5µ sections were stained using standard H&E protocol and imaged with an upright Nikon Eclipse E600 using PAXIT! Software. Sections were scored for epithelial and perivascular inflammation using a 0-4 point scale, as described in the supplementary data. For analysis of mucin, 5µ sections were cut from paraffin embedded lung and stained with Alcian Blue for 30 mins (to identify mucin) and neutral red for 5 mins (to identify nuclei). Sections were mounted with resinous mounting media and slides were imaged as described above.

Smooth Muscle Relaxation and Airway hyperresponsiveness. These methods have been described previously (20) (3) (4) (5). Briefly, airway responsiveness in mice was measured using the forced oscillation technique (FOT), an invasive method that measures

lung impedance. From this measure Newtonian resistance, an indicator of airway luminal diameter, was calculated according to the constant-phase model.

Data and Statistical Analysis. Statistical significance determined using one-way analysis of variance and Tukey-HSD post-tests to compare treatment groups. Data, graphs and statistical analyses performed using FACS DIVA, FlowJo, Microsoft Excel 2003 or GraphPad Prism 5.0 (6).

Section 2.3

Introduction

Currently, 300 million people suffer from asthma resulting in nearly 250,000 asthma-related deaths reported annually, approximately 80% occurring in low- and lower-middle income regions. The development of new medications that inhibit cellular inflammation may reduce morbidity rates and attempts to manage this disease have identified proteinase-activated-receptor-2 (PAR_2) as an attractive new target(7). PAR_2 is a G-protein coupled receptor (GPCR) that is widely expressed in bronchial epithelial cells, leukocytes and airway smooth muscle, where it may be activated by proteinases secreted from invading pathogens, inhaled proteinases, or by locally released proteinases such as tissue kallikreins or tryptase (8-11). Serine proteinases activate PAR_2 by cleaving its N-terminus, revealing a tethered ligand (SLIGRL/SLIGKV, human/mouse) that self-activates the receptor, leading to G-protein coupling and β-arrestin recruitment. Peptides corresponding to the tethered ligand and peptidomimetics such as 2-furoyl-LIGRL-ornithine-NH_2 (2fAP) are commonly used to activate PAR_2, both in cultured cells and *in* vivo (12, 13). We previously reported that PAR_2 can activate two independent signaling pathways, one transduced by 'classical' G-protein-coupled signaling and the other by a G-protein-independent, β-arrestin-mediated signaling pathway (2, 14-17). Although the two signaling pathways can target common downstream effectors, the outcomes can be distinct and even opposing. For example, β-arrestins can scaffold the actin severing protein, cofilin, with its upstream activator (Chronophin) while inhibiting its negative regulator (LIMK). This scaffold has been identified in fibroblasts, tumor cells and

primary mouse leukocytes, and is crucial for PAR_2-stimulated chemotaxis. Downstream of the G-protein-coupled pathway, this same process is inhibited (2, 17).

In keeping with its opposing signals in vitro, studies done in vivo suggest that PAR_2 activation can play diametrically opposed roles in allergic asthma. In favor of a pro-inflammatory role for PAR_2, the recruitment of leukocytes to the lungs in a murine ovalbumin (OVA) model of allergic inflammatory airway disease was reduced in $PAR_2^{-/-}$ mice and increased in PAR_2 over-expressing mice (18, 19). The inflammatory response to OVA is also enhanced by the intranasal administration of PAR_2 peptide agonists in wild type mice (1). These inflammatory responses involve cell migration, leading to the hypothesis that they are β-arrestin-dependent. β-arrestins are also required for leukocyte chemotaxis downstream of a number of other GPCRs, including several chemokine receptors known to be involved in allergic asthma (2, 17, 20). In favor of a protective role, administration of PAR_2 agonists promotes prostanoid-induced cytoprotection in rodent and human airways, and bronchoconstriction is elevated in $PAR_2^{-/-}$ mice (21). Prostaglandin E2 (PGE2) production also inhibits eosinophil migration and degranulation(21, 22). PAR_2-induced PGE2 production utilizes a Gαq-Ca^{2+}-coupled mechanism that we hypothesize is independent of β-arrestins (23, 24). Depending on the balance of PAR_2 signals between G-protein and β-arrestin-dependent pathways, PAR_2 agonists may be capable of either compounding or curbing allergic asthma. This study examines the potential role of the β-arrestin signaling pathway in the proinflammatory and protective actions of PAR_2 in the airway (6).

Results

Section 2.4

PAR_2 induces cellular airway inflammation in wt but not β-arrestin-$2^{-/-}$ mice

To assess a role for β-arrestin-2 in PAR_2-induced cellular airway inflammation, we used a modification of a previously described OVA-induced murine model of allergic asthma (1). Mice were sensitized with an intraperitoneal (IP) injection of saline (as a negative control) or OVA/alum, on days 1 and 6. This was followed on days 12 and 14 by an intranasal (IN) challenge of saline or OVA plus either a PAR_2 agonist (2 furoyl-LIGRLO-$_{NH2}$, 2fAP) or a scrambled negative control peptide (2-furoyl-OLRIGL-$_{NH2}$, CP). The mice were sacrificed on day 15, bronchoalveolar lavage fluid (BALF), lungs were collected, and cellular inflammation was assessed (Figure 2.1A). In this short term model the response to OVA alone is less pronounced, and the additive effect of PAR_2 on OVA-induced inflammation is more evident. Analysis of H&E stained lung tissue sections reveals that influx of inflammatory cells into the perivascular and peribronchial regions from all wild type mice treated with OVA (receiving either CP or 2fAP with the IN challenges) was increased compared with saline controls (Figure 2.1B). Lung sections were scored for characteristics associated with acute and chronic inflammation revealing that the inflammation index was 1.5-fold greater for OVA+2fAP-treated wild type compared with the OVA+CP treated mice (Figure 2.1C). In contrast, OVA+2fAP treatment was unable to exacerbate inflammation in β-arrestin-$2^{-/-}$ mice. Although OVA+CP treatment elevated total BALF cells for both wild type and β-arrestin-$2^{-/-}$ mice relative to their respective saline controls, the effect was significantly less in

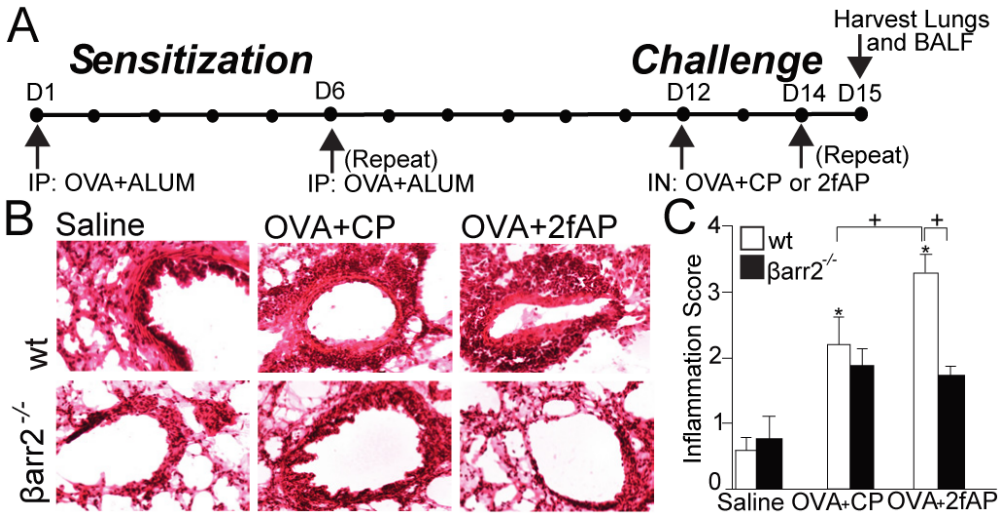
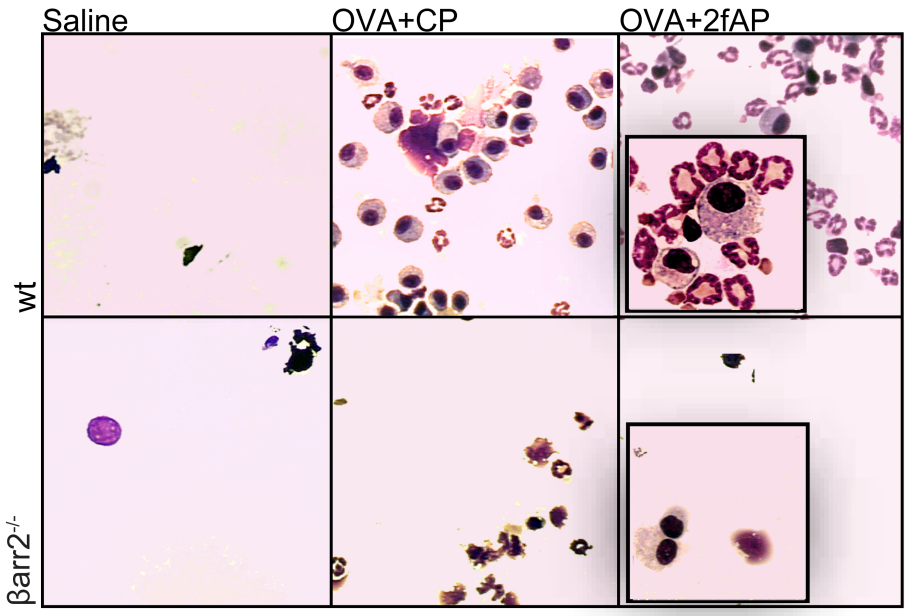


Figure 2.1 (Top): PAR_2-induced lung inflammation is reduced in β-arrestin-$2^{-/-}$ mice. A. Timeline of OVA-induced sensitization and PAR_2 challenge in wt and β-arrestin-$2^{-/-}$ mice (24 per OVA treatment group, 18 per saline treatment group, 128 total mice). **B.** Representative images of Hematoxylin/Eosin-stained frozen lung sections from mice treated as described in Fig 2.1A. **C.** Histological inflammation scoring for peribronchial thickness and leukocyte invasion on a scale from 0-4 (see supplement for details; 20 sections from each mouse, 4 separate experiments, scored double-blinded)**.** +Significant difference between groups ($p<.01$). All groups differed significantly from saline treated mice ($p<.05$, $n=15$ for saline $n=20$ for OVA+CP and OVA+2fAP). Statistics in this and all subsequent figures determined by ANOVA with Tukey HSD post-tests.

Figure 2.2-1 (Bottom): Representative Cytospin images of BAL Fluid. A. Images illustrate increase in inflammatory cells recruited to lungs in wild type mice challenged with OVA+2fAP. Of equal importance is the augmented recruitment of eosinophils, an indicator of allergic disease, in the aforementioned mice (see zoom inlay). Note the reduced total cell number and absence of eosinophils in β-arrestin-2-/- mice also challenged with OVA+2fAP (6)



β-arrestin-$2^{-/-}$ mice, consistent with previous reports (25). Challenge with OVA+2fAP in wild type animals augmented the influx of inflammatory cells (16.6±3.5-fold increase over saline), but in β-arrestin-$2^{-/-}$ mice, no augmentation of total cells was observed with 2fAP (Figure 2.2-2A). Differential counts, performed from cytospin preparations (Figure 2.2-1), revealed challenge of wild type mice with OVA+CP alone increased lung recruitment of both eosinophils (Figure 2.2-2B) and lymphocytes (Figure 2.2-2C) compared with saline controls. Challenge with OVA+2fAP further increased numbers of both cell types in the BALF of wild type mice by 3- and 2.5-fold, respectively, over those observed with OVA+CP. In contrast the numbers of eosinophils and lymphocytes recovered from the BALF of β-arrestin-$2^{-/-}$ mice receiving OVA+CP was significantly lower than those in similarly treated wild type mice and the pro-inflammatory effect of 2fAP was not observed in β-arrestin-$2^{-/-}$ mice. Flow cytometric analysis, using forward and side scatter (FSC/SSC) and expression of cell surface markers CCR3 and CD3 to calculate the percentage of CD45+ BALF cells that were either eosinophils or lymphocytes, respectively (Figure 2.2-2 D, E), revealed that BALF from OVA+CP-treated wild type mice contained approximately 50% eosinophils and 9% lymphocytes. In contrast, the inflammatory response to OVA+CP in β-arrestin-$2^{-/-}$ mice was significantly muted (19% CCR3+; less than 5% CD3+). Whereas PAR_2 activation by 2fAP elevated BALF levels of both eosinophils (72%) and lymphocytes (22%) in wild type mice, no such effect was observed in similarly treated β-arrestin-$2^{-/-}$ mice. Similar results were observed in digested lung tissue (data not shown).

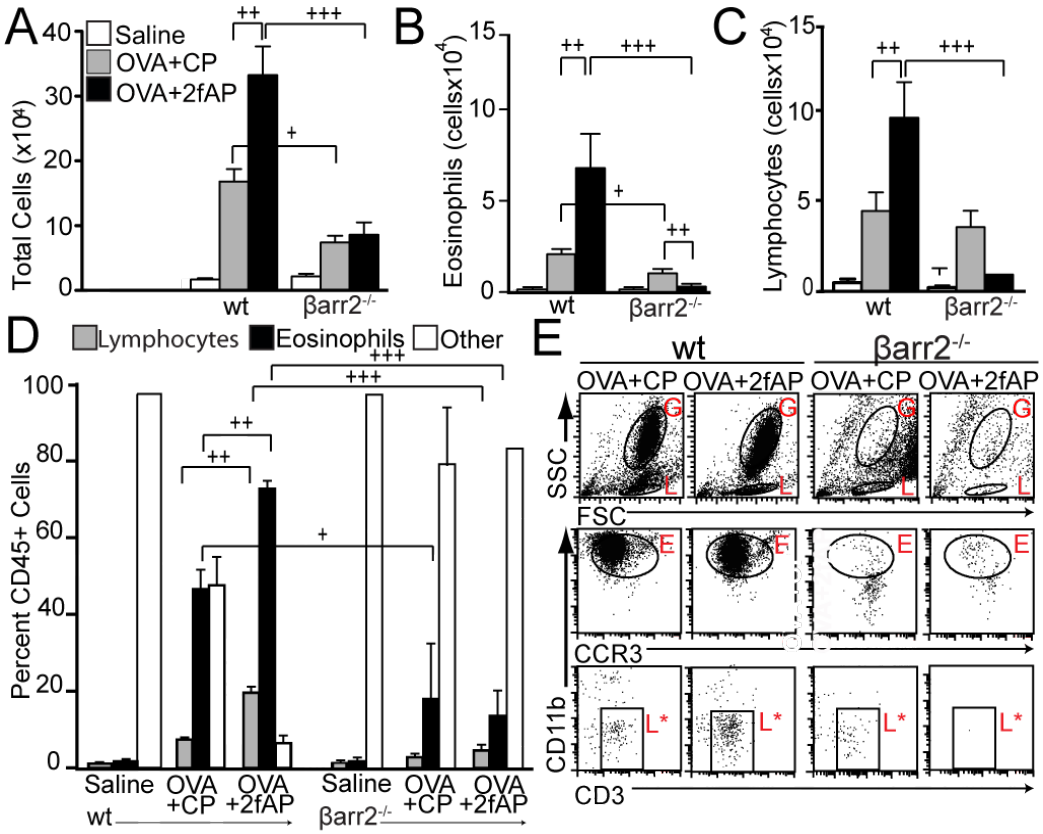


Figure 2.2-2: PAR_2-induced recruitment of leukocytes to the lungs requires β-arrestin-2. Bar graphs depict cell numbers in BALF: total cells **(A)**, eosinophils **(B)**, and lymphocytes **(C)**. All OVA-treated mice were statistically different from saline controls except eosinophils in β-arrestin-$2^{-/-}$ treated with OVA+2fAP. **D.** Flow cytometric determination of the percentage of CD45+ cells that were lymphocytes, eosinophils or other cells. Significant differences between bracketed groups are indicated: +($p<.05$), ++ ($p<.01$), and +++ ($p>.001$). **E.** Representative scatter plots of flow cytometric analysis of BALF cells. High FSC/SSC, CD45+ granulocyte populations (G) were analyzed for the presence of CD11b and CCR3 (eos, E). Low SSC, CD45+ lymphocyte (L) populations were further analyzed for the presence of the T-lymphocyte marker, CD3 (L*) (n=18 for saline, n=24 each for OVA+CP and OVA+2fAP).

