PAK1'S REGULATION OF EOSINOPHIL MIGRATION AND IMPLICATIONS FOR ASTHMATIC INFLAMMATION

Muithi Mwanthi

Submitted to the faculty of the University Graduate School
in partial fulfillment of the requirements
for the degree
Doctor of Philosophy
in the Department of Microbiology and Immunology,
Indiana University

June 2013

Accepted by the Faculty of Indiana University, in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

_____

D. Wade Clapp, MD, Chair

_____

Janice S. Blum, PhD

Doctoral Committee

_____

Susan Gunst, PhD

July, 6th, 2012

_____

David S. Wilkes, MD

_____

Feng-Chun Yang, MD/ PhD

ACKNOWLEDGEMENTS

First, I sincerely thank Dr. Wade Clapp for his mentorship. Dr. Clapp aggressively recruited me to Indiana University's MSTP program, frequently checked on my school progress, kindly drafted me to work on a project that matched my scientific interest, defended me, and consistently provided career-building opportunities, as well as modeled the physician-scientist I aspire to become. Dr. Clapp selflessly and liberally invested in my career and always set a high yet fair standard that focused entirely on my development. I look forward to our interactions for years to come. I also deeply thank my thesis committee of distinguished scientists with diverse expertise who generously co-mentored my development. They often identified potential in my project and career before I saw it and that was only eventually realized by their nurturing guidance.

I thank past and present members of the Clapp, Yang and Hanenberg labs for their support. I especially am grateful to Dr. Su Jung Park who patiently monitored and mentored my progress in the laboratory. I am indebted to many individuals for the collaborative effort and support culminating in the findings of this thesis research project. I thank all our collaborators for their tireless efforts towards this project. I also thank the faculty, staff, and students in the Microbiology and Immunology department as well as the MSTP program who have encouraged my pursuit of this degree and with whom I have fostered many-a-lifelong relationship.

I also thank my parents, Japheth and Sarah, as well as my siblings, Wambua, Caroline, John, and Mwende for their love, guidance, and great examples of industry. I cannot thank my loving wife, Hannah, enough for her forbearance, kind disposition, and unselfish support toward my graduate school experience. Last and most importantly, I thank God for orchestrating every circumstance that brought about the successful completion of this thesis project “for in Him we live and move and have our being” (Acts 17:28) and for His guidance throughout graduate school “for his mercy endures forever” (1 Chronicles 16:34).

ABSTRACT

Muithi Mwanthi

PAK1'S REGULATION OF EOSINOPHIL MIGRATION AND IMPLICATIONS FOR ASTHMATIC INFLAMMATION

More than 300 million people world-wide suffer from breathlessness, wheezing, chest tightness, and coughing characteristic of chronic bronchial asthma, the global incidence of which is on the rise. Allergen-sensitization and challenge elicits pulmonary expression of chemoattractants that promote a chronic eosinophil-rich infiltrate. Eosinophils are increasingly recognized as important myeloid effectors in chronic inflammation characteristic of asthma, although few eosinophil molecular signaling pathways have successfully been targeted in asthma therapy. p21 activated kinases (PAKs), members of the Ste-20 family of serine/threonine kinases, act as molecular switches in cytoskeletal-dependent processes involved in cellular motility. We hypothesized that PAK1 modulated eosinophil infiltration in an allergic airway disease (AAD) murine model. In this model, *Pak1* deficient mice developed reduced inflammatory AAD responses *in vivo* with notable decreases in eosinophil infiltration in the lungs and broncho-alveolar lavage fluids (BALF). To test the importance of PAK1 in hematopoietic cells in AAD we used complementary bone marrow transplant experiments that demonstrated decreased eosinophil inflammation in hosts transplanted with *Pak1* deficient bone marrow. In *in vitro* studies, we show that eotaxin-signaling through

PAK1 facilitated eotaxin-mediated eosinophil migration. Ablating PAK1 expression by genetic deletion in hematopoietic progenitors or siRNA treatment in derived human eosinophils impaired eotaxin-mediated eosinophil migration, while ectopic PAK1 expression promoted this migration. Together these data suggest a key role for PAK1 in the development of atopic eosinophil inflammation and eotaxin-mediated eosinophil migration.

Wade Clapp, MD, Chair

TABLE OF CONTENTS

ABBREVIATIONS

Axβy:	Integrins (in the form alpha x and beta y)
AAD:	Allergic airway disease
AAI:	Allergic airway inflammation
ADF:	Actin depolymerizing factor
FLNA:	Filamin
AHR:	Airway hyperresponsiveness
ANOVA:	Analysis of Variance
APC:	Allophycocyanin
ARC:	Activity-regulated cytoskeleton-associated protein
ARP2/3:	Actin related protein 2/3 complex
ASM:	Airway Smooth muscle
ATP:	Adenosine Triphosphate
BALF:	Broncho-alveolar Lavage Fluid
BCA:	Bicinchoninic acid
bmEos:	Bone marrow derived eosinophils
BSA:	Bovine serum albumin
CCL:	Chemokine (C-C motif) ligand
CCR3:	C-C chemokine receptor type 3
CD:	Cluster of Differentiation
CDC:	Center for Disease Control
CDC42:	Cell division control protein 42
cDNA:	complementary DNA
C-ERMAD:	COOH-terminal ERM association domain
c/EBP:	CEBPA CCAAT/enhancer binding protein
CLC:	Charcot Layden Crystal
CRIB:	Cdc42/Rac-interactive binding
DAPI:	4',6-diamidino-2-phenylindole
DC:	Dendritic cell
DMEM:	Dulbecco’s Modified Eagle Medium
DMSO:	Dimethyl Sulfoxide
DNP:	2,4-Dinitrophenol
ECP:	Eosinophil Cationic Protein
EDN:	Eosinophil-derived neurotoxin
ELISA:	Enzyme Linked ImmunoSorbent Assay
EPO:	Erythropoietin
ERK:	Extracellular regulated kinase
ERM:	Ezrin/Radixin/Moiesin
ESC:	Embryonic Stem Cell
EZ KO:	Ezrin knockout
EZ WT:	Ezrin wildtype
FACS:	Flourescence Activated Cell Sorting
F-actin:	Filamentous actin
FBS:	Fetal Bovine Serum

FERM:	4.1 protein, ezrin, radixin and moesin
FcεRI:	Fc epsilon receptor I/ high-affinity IgE receptor
FITC:	Fluorescein isothiocyanate
Flt3L:	Fms-like receptor tyrosine kinase 3 ligand
G-CSF:	Granulocyte-colony stimulating factor
GATA1:	GATA binding protein 1
GM-CSF:	Granulocyte-macrophage-colony stimulating factor
GAP:	GTPase activating protein
GAPDH:	Glyceraldehyde 3-phosphate dehydrogenase
GDP:	Guanosine diphosphate
GFP:	Green Fluorescent Protein
GMP:	Granulocyte-macrophage progenitor
GTP:	Guanosine triphosphate
H&E:	Hematoxylin and Eosin
HEPES:	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HL-60:	Human promyelocytic leukemia cells
HPGK:	Human phosphoglycerate kinase 1
HPPC:	High proliferation potential cell
HSC:	Hematopoietic stem cell
Hst:	Hoechst
ICAM:	Intercellular adhesion molecule 1
IgE:	Immunoglobulin E
IL-:	Interleukin-
IMDM:	Iscove's Modified Dulbecco's Medium
I.P.:	Intraperitoneal
I.V.:	Intravenous (tail vein)
KO:	Knockout
LDMNC:	Low density mononuclear cell
LFA-1:	Lymphocyte function-associated antigen 1/ CD11a:CD18
LIMK:	Lim Kinase
Mac1:	Macrophage-1 antigen/ αmβ2/ CD11b:CD18/ CR3
MadCAM:	Mucosal vascular addressin cell adhesion molecule
MAPK:	Mitogen activated protein kinase
MBP:	Major basic protein
MCS:	Multiple cloning site
M-CSF:	Macrophage-colony stimulating factor
MCP-1:	Monocyte chemotactic protein 1
Mek:	MAP kinase / Erk Kinase
MEP:	Megakaryocyte-erythroid progenitor
MLCK:	Myosin light-chain kinase
MW-U:	Mann-Whitney U test
NaN3:	Sodium azide
NF1:	Neurofibromatosis type 1
NIH:	National Institutes of Health
OVA:	Ova-albumin
PAKs:	p-21 activated kinases

PAMP:	Pathogen-associated molecular patterns
PAS:	Periodic acid-Schiff
PBD:	p-21 binding domain
PBS:	Phosphate buffered saline
PCR:	Polymerase chain reaction
PE:	Phycoerythrin
PEI:	Polyethyleneimines
PerCP:	Peridinin chlorophyll protein
PU.1/SPI1:	Spleen focus forming virus (SFFV) proviral integration oncogene spi1
PVDF:	Polyvinylidene fluoride
RAC1:	Ras-related C3 botulinum toxin substrate 1
RBC:	Red blood cell
ROCK:	Rho-associated protein kinase
RPMI:	Roswell Park Memorial Institute (culture medium)
SCF:	Stem cell factor
SDS:	Sodium dodecyl sulfate
SDS-PAGE:	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SNARE:	SNAP (Soluble NSF Attachment Protein) REceptor
TGF:	Transforming growth factor
Th2:	T-helper 2
TLSP:	Thymic stromal lymphopoietin
TNF-α:	Tumor necrosis factor alpha
VAMP:	Vesicle-associated membrane protein
VCAM:	Vascular cell adhesion protein
VLA-4:	Very Late Antigen-4 aka α4β1/CD49d:CD29
WHO:	World Health Organization
WT:	Wild-type

BACKGROUND AND SIGNIFICANCE

Asthma

Approximately 250 million people world-wide suffer from breathlessness, wheezing, chest tightness, and coughing characteristic of chronic bronchial asthma. Asthma's staggering 12% US population prevalence continues to rise, paralleling hospitalizations for severe asthmatic attacks, especially in women, children, minority, and low-income populations [1, 2]. Asthma increases morbidity and health care costs, and, in severe cases, causes death. Chronic asthma also synergizes with comorbid cardiopulmonary diseases, significantly compromising the patient's respiratory system [3]. While patients need essential education on allergen–avoidance, the management of asthma primarily centers on pharmacotherapy. The 2009 Asthma Insight and Management survey of treatment modalities in the US reveals unsatisfactory asthma control assessment and implementation of current treatment guidelines [4]. Current global management of asthma symptoms with corticosteroids and β2 agonists has done little to stop disease morbidity or thousands of annual asthma attack fatalities (WHO) and clinical asthmatic care and outcomes in the US have idled in the past decade [5]. General immunosuppressive strategies which cause adverse systemic effects or novel therapies with narrow target population specificity, fail to effectively deal with the heterogeneity among asthma patients. This therapeutic stagnation warrants focused investigation into deregulated inflammatory pathways within effector cells central to asthma pathogenesis. The

mitogen-activated protein kinases and their effector molecules like the p-21 activated kinases (PAKs) are molecular switches that amplify intracellular proinflammatory signals in immune cells that may provide promising targets to modulate asthmatic inflammation.

Bronchial asthma is a clinically heterogeneous disease with broad impact, multi-factorial origin, and varied manifestation. Clinically, asthma is characterized by airway hyper-responsiveness (AHR) to muscarinic agonists, reversible airway obstruction, chronic airway inflammation, and excessive mucus production [6, 7]. Atopic asthma is triggered by exogenous antigens. Clinicians delineate atopic asthmatic subtypes based on the cell infiltrate nature, triggering factors, and response to corticosteroid treatment [6, 7]. Asthmatic inflammatory processes occur in three recognized phases. On allergen exposure the patient experiences rapid onset of local early-phase chest tightness, wheezing, and breathlessness (1-30 minutes) mediated by mast cell degranulation in airway epithelium and smooth muscle. The allergen-challenged individual subsequently suffers prolonged obstructive and pruritic late-phase symptoms (2-9 hours) provoked by systemic lipid mediators [8]. Repeated and continuous allergen-exposure establishes a chronic inflammatory lung environment exhibiting pathognomonic structural changes: distended airways with mucus plugs, eosinophil infiltration, charcot-laden crystals (CLCs), basement membrane fibrosis, airway smooth muscle (ASM) hypertrophy, hyperplasia, and mucus airway epithelium metaplasia (weeks, months, and years) [6]. These morpho-pathological findings

implicate deregulated intercellular interactions among multiple hematopoietic cell lineages and pulmonary environment cell types critical to pathogenesis [9, 10].

Development of murine models of allergic airway inflammation

Animal model design mimics the recognized features of human disease, highlights disease mechanisms, and drives the development of therapeutics. Several animal species including rats, guinea pigs, and even several non-human primate species have provided informative models for allergic airway disease. However, most current field-advancing asthma research is performed in mice primarily because of the ease of genetic manipulation, availability of a broad variety of immunological assays, and practical merits of murine colony management [11]. Initial murine allergic airway disease models developed to investigate hyper-reactive smooth muscle mediated bronchoconstriction, as well as hyperpnea promoted the use of β_2 agonists for asthma treatment [12]. For the last three decades, allergic airway disease (AAD) animal models reflect the increased recognition of the role chronic inflammation plays in the pathogenesis of the human disease of asthma. This recognition initiated the dichotomy of AHR versus inflammatory murine models of allergic airway inflammation (AAI).