Lymphocytes, identified by SSC, were further analyzed for expression of lineage-specific markers (Figure 2.3-1 A). OVA+2fAP treatment increases numbers of CD4+ and CD8+ cells found in BALF obtained from in wt mice. Consistent with the other BALF data, OVA+CP treatment reduced the inflammatory effect on β-arrestin-2$^{-/-}$ mice relative to wild types (Figure 2.3-1 B). These two cell types were increased with PAR_2 activation in wild type but not β-arrestin-2$^{-/-}$ mice and this may reflect a more global requirement for β-arrestins. Both wild type and β-arrestin-2$^{-/-}$ mice, challenged with either OVA+CP or OVA+2fAP, showed a similar increase in production of OVA-IgE, compared with saline-treated controls (data not shown), suggesting that the differences in cellular inflammation observed in β-arrestin-2$^{-/-}$ mice were not due to their inability to become sensitized to ovalbumin. To determine the extent to which 2fAP is able to access cells expressing PAR_2 within the pleural cavity, we examined the labeling of airway cells with rhodamine-conjugated-2fAP (Rh-2fAP) 24 hours after intranasal administration. We observed Rh-2fAP labeled cells distributed along both the basement membrane and apical surface of airway epithelia in wild type mice, but not $PAR_2^{-/-}$, mice (Figure 2.3-2). This suggests that PAR_2 expressed on leukocytes mediates their chemotaxis into the lungs. This is supported by the observation that the cellular inflammation in response to OVA+2fAP is partially mediated by hematopoietic PAR_2. As OVA+2fAP-induced airway inflammation was reduced after adoptive transfer of $PAR_2^{-/-}$ bone marrow cells into wild type mice (Figure 2.3-3). Conversely, wild type bone marrow was transplanted into $PAR_2^{-/-}$ mice and these mice were challenged with OVA+2fAP, recruitment of immune cells to the lungs was partially restored (6).

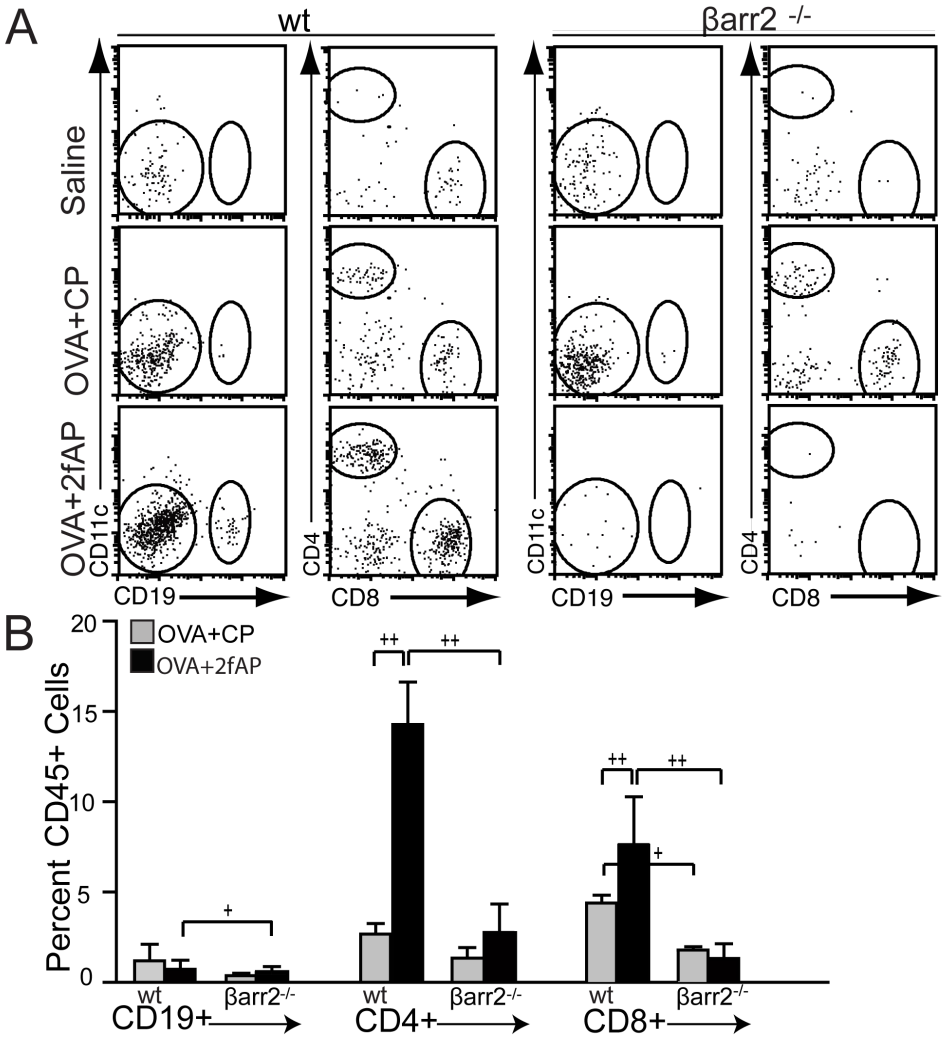


Figure 2.3-1. PAR_2 promotes β-arrestin-dependent recruitment of CD4+ T-cells. **A.** Representative scatter plots of flow cytometric analysis. CD45+/low SSC cells were analyzed for immunoreactivity to CD19+/CD11c- (CD19+ B-cells), CD19-/CD4+/CD8- (CD4 T-cells) and CD19-/CD4-/CD8+ (CD8 T-cells). **B.** Graph showing the %CD45+ cells that were CD19+, CD4+ or CD8+. + ($p<.05$) and ++($p<.01$) show significant difference between bracketed groups. n=15 for saline, n=20 each for OVA+CP and OVA+2f-AP (6).

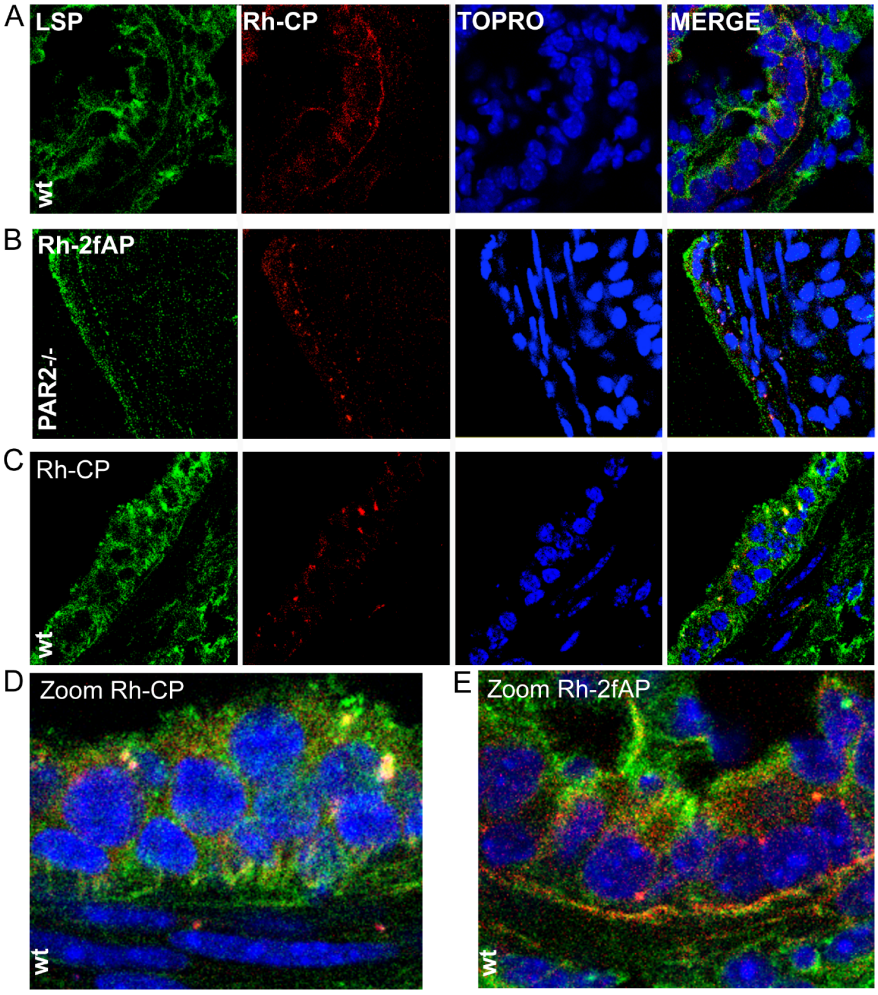
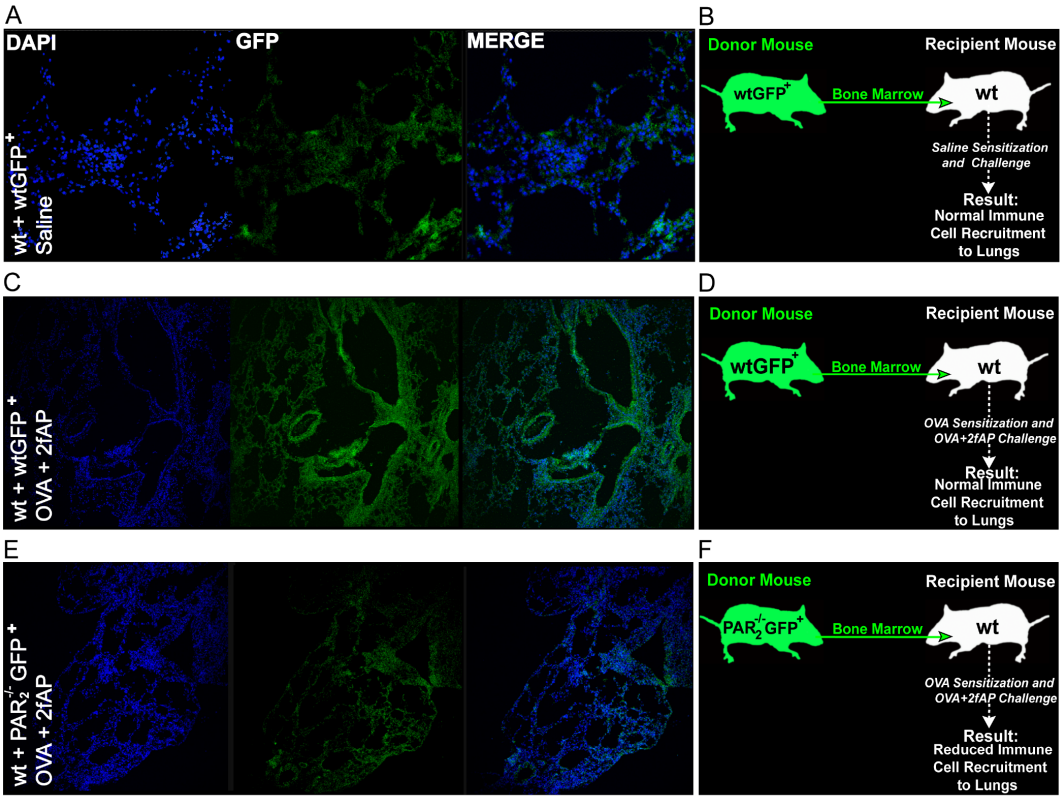


Figure 2.3-2: Intranasally administered PAR_2 activating peptide can access the basolateral side of the airways. Wild type and $PAR_2^{-/-}$ mice were given Rhodamine-labeled 2fAP (Rh-2fAP), or Rhodamine labeled control peptide (Rh-CP), intranasally and lungs harvested 2 hours after administration. Frozen sections were stained with Lung Surfactant protein (LSP, in green) and TOPRO 3 to visualize nuclei (in blue) and then imaged by confocal microscopy using a ZEISS LSM510, 100X objective. Representative images showing Rh-2fAP labeling (red) throughout the airway epithelium and serosa in wild type (**A**) but not $PAR_2^{-/-}$ mice (**B**) or wt mice given Rh-CP **(C)**, are shown. Zoomed images (3X) of Rh-CP treated wild type (**D**) and Rh-2fAP-treated wt (**E**) lungs are shown (6).



2.3-3 Adoptive transfer of wtGFP+ hematopoietic cells restores normal inflammatory response. (A) wtGFP+ mice saline-treated have little inflammation in perivascular or peribronchial regions. (B) Cartoon illustration of adoptive transfer protocol identifying donor and recipient. (C and D) WT recipients of wtGFP+ hematopoietic cells and challenged with OVA+2f-AP show increased inflammation in the region of interest. (E and F) WT recipients of PAR_2-/-/GFP+ hematopoietic cells show little inflammation even after OVA+2f-AP treatment (6).

Section 2.5

PAR_2-induced cytokine production is partially dependent on β-arrestin-2

Allergic asthma involves a Th2 response orchestrated by cytokines and chemokines secreted by epithelial cells, T-cells and other invading leukocytes. Using a cytometric bead array to assay cytokine levels, we observed increased IL-4, IL-6, IL-13, TNFα, IL-10, and IL-12p70 levels in OVA+2fAP- challenged compared to OVA+CP-treated wild type mice (Figure 2.4). In β-arrestin-$2^{-/-}$ mice, no PAR_2-mediated increase in IL-4, IL-6, IL-13 and TNFα levels was observed, but levels of IL-10 and IL-12p70 were still increased with 2fAP treatment (Figure 2.4 E,F). Similar to what was reported previously (1), in this short-term model of airway inflammation, no significant changes in cytokine levels other than IL-6 were seen between animals challenged with OVA+CP and saline-treated controls (Figure 2.4B).

One of the upregulated cytokines, IL-13, is known to play a key role in goblet cell hyperplasia and increased mucin production during asthma (26). To determine whether PAR_2 increased mucin production via a β-arrestin-dependent mechanism, lung sections were stained with alcian blue to identify goblet cells and goblet cell numbers were quantified. OVA+2fAP significantly increased mucin production above that observed in saline-treated and OVA+CP-treated animals. Consistent with the dependence of IL-13-mediated mucin production on β-arrestin-2, PAR_2 stimulated mucin production was also abolished in the β-arrestin-$2^{-/-}$ animals (Figure 2.5) (6).

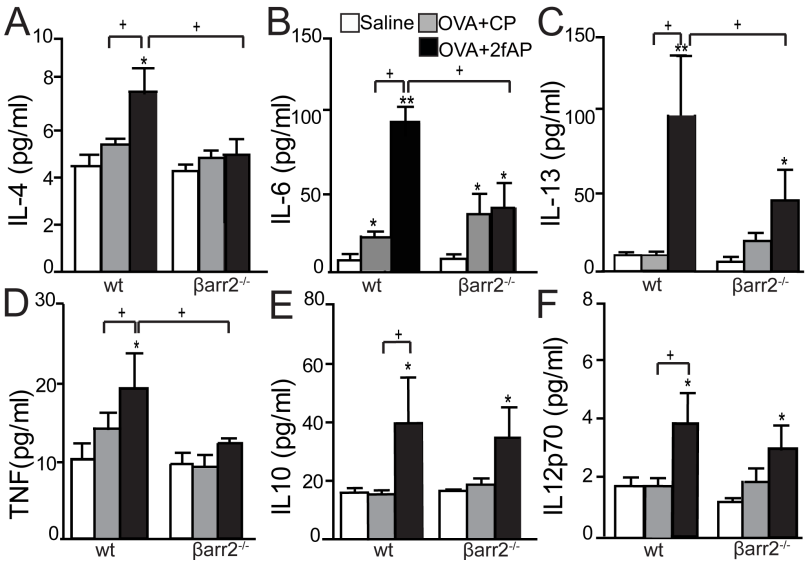
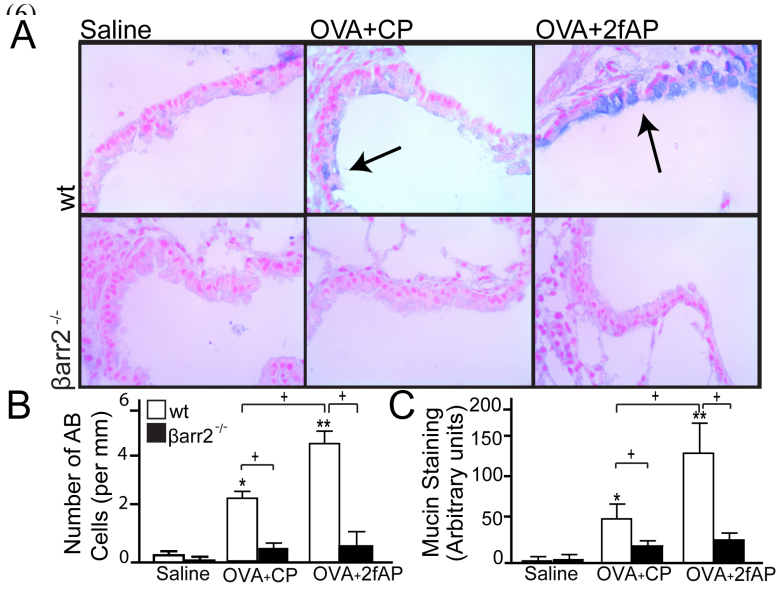


Figure 2.4. (Top) PAR_2-induced cytokine production in wild type and β-arrestin-2$^{-/-}$ mice. BALF supernatants from mice, treated as described in Fig 1A, were analyzed by cytometric bead array for the presence of IL-4**(A)**, IL-6 **(B)**, IL-13 **(C)**, TNFα **(D)**, IL-10 **(E)** and IL-12p70 **(F)**. *, **Statistically significant differences from saline controls *(p<.01) and **(p<.001), +Statistically significant differences between bracketed groups (p<.01) (n=15 for saline, n=20 each for OVA+CP and OVA+2fAP).

Figure 2.5. (Bottom) Goblet cell hyperplasia and mucin production in response to PAR_2 is abolished in β-arrestin-2-/- mice. **A.** Lung tissue from each of the treatment groups shown in Fig 1A was stained with Alcian blue to reveal acidic mucins and co-stained with neutral red. Arrows indicate mucin-producing goblet cells. **B**. Double-blind quantification of the number of mucin-producing cells per mm of basement membrane. **C.** Quantification of intensity of Alcian Blue stain. Significant differences between bracketed groups are indicated, + (p<.05). 20 images of at least 2 mice from 3 independent experiments (120 total) were analyzed for B and C (6).



Section 2.6

PAR_2-mediated bronchiolar relaxation is independent of β-arrestin-2

The major protective effect reported for PAR_2 is smooth muscle relaxation, which is mediated by prostaglandins (e.g.PGE2) released from airway epithelial cells. PGE2 levels were significantly increased in the BALF of both wt and β-arrestin-$2^{-/-}$ mice receiving OVA+2fAP (Table 2.1), suggesting β-arrestin-2 is not required for this PAR_2-mediated response. We next investigated 2fAP-induced smooth muscle relaxation from wild type and β-arrestin-$2^{-/-}$ mice. Treatment of first-order bronchiolar rings with 2fAP caused a rapid relaxation response; the magnitude and duration of which was similar in wild type and β-arrestin-$2^{-/-}$ bronchioles (Fig 2.6 A, B). In keeping with previous reports (27), relaxation in both animal groups was abolished by selective inhibitors of either COX1 or COX2 (Figure 2.6A). We conclude that PAR_2-induced smooth muscle relaxation is independent of β-arrestin-2. Consistent with a prior report (25), an increase in AHR was observed in wild type animals receiving intranasal challenges of OVA+CP, but this effect was absent in β-arrestin-$2^{-/-}$ mice, (Figure 2.6D). Interestingly, both baseline airway resistance and methacholine-induced AHR were significantly reduced in wild type mice treated with OVA+2fAP (Fig 2.6C, D), consistent with the protective effects of PAR_2 reported by others (21). The already low level of baseline airway resistance in OVA-treated β-arrestin-$2^{-/-}$ mice was decreased with the addition of 2fAP suggesting that the protective effect of PAR_2 on airway smooth muscle relaxation is independent of β-arrestin-2 (Figure 2.6C). However, 2fAP was not able to lower the airway response to methacholine in β-arrestin-$2^{-/-}$ mice, perhaps owing to the already

reduced magnitude of the response in these mice (Figure 2.6D). The abrogation of OVA-induced AHR in β-arrestin-2$^{-/-}$ animals has been reported previously and studies with chimeric mice suggest that this effect is independent of the cellular inflammation (28) (6).

Mouse Strain	Sensitization	Challenge	PGE_2 Levels
wt	None	Saline	1.4 ± .23pg/ml
wt	OVA+Al(OH_3)	OVA + CP	2.2 ± .18pg/ml
wt	OVA+Al(OH_3)	OVA + 2fAP	*3.4 ± .15pg/ml
βarr2$^{-/-}$	None	Saline	1.8 ± .31pg/ml
βarr2$^{-/-}$	OVA+Al(OH_3)	OVA + CP	2.2 ± .29pg/ml
βarr2$^{-/-}$	OVA+Al(OH_3)	OVA + 2fAP	**3.2 ± .06pg/ml

Table 2.1. Prostaglandin (PGE2) levels in BALF after OVA + 2fAP-induced airway inflammation model. BALF supernatants were assayed for PGE2 levels using Prostaglandin E2 Express AChE tracer ELISA assay. Values are mean ± SEM, n=8. Statistically significant differences from control group are indicated, *p=.0003, **p=.0001 (n=15 for saline, n=20 for OVA+CP and OVA+2fAP) (6).

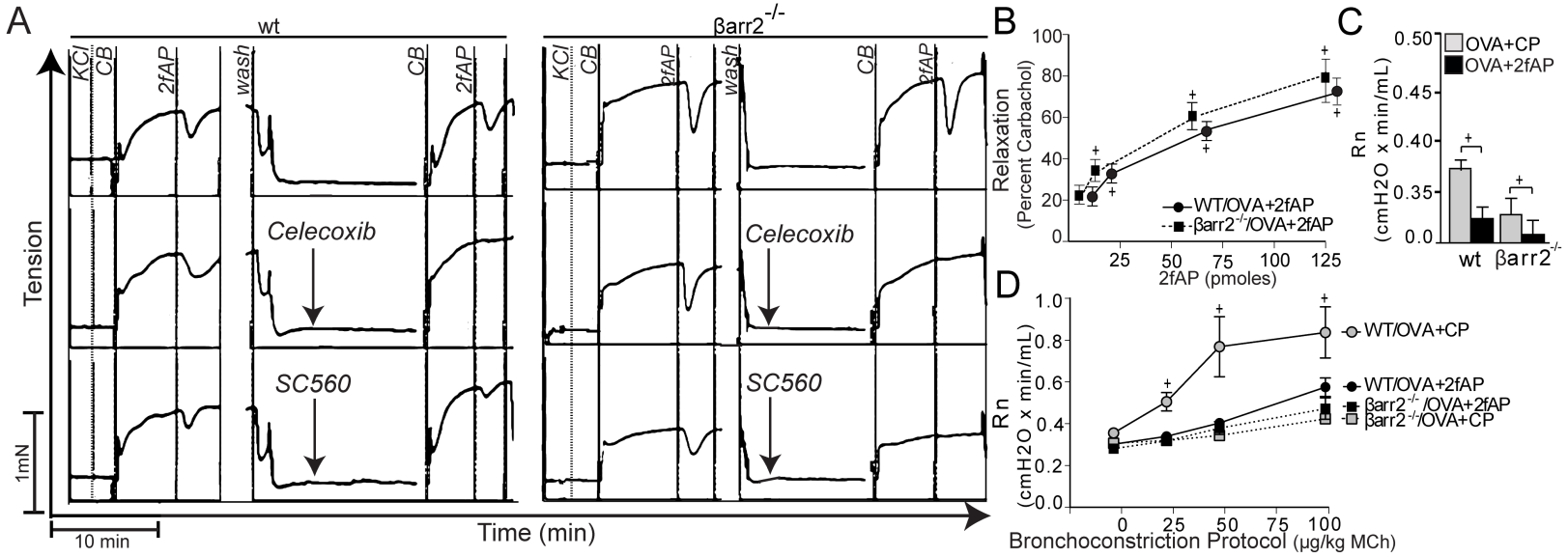


Figure 2.6 PAR_2-induced smooth muscle relaxation is maintained in β-arrestin-2-/- mice. A. Representative myograph traces of tension in bronchiole smooth muscle from wt (left) and β-arrestin-2-/- mice (right). Treatments with KCl (to assess viability), carbachol (CB) and 2fAP are indicated. Samples were washed and pretreated with vehicle (top panel), COX-2 inhibitor (Celecoxib, .2μM) (middle panel) or COX1 inhibitor (SC-560, .1μM) (bottom panel). **B.** Average relaxation (percentage of the CB-induced tension) from 3 independent experiments is graphed as a function of [2fAP]. +Significant differences in tension with 2fAP ($p<0.01$). Mice, treated as described in Fig 1A, were bronchoconstricted with indicated doses of methacholine (MCh), **C.** Graph of baseline Newtonian resistance values (Rn) and **D**. Methacholine-induced AHR in wt and β-arrestin-$2^{-/-}$ mice that received OVA+CP or OVA+2fAP. +Significant difference between bracketed groups (Fig 6D) ($p<.01$ n=6). D. +Significant differences in MCh responsiveness compared to baseline ($p<0.05$).

Section 2.7

Discussion:

Efforts directed at exploiting the potential 'protective' bronchodilator effects of PAR_2 agonists for the treatment of asthma have been hindered by studies demonstrating that PAR_2 agonists can cause inflammatory responses in various tissues (18, 21, 29). The debate as to whether PAR_2 is a protective or pro-inflammatory receptor in allergic inflammatory airway disease resembles the continued controversy surrounding the function of the β_2-AR, the prototypical GPCR, in asthma. Although β-agonists are bronchiodilators of choice to alleviate acute bronchospasm, their chronic use is associated with loss of bronchoprotection, worsening of asthma control and asthma-related death(30). In murine models, the asthma phenotype is significantly promoted by chronic β-agonist treatment (5, 31) or impaired in mice having genetic or pharmacologic ablation of β_2-ARs(32, 33). Similarly, mice lacking β-arrestin-2 do not develop the asthma phenotype (25), placing β-arrestin-2 downstream of GPCRs in the pro-asthmatic signaling pathway. Thus, the seemingly paradoxical effects of PAR_2 and β_2-AR agonists on the asthma phenotype can likely be explained by the activation of dual signaling pathways; a pro-inflammatory β-arrestin-dependent signaling pathway in addition to the classical G protein-mediated bronchorelaxation pathway(6).

Our studies provide new insight to explain the apparently opposing responses of PAR_2 activation. We, and others, have previously shown that in a 25-day murine multiple-OVA-challenge model development of the asthma phenotype is significantly impaired in mice lacking either β-arrestin-2 or PAR_2 (18, 19, 25). To differentiate

between PAR_2-specific effects that are mediated by β-arrestin-2, and the general β-arrestin-2-OVA-induced allergic responses also we employed a 15-day OVA model in the current study. At this time point, the magnitude of the asthma phenotype induced by OVA is low, whereas the PAR_2 agonist exacerbating effects are high (1), allowing the asthma phenotype-inducing effects of PAR_2 to be highlighted. We show that the 'inflammatory' leukocyte infiltration response in our asthma model depends on β-arrestin-2-mediated signaling, whereas many of the 'protective' bronchodilator effects of PAR_2 activation (such as PGE2 production and subsequent bronchiolar smooth muscle relaxation) do not. The results shown here are consistent with previous studies demonstrating two independent signaling pathways downstream of PAR_2: a β-arrestin-2-dependent pathway leading to actin cytoskeletal changes and cell migration, and a G-protein-dependent one that promotes Ca^{2+} mobilization and diacylglycerol formation(16, 17). We have previously shown formation of a β-arrestin scaffold containing the actin filament-severing protein cofilin and its upstream activator in primary bone marrow leukocytes, and have demonstrated that this signaling pathway is crucial for PAR_2-stimulated cell migration (2, 17). PAR_2 has also been reported to increase PGE2 levels by multiple mechanisms, some resulting in rapid, and others delayed, PGE2 release, all of which appear to occur through Gαq-dependent, β-arrestin-independent signaling (14-17, 22, 24). Our data suggest that both COX1 and COX2 appear to be interlinked to generate the relaxant response, since a selective inhibition of either enzyme blocked the relaxant response. Taken together, these results indicate that this protective signaling axis is independent of β-arrestin-2, despite reports that IL-1 and Angiotensin II can increase

PGD2 and PGE2 levels via a β-arrestin-dependent pathway in vitro (34, 35). The signaling pathway activated by a PAR_2 mechanism that does not involve β-arrestin-2 merits further investigation. Taken together, these studies support the hypothesis that these two independent PAR_2 signaling pathways may direct opposing responses in vivo.

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

Like human asthma, one of the defining features of allergic inflammatory airways disease in mice is airway hyperresponsiveness (AHR), a measure of the sensitivity and reactivity of airway narrowing to a bronchoconstrictor. We assessed airway reactivity, the more clinically relevant component of AHR (36), by measuring the resistance response to multiple increasing concentrations of the bronchoconstrictor, methacholine. Wild type mice treated with 2fAP in the context of OVA displayed a statistically significant and physiologically relevant decrease in airway reactivity relative to those treated with CP. Because the effect of OVA treatment on AHR in β-arrestin-$2^{-/-}$ mice is so muted, it is

difficult to assess whether this protective effect of PAR_2 in vivo requires β-arrestins; however a significant reduction in baseline resistance was observed in mice receiving OVA+2fAP compared with OVA+CP in both wild type and β-arrestin-$2^{-/-}$ animals, suggesting that PAR_2-mediated airway smooth muscle relaxation is independent of β-arrestin-2. The protective effect of 2fAP on AHR in mice appears to be dominant over the pro-inflammatory effect since AHR decreased despite the concomitant enhanced airways inflammation and mucin phenotypes, which are typically associated with increased AHR. Our data support the notion that the paradoxical effects of PAR_2 activation are mediated by dual signaling pathways. Separation of these dual pathways in vivo can be challenging, especially if the receptor activates opposing outcomes. For example, in guinea pig bronchial preparations the prostanoid-mediated epithelium-dependent bronchodilator action of PAR_2 is masked by the concurrent PAR_2–mediated triggering of airway hyperresponsiveness and only revealed in the presence of indomethacin (37). PAR_2-mediated production of airway prostaglandins likely underlies the protective effect of 2fAP on AHR observed here as well. To definitively determine whether the protective effects of PAR_2 on AHR are independent of β-arrestins in vivo will likely require using a model that results in a more robust AHR phenotype in β-arrestin-$2^{-/-}$ mice (6).