Murine ovalbumin (OVA)-sensitization and challenge provides a model to study specific cell and cytokine regulation of allergic airway inflammation and has informed the development of many specific therapies. The OVA murine model has been the linchpin in studying the initial development of allergic airway

inflammation [7]. Lessons learned via the OVA model of allergic airway inflammation have provided insights resulting in oral and inhaled corticosteroids complementing β_2 agonists in human bronchial asthma management [13-16]. Currently β_2 agonist monotherapy without corticosteroids is uncommon practice [17]. On the other hand, in murine models corticosteroids have also been shown to cause significant immunosuppression and osteoporosis [16, 18]. Moreover, corticosteroids do not alter the natural course of the disease [19]. The OVA model, however, is limited by inaccuracy in recapitulating chronic inflammation and tissue remodeling, along with an allergen-induced tolerance induced by chronic OVA challenge that are not characteristic of human asthma [20, 21]. However, the OVA murine AAD model has several limitations as a tool for studying human asthma. Unlike many human allergens, pure OVA lacks intrinsic protease activity and contaminating environmental adjuvants like house dust mite extracts; therefore OVA is commonly used in conjunction with the adjuvant aluminum hydroxide/ALUM or low LPS levels in mice to bolster the inflammatory AAD response [22-25]. Similarly age, gender, strain, and OVA-dose concerns also beleaguer the utility of any murine AAD model [26-29].

Development of murine allergic airway inflammation

Allergic airway inflammation, an adaptive immune response, entails antigenic sensitization and subsequent challenge of the immune system. Allergens, innocuous environmental antigens, sensitize the immune system of atopic individuals. In airway sensitization, allergens degrade epithelial tight junctions,

enhance epithelial blood flow or penetrate the airway epithelial barrier by other mechanisms [30-32]. Tobacco smoke, other air pollutants, genetic factors, and infectious agents may also compromise the integrity of the airway epithelial barrier exposing immunosurveillant airway dendritic cells (DCs) to higher doses of environmental allergens [22, 33-35]. At the basolateral surface of the epithelium, airway DCs usually sample and induce immune tolerance to airway-invasive allergens in adjacent lymph nodes. However, some antigens can activate airway DCs by pattern recognition receptors and protease-activated receptors to induce a potent adaptive immune response [36-38]. Activated airway DCs secrete IL-4 that directs the maturation of naïve T cells to a Th2 phenotype [39]. Airway DCs also secrete chemokines like CCL17 and CCL22 that attract Th2 cells to the lung to orchestrate AAI [40]. A schematic of cellular interactions in the development of human asthmatic inflammation is illustrated in Figure 1.

For decades, researchers studying atopy have considered CD4+ T-helper II (Th2) cell polarization as the central cell dysregulation in allergic diseases and asthma although the crucial role of the inflammatory environment is increasingly gaining recognition [7, 8, 25, 41, 42]. The mature Th2 cell synthesizes and secretes IL-3, IL-4, IL-5, IL-9, IL-13, and granulocyte-macrophage colony stimulating factor (GM-CSF); these molecules direct asthmatic inflammation [43]. Moreover, depletion of murine CD4+ T cells prevents development of AAI and only adoptive transfer of Th2 cells restores allergic inflammatory phenotype [44-

47]. However, specific IL-4, IL-5, and IL-13 anti-therapies have shown varied and limited efficacy in clinical trials [41].

Atopic asthma, a type I hypersensitivity, classically centers around the Th2 cell regulation of B cell antibody isotype switching to antigen-specific IgE molecules [41, 48]. Th2 derived IL-4 and IL-13 promote B cell maturation to allergen-specific IgE secreting plasma cells and memory cells, while, IL-3, IL-4, and IL-13 govern basophil maturation as well as mast cell maturation and pulmonary recruitment [7, 49, 50] . Allergen-specific IgE can then bind to the high affinity F_c epsilon receptor type I (FcεRI) on basophils and mast cells and subsequent allergen exposure crosslinks the IgE-bound receptors inducing immediate degranulation [8]. The mast cell preformed degranulation mediators, histamine, proteoglycans, and serine proteases rapidly increase vascular permeability and bronchoconstriction provoking the characteristic obstructive symptoms of the early phase of asthma [8]. IgE also negatively feeds back on IgE production and signaling via CD23 (FcεRII) crosslinking on B and non-B cells [49-51]. Moreover, AAD studies in immunoglobulin deficient (J_HD) mice show B cells play an immunotolerizing role to allergens [52]. Additionally, anti-IgE treatment in mice reduces several hallmarks of AAI [53]. Mast cell deficient mice ($Kit^{W/W-v}$ & $Kit^{W-sh/W-sh}$) have a significantly attenuated overall airway inflammatory response indicating mast cells are not only crucial in the early-phase but also the late and chronic phases of asthma [54]. Similarly, c-kit silencing in mice ablated this airway inflammation [55]. IgE-bound FcεRI crosslinking also initiates mast-cell

and basophil synthesis of lipid mediators important in the late phase of an asthmatic attack [8].

Another player, the airway epithelium fuels asthmatic inflammation by secreting critical factors to chronic inflammation [25]. Airway epithelium–Th2 interactions are pivotal in chronic airway remodeling characteristic of human bronchial asthma. Th2 IL-13 induces goblet cell metaplasia, airway epithelial hyperplasia, increased epithelial secretions, and ASM hyper-responsiveness [56-60]. In contrast to its barrier function, the airway epithelium also secretes cytokines and growth factors that actively contribute to the development of chronic asthmatic inflammation. Epithelial cell-derived cytokines and growth factors IL-25, IL-33, thymic stromal lymphopoeitin (TLSP), GM-CSF, and eotaxins promote the development of chronic asthmatic inflammation [25]. Epithelial TLSP secreted in response to ligation of pathogen-associated molecular pattern (PAMP) receptors amplifies the Th2 response directly, by enhancing antigen presentation, or by acting on other hematopoietic cells [25, 61-64]. Epithelial IL-25 stimulates Th2 cytokine secretion from both T cells and non-T cells and induces goblet cell metaplasia and eosinophilia [65-68]. Similarly, IL-33 activates hematopoietic cells and can generate Th2 cells by toll-like receptor, ST2, ligation independent of STAT 6. Furthermore, OVA-sensitized and challenged ST2 deficient mice have decreased parameters of inflammation [69-71]. Critically, epithelial GM-CSF and eotaxins promote development, trafficking, and lipid mediator synthesis of eosinophils

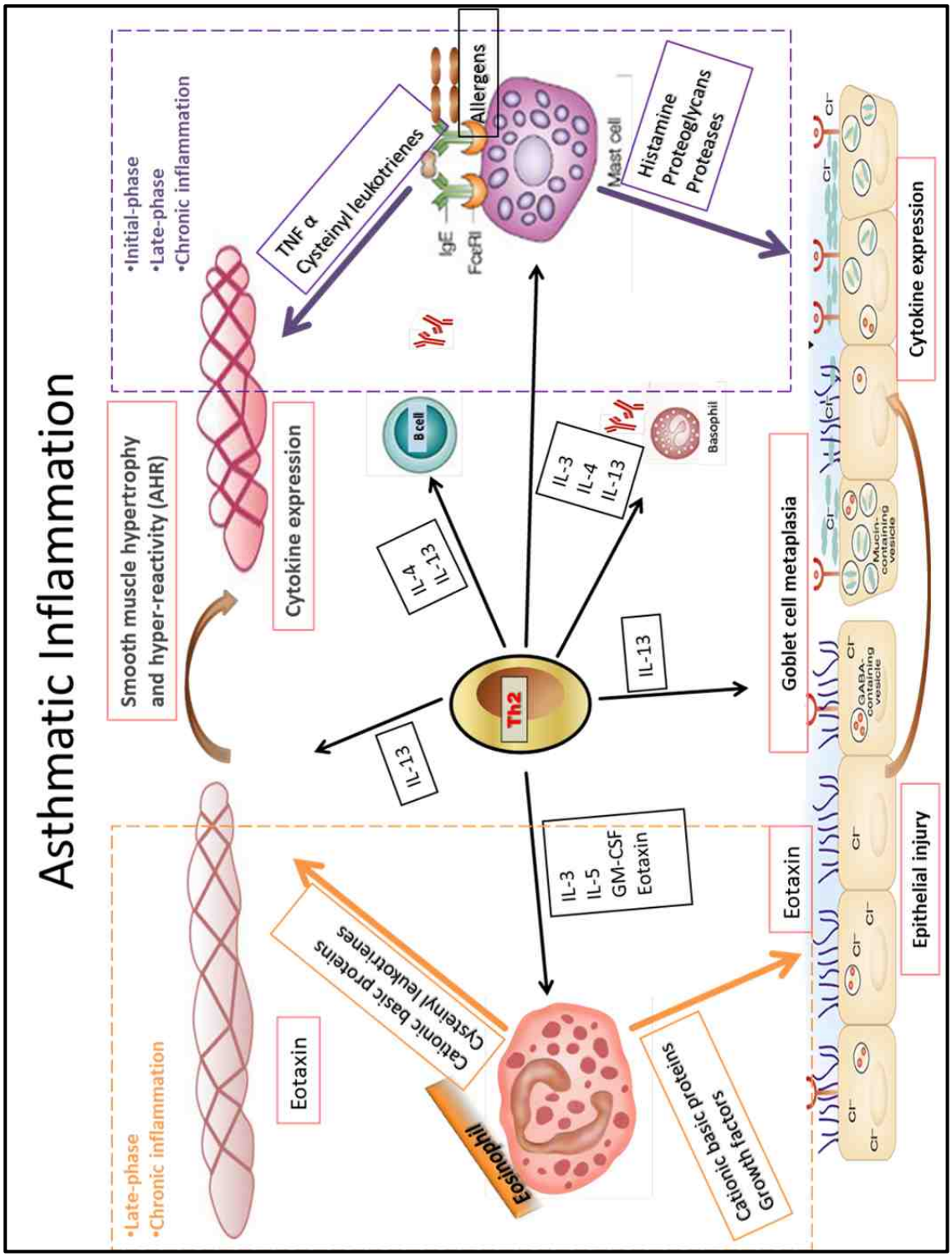

Asthmatic Inflammation
•Initial-phase
•Late-phase
•Chronic inflammation
Allergens
IgE
FcεRI
Mast cell
TNF α
Cysteinyl leukotrienes
Histamine
Proteoglycans
Proteases
Smooth muscle hypertrophy
and hyper-reactivity (AHR)
Cytokine expression
B cell
IL-4
IL-13
IL-3
IL-4
IL-13
Basophil
Th2
IL-13
IL-13
Goblet cell metaplasia
Mucin containing vesicle
Cytokine expression
IL-3
IL-5
GM-CSF
Eotaxin
Eotaxin
GABA containing vesicle
Epithelial injury
•Late-phase
•Chronic inflammation
Eotaxin
Cationic basic proteins
Cysteinyl leukotrienes
Eosinophil
Cationic basic proteins
Growth factors
Cl⁻

Figure 1

Figure 1: Schematic illustrating cellular interactions promoting chronic inflammation and remodeling in asthma. Th2 cytokines regulate the inflammatory changes in multiple hematopoietic and airway cell types. Th2-eosinophil-airway tissue interactions critically promote chronic asthmatic inflammation and promote airway hyper-reactivity, cytokine expression, injury, smooth muscle hypertrophy and consequent airway remodeling. This figure summarizes content from Galli, Nature reviews, 2008 [8].

The eosinophil and the inflammatory environment in asthma

As current understanding of allergic mechanisms does not fully account for the great variety in asthmatic phenotype, human asthma persists as a clinically-defined disease of elusive mechanistic complexity. Traditionally asthmatic inflammation was regarded primarily as a Th2 cell mediated hypersensitivity. Mounting evidence, however, suggest fundamental modulatory roles for other myeloid cells, and the airway environment [7, 25, 41, 72]. Granulopoiesis accelerates because of cytokine secretion during asthmatic inflammation [73, 74]. All major myelocytes (neutrophils, mast cells, and especially eosinophils) are reported to modulate asthmatic inflammation [25, 75]. Clinically, atopic asthma with predominant eosinophil lung infiltration and inflammation is the most common asthmatic subtype [6] in which eosinophils are the most numerous cell type recruited to the asthmatic lung [76].

Tissue eosinophils are found in the gastrointestinal tract, bone marrow, blood, and other tissues, excluding the lung under non-inflammatory homeostatic conditions. Recent reports, however, demonstrate eosinophil-lineage committed cells undergo proliferation locally in the inflamed asthmatic lung [74]. However, eosinophils have classically been considered effectors of Th2 cell inflammation. Th2 eosinophilopoietins (GM-CSF, IL-3, and critically IL-5) direct differentiation and maturation of eosinophil progenitor cells in the bone marrow as well as mobilization of eosinophils and eosinophil progenitors into the blood [77-79]. Bone marrow of the eosinophil lineage is coordinately controlled by GATA-1 and

PU.1 [80]. GATA-1 drives CCR3, major basic protein (MBP) and IL-5Rα eosinophil-specific gene expression, and functional deletion of GATA (dlb GATA) or transgenic co-expresssion of GATA with diphtheria toxin (PHIL) ablates the eosinophil lineage and AAD hallmarks in allergen-sensitized mice [81-83]. Similarly, PU.1 regulates expression of many eosinophil cationic proteins [84].

Repeated allergen-challenge promotes a chronic eosinophil-rich pulmonary infiltrate in humans and mice. GM-CSF, IL-3, and IL-5 in concert with allergen-induced Th2 cell, ASM, and airway epithelial-derived eotaxins elicit rapid eosinophil vascular egression and selective pulmonary trafficking of eosinophils via CCR3 G-protein signaling [76, 78, 85-87]. Moreover, IL-5 deficient mice and patients with a genetic polymorphism decreasing eotaxin expression demonstrate decreased allergen–induced eosinophilic lung infiltration [84, 88]. On the contrary, CCR3 ligation by inhibitory ligand can inhibit migration [89]. Emerging evidence further suggests that allergen incited inflammatory cytokines recruit hematopoietic progenitor cells from bone marrow to the lung where they can undergo further proliferation and final maturation, intensifying the degree of tissue inflammation [74]. While the genetic evidence for IL-5 and eotaxin control of eosinophil lung accumulation in AAD is compelling, anti-IL-5 therapy in asthma patients reduced eosinophilia and eosinophil TGFβ expression in the lung, but only modestly reduced AHR [90]. In contrast to these studies, other evidence suggests eosinophil infiltration of tissues can be T-helper cell-independent. Helper T cells were dispensable in eosinophil infiltration in a non-AAD murine

model [91]. Rather, eosinophil transfers were necessary to restore Th2 cytokine secretion in IL-5 and eotaxin deficient mice [92].