PAR_2 is up-regulated in the airways of patients with chronic asthma (38), which along with the reported pro-inflammatory effects of PAR_2, has led to an interest in inhibitors of PAR_2-activating proteinases or of the receptor itself as therapeutic agents for treating asthma (39). In contrast, the protective effects of PAR_2 have also generated

interest in PAR_2 agonists as therapeutic agents for asthma, with the idea being that agonists of PAR_2 might promote bronchodilatation (8, 21, 40, 41). Although these same agonists can exacerbate other aspects of lung inflammation (1, 18, 19, 42), our work reveals the potential of developing 'biased' PAR_2 agonists or antagonists that can selectively attenuate the β-arrestin-2-dependent signal pathway and optimize the 'protective' PAR_2 G protein-mediated signaling responses. Studies identifying naturally occurring biased PAR_2 signaling by cryptic proteolytic cleavage and mutant tethered ligands point to the feasibility of developing such agonists(7, 43, 44). Recent identification of pepducins that specifically inhibit PAR_2 raises the possibility that development of small molecule β-arrestin-specific PAR_2 antagonists might be possible(45). Pharmacologic manipulation of biased signaling may underlie the mortality advantage observed in congestive heart failure (CHF) patients treated with carvedilol versus metoprolol (46, 47). Perhaps PAR_2, like the β_1-AR, as a GPCR capable of eliciting G protein- and β-arrestin-dependent signals that regulate organ system function and disease features in disparate ways. However, in this instance we have PAR_2 -mediated β-arrestin signaling driving airway pathology and G-protein signaling combating it, whereas in heart failure β_1-AR-mediated G protein signaling is pathogenic and β-arrestin signaling is cardioprotective. Taken together, direct targeting of the β-arrestin-2-dependent signaling pathway may hold tremendous therapeutic promise in the treatment of asthma. The potential to modulate PAR_2-specific signaling pathways that mediate protective or pro-inflammatory effects sets the stage for development of pathway-specific therapeutic agents for the treatment of asthma and other inflammatory diseases (6).

Section 2.8

References

1. Ebeling C*, et al.* (2005) Proteinase-activated receptor 2 activation in the airways enhances antigen-mediated airway inflammation and airway hyperresponsiveness through different pathways. (Translated from eng) *J Allergy Clin Immunol* 115(3):623-630 (in eng).
2. Zoudilova M*, et al.* (2010) beta-Arrestins scaffold cofilin with chronophin to direct localized actin filament severing and membrane protrusions downstream of protease-activated receptor-2. (Translated from eng) *J Biol Chem* 285(19):14318-14329 (in eng).
3. Schuessler TF & Bates JH (1995) A computer-controlled research ventilator for small animals: design and evaluation. (Translated from eng) *IEEE Trans Biomed Eng* 42(9):860-866 (in eng).
4. Proskocil BJ & Fryer AD (2005) Beta2-agonist and anticholinergic drugs in the treatment of lung disease. (Translated from eng) *Proc Am Thorac Soc* 2(4):305-310; discussion 311-302 (in eng).
5. Lin R*, et al.* (2012) Chronic treatment in vivo with beta-adrenoceptor agonists induces dysfunction of airway beta(2) -adrenoceptors and exacerbates lung inflammation in mice. (Translated from eng) *Br J Pharmacol* 165(7):2365-2377 (in eng).
6. Nichols HL*, et al.* (2012) beta-Arrestin-2 mediates the proinflammatory effects of proteinase-activated receptor-2 in the airway. (Translated from eng) *Proc Natl Acad Sci U S A* 109(41):16660-16665 (in eng).
7. Ramachandran R, Noorbakhsh F, Defea K, & Hollenberg MD (2012) Targeting proteinase-activated receptors: therapeutic potential and challenges. (Translated from eng) *Nat Rev Drug Discov* 11(1):69-86 (in eng).
8. Cocks TM & Moffatt JD (2001) Protease-activated receptor-2 (PAR2) in the airways. (Translated from eng) *Pulm Pharmacol Ther* 14(3):183-191 (in eng).
9. Oikonomopoulou K*, et al.* (2006) Proteinase-mediated cell signalling: targeting proteinase-activated receptors (PARs) by kallikreins and more. (Translated from eng) *Biol Chem* 387(6):677-685 (in eng).
10. Arizmendi NG*, et al.* (2011) Mucosal allergic sensitization to cockroach allergens is dependent on proteinase activity and proteinase-activated receptor-2 activation. (Translated from eng) *J Immunol* 186(5):3164-3172 (in eng).
11. Boitano S*, et al.* (2011) Potent Agonists of the Protease Activated Receptor 2 (PAR2). *Journal of Medicinal Chemistry* 54(5):1308-1313.
12. Hollenberg MD*, et al.* (2008) Derivatized 2-furoyl-LIGRLO-amide, a versatile and selective probe for proteinase-activated receptor 2: binding and visualization. (Translated from eng) *J Pharmacol Exp Ther* 326(2):453-462 (in eng).
13. McGuire JJ, Saifeddine M, Triggle CR, Sun K, & Hollenberg MD (2004) 2-furoyl-LIGRLO-amide: a potent and selective proteinase-activated receptor 2 agonist. (Translated from eng) *J Pharmacol Exp Ther* 309(3):1124-1131 (in eng).

14. DeFea KA, *et al.* (2000) beta-arrestin-dependent endocytosis of proteinase-activated receptor 2 is required for intracellular targeting of activated ERK1/2. (Translated from eng) *J Cell Biol* 148(6):1267-1281 (in eng).
15. Ge L, Ly Y, Hollenberg M, & DeFea K (2003) A beta-arrestin-dependent scaffold is associated with prolonged MAPK activation in pseudopodia during protease-activated receptor-2-induced chemotaxis. (Translated from eng) *J Biol Chem* 278(36):34418-34426 (in eng).
16. Wang P & DeFea KA (2006) Protease-activated receptor-2 simultaneously directs beta-arrestin-1-dependent inhibition and Galphaq-dependent activation of phosphatidylinositol 3-kinase. (Translated from eng) *Biochemistry* 45(31):9374-9385 (in eng).
17. Zoudilova M, *et al.* (2007) Beta-arrestin-dependent regulation of the cofilin pathway downstream of protease-activated receptor-2. (Translated from eng) *J Biol Chem* 282(28):20634-20646 (in eng).
18. Schmidlin F, *et al.* (2002) Protease-activated receptor 2 mediates eosinophil infiltration and hyperreactivity in allergic inflammation of the airway. (Translated from eng) *J Immunol* 169(9):5315-5321 (in eng).
19. Takizawa T, *et al.* (2005) Abrogation of bronchial eosinophilic inflammation and attenuated eotaxin content in protease-activated receptor 2-deficient mice. (Translated from eng) *J Pharmacol Sci* 98(1):99-102 (in eng).
20. DeFea KA (2007) Stop that cell! Beta-arrestin-dependent chemotaxis: a tale of localized actin assembly and receptor desensitization. (Translated from eng) *Annu Rev Physiol* 69:535-560 (in eng).
21. Cocks TM, *et al.* (1999) A protective role for protease-activated receptors in the airways. (Translated from eng) *Nature* 398(6723):156-160 (in eng).
22. Henry PJ (2006) The protease-activated receptor2 (PAR2)-prostaglandin E2-prostanoid EP receptor axis: a potential bronchoprotective unit in the respiratory tract? (Translated from eng) *Eur J Pharmacol* 533(1-3):156-170 (in eng).
23. Kawao N, *et al.* (2005) Signal transduction for proteinase-activated receptor-2-triggered prostaglandin E2 formation in human lung epithelial cells. (Translated from eng) *J Pharmacol Exp Ther* 315(2):576-589 (in eng).
24. Nagataki M, Moriyuki K, Sekiguchi F, & Kawabata A (2008) Evidence that PAR2-triggered prostaglandin E2 (PGE2) formation involves the ERK-cytosolic phospholipase A2-COX-1-microsomal PGE synthase-1 cascade in human lung epithelial cells. (Translated from eng) *Cell Biochem Funct* 26(2):279-282 (in eng).
25. Walker JKL, *et al.* (2003) β-Arrestin-2 regulates the development of allergic asthma. *The Journal of Clinical Investigation* 112(4):566-574.
26. Izuhara K, *et al.* (2009) The mechanism of mucus production in bronchial asthma. (Translated from eng) *Curr Med Chem* 16(22):2867-2875 (in eng).
27. McGuire JJ, Hollenberg MD, Andrade-Gordon P, & Triggle CR (2002) Multiple mechanisms of vascular smooth muscle relaxation by the activation of proteinase-activated receptor 2 in mouse mesenteric arterioles. (Translated from eng) *Br J Pharmacol* 135(1):155-169 (in eng).

28. Hollingsworth JW, *et al.* (2010) Both hematopoietic-derived and non-hematopoietic-derived {beta}-arrestin-2 regulates murine allergic airway disease. (Translated from eng) *Am J Respir Cell Mol Biol* 43(3):269-275 (in eng).
29. Vergnolle N, Hollenberg MD, Sharkey KA, & Wallace JL (1999) Characterization of the inflammatory response to proteinase-activated receptor-2 (PAR2)-activating peptides in the rat paw. (Translated from eng) *Br J Pharmacol* 127(5):1083-1090 (in eng).
30. Walker JKL, Penn RB, Hanania NA, Dickey BF, & Bond RA (2011) New perspectives regarding β2-adrenoceptor ligands in the treatment of asthma. *British journal of pharmacology* 163(1):18-28.
31. Nguyen LP, *et al.* (2008) Chronic exposure to beta-blockers attenuates inflammation and mucin content in a murine asthma model. (Translated from eng) *American journal of respiratory cell and molecular biology* 38(3):256-262 (in eng).
32. Nguyen LP, *et al.* (2009) Beta2-adrenoceptor signaling is required for the development of an asthma phenotype in a murine model. (Translated from eng) *Proc Natl Acad Sci U S A* 106(7):2435-2440 (in eng).
33. Callaerts-Vegh Z, *et al.* (2004) Effects of acute and chronic administration of beta-adrenoceptor ligands on airway function in a murine model of asthma. (Translated from eng) *Proc Natl Acad Sci U S A* 101(14):4948-4953 (in eng).
34. Mathurin K, *et al.* (2011) An interaction between L-prostaglandin D synthase and arrestin increases PGD2 production. (Translated from eng) *J Biol Chem* 286(4):2696-2706 (in eng).
35. Kendall RT, *et al.* (2011) The beta-arrestin pathway-selective type 1A angiotensin receptor (AT1A) agonist [Sar1,Ile4,Ile8]angiotensin II regulates a robust G protein-independent signaling network. (Translated from eng) *J Biol Chem* 286(22):19880-19891 (in eng).
36. Busse WW (2010) The relationship of airway hyperresponsiveness and airway inflammation: Airway hyperresponsiveness in asthma: its measurement and clinical significance. (Translated from eng) *Chest* 138(2 Suppl):4S-10S (in eng).
37. Barrios VE, Jarosinski MA, & Wright CD (2003) Proteinase-activated receptor-2 mediates hyperresponsiveness in isolated guinea pig bronchi. (Translated from eng) *Biochem Pharmacol* 66(3):519-525 (in eng).
38. Knight DA, *et al.* (2001) Protease-activated receptors in human airways: upregulation of PAR-2 in respiratory epithelium from patients with asthma. (Translated from eng) *J Allergy Clin Immunol* 108(5):797-803 (in eng).
39. Kanke T, *et al.* (2009) Novel antagonists for proteinase-activated receptor 2: inhibition of cellular and vascular responses in vitro and in vivo. *British journal of pharmacology* 158(1):361-371.
40. De Campo BA & Henry PJ (2005) Stimulation of protease-activated receptor-2 inhibits airway eosinophilia, hyperresponsiveness and bronchoconstriction in a murine model of allergic inflammation. *British journal of pharmacology* 144(8):1100-1108.

41. D'Agostino B, *et al.* (2007) Activation of protease-activated receptor-2 reduces airways inflammation in experimental allergic asthma. *Clinical & Experimental Allergy* 37(10):1436-1443.
42. Alshurafa HN, *et al.* (2004) A protease activated receptor-2 (PAR-2) activating peptide, tc-LIGRLO-NH2, induces protease release from mast cells: role in TNF degradation. (Translated from eng) *BMC Pharmacol* 4:12 (in eng).
43. Ramachandran R, *et al.* (2009) Agonist-biased signaling via proteinase activated receptor-2: differential activation of calcium and mitogen-activated protein kinase pathways. (Translated from eng) *Mol Pharmacol* 76(4):791-801 (in eng).
44. Ramachandran R, *et al.* (2011) Neutrophil elastase acts as a biased agonist for proteinase-activated receptor-2 (PAR2). (Translated from eng) *J Biol Chem* 286(28):24638-24648 (in eng).
45. Sevigny LM, *et al.* (2011) Interdicting protease-activated receptor-2-driven inflammation with cell-penetrating pepducins. (Translated from eng) *Proc Natl Acad Sci U S A* 108(20):8491-8496 (in eng).
46. Remme WJ, *et al.* (2007) Effect of carvedilol and metoprolol on the mode of death in patients with heart failure. (Translated from eng) *Eur J Heart Fail* 9(11):1128-1135 (in eng).
47. Wisler JW, *et al.* (2007) A unique mechanism of beta-blocker action: carvedilol stimulates beta-arrestin signaling. (Translated from eng) *Proc Natl Acad Sci U S A* 104(42):16657-16662 (in eng).

Chapter 3:

Protease-activated-Receptor-2-Induced Signaling through β-Arrestin-2 Mediates BG and AltA-induced Airway Inflammation

Section 3.1

Abstract

Protease Activated Receptor 2 (PAR_2) is a well-characterized receptor omnipresent in lung tissues, that is activated by a variety of trypsin-like proteases(1-3). Recent studies have shown that proteases found in common allergens such as feces of the German cockroach *Blatella germanica* (BG) and fungal spores of *Alternaria alternata* (AltA) can activate PAR_2 in the airways(4). Furthermore, these studies indicate that PAR_2 is crucial to the ability of these allergens to promote asthma. We have previously reported that the pro-inflammatory effects of PAR_2 in the airway are dependent upon signaling through β-arrestin-2 and that influx of immune cells into the airway involves PAR_2-induced chemotaxis-a pathway known to require β-arrestins(5). However, the role of β-arrestin-dependent signaling and chemotaxis in BG- and AltA-induced asthma has not been investigated. Here we show that BG and AltA promote recruitment of β-arrestins to PAR_2, leading to activation of cofilin and chemotaxis-both hallmarks of β-arrestin-dependent signaling. We demonstrate that exposure to BG or AltA increases damage to airway epithelial tissue and cellular airway inflammation in wild type but not β-arrestin-2-/- mice. Analyses of inflammatory cells in Broncho Alveolar Lavage (BAL) and digested lung tissues highlight an aero-allergen induced increase in neutrophils, eosinophils and CD4+ T-cells, which distinguishes these models from models of PAR_2 dependent airway inflammation which are primarily eosinophilic. Additionally, histological examination reveals that the number of goblet cells is increased and mucin-filled

airways are greatly increased in wt but not β-arrestin-2$^{-/-}$ mice. Recruitment of eosinophils and lymphocytes in response to BG is impaired in wild type mice transplanted with PAR$_2$$^{-/-}$ bone marrow, suggesting PAR$_2$ signaling through β-arrestins in the immune cells themselves is important for their migration into the lungs. Both BG and These studies demonstrate that proteases found in common household aeroallergens can activate PAR$_2$ signaling through β-arrestin-2, resulting in a profound asthma phenotype, pointing to the potential use of β-arrestin biased PAR$_2$ antagonists in the treatment of asthma.

Section 3.2

Methods and Materials:

Mouse Inflammation Models: B. germanica: Cockroach extract purchased from Greer Labs. Intranasal protocol followed as previously published (6). *A. alternata:* For these experiments we used *AltA* filtrate provided by our collaborator, Dr. Scott Boitano. Filtrate is collected from fungal tissue, standardized for protein and protease content, and at 650 μg/ml (in Hanks Buffered Saline Solution/ HBSS) is used as a standard concentration in all assays (4)

H&E stain and quantification: staining was performed on paraffin embedded sections. 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 will be stained with Alcian blue (to stain mucin) and fast nuclear red (to identify cell nuclei).

BALF Collection and Differential Cell Count: As previously published. Briefly, 100ul of BALF is spun on to glass slides using Shandon Cytospin. Cells stained using Hema 3 stain kit following manufactures protocol. Differential counts obtained by counting 200 using and categorized based on morphological criteria.

BALF Flow Cytometry Gating for granulocytes and lymphocytes: We used forward and side scatter (FSC/SSC) and expression of cell surface markers CCR3 for eosinophils, CD3+ for lymphocytes from percentages of CD45+ cells in BALF. High SSC/Gr1+ cells were designated as neutrophils. Specific subsets of lymphocytes were gated using antibodies that recognized expression of CD4, CD8 or CD19.

BRET Recruitment of arrestins: BRET, in which the energy emitted when luciferase oxidizes its substrate will excite an acceptor fluorophore (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-1 or 2 are plated in 96-well microplates and either vehicle or agonist is added along with the Luc substrate coelenterazine. Readings are collected using a Multilabel Reader Tristar9640 from Berthold, and emission is detected at 480 nm (Luc) and 535 nm (YFP). The BRET signal is expressed as the signal at 535nm over that at 480nm. To eliminate the possibility that observed signals represent non-specific interactions, we include a donor only control (β-arrestin-luc only) and β-arrestin-Luc + YFP in every experiment. Values observed with donor only controls are subtracted from experimental values to give the "net BRET" signal. Dose curves, ranging from 6.5μg/ml to 650μg/m are used to evaluate differences in the EC50's of different PAR_2-induced interactions.

Transwell Assay: To isolate lymphocytes**,** age and sex-matched wild type and β-arrestin-$2^{-/-}$mice will be sacrificed and spleens immediately removed, minced into small pieces, homogenized in 10mls of PBS, filtered through sterile nylon mesh. Cells will be 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. Red blood cells will be removed by NH_4Cl lysis (0.83% ammonium chloride, 2 min, at 4^oC). Approximately 10^5 cells plated onto collagen-coated membrane inserts with 5μm pores. Cells allowed to attach and then treated with 2fAP or

allergens in the bottom chamber. Cells were allowed to migrate for 2 hours after which migration was analyzed two ways: attached cells identified numbers of crystal violet stained cells on bottom of filters after removing non-migratory cells from the upper side with a cotton swab. Attached migrated cells counted using a bright field compound microscope in 4 fields of vision. Non-attached migratory cells will be identified by collecting the media from the lower chamber, pelleting the cells and counting them in a hemocytometer. For lymphocytes, CD4+ T-cells were confirmed by flow cytometry using anti-CD3, CD4, CD8 and CD19 antibodies and gating based on FSC/SSC, CD3+/CD4+ cells and expressed as fold change over baseline migration to HBSS.

Adoptive Transfer: Donor mice were litter-mates of recipient mice, from heterozygous matings between $GFP^{+/-}$ mice and $PAR_2^{-/-}$. Recipient mice were all $PAR_2^{+/+}GFP^{-/-}$ *and* donor mice were $PAR_2^{+/+}GFP^{+/-}$ or $PAR_2^{-/-}GFP^{+/-}$. A control set, in which $GFP^{+/-}$ bone marrow is transplanted into wt mice, will be included in every experiment, which should give data similar to what was obtained with wild type mice previously. All cells except red blood cells from $GFP^{+/-}$ mice are green. Recipient mice were given ~10^7 bone marrow cells transferred in 250-300µl by retro-orbital injection (under anesthesia). Three days post injection, BG-induced asthma was initiated. The same parameters measured previously were used to evaluate inflammation.

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 3.3

Introduction

Allergic asthma, atopic dermatitis (eczema), and allergic rhinitis (hay fever) are referred to as an allergic triad of symptoms that plague atopic individuals and PAR_2 has been shown to play a role in exacerbation of inflammation present in each case (7, 8). The triad of symptoms disproportionately affects members of lower socioeconomic level (9, 10) and is deserving of further medical attention. Both agonists and antagonists of PAR_2 have long-been proposed as therapeutics in airway inflammation, mostly mediated by prostaglandins, which may be beneficial in treating atopic individuals (11, 12). Here we further investigate PAR_2-induced protective and pro-inflammatory effects in medically-relevant, robust models of allergic asthma. Recently, cockroach frass from *Blatella germanica* (BG) and fungal spores from *Alternaria alternata* (AltA) were published as inducing a PAR_2- dependent airway inflammation and hyperresponsiveness (4, 6). In these studies it was demonstrated that serine proteases present in the AltA and BG extracts cleave the receptor N-terminus to promote canonical PAR_2 signaling such as Ca2+ mobilization. However, β-arrestin-dependent signaling was not examined. As discussed in Chapter 2, PAR_2 also acts as anti-inflammatory receptor in the airway by increasing PGE2 production, which promotes smooth muscle relaxation (13-15). We have previously published studies in a PAR_2 dependent-OVA model of allergic inflammation that suggests that recruitment of β-arrestins may serve as the molecular switch that enables PAR_2 to act as an inflammatory receptor, with the protective effects remaining intact(1, 16).

Therefore, we were interested to know how PAR_2/β-arrestin-2 signaling in these two robust inflammatory models might modulate the inflammatory response in altering the epithelial release of cytokines and chemokines or the difference seen in the distribution or quantity of invading leukocyte subtypes.

Section 3.4

Results

We investigated the role of β-arrestins in the *Blatella germanica* (BG) and *Alternaria alternata*-induced airway inflammation (AltA) that were previously shown to be PAR_2-dependent. To elicit the BG-induced airway inflammation, we administered an intra-nasal dose of BG extract or saline on days 1-5 and then again on days 11-14 to both wild type and β-arrestin-$2^{-/-}$ mice or AltA on days 1, 4, 8 and 9 (Figure 3.1 A.,G.). 24-48 hours after the final intra-nasal event, we collected BAL and lung tissue for further analysis. In our prior work in the OVA-model, we showed that β-arrestin-$2^{-/-}$ mice were capable of processing and presenting antigen and they produce comparable levels of ova-specific IgE to wt mice(5). Examination of H&E stained lung from either allergen-induced inflammatory model shows an increase in epithelial damage in the form of epithelial sluffing and denuded epithelium (Figure 3.1B, G). As both BG and AltA have been shown to contain proteases, it seems likely that they contribute to the epithelial damage observed. We also counted the number of epithelial cells found in the BALF from BG- or AltA-treated wt and observed a 4.8-fold and 10-fold increase over β-arrestin-$2^{-/-}$ mice with same allergen exposure. Only allergen-treated wild type mice significantly increased in cell number compared to saline controls. The damaged

epithelium is likely a result of BG exposure as the damage is not manifest in β-arrestin-2$^{-/-}$ mice and points to an additional route the PAR_2-dependent aeroallergens may gain access to the basolateral cell region, immune cells in the submucosa or endothelial cells; all of which abundantly express PARs. The morphological changes observed in the lung of allergen-treated mice were quantified by the thickness of the epithelial basement membrane and the number of inflammatory cells surrounding the bronchial regions. There was a two-fold increase in thickness of the basement membrane in wt mice treated with either allergen, compared with allergen-exposed β-arrestin-2$^{-/-}$ mice and saline controls (3.1 D. and I.). Changes to the basement membrane of the epithelial tissue can result from deposition of extracullular matrix components as a result of communication between structural cells and immune cells. The pseudothickening observed, more commonly known as airway remodeling is sometimes thought of as a protective mechanism of the lung-to compensate for epithelial tissue by serving as a second barrier, but studies suggest this is not the case (17). Instead it may promote allergic sensitization and contribute to airway hyperresponsiveness. We quantified the number of cells surrounding the bronchial epithelial tissue. The H&E stained tissues reflect a 2-fold inflammatory score increase in BG-treated wt mice over BG-treated β-arrestin-2$^{-/-}$ mice and saline controls (Figure 3.1 E.). The AltA model appears to induce an inflammatory response as we observed a 3-fold increase in inflammatory cells in AltA-treated wt mice over Alta-treated β-arrestin-2$^{-/-}$ mice and HBSS controls (Figure 3.1 J). The apparent β-arrestin-dependent recruitment of inflammatory cells observed in lung tissue reflects the severity of the inflammatory response.

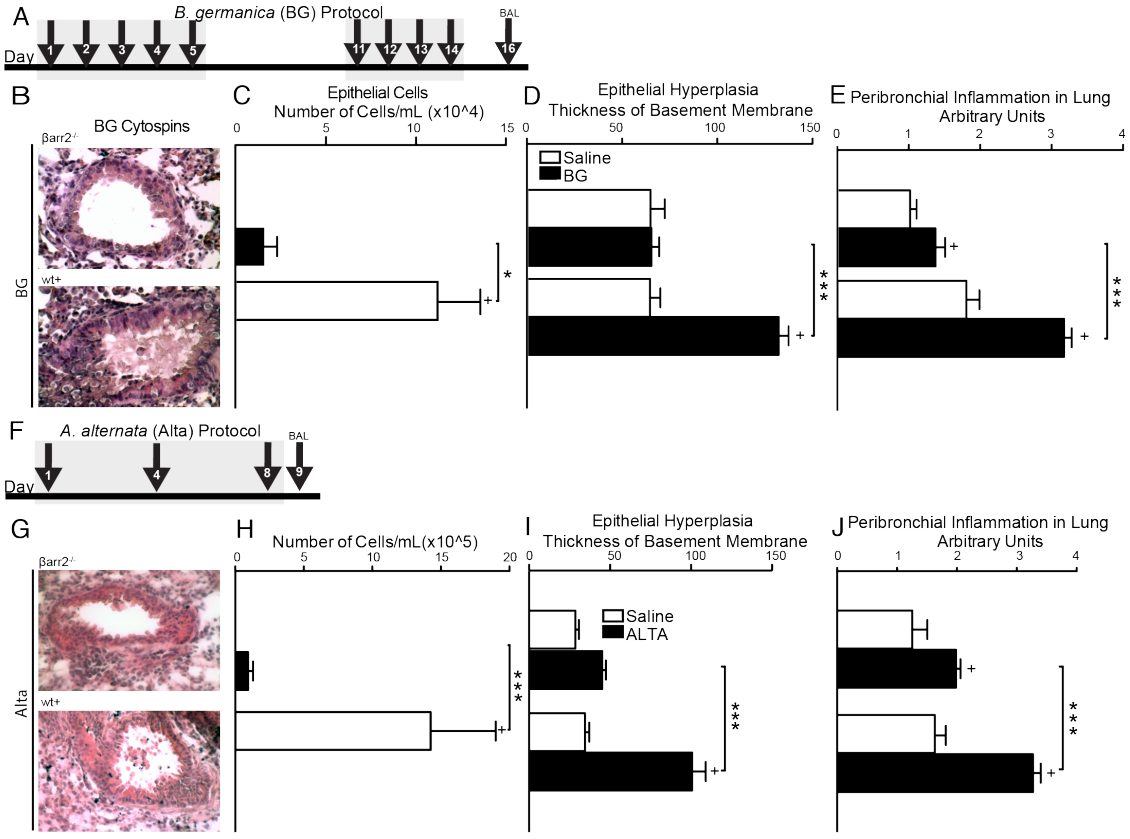


Figure 3.1: Inflammation in lung tissue of mice treated with BG or AltA

A. and **F**. Timeline for BG-induced or Alta-induced airway inflammation. H&E stained lung sections from BG-treated (**B.**) or Alta-treated (**G.**) wt show a visible increase in epithelial damage and inflammation. As a measure of damage, we counted the number of epithelial cells in the BAL cytospins (**C.** and **H.**), and determined a 4-fold and 10-fold increase in cell numbers. Further, we observed nearly 2-fold width increase in the basement membrane of BG-treated (**D.**) and Alta-treated **(E.)** –wt mice over βarr2-/- mice given either PAR_2 dependent allergen and saline controls. The H&E stained tissues from BG-treated (**E.**) or Alta-treated (**J.**) wt mice also reveal elevated numbers of inflammatory cells in peribronchial regions compared with Barr2-/- mice or saline controls. *, ($p<.05$) and ($p<.001$) *** statistically significant differences from treated β-arrestin2-/- mice or sham controls.

Mucin is a high molecular weight mucous glycoprotein secreted by lung epithelial tissue under normal conditions to trap and remove particulate matter, and protects delicate epithelial tissue (18). However, increased mucin production in airway inflammation blocks bronchioles, impeding airflow, correlates with severity in asthma and may contribute to airway hyperresponsiveness. To elucidate the role PAR_2/β-arrestin signaling in airway epithelia, we sought to semi-quantify the number of goblet cells present in airway epithelium and amount of mucin found in the airways by using Alcian blue as a marker of acidic proteoglycans present in mucin and goblet cells (19). As a result BG- or AltA exposure, wt mice exhibit a massive increase in amount of mucin produced reducing the airway conduit (Figure 3.2 A. and B.). Visual analysis of lung tissue from β-arrestin-$2^{-/-}$ mice reveals a marked reduction in blue-stained mucin or goblet cells in these mice (Figure 3.2 A, B).

Examination of both cell concentration and inflammation subtype in differential cell counts and cytometric analysis reveal a change in both total number of cells and the relative distribution of immune cell types with either BG or AltA-induced inflammation. BAL counts of eosinophils, neutrophils and lymphocytes highlight a striking increase in numbers of each from BG-treated (Figure 3.3 A, B, C) and AltA treated (Figure 3.3 D, E, F) wild type mice compared with sham controls. Recruitment of all three cell types is reduced in β-arrestin-$2^{-/-}$ mice although inflammatory cell counts are not completely abolished in BG-treated β-arrestin$2^{-/-}$ mice compared with BG-treated wt mice as we had previously seen in our PAR_2-dependent OVA model of inflammation.