Granulocyte trafficking from bone marrow to sites of inflammation involves relative eosinophil-endothelial rolling, adhesion, and transmigration. CCR3: chemokine-induced eosinophil adhesion and transmigration depends on interactions between eosinophil and the vessel wall. Expression and ligation of eosinophil alpha class of integrins LFA-1, VLA-4, α4β7, and Mac1 by endothelial ICAM, VCAM, and MadCAM are implicated in eosinophil accumulation *in vivo* [93-95]. In addition, Siglec F seems critical to egression and survival of eosinophils at sites of eosinophil inflammation [96]. A coordinated balance among adhesive, motile, and structural forces in the eosinophil are responsible for its consequent diapedesis through vascular epithelium and infiltration of local sites of airway inflammation. These processes are illustrated in Figure 2. While eosinophil infiltration is crucial to the development of asthmatic inflammation, the exact intrinsic eosinophil mechanisms by which eosinophils traffic to the lung in asthma and AAD remain incompletely understood.

Accumulating evidence suggests eosinophils can regulate production and secretion of a variety of immunomodulatory molecules. Critically, eosinophil mediators, cytokines, and growth factors modulate asthmatic late phase and chronic airway inflammation. Eosinophils are the main source of cysteinyl leukotrienes that amplify the late phase response [97]. In concert with Th2

cytokines, lipid mediators promote exacerbated systemic vascular leak and pulmonary inflammation [8]. Moreover, currently prescribed cysteinyl leukotriene receptor blockers and phosphodiesterase inhibitors have been moderately successful at attenuating asthma [41]. Eosinophil granules contain toxic cationic proteins that cause tissue injury, remodeling and chronic inflammation in the asthmatic lungs [90, 98]. Airway tissue samples from asthmatic patients frequently demonstrate eosinophil degranulation products in CLCs [99]. The cationic granule proteins, MBP, eosinophil peroxidase (EPO), eosinophil cationic protein (ECP) and eosinophil-derived neurotoxin (EDN) wound airway epithelial cells, parasympathetic nerve endings, basement membrane, and smooth muscle promoting airway remodeling [100-104]. Degranulation products discovered around vagal nerve ganglia that innervate smooth muscle are hypothesized to cause AHR [105]. A piece-meal exocytotic mechanism governs release of human eosinophil preformed granule proteins and mediators, which allows for selective mobilization of granule proteins [106, 107]. Eosinophil degranulation and mediator release is controlled by cytoskeleton-facilitated Soluble N-ethylmaleimide-sensitive factor Attachment Protein Receptor (SNARE)/Vesicle-associated membrane protein (VAMP) interactions and intracellular trafficking [103]. Additionally on allergen-sensitization, eosinophils synthesize and secrete cytokines, chemokines, and growth factors. These pro-inflammatory molecules including IL-25, TGFα, TGFβ, metalloproteinases, pro-fibrotic, and angiogenic factors establish a chronic inflammatory milieu in the airway epithelium and facilitate injury and remodeling in asthma [90, 108-112].

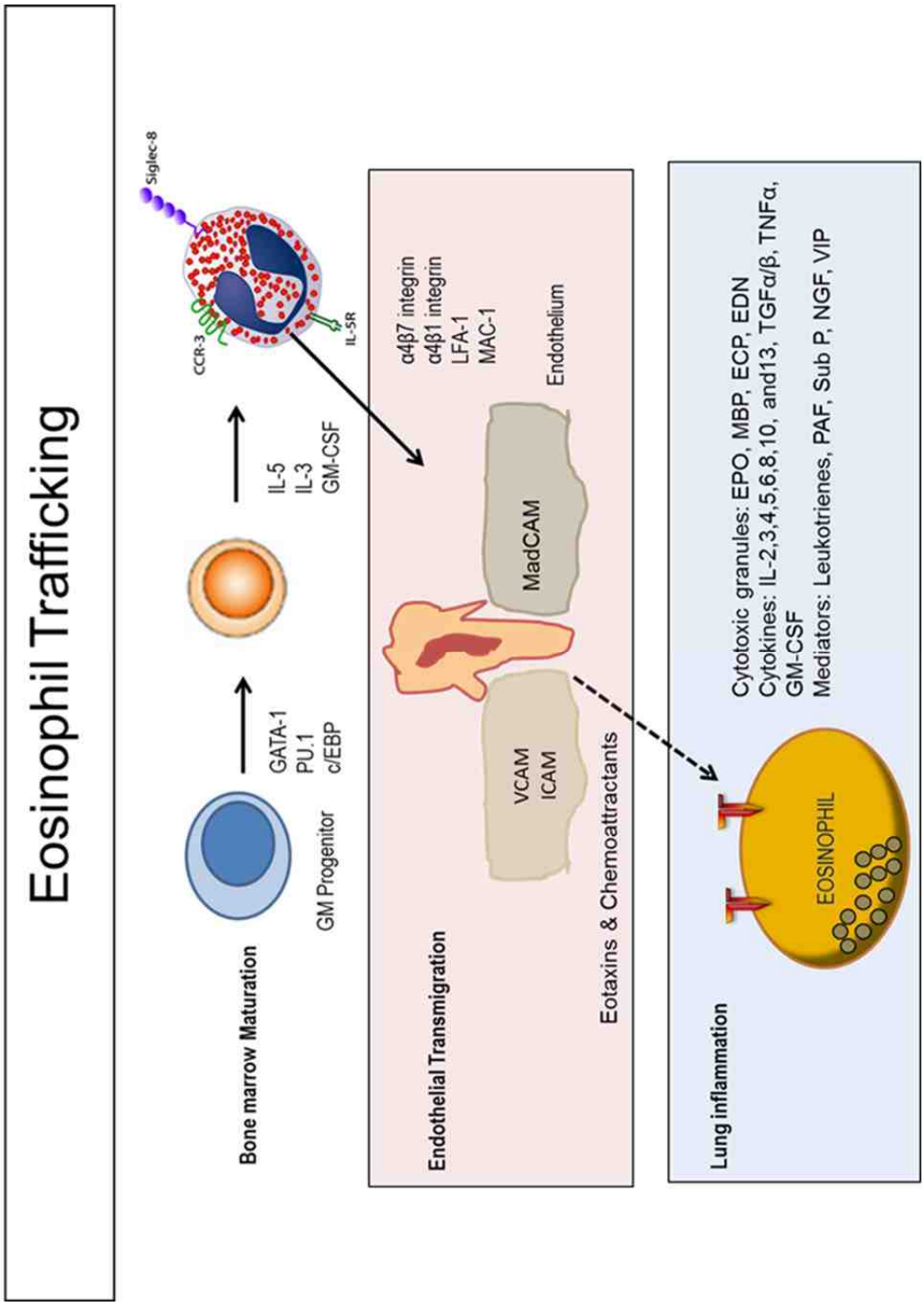

Eosinophil Trafficking
Siglec-8
CCR-3
IL-5R
IL-5
IL-3
GM-CSF
GATA-1
PU.1
c/EBP
GM Progenitor
Bone marrow Maturation
α4β7 integrin
α4β1 integrin
LFA-1
MAC-1
Endothelium
MadCAM
VCAM
ICAM
Endothelial Transmigration
Eotaxins & Chemoattractants
Cytotoxic granules: EPO, MBP, ECP, EDN
Cytokines: IL-2,3,4,5,6,8,10, and13, TGFα/β, TNFα, GM-CSF
Mediators: Leukotrienes, PAF, Sub P, NGF, VIP
EOSINOPHIL
Lung inflammation

Figure 2

Figure 2: Schematic illustrating selective eosinophil interactions in bone marrow eosinophilopoiesis, egress into the blood, transmigration into the asthma-inflamed lung, and inflammation. Eosinophil lineage differentiation and maturation is coordinately controlled by GATA-1, PU.1, and c/EBP driven eosinophil gene expression as well as IL-3, IL-5, and GM-CSF signaling (top panel). Mature eosinophils or committed progenitors are mobilized in response to IL-5. Eotaxin concentration gradients induce the relative adhesion and diapedesis of eosinophils through the leaky endothelial wall (middle panel). Eotaxin-stimulated eosinophils release cationic proteins, cytokines, and inflammatory mediators that modulate chronic airway inflammation and remodeling. This figure was adapted and simplified from a review published by Rothenberg and Hogan in Annu. Rev. Immunol. 2006 [103, 113].

Identifying novel asthma targets

While we are also beginning to understand the intercellular cytokine and mediator signaling cascades in asthma, no new drugs have been developed to replace corticosteroids in managing bronchial asthma. This stagnation in effective new asthma therapy warrants a broader investigative approach that seeks to identify putative molecular instigators of inflammation in multiple inflammatory processes. The mitogen-activated protein kinases (MAPKs) are kinases that modulate and amplify many intracellular signals important to the inflammatory process in multiple cell lineages. Moreover, mice treated with inhaled inhibitors of p38 MAPK have shown promising attenuation of eosinophilic inflammation [39, 114, 115]. Furthermore p38 MAPK, ERK, and JNK inhibitors have shown some efficacy at inhibiting eosinophil chemotaxis and pro-inflammatory mediator synthesis and release [116-118]. p38 MAPK are known to modulate cytoskeletal remodeling and cell motility [119, 120]. p38 MAPK transduces migratory signals from multiple upstream molecular switches. PAKs, one such group of kinases, indirectly activate p38 MAPK and induce hematopoietic and endothelial cell migration [121, 122]. PAKs, modulators of p38 MAPK and other MAPK proteins, may also promote chronic asthmatic inflammation and provide attractive alternative therapeutic targets.

The Rho-family of GTPase proteins, the classic activators of PAKs, are implicated in the pathophysiology of bronchial asthma. Rac GTPase levels and activity and Rho-kinase (ROCK) activity are both elevated in smooth muscle

hyper-responsiveness and eosinophilia in models of chronic AAI [123-125]. Furthermore, Simvastatin decreases human AHR by inhibiting Rac1 [126]. In a murine model of AAD, ROCK small molecule inhibitors attenuated asthmatic eosinophilia and AHR [127]. Racs also modulate SCF-kit induced mast cell migration [128, 129]. Moreover, expression of a dominant negative construct of Rac 1 decreases mast cell synthesis and secretion of cysteinyl-leukotrienes to IgE stimulation [130].

p-21 activated Kinases (PAKs)

PAKs, members of the Ste-20 family of serine/threonine kinases, regulate many cellular physiological processes. Human tissues vary widely in their expression of the six isoforms of PAKs [131]. PAK1-3, Group 1 PAKs are structurally comprised of an N-terminal p21-binding domain, an overlapping regulatory region that includes an autoinhibitory domain, and a C-terminal catalytic kinase domain. They exist as auto-inhibited trans-homodimers under basal conditions [132-135].

First described as binding partners of the Rho-family proteins CDC42 and Racs in murine brain tissue, Group 1 PAKs were found at 68, 65, and 62 kDa and were later designated PAK1 (α-PAK), PAK3 (β-PAK), and PAK2 (γ-PAK) respectively [134]. Their activation requires the CDC42/Racs-dependent dismantling of the inhibited dimeric complex into active monomeric units [136]. Classically, the active GTP-bound Rho-family GTPases, CDC42 or Rac, bind and

remove the CDC42/Rac binding domain (CRIB/PBD), that liberates the kinase domain's autocatalytic function, and induces a complex conformational change that permits further phosphorylation events [136-140]. The autophosphorylated PAK1 intermediate is phosphorylated at several residues including the signal residue threonine 423 [141, 142]. Many further phosphorylation events disassemble the dimeric complex into two active monomers of PAK1. A general schematic of these events is depicted in Figure 3.

Independent and distinct from GTPase activation, sphingosine and other cell membrane lipids important in asthma can directly activate PAK1 and are implicated in AAI [138, 143, 144]. Nck and Grb2 adaptor proteins can chaperone inactive PAK1 to the cortical membrane where Rho-family GTPases enhance PAK1 catalytic activity up to 20-fold [138, 145-147].

PAK2, another isoform of PAKs and member of Group I PAKs, is ubiquitously expressed in human tissue including hematopoietic cells [134]. PAK2 shares 77% protein sequence homology with PAK1 [148]. PAK2 in addition to GTPase-dependent activation can additionally undergo proteolytic cleavage and activation by caspase 3 during apoptosis [149]. Both PAK2 and PAK1 are expressed in human and murine hematopoietic cells. However, while PAK1 has largely a similar substrate specificity and function to PAK2 *in vitro* [150], the differential roles of PAK1 and PAK2 in hematopoietic function are incompletely understood.