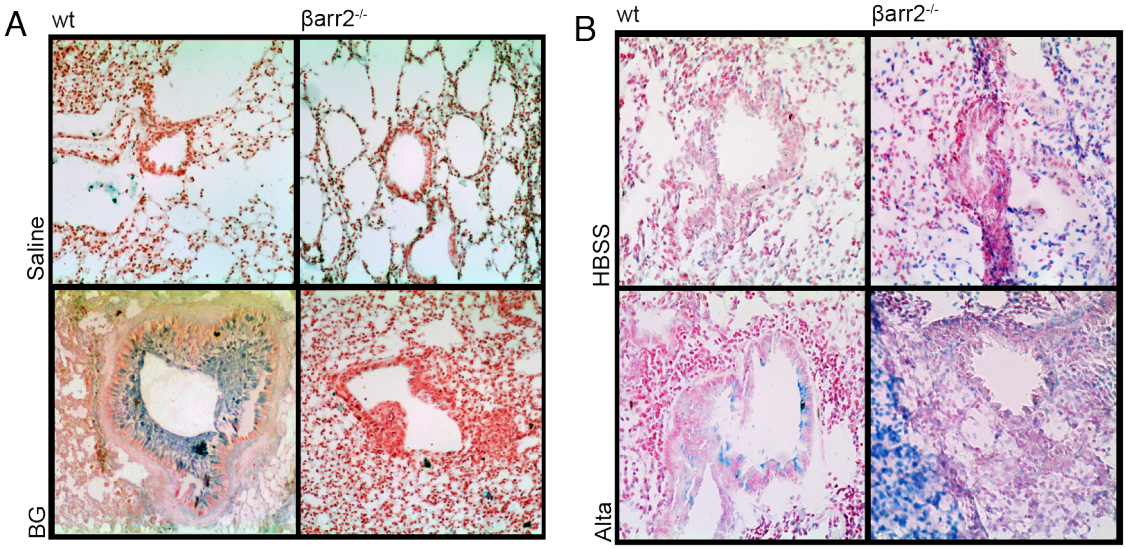


Figure 3.2: Alcian-blue stained mucin in BG- or AltA-treated mice.
Alcian Blue dyes acidic mucins and goblet cells seen in the epithelial tissue of airways. This is greatly increased in BG-treated **(A.)** and Alta-treated **(B.)** Wild type mice and reflects another measure of the allergen-induced exacerbated inflammatory response.

A striking difference in these models is the contribution of neutrophils to the overall inflammation, as neutrophilia was not observed in the PAR_2-dependent OVA model used previously. We further characterized cell populations seen in the inflammatory response with flow cytometric analysis. Using forward and side scatter (FSC/SSC) and expression of cell surface markers CCR3 and CD3 to calculate the percentage of CD45+ BALF cells that were either eosinophils or lymphocytes, respectively (Figure 3.3 C. D. and G.), we observed that 20% of total immune cells in the BAL after BG treatment were positive for neutrophil marker, GR1, ~ in wild type mice and this was reduced only slightly β-arrestin-$2^{-/-}$ mice.

Percentages of eosinophils and lymphocytes were reduced by approximately 50% in the β-arrestin-$2^{-/-}$ mice compared with wt controls. In contrast, in the AltA model, percentages of all three cell types were reduced by approximately 50% in the β-arrestin-$2^{-/-}$ mice. Thus, β-arrestin-2 appears to be crucial for recruitment of cells to the BALF but there is also a β-arrestin-2-independent component to the cellular inflammation. Additionally, neutrophils may be less sensitive to β-arrestin-2 deletion than eosinophils and neutrophils suggesting that neutrophil recruitment may be β-arrestin-independent (Figure 3.3 G). It is noteworthy that in the AltA model, and not BG model, we see a significant difference in proportions of neutrophils between wt and β-arrestin-$2^{-/-}$ mice.

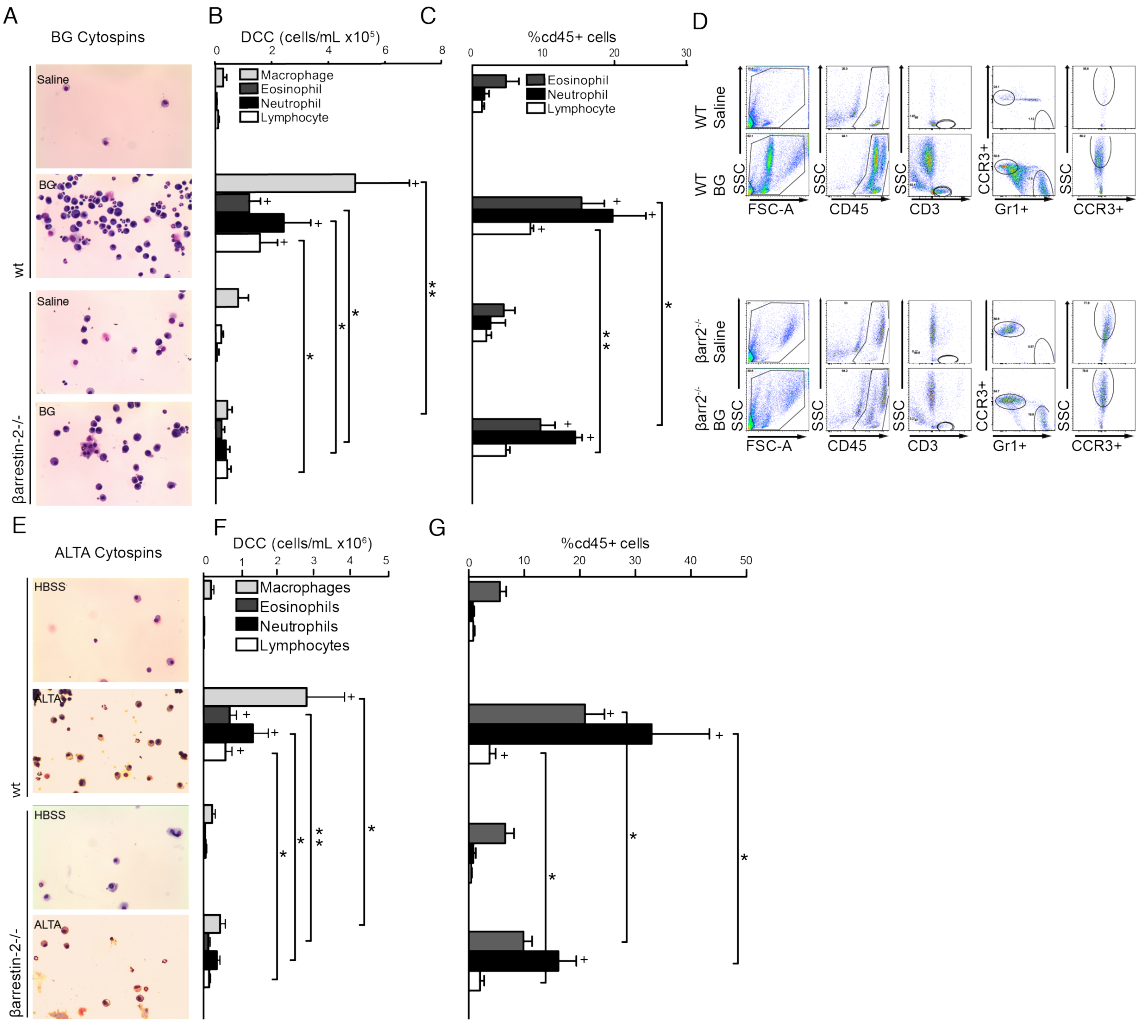


Figure 3.3: Analysis of cell type and distribution obtained from BALF of allergen-treated mice. Both cell numbers and proportion of cell types in the population are altered in allergen-treated mice. Cytospins of BAL obtained from BG- and Alta-treated wt mice **(A., B.; E., F.)** show a statistically significant increase in macrophages, eosinophils, neutrophils and lymphocytes compared to allergen-treated-β-arrestin-2$^{-/-}$ mice, which are similar to saline controls. Cytometric analysis of BAL from wt mice treated with either allergen **(C. and G.)** show significant increases in percentages of eosinophils and lymphocytes, whereas BG-treated wt mice do not show a significant difference in number of neutrophils. **D.** Representative gating strategy used to determine proportions of cd45+ cells. *, ($p<.05$), **, ($p<.01$),and ($p<.001$) *** statistically significant differences from treated β-arrestin2-/- mice. # ($p<.001$) statistically difference from sham controls.

Many cell types are involved in the pathophysiology of asthma. Once an antigen such as BG or AltA is presented by an antigen presenting cell to lymphocyte, a Th2 response, which is a hallmark feature of allergic asthma, is initiated with production of IL-4,-5,-6,-10 and -13. The cytokines orchestrate the Th2 response with lymphocyte recruitment and further release of cytokines. While many cell types are involved in the pathophysiology of asthma, it has been shown previously that lymphocytes exhibit β-arrestin-dependent migration (1, 20) and β-arrestin-2$^{-/-}$ mice display a reduced level of Th2 cytokines, have reduced recruitment of eosinophils/lymphocytes and absence of AHR (5, 21). We sought to characterize the lymphocyte response with antibodies specific to cd4+, cd8+ and cd19+. Both cd4+ and cd8+ subtypes demonstrate complete dependence on β-arrestin-2 as both cell types are completely abolished in BG-treated β-arrestin-2$^{-/-}$ animals (Figure 3.4). CD19+ cells were not significantly different than saline or HBSS-treated mice. These data suggest that cd4+ and cd8+ lymphocytes are essential in the airway inflammatory response.

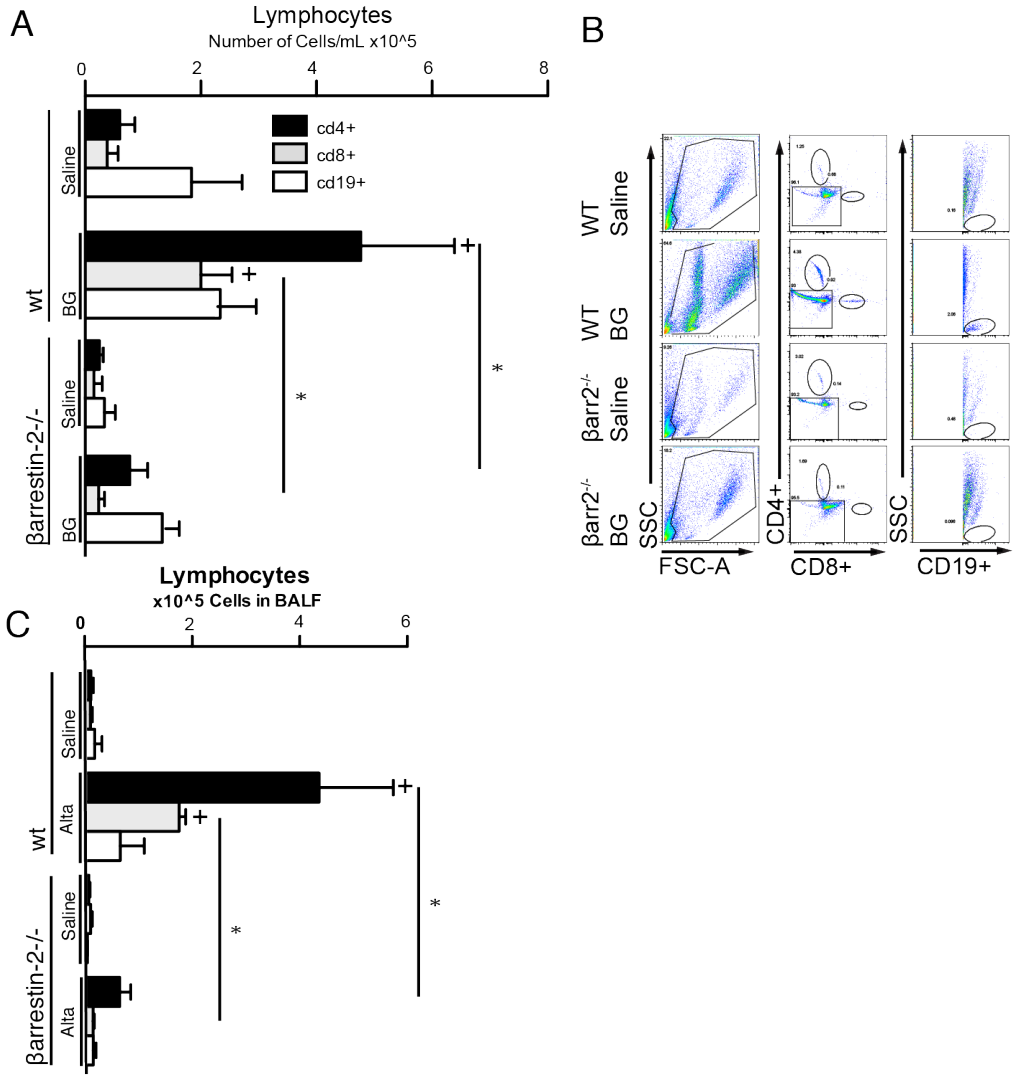


Figure 3.4 Lymphocytes exhibit βarrestin-dependent distribution in cell populations.
Using the representative gating seen in **B**., flow cytometric determination of specific lymphocyte populations. Bar graphs depict numbers of CD4+, CD8+ and CD19+ t cells found in BAL. Either allergen induces a β-arrestin-2$^{-/-}$ -dependent increase in CD4+ and CD8+ t cells **(A. and C.)**, but not CD19+ T-cells. *, ($p<.05$), statistically significant differences from treated β-arrestin-2$^{-/-}$ mice. # ($p<.001$) statistically difference from sham controls.

To determine if the reduced numbers of cd4+ T-cells might be due to their impaired ability to migrate we seeded transwells with isolated splenocytes from either wild type or β-arrestin-$2^{-/-}$. After allowing cells to attach, we added increasing concentrations of 2fAP to the underlying chamber. Numbers increased of cd4+ T-cells from wt mice that migrated through the filter exposed to AltA, BG or 2fAP. No change in migration index is seen cd4+ T-cells from β-arrestin-$2^{-/-}$ mice at any treatment (Figure 3.5 A., B.). Interestingly, we reserved ~10^6 of both wild type and β-arrestin-$2^{-/-}$ splenocytes and treated with BG or AltA for increasing time interval. SDS-PAGE separation of lysates immunoblotting for p/t cofilin reveals an increase in dephosphorylated, active cofilin. (not shown).

Taken together, these data suggest that cd4+ T-cells exhibit PAR_2 / β-arrestin-2-dependent chemotaxis (22, 23) entirely independent of G-proteins. These data beg further validation, but the sequential analysis and attention given to understanding the mechanisms of cd4+ T-cell migration serve as an example in identifying other PAR_2/β-arrestin-2 dependent cell signals and the development of targeted PAR_2 therapeutics.

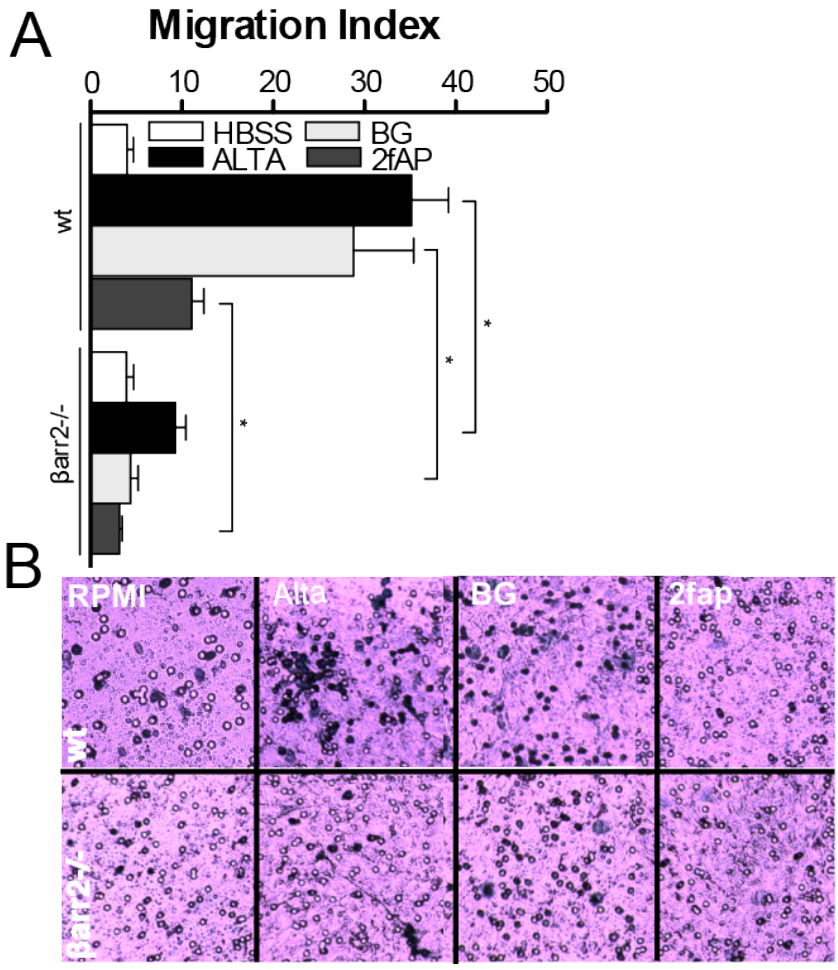


Figure 3.5 BG, Alta and 2fAP induce β-arrestin-2$^{-/-}$ dependent lymphocyte migration. Splenocytes were seeded onto transwells which were submergered in chamber contained either allergen or 2fAP for 90 minutes. The bar graphs **(A.)** depicts number of cells attached to the underside of the transwell at the end of the time course. Both allergens induced a response in wt-cells double that induced by 2fAP only. Images of transwells used to determine migration index **(B.)**. *, ($p<.05$), statistically significant differences from treated β-arrestin-2$^{-/-}$ mice. # ($p<.001$) statistically difference from sham controls.

One of the overarching goals of the BG- and AltA-induced inflammation was to determine if PAR_2 induced recruitment of lymphocytes to the lungs is a direct consequence of PAR_2/β-arrestin signaling on the immune cells themselves (24, 25), or an indirect response to chemotactic factors secreted from the airway epithelium in response to PAR_2. To explore this idea, we transferred marrow from donor mice: $PAR_2^{+/+}GFP^{+/-}$ or $PAR_2^{-/-}GFP^{+/-}$ to recipient mice $PAR_2^{+/+}GFP^{-/-}$. See Figure 2.3-3 for a cartoon depicting the transfer. As all cells except red blood cells from $GFP^{+/-}$ mice are green, the transplanted cells will be readily identifiable by flow cytometry or fluorescence microscopy.

Three days post retro-orbital transplantation of hematopoietic cells we began the BG-induced model allergic inflammation (Figure 3.1). Preliminary data suggests that BG-treated $PAR_2^{-/-}GFP^{+/-}$ marrow recipient mice had a reduction in all cell types found in the BAL (Figure 3.6 A, B, C) though the inflammation appears to be maintained in BG-treated $PAR_2^{+/+}GFP^{+/-}$ marrow recipient mice. As previously discussed, PAR_2 expressed on lymphocytes and eosinophils contribute to the inflammatory responses in vivo as mice with inflammatory cells lacking PAR_2 have reduced numbers of cells, though we did not see the same reduction in number in neutrophil or macrophage cell types as $PAR_2^{+/+}GFP^{+/-}$ or $PAR_2^{-/-}GFP^{+/-}$ recipient mice had similar levels of these cell types.

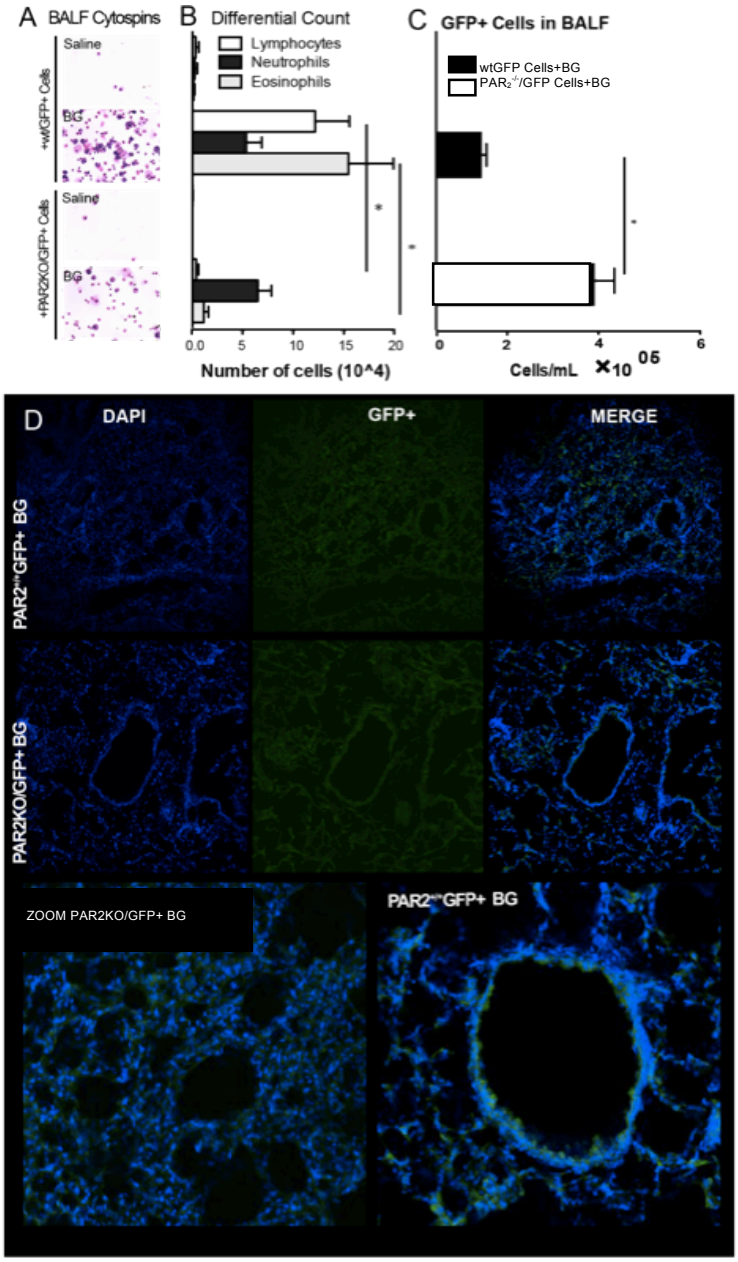


Figure 3.6 Analysis of adoptive transfer experiments shows increased inflammatory subtype recruitment to lungs of BG-treated wt mice as seen in cytospins **(A)** and this is reflected in differential cell counts **(B)** and in flow cytometry analysis of GFP+ cells **(C.).** Confocal microscopy of lung tissue does show an increase in inflammation in intraepithelial tissue in BG-treated wt mice **(D).** *, ($p<.05$), statistically significant differences from treated β-arrestin-2-/- mice. # ($p<.001$) statistically difference from sham controls.

We evaluated BG and AltA-stimulated recruitment of β-arrestin-1 and β-arrestin-2 using BRET (Bioluminesce Resonance Energy Transfer) to confirm the involvement in PAR_2 induced inflammation (26). The DeFea Lab uses BRET to monitor β-arrestin recruitment to PAR_2, in response to trypsin and the peptidomimetic agonist used in our in vivo studies (2fAP) (27). BRET provides a means for monitoring direct actions in live cells and the "net BRET" signal in the dose curves illustrates differences in the binding or EC50's of different PAR_2-induced interactions. Interestingly, the dose response curves of 2fAP (fig 3.7 A.) a PAR_2 specific peptidomimetic and AltA (Figure 3.7 C.) are surprisingly similar while trypsin (Figure 3.7 B.) and BG (Figure 3.7 D.) exhibit similar dose-response curves. This may reflect possible differences in the β-arrestin binding by BG or AltA -induced interactions or spatial-temporal recruitment of β-arrestin-2 to the C-terminus of PAR_2.

Section 3.5

Discussion

PAR_2 is activated by proteases associated with allergy-inducing pathogens such as dust mites, cockroaches *B. germanica* and the fungus *A. alternata* (AltA). AltA is of particular interest because it is one of the most common fungi worldwide and is highly correlated with early onset allergic asthma in more arid regions of the globe. BG feces contain proteases also known for a PAR_2-dependent signal.

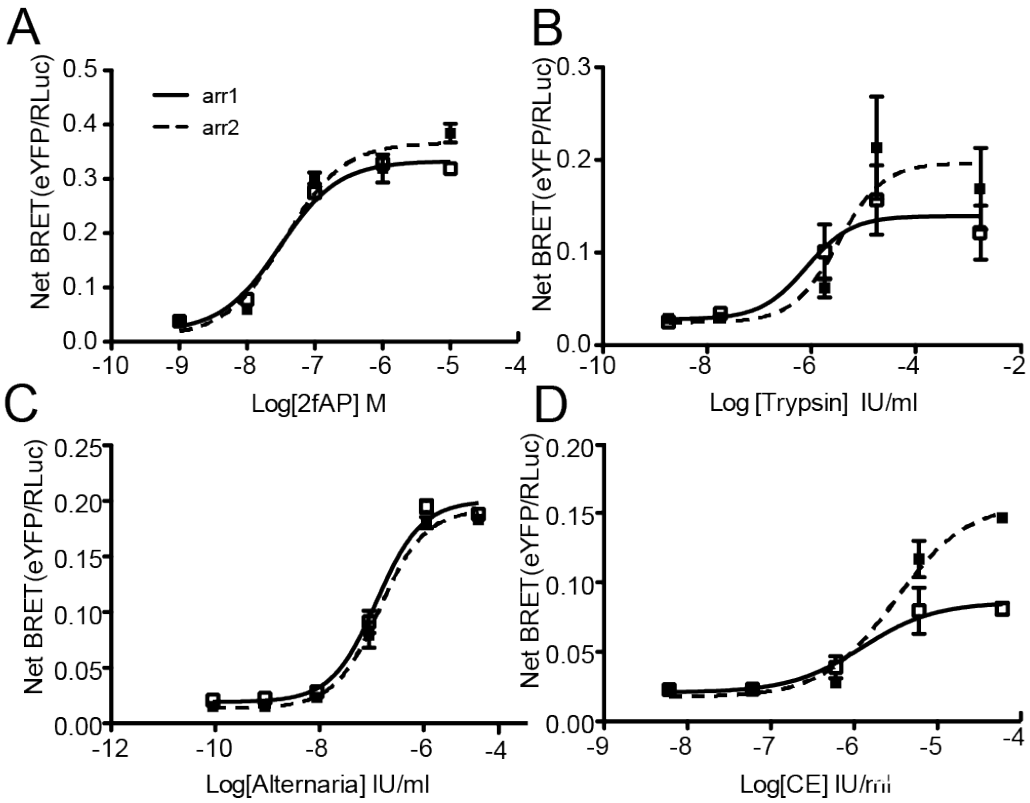


Figure 3.7 Measuring recruitment of βarrestins to allergen-activated PAR_2 using BRET

Bioluminescence resonance energy transfer (BRET) measures recruitment of β-arrestin-1 and β-arrestin-2 to PAR_2 in cells treated with 2fAP **(A.),** Trypsin **(B.),** AltA filtrate **(C.)** or BG **(D.).** *, ($p<.05$), statistically significant differences from treated β-arrestin-2-/- mice. # ($p<.001$) statistically difference from sham controls.

Both physiological proteases require further characterization in terms of signaling downstream of PAR_2. Well-known for G-protein dependent signals via Gαq, Gαi or Gα12/13 pathways and G-protein independent signals (or β-arrestin-$2^{-/-}$ dependent signals) Furthermore, upregulation of PAR_2 has been observed in patients with chronic asthma. Paradoxically, PAR_2 has also been reported to orchestrate protective effects in the airway by promoting prostaglandin E2 [PGE2]-mediated bronchiolar smooth muscle relaxation leading to bronchodilatation. These seemingly contradictory findings have led to the current interest in developing both agonists and antagonists of PAR_2 as putative therapeutics for asthma (27). However, a resolution to the question of whether PAR_2 is friend or foe in the airway is essential to the success of such endeavors, which mandates that the molecular mechanisms underlying the two possibilities be delineated.

Furthermore, environmental allergens such as AltA activate PAR_2 via proteolytic cleavage. In contrast studies examining both the protective effects of PAR_2 and the enhanced inflammation in the presence of PAR_2 activation use nonproteolytic mechanisms to activate PAR_2. Although all of the studies showing β-arrestin-specific PAR_2 signaling have usedboth trypsin and peptide agonists, recent studies have suggested that not some proteases capable of activating PAR_2 show signaling bias. Because PAR_2 can signal through multiple pathways, it is important to understand whether the peptide versus proteolytic PAR_2-induced airway inflammation utilize the same signaling pathways.

We intend to pursue the nuances in leuckocyte recruitment and their dependence on PAR_2/β-arrestin signaling. Whereas eosinophils predominated in our OVA+2fAP model of inflammation, and neutrophils were only sparingly observed, we now see striking increases in neutrophils-perhaps reflecting an increase in severity of airway inflammation. The neutrophilia may also explain the dramatic increase in width of the basement membrane seen in the allergen-treated wt mice as they are the first granulocyte to respond to the intranasal exposure. They arrive within 30 minutes after exposure and are the first granulocyte to infiltrate the lung as they are equipped to recognize cell injury and activated endothelium for adhesion. Once upon the scene, they release preformed mediators such as metalloproteinases, elastase, lactoferrin, and myeloperoxidase all of which correlate with decreased pulmonary function (28). While airway eosinophilia often characterizes allergic asthma, it is widely recognized that neutrophils are found in patients with acute severe asthma (29, 30).

Our investigation of the lung histology demonstrated a pseudothickening with allergen treatment. Changes to the basement membrane of the epithelial tissue can result from deposition of extracullular matrix components as a result of communication between structural cells and immune cells. The pseudothickening observed, more commonly known as airway remodeling is sometimes thought of as a protective mechanism of the lung-to compensate for epithelial by serving as a second barrier, but studies suggest this is not the case (17). Instead it may promote allergic sensitization and contribute to airway hyperresponsiveness.