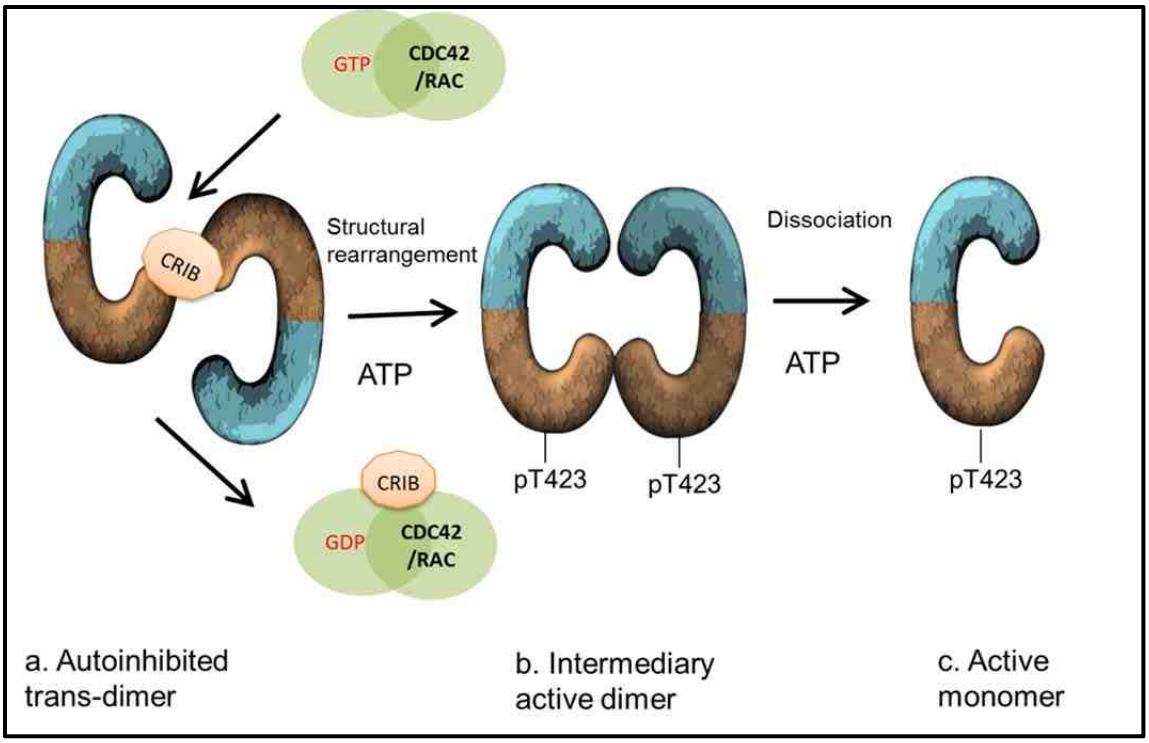


Figure 3. adapted from Eswaran, J *et al.*, Trends Biochem Sci, 2008

Figure 3: Schematic showing the activation of p-21 activated Kinase 1 protein. A. PAK1 exists as a *trans*-inhibited homodimer associated at their CRIB domains (shown in orange). GTP-bound CDC 42 or Rac binding activates this complex by reorientating the dimeric complex and disinhibiting the kinase activity of the catalytic subunits (shown in brown). The activated catalytic domains are phosphorylated at many residues including the signal pT423 residue. This figure was adapted from the version first published by Eswaran *et al* in Trends Biochem Sci, 2008 [151].

PAK1 in cytoskeletal regulation and cell motility

PAKs modulate many cellular processes by regulating cytoskeletal function. PAK1 modulates remodeling of the F-actin cytoskeleton involved in cell migration. On cell stimulation, PAK1 relocates to various F-actin cytoskeletal structures. PAK1 localizes to focal adhesion complexes at leading cell edges [133, 152-154], lamellipodia [155], filopodia, membrane ruffles, retracting cell edges [156], and vesicles [157]. Furthermore, PAK1 assists in recycling focal adhesions and actin stress fibers [156, 158]. In lower organisms, embryological, neurogenic, and metabolic evidence supports a role for PAKs homologs in modulating the actin cytoskeleton [159-165].

PAK1 modulates cytoskeletal remodeling by both kinase-dependent and -independent mechanisms. In general, PAK1 binds CDC42/Racs, mediating activation of its catalytic activity, whereas PAK1 binding of adaptor proteins like PI mediates its translocation and binding to several transmembrane receptors [166]. Reports utilizing ectopically expressed activating and deactivating mutant constructs of PAK1 have clarified some of these effects [133, 142, 167]. Full length PAK1 as well as a constitutively active kinase mutant, L107F, promote F-actin turnover and cell motility, whereas a kinase dead mutant of PAK1, K299R, suppressed these phenotypes [156, 158, 168].

PAK1 catalytically modulates F-actin remodeling in events essential to cell motility through multiple downstream effectors as summarized in Figure 4. PAK1 activates the LIM Kinase inhibiting ADF/cofilin (cofilin) and allowing for the

persistence of polymerized actin filaments in cell protrusions and other actin sub-cellular structures [169]. Cofilin severs F-actin filaments, generating free actin barbed ends, the building blocks for polymerizing new filaments of actin [170]. Genetic and overexpression studies support a role of cofilin in F-actin dependent cell motility [171-173]. PAK1 regulates cofilin activity both directly and by downstream effectors; however, this temporal-spatial cofilin control is incompletely understood [174-176].

Importantly this kinase also localizes with, binds, phosphorylates, and activates p41-ARC, a subunit of the ARP2/3 complex *in vivo* and *in vitro* [177]. This complex directs nucleation and branching of new filaments of actin by using filaments with free barbed ends [178, 179]. Additionally ARP2/3 contributes to F-actin motility structures and force-generation at the cell's cortical membrane, and consequent cell migration; however, its exact regulation of motility is incompletely understood [145, 177, 180, 181].

Recent work that we have done suggests that PAK1 regulates Ezrin's actin regulation. Ezrin/Radixin/Moeisin (ERM) proteins act as linkers for polymerizing F-actin to multiple transmembrane receptors [182, 183]. Ezrin and the other ERM proteins exist under homeostatic conditions in a closed autoinhibited conformation with tight binding between its N-terminal plasma membrane associated (FERM) and C-terminal ERM-association (C-ERMAD) domains. Receptor tyrosine kinase induced signaling events break the association between these domains and facilitate binding of the underlying actin cytoskeleton [182]. Ezrin has no intrinsic enzymatic activity but draws the actin cytoskeletal

signaling complexes in close apposition to membrane-bound receptors facilitating signal transduction [182, 183]. As such, Ezrin's importance has been demonstrated in actin reorganization necessary for apical-basal organization of epithelia, T-cell immunological synapse, and hematopoietic cell adhesion to the extracellular matrix [184-187]. Moreover, tissue-specific murine *Ezrin* deficiency has been linked to defects in epithelial morphogenesis [188, 189]. Several studies link Ezrin deregulated expression and activation with metastatic invasion in human epithelial-derived and hematopoietic cancers [190, 191]. Our work demonstrates for the first time that PAK1 in mast cells regulates F-actin depolymerization in IgE/DNP-mediated degranulation [192].

PAK1 also phosphorylates and activates Filamin A, an actin binding protein. Filamin A crosslinks F-actin into networks and anchors the actin cytoskeleton to transmembrane proteins in cellular adhesion, morphology, and motility. Studies in *Filamin A/FLNA* deficient embryos and human cell-lines demonstrate an important role for Filamin A in cellular migration and maintenance of intercellular junctions in organogenesis [193, 194]. PAK1 can elicit cortical actin assembly by direct phosphorylation of Filamin A [195]. In cancer cell-lines, PAK1 transduces signals through Filamin A crucial for lamellipodia formation and cell migration [196, 197]. In muscle, PAK1 additionally phosphorylates and inhibits myosin light chain kinase decreasing the actin-myosin filament mediated cell tension [198, 199]. PAK1 can further modify the tubulin cytoskeleton by phosphorylating and deactivating Op18/Stathmin at Serine 16, stabilizing microtubules [200, 201].

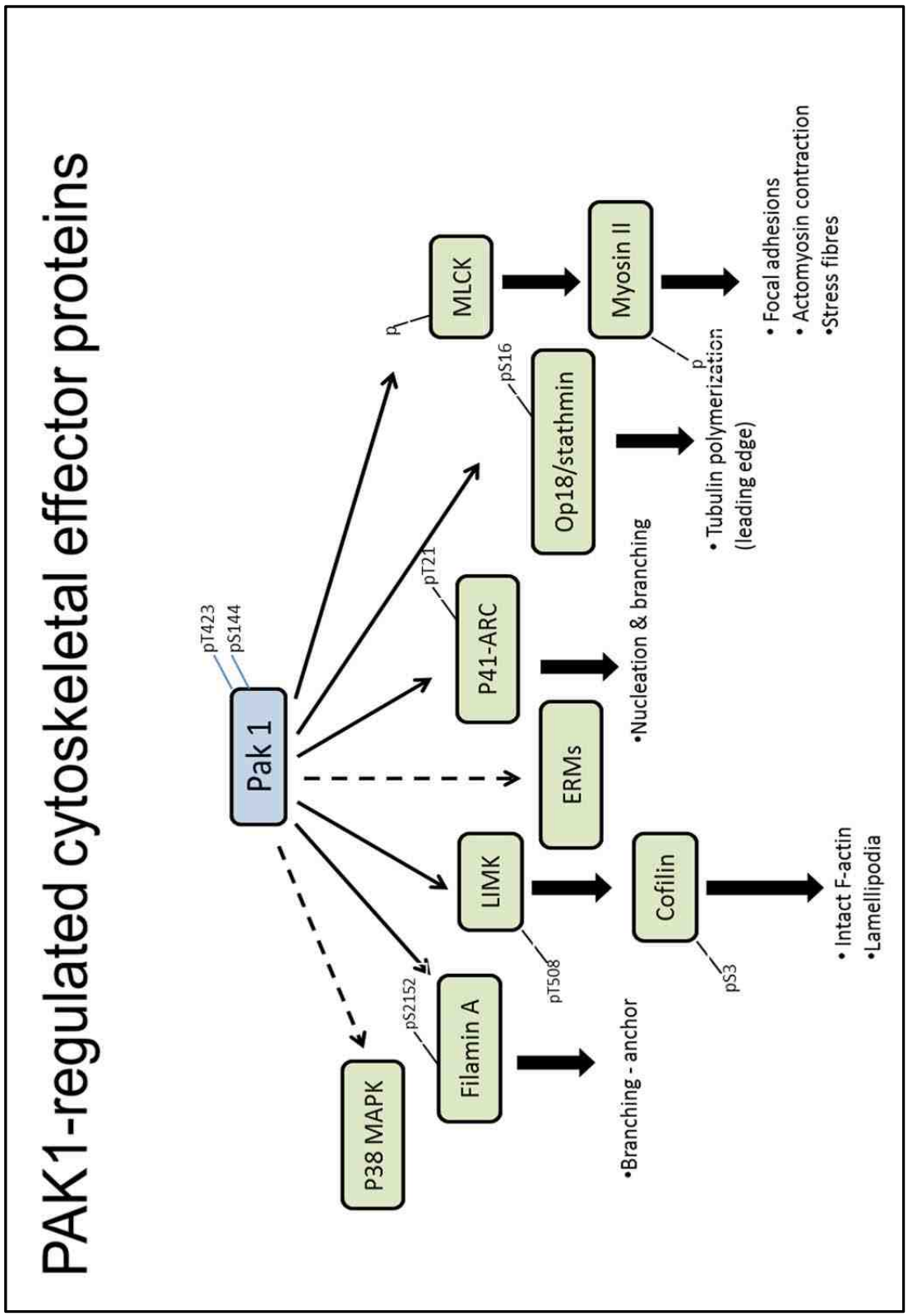

PAK1-regulated cytoskeletal effector proteins
Pak 1
pT423
pS144
P38 MAPK
Filamin A
pS2152
LIMK
pT508
ERMs
P41-ARC
pT21
MLCK
p
Op18/stathmin
pS16
Myosin II
p
Cofilin
pS3
•Branching - anchor
•Nucleation & branching
• Tubulin polymerization (leading edge)
• Focal adhesions
• Actomyosin contraction
•Stress fibres
• Intact F-actin
•Lamellipodia

Figure 4

Figure 4: PAK1 regulation of cytoskeletal processes by downstream cytoskeletal binding and remodeling effector proteins. PAK1 directly phosphorylates p41ARC of the ARP2/3 complex, LIM Kinases (LIMKs), Filamin A Myosin Light Chain Kinase (MLCK), and Op18/Stathmin. PAK1 also indirectly induces phosphorylation changes in cofilin, p38 MAPK and Ezrin/Moeisin/Radixin (ERM) proteins.

PAK1 as a potential target in allergic airway disease

PAK1 and other isoforms regulate chemoattractant-directed cell migration, a process that heavily involves the actin cytoskeleton. Inhibiting PAK1 kinase activity in NIH-3T3 fibroblasts alters cell motility, whereas enhancing PAK1 activity by expression of a PAK1 L107F plasmid construct increased fibroblast migration to collagen [155]. Moreover, other constitutively active *Pak1* constructs rescue decreased chemoattractant-induced formation of lamellipodia and cytoskeletal rearrangement in *Akt-/-* fibroblasts [202]. PAK1 also modulates human endothelial cell, macrophage, and neutrophil motility [203, 204]. Dysregulated PAK1, which promotes cell motility and tissue invasion in cancer metastasis, localizes at the protrusions on migrating COS-7 cancer cells *in situ* [205].

PAK1 is a well-known regulator of the F-actin cytoskeleton and cell migration, although work has not been done in eosinophils. p38 MAPK one putative downstream effector of PAK1 has been implicated in migration and F-actin modulation and motility in multiple hematopoietic cells. In contrast, although cofilin, ARP2/3, and Ezrin are individually implicated in F-actin remodeling in other cell systems, their role in eosinophil F-actin-mediated processes remains unexplored.

PAKs dysregulation in multiple human cancer types is well-documented. Uncontrolled PAK1 signaling in human breast adenocarcinoma confers tamoxifen resistance [206-208]. PAK1 levels are also elevated in colonic adenocarcinoma

[209], ovarian carcinoma [210, 211], and renal cell carcinoma [212]. Similarly, *Pak1* gene amplification has been detected in transitional cell carcinoma [168], high-grade ovarian carcinoma [211, 213, 214] and T cell lymphoma [215, 216]. Our multi-disciplinary research group has recently shown that PAK1 modulates mast cell pro-inflammatory functions in neurofibromatosis 1 [121]. Few studies, however, have focused on the effect PAK1 has in chronic AAD.

Our collaborators and others have delineated the PAKs-F-actin cell-physiological changes in contracting ASM [217-219]. Their data suggest that dysregulated PAK1 signaling permits cellular processes culminating in AHR [219-222]. We have recently established that genetic deletion of *Pak1* attenuates the SCF/c-kit induced migration of mast cells [121]. Furthermore, PAK1 is important in immediate allergen/IgE induced calcium-flux changes and F-actin cytoskeletal-mediated depolymerization in mast cell degranulation [223].These reports taken together support studies investigating the role PAK1 plays in allergic airway disease. We hypothesized that a Racs/CDC42/PAK1 signaling axis modulates the development of chronic AAI by modifying cytoskeletal dependent cellular processes.