Section 3.6

References:

1. Zoudilova M*, et al.* (2010) beta-Arrestins scaffold cofilin with chronophin to direct localized actin filament severing and membrane protrusions downstream of protease-activated receptor-2. (Translated from eng) *J Biol Chem* 285(19):14318-14329 (in eng).
2. Cho HJ*, et al.* (2010) House dust mite extract activates apical Cl(-) channels through protease-activated receptor 2 in human airway epithelia. (Translated from eng) *J Cell Biochem* 109(6):1254-1263 (in eng).
3. Kondo S, Helin H, Shichijo M, & Bacon KB (2004) Cockroach allergen extract stimulates protease-activated receptor-2 (PAR-2) expressed in mouse lung fibroblast. (Translated from eng) *Inflamm Res* 53(9):489-496 (in eng).
4. Boitano S*, et al.* (2011) Alternaria alternata serine proteases induce lung inflammation and airway epithelial cell activation via PAR2. (Translated from eng) *Am J Physiol Lung Cell Mol Physiol* 300(4):L605-614 (in eng).
5. Walker JK*, et al.* (2003) Beta-arrestin-2 regulates the development of allergic asthma. (Translated from eng) *J Clin Invest* 112(4):566-574 (in eng).
6. Arizmendi NG*, et al.* (2011) Mucosal allergic sensitization to cockroach allergens is dependent on proteinase activity and proteinase-activated receptor-2 activation. (Translated from eng) *J Immunol* 186(5):3164-3172 (in eng).
7. Yosipovitch G (2004) Dry skin and impairment of barrier function associated with itch - new insights. (Translated from eng) *Int J Cosmet Sci* 26(1):1-7 (in eng).
8. Zheng J*, et al.* (2012) Suppression of connexin 26 is related to protease-activated receptor 2-mediated pathway in patients with allergic rhinitis. (Translated from eng) *Am J Rhinol Allergy* 26(1):e5-9 (in eng).
9. Gary L Evans eKaMS (2008) Socioeconomic Status and Health: The Potential Role of Suboptimal Physical Environments. in *Research Network on SES and Health* (San Francisco).
10. Schafer T RJ (1997) Epidemiology of allergic diseases. *Allergy* 57:14-22.
11. Ramachandran R, Noorbakhsh F, DeFea K, & Hollenberg MD (2012) Targeting proteinase-activated receptors: therapeutic potential and challenges. *Nat Rev Drug Discov* 11(1):69-86.
12. Cocks TM*, et al.* (1999) A protective role for protease-activated receptors in the airways. (Translated from eng) *Nature* 398(6723):156-160 (in eng).
13. Henry PJ (2006) The protease-activated receptor2 (PAR2)-prostaglandin E2-prostanoid EP receptor axis: a potential bronchoprotective unit in the respiratory tract? (Translated from eng) *Eur J Pharmacol* 533(1-3):156-170 (in eng).
14. Nagataki M, Moriyuki K, Sekiguchi F, & Kawabata A (2008) Evidence that PAR2-triggered prostaglandin E2 (PGE2) formation involves the ERK-cytosolic phospholipase A2-COX-1-microsomal PGE synthase-1 cascade in human lung epithelial cells. (Translated from eng) *Cell Biochem Funct* 26(2):279-282 (in eng).

15. Kawabata A*, et al.* (2004) Proteinase-activated receptor-2-mediated relaxation in mouse tracheal and bronchial smooth muscle: signal transduction mechanisms and distinct agonist sensitivity. (Translated from eng) *J Pharmacol Exp Ther* 311(1):402-410 (in eng).
16. Heddie L. Nichols1 MS, Barbara S. Theriot4, Akhil Hegde4, Daniel Polley2, Tamer El-Mays3, Harissios Vliagoftis5, Morley D. Hollenberg2, Emma H. Wilson1, Julia K. L. Walker4 and Kathryn A. DeFea1, * (2012) β-arrestin-2 mediates the pro-inflammatory effects of proteinase-activated-receptor-2 in the airway *Proceedings of the National Academy of Sciences*.
17. Lambrecht BN & Hammad H (2012) The airway epithelium in asthma. (Translated from eng) *Nat Med* 18(5):684-692 (in eng).
18. VERDUGO P (1990) Goblet Cells Secretion and Mucogenesis. *ANNUAL REVIEW OF PHYSIOLOGY* Volume: 52 157-176
19. Anonymous (2008) *The Pulmonary Epithelium in Health and Disease*.
20. Nichols HL*, et al.* (2012) beta-Arrestin-2 mediates the proinflammatory effects of proteinase-activated receptor-2 in the airway. (Translated from eng) *Proc Natl Acad Sci U S A* 109(41):16660-16665 (in eng).
21. Hollingsworth JW*, et al.* (2010) Both hematopoietic-derived and non-hematopoietic-derived {beta}-arrestin-2 regulates murine allergic airway disease. (Translated from eng) *Am J Respir Cell Mol Biol* 43(3):269-275 (in eng).
22. Ge L, Ly Y, Hollenberg M, & DeFea K (2003) A beta-arrestin-dependent scaffold is associated with prolonged MAPK activation in pseudopodia during protease-activated receptor-2-induced chemotaxis. (Translated from eng) *J Biol Chem* 278(36):34418-34426 (in eng).
23. Ge L, Shenoy SK, Lefkowitz RJ, & DeFea K (2004) Constitutive protease-activated receptor-2-mediated migration of MDA MB-231 breast cancer cells requires both beta-arrestin-1 and -2. (Translated from eng) *J Biol Chem* 279(53):55419-55424 (in eng).
24. Leong KP & Huston DP (2001) Understanding the pathogenesis of allergic asthma using mouse models. (Translated from eng) *Ann Allergy Asthma Immunol* 87(2):96-109; quiz 110 (in eng).
25. Moffatt JD, Jeffrey KL, & Cocks TM (2002) Protease-activated receptor-2 activating peptide SLIGRL inhibits bacterial lipopolysaccharide-induced recruitment of polymorphonuclear leukocytes into the airways of mice. (Translated from eng) *Am J Respir Cell Mol Biol* 26(6):680-684 (in eng).
26. Marullo S & Bouvier M (2007) Resonance energy transfer approaches in molecular pharmacology and beyond. (Translated from eng) *Trends Pharmacol Sci* 28(8):362-365 (in eng).
27. Ramachandran R*, et al.* (2011) Neutrophil elastase acts as a biased agonist for proteinase-activated receptor-2 (PAR2). (Translated from eng) *J Biol Chem* 286(28):24638-24648 (in eng).
28. Monteseirin J (2009) Neutrophils and asthma. (Translated from eng) *J Investig Allergol Clin Immunol* 19(5):340-354 (in eng).

29. ORDO√ëEZ C, FERRANDO R, HYDE DÄ, WONG HÄ, & FAHY JÄ (2000) Epithelial Desquamation in Asthma. *American Journal of Respiratory and Critical Care Medicine* 162(6):2324-2329.
30. Vooght VD*, et al.* (2012) Neutrophil and eosinophil granulocytes as key players in a mouse model of chemical-induced asthma. (Translated from Eng) *Toxicol Sci* (in Eng).

Chapter Four

GPCRs and β-Arrestins Signaling and Their Therapeutic Potential

Section 4.1

Conclusions and Perspectives

The primary objective of my research was to take the next logical step. For nearly 10 years, the PAR_2/βarrestin- induced scaffolding had been investigated with a combination of basic biochemistry techniques. They began with investigations of the role of βarrestin-containing scaffolding complexes in the activation and subcellular localization of ERK1/2 (1). In subsequent years, β-arrestin-dependent scaffolds were shown to associate with MAPK in pseudopodia during PAR_2-induced chemotaxis in multiple cell lines (2), and that both β-arrestin-1 and -2 are involved. Further exploration of the signal transduction pathways involved in PAR_2-induced G-protein independent, but arrestin-dependent migration identified scaffolding of the key players in actin reorganization: cofilin and its phosphatase (Chronophin) as well as the upstream kinase, LIMK (3). β-arrestins spatially regulate cofilin activity to facilitate the formation of a leading edge and this is important for PAR_2-stimulated immune cell migration. In 2010, the DeFea Lab published data using size exclusion chromatography and co-immunoprecipitation to illustrate PAR_2-induced β-arrestin scaffolds of actin migration machinery from cultured cells and primary leukocytes (4). These careful observations served as an impetus to incorporate an animal model that allowed us to investigate chemotaxis in a physiological setting.

There are many potential physiological roles, with corresponding animal models, that investigate PAR_2 including: endothelial cell function, intestinal function, skin pigmentation, tumour cell growth and metastasis and allergic asthma (4, 5). Investigation of the latter allowed examination of a long-standing controversial role behind PAR_2 in the airways. The controversy refers to seemingly opposite and opposing responses, or, protective and proinflammatory responses with exposure to PAR_2 activating peptide. For example PAR_2-induced protective mechanisms such as bronchorelaxation of isolated airways (6) were demonstrated in addition to PAR_2-induced proinflammatory effects such as eosinophilia (7) and airway hyperresponsiveness (8). We sought to understand how a single receptor might be responsible for both pro- and anti- inflammatory events in the airways and previous research in the DeFea Lab on g-protein dependent and β-arrestin-dependent pathways downstream of PAR_2 activation served as a starting hypothesis.

In my investigation of a mouse model of ova-induced airway inflammation, we observed that 2fAP, administered in the face of standing ova/alum-sensitization, exacerbated inflammation in wt mice. Inflammation was characterized by eosinophilia, proinflammatory cytokines, leukocyte recruitment and mucin production. However these signs were completely abolished in our β-arrestin-$2^{-/-}$ mice, indicating their β-arrestin-dependence (9). In contrast, PAR_2-mediated PGE2 production, smooth muscle relaxation and decreased baseline airway resistance were independent of β-arrestin-2.

Our data also suggest that the PAR_2-enhanced inflammatory process is β-arrestin-2-dependent, whereas the protective anti-constrictor effect of bronchiαλ epithelial PAR_2 may be β-arrestin independent.

During our investigation of PAR_2 exacerbation of ova-induced allergic asthma, two physiologically relevant models of airway inflammation were shown to be PAR_2 dependent. The first administered extract from cockroach feces intranasally (10) and the other was a fungal model using filtrate from *Alternaria alternata*. (11). We investigated the role of β-arrestin-dependent signaling and chemotaxis in BG and AltA-induced asthma and demonstrate that exposure to BG or AltA increases damage to airway epithelial tissue and airway inflammation in wild type but not β-arrestin-$2^{-/-}$ mice.

One goal in the mouse model of PAR_2 induced airway inflammation was to elucidate the physiological processes that result from direct activation of PAR_2. Identification of the G-protein dependent and β-arrestin-dependent mechanisms may enable us to pharmacologically target and inhibit deleterious PAR_2 signaling or activate protective responses. Like PAR_2, many GPCRs have multiple signaling pathways downstream of the same receptor depending on the activating ligand. When a ligand preferentially activates one pathway over another, it is called a biased agonist(5). The growing awareness of ligand bias creates the opportunity to develop therapeutic agents that can control intracellular signaling with much greater precision while keeping the exquisite specificity (determined by receptor binding) associated with GPCR ligands. It is therefore important to decipher the molecular basis of pharmacological bias of GPCR ligands (12).

There are multiple molecular readouts used to determine bias of a ligand (i.e. arrestin vs. g-protein coupling). Two emphasized in the DeFea Lab are BRET and examination of the crystal structure of arrestins and identifying binding sites on the scaffolding molecules themselves. As previously mentioned, BRET monitors the conformational changes of β-arrestin-2 in living cells while crystal structures used to identify binding sites might shed insight on allosteric modulation for therapeutic manipulation (13).

As mentioned previously, PAR_2 has been proposed as a therapeutic target in allergic airway inflammation and our data suggests that β-arrestin coupling with the receptor directs the proinflammatory response, while G-protein signaling directs the protective effects mediated by prostaglandins. While targeting the g-protein dependent pathway is not always preferred, one attractive therapeutic option in drug development would produce a ligand to bind PAR_2, induce allosteric modulation so the G-protein pathway (and thus protective) prevails. In some situations β-arrestin bias confers positive effects, whereas G protein–dependent signaling may cause side effects (14). An example of beneficial β-arrestin-dependent effects is provided by the β-blocker cervedilol, which induces β-arrestin-biased signaling in both the β_1-AR and β_2-ARsubtypes. The effect of the arrestin-bias is cardioprotective as it stimulates epidermal growth factor receptor transactivation and ERK phosphorylation in a G protein–independent manner while simultaneously inhibiting the G-protein dependent pathway.

In this situation, it appears that β-arrestin signaling, provides additional curative options that are very specific, as compared with other antagonists that block all β-AR signaling that are often used in treatment of heart failure (12).

Biased ligands are not exclusive to airway inflammation and nor heart failure. A review published in 2011 by Nobel Laureate Robert J. Lefkowitz identifies the therapeutic potential of β-arrestin- and G protein-biased agonists in treatment of pathologies in virtually every physiologic system including, but not limited to: renal, rheumatology, immunology, metabolic and bone homeostasis endocrinology, neurology and oncology (14). As these exciting themes in medicine are further investigated in clinical trials, specific therapeutic options targeting GPCRs may become more widely available.

.

Section 4.2

References

1. DeFea KA*, et al.* (2000) beta-arrestin-dependent endocytosis of proteinase-activated receptor 2 is required for intracellular targeting of activated ERK1/2. (Translated from eng) *J Cell Biol* 148(6):1267-1281 (in eng).
2. Ge L, Ly Y, Hollenberg M, & DeFea K (2003) A beta-arrestin-dependent scaffold is associated with prolonged MAPK activation in pseudopodia during protease-activated receptor-2-induced chemotaxis. (Translated from eng) *J Biol Chem* 278(36):34418-34426 (in eng).
3. Zoudilova M*, et al.* (2007) Beta-arrestin-dependent regulation of the cofilin pathway downstream of protease-activated receptor-2. (Translated from eng) *J Biol Chem* 282(28):20634-20646 (in eng).
4. Zoudilova M*, et al.* (2010) beta-Arrestins scaffold cofilin with chronophin to direct localized actin filament severing and membrane protrusions downstream of protease-activated receptor-2. (Translated from eng) *J Biol Chem* 285(19):14318-14329 (in eng).
5. Ramachandran R, Noorbakhsh F, DeFea K, & Hollenberg MD (2012) Targeting proteinase-activated receptors: therapeutic potential and challenges. *Nat Rev Drug Discov* 11(1):69-86.
6. Moffatt JD, Jeffrey KL, & Cocks TM (2002) Protease-activated receptor-2 activating peptide SLIGRL inhibits bacterial lipopolysaccharide-induced recruitment of polymorphonuclear leukocytes into the airways of mice. (Translated from eng) *Am J Respir Cell Mol Biol* 26(6):680-684 (in eng).
7. Schmidlin F*, et al.* (2002) Protease-activated receptor 2 mediates eosinophil infiltration and hyperreactivity in allergic inflammation of the airway. (Translated from eng) *J Immunol* 169(9):5315-5321 (in eng).
8. Ebeling C*, et al.* (2005) Proteinase-activated receptor 2 activation in the airways enhances antigen-mediated airway inflammation and airway hyperresponsiveness through different pathways. (Translated from eng) *J Allergy Clin Immunol* 115(3):623-630 (in eng).
9. Nichols HL*, et al.* (2012) beta-Arrestin-2 mediates the proinflammatory effects of proteinase-activated receptor-2 in the airway. (Translated from eng) *Proc Natl Acad Sci U S A* 109(41):16660-16665 (in eng).
10. Arizmendi NG*, et al.* (2011) Mucosal allergic sensitization to cockroach allergens is dependent on proteinase activity and proteinase-activated receptor-2 activation. (Translated from eng) *J Immunol* 186(5):3164-3172 (in eng).
11. Boitano S*, et al.* (2011) Alternaria alternata serine proteases induce lung inflammation and airway epithelial cell activation via PAR2. (Translated from eng) *Am J Physiol Lung Cell Mol Physiol* 300(4):L605-614 (in eng).

12. Reiter E, Ahn S, Shukla AK, & Lefkowitz RJ (2012) Molecular mechanism of beta-arrestin-biased agonism at seven-transmembrane receptors. (Translated from eng) *Annu Rev Pharmacol Toxicol* 52:179-197 (in eng).
13. Wang CI & Lewis RJ (2012) Emerging opportunities for allosteric modulation of G-protein coupled receptors. (Translated from Eng) *Biochem Pharmacol* (in Eng).
14. Whalen CN & Case-Smith J (2012) Therapeutic effects of horseback riding therapy on gross motor function in children with cerebral palsy: a systematic review. (Translated from eng) *Phys Occup Ther Pediatr* 32(3):229-242 (in eng).