THESIS OVERVIEW

In sum, existing therapeutic modalities with adverse systemic effects or new drugs with narrow target population specificity, fail to effectively impact the increasing asthma incidence and morbidity. Current management of asthma symptoms with corticosteroids and β2 agonists has done little to stop the natural progression and lung remodeling of asthma. In response to allergen-induced eotaxins and other chemoattractants, eosinophils infiltrate the allergen-sensitized and challenged airways *en masse* and promote this remodeling. Furthermore, Th2-airway tissue-eosinophil reciprocal signaling and interactions orchestrate the development of asthmatic inflammation. Directed research into these synergistic cell signaling networks can identify specific targets involved in multiple processes in this inflammatory cell environment.

PAK1 classical activators Racs, as well as PAK1-interacting MAPK proteins both regulate eosinophilia in animal models as well as eotaxin-mediated eosinophil migration *in vitro*. However, genetic studies testing the mechanism by which PAK1 may regulate eotaxin-mediated eosinophil pro-inflammatory functions have not been explored. In these studies, we formally assess PAK1 as a novel anti-eosinophil inflammation target. We use a *Pak1* deficient AAD model to test PAK1's role in eosinophil inflammation characteristic of asthma. Furthermore, we utilize bone-marrow derived eosinophils to evaluate the consequence of *Pak1* deletion on eotaxin-mediated eosinophil adhesion, migration, and degranulation.

MATERIALS AND METHODS

Animals used

Pak1-/- mice were generated by Dr. Jonathan Chernoff's laboratory at Fox Chase as described [223] and were backcrossed at an Indiana University Laboratory Animal Research Center facility for nine generations onto a C57BL/6 strain background using mice obtained from Jackson Laboratories.

Ezrin $^{flox/flox}$ mice on a 129/SV strain background were generated by Dr. Andrea McClatchey's laboratory at Harvard Medical School as described [188] and bred with *Mx1Cre* transgenic (*MxCre*$^{+}$) mice on a C57BL/6J strain background to yield *Mx1Cre*$^{+}$ and *Mx1Cre*$^{-}$ *Ezrin* $^{flox/+}$ mice. These F1 mice were bred to yield stable *Mx1Cre*$^{+}$ and *Mx1Cre*$^{-}$ *Ezrin* $^{flox/flox}$ breeders and experimental mice. The *Ezrin* $^{flox/flox}$ and *Mx1Cre* alleles are genotyped to yield 251 and 217 base pair bands respectively in reactions as previously described [188, 224, 225].

Mice were housed in pathogen-free conditions at Indiana University School of Medicine according to the Institutional Animal Care and Use Committee and Institutional Review Board guidelines.

Mx1Cre induction

Poly I poly C (PolyIC), a synthetic dsRNA analog, induces IFN-β production and release *in vivo*. Downstream JAK-STAT signaling acting at the Mx1 gene promoter induces Cre expression in *Mx1Cre*$^{+}$ mice. Hematopoietic stem and

progenitor cells from *Mx1Cre*$^{+}$ mice express Cre following polyIC administration [226]. Ezrin deletion (*Ezrin-/-*) was achieved in *Mx1Cre*$^{+}$ *Ezrin* $^{flox/flox}$ mice after the administration of five intraperitoneal injections of polyIC (Sigma, Saint Louis, MO) dissolved in sterile PBS. Minimal *in vivo* adverse effects from PolyIC treatment were achieved using a tapered dosing scheme as follows; two 15 µg, two 20 µg, and one 25 µg injections per gram of body weight Poly IC injections on alternate days over a ten day period. *MxCre*$^{-}$ *Ezrin* $^{flox/flox}$ control mice for these experiments also received the same doses of PolyIC prior to experimentation.

Eosinophil infiltration murine models

Cohorts of age and sex matched *Pak1-/-* and *Pak1+/+* mice were injected with a 1 mL intraperitoneal dose of 2 µg murine recombinant eotaxin ('mreotaxin', R&D systems) or PBS. Two hours later, mice were euthanized by CO_2 asphyxiation and their peritoneal cavities lavaged with 5 mL PBS to retrieve peritoneal cells as described [227]. Total cells were counted by hemocytometer, cell populations visualized by giesma-stained slides, and eosinophils quantified by fluorescent cytometry of CCR3.

Alternatively, cohorts of age and sex matched anesthetized *Pak1-/-* and *Pak1+/+* mice were injected with a 25µL intratracheal dose containing 10µg mreotaxin (Peprotech) or saline as described [228, 229]. The mice were maintained under anesthesia for four hours [228].The mice were euthanized by CO_2 asphyxiation

and broncho-alveolar lavage fluid (BALF) collected at 4°C for hemocytometer counts, giesma staining, and fluorescence cytometric analysis of eosinophils.

OVA-induced allergic airway disease model

Cohorts of male 10-14 week *Pak1-/- and Pak1+/+* mice were sensitized and challenged in a murine model of allergic airway inflammation (AAI). Mice of both genotypes were sensitized by two intraperitoneal injections, seven days apart with 100 µg purified Ovalbumin ('OVA'/ Sigma) adsorbed to 2 mg of the adjuvant Aluminum hydroxide ('Alum'/ Sigma). On the 14th day, OVA-sensitized mice were anesthetized using aerosolized isoflurane (Webster Veterinary) daily for five consecutive days to prepare them for intranasal OVA challenge. Anesthetized mice were intranasally challenged with 50 µg of OVA in solution with saline for five consecutive days. Twenty-four hours after the last intranasal challenge (day 19), AAD mice were euthanized by a 60 µg/g body weight lethal intramuscular injection of pentobarbital, their tracheas cannulated using a 20G 11/4 inch veterinary I.V. catheter, and broncho-alveolar lavage (BAL) samples collected at 4°C. Some of these murine tracheas and lungs for histological analysis were inflated to 20-25 cm of H_2O and fixed in formalin while tissue for cellular analysis was washed and collected in saline at 4°C.

All BAL were centrifuged at 1500 X g for 5 minutes and the first 0.5 mL rinse stored at -80°C for cytokine analysis by ELISA. The BAL cells from the rinses were resuspended and pooled then red blood cells lysed in 300 µl of RBC lysis

buffer (Qiagen) until the cell pellet blanched at room temperature. Cell pellets recovered from each mouse were resuspended in 100 µL of FACS buffer (2% BSA solution with PBS and 0.09% NaN_3) and 10 µL aliquots taken for hemocytometer BAL cell counts and slide preparation. Resuspended cell aliquots were counted in duplicate in solutions of 1 part cells diluted in 10 or 20 parts trypan blue (Mediatech, Inc.) by hemocytometer. *Pak1-/- and Pak1+/+* total BAL cell counts were compared for significant differences. BAL cell aliquots for slide preparation were diluted in PBS, spun onto glass slides at 1200 rpm for 10 minutes, and Giesma-stained (HEMA-TEK 2000, Bayer). Representative digital pictures per sample were taken by a digital camera slide microscope system (SPOT, Diagnostic Instruments, Inc.).

Peripheral immune responses in Pak1+/+ and Pak1-/- OVA- sensitized and challenged mice were evaluated by OVA-specific splenocyte cytokine analysis. Splenocytes were harvested from mice of both genotypes and challenged with 100 µg/ml OVA for 72 hours as previously described [230]. Cell-free supernatants were collected and analyzed for cytokines by multiplex ELISA (reagents from Millipore).

Histological analysis and inflammation scores

Fixed lung tissue was embedded in paraffin, and cut into 4-8 µm sections by our histology core. Paraffin-embedded tissue sections were mounted onto glass slides and stained with Hematoxylin and Eosin ('H&E', Sigma), Masson's

Trichrome (Fisher Scientific), and Periodic acid-Schiff using standard histological techniques. Slides were visualized by light microscopy, and representative 20 and 40X images captured with a digital camera (SPOT, Diagnostic Instruments, Inc.). In addition, blinded observers assigned inflammation scores to H&E tissue sections from 0-4 with 0 being uninflamed lung and 4 severely inflamed lung. Inflammation scores were compared for both genotypes and treatments and differences determined.

Fluorescence cytometric analysis of cell populations

Aliquots from BAL samples were pooled to provide at least 10^6 cells per cell sample for control staining. At least 10^5 cells for each sample were probed with leukocyte antigen conjugate fluorescent antibodies at final concentrations of 5 μg/mL anti-CD45, a generic leukocyte antigen, 2 μg/mL B220-FITC, 10 μg/mL CD3-FITC, 0.625 μg/mL CCR3-PE (R&D systems), 2.5 μg/mL MHCII-PerCP, and 2 μg/mL CD11c-APCcy7 as well as 6 μg/mL anti mouse CD16/32, Fc receptor blocking antibody [230, 231]. Alternatively, 10 μg/mL CD4-FITC, and 10 μg/mL CD8-FITC were used instead of CD3-FITC, 10 μg/mL SiglecF-PE instead of CCR3-PE, and 10 μg/mL APCcy7 CD11b instead of CD11c-APCCy7. All antibodies were obtained from BD pharmingen unless otherwise indicated in parenthesis. All control and test samples were incubated with antibodies for at least 30 minutes in the dark at 4°C before a wash and resuspension in FACS buffer for cell population data acquisition (BD FACSCalibur) using CellQuest (BD Immunocytometry Systems). On analysis, leukocyte population gates (all CD45+)

were set to distinguish the following populations: Eosinophils, FSC_{lo} B220/CD3 (-), CCR3/SiglecF (+), Neutrophils, FSC_{lo} B220/CD3 (-) CCR3/SiglecF(-), Lymphocytes FSC_{lo} B220/CD3 (+), CCR3/SiglecF (-), and alveolar macrophages, $FSC_{hi,}$ CDIIc or CD11b (+), MHCII (+). Total leukocyte population numbers were computed from the analysis percentages and significant differences ($p<0.05$) between *Pak1-/- and Pak1+/+* populations compared.

Adoptive bone marrow transplantation model

For bone marrow transplantation assays, low density bone marrow mononuclear cells were harvested from CD45.2 *Pak1-/- and Pak1+/+* mice on a C57BL/6J strain background as described in "Bone marrow isolation of LDMNCs". These cells were transplanted into *Pak1+/+* F1 mice which expressed both CD45.1 and CD45.2 common leukocyte antigens (CD45.1/2+) obtained from the Indiana University School of Medicine *In Vivo* Therapeutics Core as described [232]. Briefly, approximately two million viable *Pak1-/- and Pak1+/+* bone marrow cells were injected with a 30 gauge 0.5 inch needle into the lateral tail vein of lethally-irradiated (1100 cGy, split dose [700cGy and 400cGy with a 5 hour interval], cesium isotope source) CD45.1/2+ *Pak1+/+* mice. Transplanted mice were housed in a pathogen-free environment to allow for stem and progenitor cell engraftment. Mice were assessed for hematopoietic reconstitution at six months post-transplantation by quantifying peripheral blood CD45.2 versus CD45.1/2 chimerism. Mice were utilized in an OVA-induced allergic airway disease model.

Orthotopic left-lung transplantation model

For lung transplantation assays, whole left lungs from donor mice were transplanted into 8-12 week old male recipient mice using non-suture microvascular anastomoses which reduces granulation tissue as previously described [233-235]. These state-of-the-art transplantation experiments were done in collaboration with Dr. David Wilkes' lab, where this model is discriminately available. *Pak1+/+* recipient mice received *Pak1-/-* lungs while *Pak1-/-* mice received *Pak1+/+* lung grafts. Following a 7-day recovery period, transplanted mice were utilized in an OVA-induced AAD model as described above. The lung grafts were procured for cell suspension preparation and analyzed for eosinophil percentages by fluorescent cytometry for murine siglec F+ eosinophil in contrast to other populations. Total eosinophils were computed and compared between transplanted groups.

Cell suspension preparation from murine lungs

Whole lungs were collected from sensitized *Pak1-/- and Pak1+/+* mice or lung transplant mice in cold PBS (4°C). Single cell suspensions were prepared for MACS separation using a gentle-MACS dissociation protocol (Miltenyi Biotec). Briefly, single mouse lungs or left-lungs were individually washed, and dissociated in HEPES buffer ph 7.4 (Sigma) supplemented with 2mg/mL collagenase D (Sigma) and 10U/mL DNAse I (Sigma) in 10mL C-tubes (Miltenyi). Total cell in these lungs were dissociated in a gentle-MACS dissociator (Miltenyi) using proprietary programming, hybridized while rotating in an oven at 37°C, and

further dissociated from parenchymal tissue. The solution was filtered through a 70μm mesh, washed in PBS, cells labelled with anti-CD45 magnetic beads, and leukocytes positively selected by passing these cells through a magnetic column using standard Miltenyi protocol (#130-052-301). These cells were stained with antibodies against lineage antigens and analyzed by fluorescence cytometry.

Multiplex ELISA assays

Several allergen-induced murine cytokines and growth factors were assayed with multiplex simultaneous quantification of fluorescent antibody capture beads directed against eotaxin-1/CCL11, RANTES, IL-4, IL-5, IL-10, IL-13, IL-17, and IFNγ. BAL samples from OVA-sensitized and challenged *Pak1-/- and Pak1+/+* mice and supernatants from OVA-stimulated *ex vivo* T-cell cultures were thawed, vortexed, and 25 μL transferred to custom ordered multiplex plates with analyte capture antibodies (Millipore Milliplex, Billerica, MA). The multiplex assay was performed according to the manufacturer's protocol with each sample tested in duplicate. Briefly, samples were incubated with analyte antibody for two hours, washed three times, incubated with streptavidin conjugated fluorescent secondary antibody, washed three additional times and suspended in 100 μL saline, and analyzed on a Luminex 200 cytometer with StarStation software (Luminex Corp, Austin, TX). Standard curves were computed using a cubic spline fit, according to the manufacturer's instruction. The quantities of cytokines were computed relative to the standard curve generated, replicates averaged, and cytokines for each group tested compared.

Bone Marrow Isolation of low density mononuclear cells (LDMNCs)

Whole bone marrow was harvested from pelves, femurs, and tibias of cohorts of 10-16 week old mice in Iscove's Modified Dulbecco's Media (IMDM, Gibco/Invitrogen), supplemented with 2% fetal bovine serum (FBS, Hyclone, ThermoScientific) using a 20-gauge one inch needle. The flushed bone marrow was resolved to obtain LDMNCs on a density gradient (Histopaque Sigma 11191) as described [121, 223]. Briefly, harvested cells were carefully layered onto an equivalent volume of Histopaque (Sigma) and centrifuged for 30 minutes at 1750 rpm on a gh-3.8 rotor (Beckman Coulter). Cells in the intermediate layer were collected washed in IMDM or other media, and enumerated as appropriate, before experimentation.

Bone Marrow IL-5 methylcellulose cultures

Non-adherent LDMNCs *Pak1+/+* and *Pak1-/-* (N=3) were cultured in 35 X 10 mm diameter tissue culture dishes (Fisher Scientific) in culture medium, which was made up of 0.9% methylcellulose (Methocult H4100, Stem Cell Technology) enriched with 20% FBS and Iscove's Dulbecco's medium (with 1% penicillin-streptomycin, 0.35% 2-ME, and 0.1% BSA) supplemented with rmIL-5 (1, 10 ng/ml/ R&D systems) with 2×10^5 LDMNCs/ml of culture media at 37°C. After 5 and 10 days, total IL-5 colonies were counted using inverse microscopy as previously described [236]. Each mouse was assessed in triplicate for each cytokine concentration and the total number of IL-5 colonies was compared between *Pak1+/+* and *Pak1-/-* groups.

pCL11eGFP PAK1 mutant generation

The pCL11eGFP lentiviral construct was a kind gift of Helmut Hannenberg at the Indiana University School of Medicine. This construct contains an eGFP cassette and a multiple cloning site (MCS) downstream of the (HPGK) promoter, which drives transcription in eukaryotic cells. The kinase-dead PAK1 mutant, K299R and the constitutively active mutant, L107F, (Addgene) were NOT1-adapted and inserted into the NOT1-cut and Klenow-blunted pCL11eGFP vector in frame at the C-terminus of the eGFP cassette using molecular cloning techniques. The integrity and orientation of the pCL11eGFPPAK1 mutant plasmid constructs were confirmed by restriction enzyme digestion and sequencing.

Lentivirus generation

To generate virus, NIH 293T cells at 60-70% confluence on 10 cm dishes were transfected with 10 µg of either pcl1PAK1eGFP, or pcl11PAK1K299R, or pcl11PAK1L107, 5 µg gag-pol expressing helper plasmid (pCD/NL-BH), and 1 µg foamy viral envelope plasmid (pcoPE01) in 6 mL Dulbecco's Modified Eagle Media (DMEM, Gibco/Invitrogen) containing 10% FBS and 0.0075 mg/mL polyethyleneimine (PEI, Sigma). After overnight transfection at 37°C, transfection media was aspirated and replaced with 5 mL fresh DMEM containing 10% FBS, 50 U/mL penicillin, 50 µg/mL streptomycin, and 2 mM L-Glutamine. After 24 hours, all supernatants were collected, pooled (to 50 mL maximum), filtered through a 0.22 µm polyethersulfone (PES) membrane Stericup unit (Millipore, Billerica, MA), and centrifuged at 16,000 x g at 4°C for 2 hours in a polycarbonate

Oak Ridge centrifuge tube (Nalgene, Rochester, NY). Supernatant was decanted, bleached, and disposed in biohazardous waste, and the viral pellet resuspended in 1 mL IMDM containing 20% FBS. Virus was stored in 1mL aliquots at -80°C and all thawed samples were either immediately used or appropriately discarded.

Viral titer (infectious particles per mL) was determined by percent GFP-positivity of serially transduced HT1080 cells, plated at 100,000 per well of a six-well plate in 1 mL DMEM/10% FBS. The serial dilution started at 10^{-2} dilution from viral frozen stock and ended with 10^{-8} dilution. Viral titer was determined by the following equation:

$$Titer\left(\frac{infectious\ particles}{mL}\right) = \frac{\%\ GFP^{+}cells\ \times 10^{5}\ total\ cells}{1\ ml\ media\ \times 10^{-x}\ dilution\ factor}$$

Lentiviral transduction

CD117/c-kit + cells were magnetically isolated in a column following labeling *Pak1+/+* and *Pak1-/-* LDMNCs with anti-c-kit/CD117 microbeads (Miletenyi Biotec). These cells enriched for stem and progenitor cells were transduced with viral supernatants as described [223]. Briefly, each well of a six-well plate was coated with 10 μg/cm^2 of recombinant fibronectin (Retronectin, Takara) in 1 mL PBS, overnight at 4°C or for four hours at room temperature. This fibronectin/PBS solution was aspirated and replaced with 50-150 infectious particles per target c-kit+ cell, diluted in a 1 mL solution of IMDM containing 20%

FBS, 50 U/mL penicillin, 50 µg/mL streptomycin, 2 mM L-glutamine supplemented with either 100 ng/mL SCF, and 10 ng/mL IL-6. After one hour of virus/fibronectin incubation at 37°C, 1-2 million target cells were added in a 100 µL volume of the aforementioned transduction media to each 1 mL solution containing well. After 16 to 24 hours of incubation at 37°C, the cells were harvested, washed in IMDM, and resuspended in eosinophil culture media.

Eosinophil culture

Bone marrow eosinophils (bmEos) were derived by culturing bone-marrow mononuclear cells *ex vivo* in eosinophil polarizing conditions. Briefly, LDMNCs were enumerated and cultured *ex vivo* in IMEM media (Gibco) with 20% fetal calf serum (Sigma), 1 U/mL penicillin/streptomycin (Lonza), glutamine (Lonza), supplemented with 1 ng/mL IL 5 (BD pharmingen), 0.25 ng/mL IL 3 (Peprotech), and 1 ng/mL GM-CSF(R&D systems) for 14 days at 37°C and 5% CO_2 as described [87, 237]. Cells were pelleted by centrifugation at 1500rpm for five minutes, and the media was changed every three days.

Alternatively LDMNCs or c-kit+ virus-transduced cells were cultured at high IL-5 concentrations to increase cell yields as described [238]. Cells were cultured at approximately 10^6 cells/mL in media containing RPMI 1640 (Gibco) with 20% FBS (Cambrex), 1 U/mL penicillin/streptomycin (Lonza), 2 mM glutamine (Lonza), 25 mM HEPES, 1 mM sodium pyruvate (Gibco), and 50 µM β-mercaptoethanol (Sigma) and supplemented with 100 ng/mL stem-cell factor

(SCF/ PeproTech) and 100 ng/mL FLT3-Ligand (FLT3-L/ PeproTech) from day 0 to day 4. On day 4, the media was replaced with eosinophil culture media supplemented with 10 ng/mL recombinant mouse interleukin-5 (rmIL-5/ R&D Systems) as the only growth factor. On day 8, the cells were moved to new flasks and maintained in fresh media supplemented with rmIL-5. On alternate days, from this point, one-half of the media was replaced with fresh media containing rmIL-5, and the cell concentration adjusted to 10^6 cells/mL. Cells were enumerated on day 0 and days requiring media replacement by hemocytometer.

Assessment of eosinophil culture purity

Eosinophil purity of *ex vivo* LDMC cultures was assessed on days 10, 12, 14 and 16 by cell cytospin and flow cytometric analysis. Approximately 200,000 cells were washed with PBS and loaded onto a plastic slide funnel mounted on a glass slide and spun for 10 minutes at 1200 rpm (Beckmann Coulter). The cells affixed to these slides were Giemsa stained (Hema-Tek 2000, Bayer) and eosinophil percentage purity determined by counts under a light microscope.

Approximately 500,000 cells from eosinophil cultures were washed and resuspended in 100 µL FACS buffer and incubated with 0.5 µg/mL PE-conjugated anti-murine CCR3 (R&D Systems) or 0.5 µg PE-conjugated anti-murine Siglec F (BD Biosciences) antibody for 30 minutes on ice as described [238]. Cells were washed in FBS/PBS and Phycoethrin (PE) intensity detected by fluorescent cytometer (FACS Calibur, BD). The data was analyzed in comparison

with PE conjugated isotype control (BD Biosciences) treated cells by Flowjo v7.6.5 software (Tree Star Inc.).

Eosinophil adhesion assay

Microfluidic 24-well plates (Fluxion Biosciences Inc.) were coated with 50 µg/mL fibronectin (Invitrogen) or gelatin for one hour at 37°C. Coating solution from each well was aspirated gently to avoid air bubble introduction and washed with 300 µL of plain RPMI media (Gibco) with 0.5% BSA (Sigma). The wash solution was steadily pumped through the microfluidic chambers at 5 dynes/cm^2 using a Bioflux 200 controller (Fluxion Biosciences Inc.). Fluid from both input and output channels was gently aspirated and cells added to each input well. bmEos were washed and resuspended in RPMI/0.5% BSA media at a cell concentration of 2 x 10^6 cells/mL. Approximately 4 x 10^6 eotaxin-treated or untreated *Pak1+/+* and *Pak1-/-* bmEos were loaded at each input and flow initiated at 2 dynes/cm^2 for two minutes to introduce a bolus of cells into the adhesion chambers, after which this volume rate was adjusted to 0.5 dynes/cm^2 for 20 minutes to mimic capillary blood flow. The input well was cleared, washed with PBS, and flow initiated for two minutes at 2 dynes/cm^2 to wash away non-adherent cells. Plates were fixed with paraformaldehyde (PFA) for imaging or stained with Coomassie blue for cell counts. 40X images of PFA stained cells were taken using (SPOT, Diagnostic Instruments, Inc.) and stained cells were counted by hemocytometer, and compared relative to genotype, treatment-group, and plate coating type.

Eosinophil migration assays

Eosinophil migration to recombinant murine eotaxin-1/mCCL11 ('rmEotaxin'/ Peprotech) was assessed using a transwell assay system. During the 10th-14th days of culture, bmEos were resuspended at cell concentrations of 2 x 10^6 cells/mL in RPMI/0.5% BSA media. To assess if this transwell setup could distinguish eotaxin-induced chemotaxis from random chemokinesis a checkerboard scheme was followed where concentrations of rmEotaxin were loaded in top or bottom chambers alone, both chambers, or neither chamber preceding the migration experiment. 100 µl of resuspended bmEos were then seeded in the top three micron-meshed polycarbonate chamber of a 24-well transwell plate (Costar 3415) and the cells allowed to migrate to the bottom chamber where they were collected and enumerated by hemocytometer. All conditions of this checkerboard migration assay format were tested in duplicate or triplicate and the cell counts averaged per experiment. To calculate the chemotactic index, the number of cells that migrated in response to chemokines was divided by the number of spontaneously migrated cells [239]. Migrating cells were compared between the two genotypes and four conditions. bmEos cultures from *Pak1*$^{+/+}$ mice were also pretreated for 1 hour at 37°C with small molecule inhibitors of PAKs, IPA3 (Chernoff, Fox Chase Cancer Center), and p38 MAPK, SB22025 (Sigma) then assayed for rmEotaxin-mediated chemotaxis. As a control, a trypan-blue exclusion count was performed on cell aliquots of bmEos pretreated with inhibitors to check for dead cell percentages.

Eosinophil degranulation assay

Eosinophil degranulation to recombinant murine rmEotaxin (Peprotech) was assessed by a colorimetric assay measuring EPO activity in cell supernatants in lieu of eosinophil degranulation. On the 14th day of culture, bmEos were resuspended at cell concentrations of 1 x 10^6 cells/mL in HBSS media (Gibco). Cells were stimulated with 100-1000 ng/mL rmEotaxin for one hour at 37°C. Cells were gently centrifuged to separate supernatant and cell pellet and kept on ice. The cell pellet was permeabilized in 0.3% Triton-X (Sigma) for five minutes at room temperature and the cell lysate supernatant collected for each sample. 100µl of cell supernatants and cell-pellet lysates were seeded in a round-bottom 96-well plate. 100 µL of EPO substrate (consisting of a solution of 1 mM H_2O_2, 1 mM O-phenylenediamine, and 0.1% Triton-X (all from Sigma) in 55 mM Tris buffer at pH of 8.0) was added to the seeded fluid and the reactants incubated in the dark for 30 minutes at 37°C. The reaction was terminated by adding 50 µL of 4 M H_2SO_4, and absorbance read on a microplate luminometer at 490 nm (). Background absorbance readings (no eosinophil products) was subtracted from the absorbance readings of each well and the EPO degranulation of each supernatant expressed as a percentage of total cell EPO content according to the following equation:

$$\%\ Degranulation = \frac{(490nmAbs\ Cell\ supernatant - 490nmAbs\ background)}{(490nmAbs\ Cell\ pellet - 490nmAbs\ background)} \times 100\%$$

HL-60 human eosinophil culture and differentiation

HL-60 clone 15 cells (CRL-1964, American Type Culture Collection) were maintained in 1 U/mL Penicillin/Streptomycin (Lonza) and 2 mM glutamine (Lonza) containing RPMI-1640 medium (Gibco) supplemented with 10% FBS (Sigma) and 25 mM HEPES pH7.6 (Sigma), at cell concentrations between 0.5-2 x 10^6 cells/mL at 37°C and 5% CO_2. Approximately 0.5 x 10^6 cells/mL were differentiated to eosinophil-like cells (HL-60 Eos) by supplementing the media with 0.5mM *n*-butyrate (Sigma) dissolved in ethanol and cultured for five to seven days as described [240, 241]. HL-60 Eos were evaluated for differentiation by Giesma staining (HEMA-TEK 2000, Bayer) of cells affixed to a glass slide.

In vitro HL-60 Eos PAK1 kinase assay

HL-60 Eos were stimulated with 100 ng/mL human recombinant eotaxin (hmEotaxin/ R&D systems) in RPMI media (Gibco) with 0.5% BSA (Sigma) at 37°C for 5, 15, and 30 minutes and the reaction terminated by addition of 1 mM Na_3VO_4 in 4°C PBS. 400 µg of whole cell lysate was prepared as previously described [242] reserving 10 µL aliquot of each sample for GAPDH detection as a loading control. The remainder of the lysate was immunoprecipitated with 2 µg/mL α-PAK1 antibody (N20, Santa Cruz Biotechnology, Inc.) at 4°C for 18 hours before incubation with protein A/G plus beads (Santa Cruz Biotechnology) for two hours. PAK1 phosphotransferase activity was assayed by incubating the immunobeads with 1 µg/reaction Mek (Millipore) and 250 µM ATP (Sigma Chemical) in 30 µL kinase buffer described [243]. Samples were separated by

10% SDS-PAGE, transferred to nitrocellulose, and probed with anti-Mek-phospho-serine 298 (1:1000, Cell signaling). Phosphorylated Mek was used *in lieu* of PAK1 activation.

PAK1 RNA interference in HL-60 eos

PAK1 knockdown in HL-60 Eos was achieved by 72 hour incubation with human PAK1 siRNA constructs in siPORT NeoFx reagent (Ambion). siPORT™ NeoFX™ Transfection Agent was diluted (1:20) in 37°C Opti-Minimal Essential I medium (Opti-MEM) and the resulting solution incubated for 10 minutes at room temperature. Three PAK1 siRNA constructs (SASI_Hs01_00087968, SASI_Hs02_00334074, and SASI_Hs01_00087970 (Sigma MISSION® siRNA) were chosen based on rank order of efficacy postulated by a siRNA design algorithm (Rosetta InPharmatics) and tested. PAK1 siRNA or scramble siRNA constructs was diluted in Opti-MEM to 30nM and mixed with incubated siPORT NeoFx reagent. After a 10 minute incubation that permitted formation of transfection complexes, 2.3 mL of transfection solution was dispensed to each well of a 6-well plate. Approximately 2 x 10^5 HL-60 cells in eosinophil differentiation media were overlaid on the transfection complexes and incubated at 37°C for 72 hours. HL-60 eos were enumerated, numbers standardized, lysed, and whole cell lysates prepared in SDS page for western blot analysis. Following optimization transfection experiments, SASI_Hs01_00087970 was chosen for further experimentation.

HL-60 Eos migration assay

HL-60 Eos, siRNA-treated HL-60 Eos, or NeoFx-only treated HL-60 eos were utilized in migration assays to no stimulus or 100 ng/mL eotaxin. On the fifth day of differentiation in *n*-butyrate supplemented media, HL-60 eos were resuspended at cell concentrations of 1 x 10^6 cells/mL in RPMI/0.5% BSA media. Approximately 100 µL of these cells were seeded in the insert of an eight micron-meshed polycarbonate chamber of a 24-well transwell plate (Costar 3422, Invitrogen) and the cells allowed to migrate to the bottom chamber where they were collected and enumerated by hemocytometer. All conditions of this checkerboard migration assay format were tested in duplicate and the cell counts averaged per experiment. To calculate the chemotactic index, the number of cells migrated in response to chemokines was divided by the number of spontaneously migrated cells. Migrating cells were compared among stimulus-treated, siRNA-treated, and non-treated groups.

Immunoblotting

Whole cell bmEos lysates were prepared by addition of 1x SDS sample buffer supplemented with 1% 2-mercaptoethanol to 1 x 10^6 cells. Cell lysates were sonicated, clarified by centrifugation, and denatured at 100°C for 5 mins. Alternatively, cells lysed with ProteoJET Mammalian Cell Lysis Reagent (Fermentas), and clarified as above, were quantified by a BCA reagent assay (ThermoScientific). Samples were separated by SDS-PAGE on a 4-12% gradient or 6% acrylamide gels (Invitrogen) and transferred to PVDF membrane (Roche Diagnostics Corporation, IN). Membrane blots were probed with primary

antibodies to p-T423 PAKs, p-S3 cofillin, cofilin, p-S2152 filamin A, p-T567 ERMs, Ezrin, p-S298 MEK, p-S16 Op/18 Stathmin (Abcam), and p34-ARC (Millipore) as well as GAPDH. All antibodies were obtained for Cell Signaling Technology, Inc. unless otherwise indicated. All blots were visualized using an HRP-conjugated anti-rabbit secondary antibody (1:5000, Amersham), and films developed using the West Dura luminal system (ThermoScientific). Immunoblotting was performed on at least three independent bmEos cultures and phosphorylated protein levels from representative blots quantified by subjecting representative autoradiographs to densitometry (NIH ImageJ).

Deconvolution confocal microscopy

For visualization, 10-14 day old cultures of *Pak1-/-* and *Pak1+/+* bmEos were resuspended in RPMI with 0.5% BSA, 50 U/mL penicillin, 50 µg/mL streptomycin, and 2 mM L-Glutamine. Cells were standardized to a concentration of 2 $x10^6$ cells/mL, and stimulated with 500 ng/mL rmeotaxin or no stimulation from 0-5 minutes at 5% CO_2/37°C. Cells were aliquoted to eppendorf tubes and the stimulation terminated by addition of 4% PFA for 15 minutes at room temperature. After fixation, cells were resuspended in 1 mL PBS. Approximately 200,000 cells were then loaded in PBS onto a cytospin funnel mounted to a glass slide and spun for 10 minutes at 1200rpm. Following cytospin, cells affixed on slides were extracted by washing in 0.3% Triton-X (Sigma) for five minutes, blocked in 5% FBS for at least one hour, and incubated with primary antibody in 0.1% BSA/PBS overnight at 4°C. The following morning, these cells were

washed three times in PBS, and subsequently incubated for one hour at room temperature with fluorophore-conjugated anti-mouse or anti-rabbit secondary antibodies (Molecular Probes, Invitrogen). After washing, one drop of a DAPI mounting solution (Molecular Probes, Invitrogen) was applied and a cover slip sealed over the samples using lacquer. Data were acquired and analyzed on a DeltaVision deconvolving microscopy system (Applied Precision, Washington). Typical controls included cells incubated with secondary (fluorophore-conjugated) antibody but not primary antibody. Intensity and localization of cells from both genotypes were compared.

Statistical analyses

All statistical analyses were performed with GraphPad Prism 5.4. One- or two-way ANOVA was performed, as appropriate, with Bonferroni post-hoc corrections. For two variable comparisons, unpaired two-tailed student's *t*-tests were used. All tests, numbers of independent replicates, and significance levels are found within the figure legends in the results section.

RESULTS

PAK1 in murine allergic airway inflammation

We tested the role PAK1 played in a model of allergic airway murine inflammation using *Pak1-/-* mice. We used a murine allergic airway disease model employing OVA-albumin sensitization and challenge with adjuvant treatment. We supplemented these studies with two chimeric models. Using both adoptive bone marrow and orthotopic lung transplantation models, we tested the relative roles of hematopoietic and pulmonary contributions to this inflammation.

***Pak1* genetic disruption**

Our multi-disciplinary research group has previously generated a novel *Pak1* global knockout mouse and demonstrated that PAK1 notably modulates mast cell inflammatory processes. A schematic illustrating disruption of PAK1 in ES cells is shown in Figure 5. Southern blot analysis of murine *Pak1-/-* ES cells showed a disrupted *Pak1* allele while western blot analysis of multiple tissues from these mice failed to detect PAK1 protein [223]. The resulting *Pak1-/-* mice are viable, undergo normal growth and development, are fertile, and have a normal lifespan. The mice used in our studies were backcrossed onto a C57BL/6J background for at least seven generations to achieve genetic uniformity with the wild-type (*Pak1+/+*) mice. The peripheral blood taken from these mice demonstrate normal blood cell composition and many cytokine levels were comparable to *Pak1+/+* mice [223].

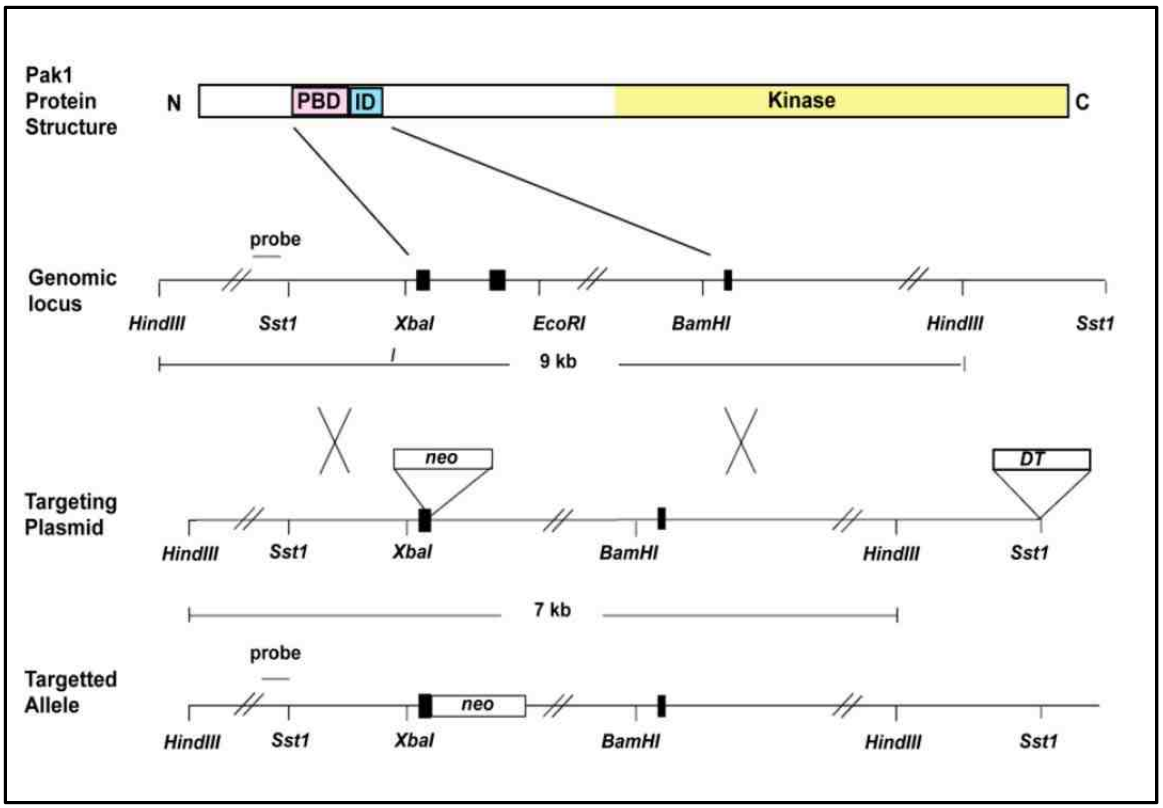

Pak1
Protein
Structure
N
PBD
ID
Kinase
C
probe
Genomic
locus
HindIII
Sst1
XbaI
EcoRI
BamHI
HindIII
Sst1
9 kb
neo
DT
Targeting
Plasmid
HindIII
Sst1
XbaI
BamHI
HindIII
Sst1
7 kb
probe
Targetted
Allele
neo
HindIII
Sst1
XbaI
BamHI
HindIII
Sst1

Figure 5

Figure 5: Schematic illustrating the genetic disruption of *Pak1* in murine ES cells to ablate PAK1 expression. We generated a *Pak1-/-* mouse by targeted disruption of the Pak1 allele on chromosome 7 in embryonic stem (ES) cells. We first published this figure in its original form in Allen, J. *et al*. in BLOOD, 2008 [223].

The *Pak1-/-* OVA-albumin allergic airway disease model

Our initial goal was to establish a model to study PAK1's effect on the development of allergen-induced airway inflammation. We utilized an ovalbumin (OVA)-sensitized and challenged murine model with aluminum hydroxide (Alum) adjuvant supplementation to elicit a potent Th2-mediated inflammatory airway response. Following optimization of the model in our mice, we employed the sensitization and challenge schedule illustrated in Figure 6. We utilized age-matched, male mice in these experiments. We sensitized mice of both genotypes using two intraperitoneal injections of an amalgam of OVA and Alum and subsequently challenged the mice by intranasal inhalation for five days. OVA-sensitized and challenged mice were utilized either for pulmonary function tests or for serum, BALF, and lung procurement to assess the inflammatory infiltrate.

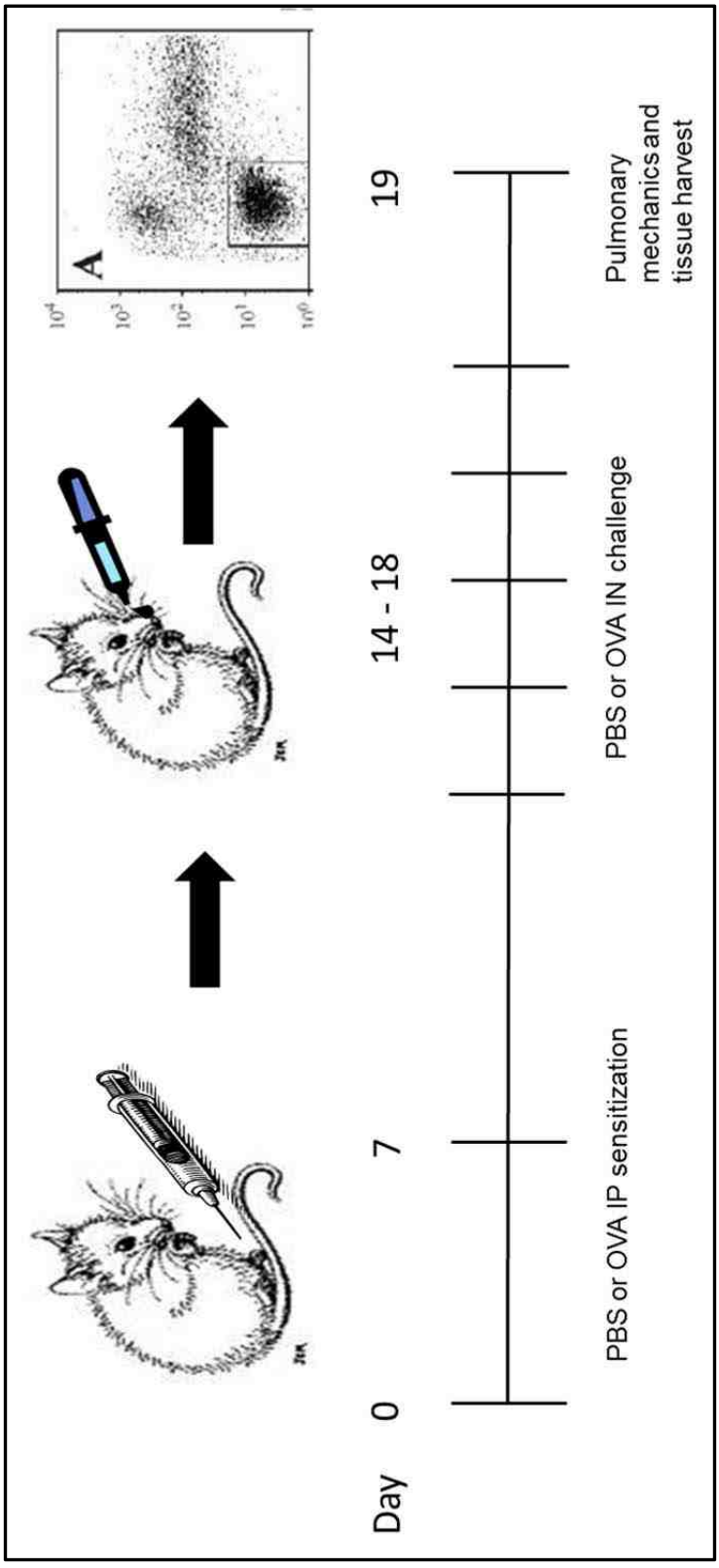
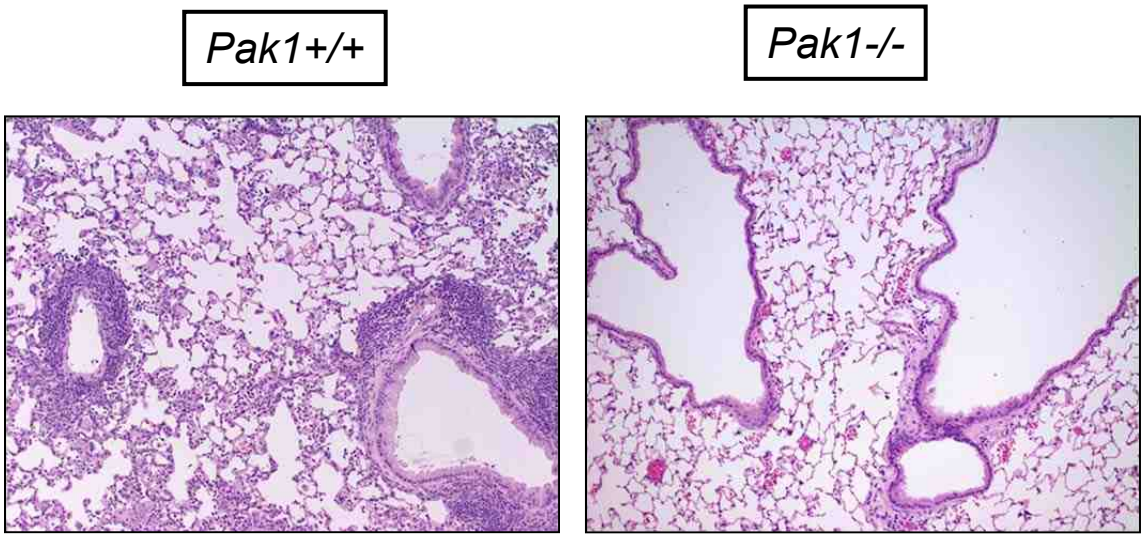

A
Day 0
7
14 - 18
19
PBS or OVA IP sensitization
PBS or OVA IN challenge
Pulmonary mechanics and tissue harvest

Figure 6

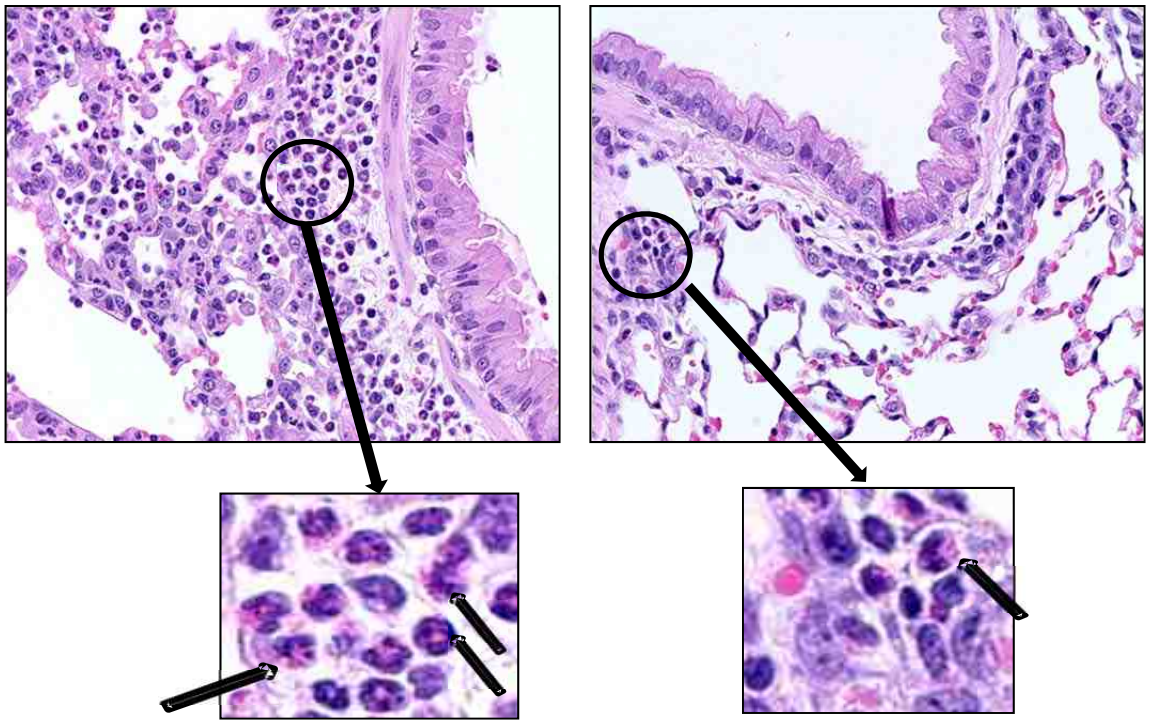
Figure 6: Schematic illustrating the *Pak1-/-* murine model of allergic airway inflammation. Age-matched male *Pak1+/+* and *Pak1-/-* mice were OVA-sensitized and challenged in a 19-day protocol and utilized for either pulmonary function tests for evaluation of AHR or cellular analysis for airway inflammation. Mice were sensitized to OVA by intraperitoneal injections on day 1 and day 8 and subsequently challenged by intranasal inhalation for five consecutive days before lungs tissue was harvested for H&E staining and analysis of leukocytes whereas BALF was assessed for leukocyte populations by fluorescent cytometry and cytokine content by ELISA.

Decreased airway eosinophil inflammation with *Pak1* deletion in a murine model of AAD

We sought to discern *in vivo* the role PAK1 played in a murine model of human bronchial asthma in which eosinophil infiltration critically contributes to the pathogenesis of the disease [9, 97]. To examine the role of the eosinophil, we scored lung eosinophilic inflammation by H&E-stained histological lung sections and eosinophil percentages by fluorescent cytometry from lung tissue harvested from *Pak1+/+* and *Pak1-/-* OVA-sensitized and challenged as well as control mice. We found that *Pak1-/-* lung samples from AAD mice showed decreased inflammatory cell infiltrate around the small airways (Figures 7a-b), interstitial inflilltration of cells (Figures 7c-d), and eosinophilic infiltrate (Figures 7e-f) compared with sensitized *Pak1+/+* lung tissue. We obtained leukocytes from lung samples of OVA-sensitized and challenged mice of both genotypes by connective tissue digestion and leukocyte magnetic bead separation, then quantified eosinophil percentages in inflamed lung tissue by fluorescence cytometry for eosinophil markers. Predictably, we found that percentages of non-lymphocyte, non-monocyte CD45+, Siglec F+ cells increased with OVA-sensitization and challenge in both genotypes, however this increase was modest in *Pak1-/-* relative to the robust eosinophil infiltrate in *Pak1+/+* AAD mice (Figure 7g).



Figures 7a-b



Figures 7c-f

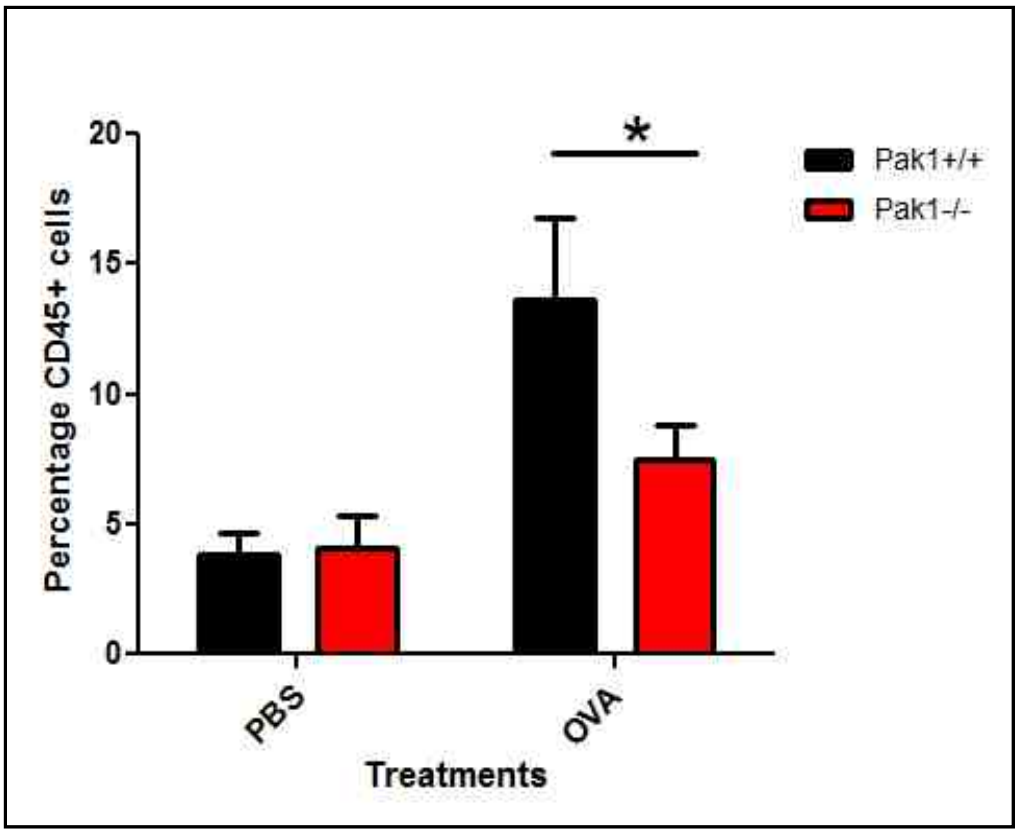

20
15
10
5
0
Percentage CD45+ cells
*
Pak1+/+
Pak1-/-
PBS
OVA
Treatments

Figure 7g

Figure 7: PAK1 promotes eosinophil infiltration of lung tissue in a murine model of AAD. *Pak1+/+* and *Pak1-/-* mice were sensitized and challenged with OVA to induce allergic airway inflammation or PBS for control. Lungs from these mice were fixed, sectioned, and stained with H & E. Micrographs are representative for at least five experiments (Figures 7a-f). Lung leukocytes from these AAD mice were obtained following enzymatic digestion, mechanical dissociation, and magnetic separation from lung tissue. Leukocytes were assessed for lineage markers by multicolor fluorescence cytometry. On analysis, leukocyte population gates (all CD45+) were set to distinguish the following populations: Eosinophils, FSC_{lo} B220/CD3 (-), CCR3/SiglecF (+), Neutrophils, FSC_{lo} B220/CD3 (-) CCR3/SiglecF(-), Lymphocytes FSC_{lo} B220/CD3 (+), CCR3/SiglecF (-), and alveolar macrophages, $FSC_{hi,}$ CDIIc or CD11b (+), MHCII (+). Population percentages were compared for significant differences ($p<0.05$) between *Pak1-/- and Pak1+/+* samples N=6 (Figure 7g). Results are averaged from three experiments and compared. * $p<0.05$, using a two-tailed student's *t*-test.

Decreased BAL eosinophil numbers with *Pak1* deletion in a murine model of AAD

We retrieved BALF from mice of both genotypes from AAD induction and assessed cell populations by fluorescence cytometry and cytokines by multiplex ELISA. Consistent with our findings in the lung tissue, BALF obtained from OVA-sensitized and challenged *Pak1+/+ and Pak1-/-* mice showed increases in cellularity and granulocyte composition. Granulocytes are depicted with red arrows (Figure 8a). These increases in cellularity and non-lymphocyte, non-monocyte Siglec F+ leukocytes were dramatically reduced in *Pak1-/-* mice relative to *Pak1+/+* AAD mice with induction of allergic airway inflammation (Figures 8 b-c). BALF from AAD-induced mice from both genotypes however, showed comparable quantities of the eosinophil chemoattractant, eotaxin, suggesting a similar allergen-induced sensitization necessary for eosinophil recruitment to the inflamed lungs.

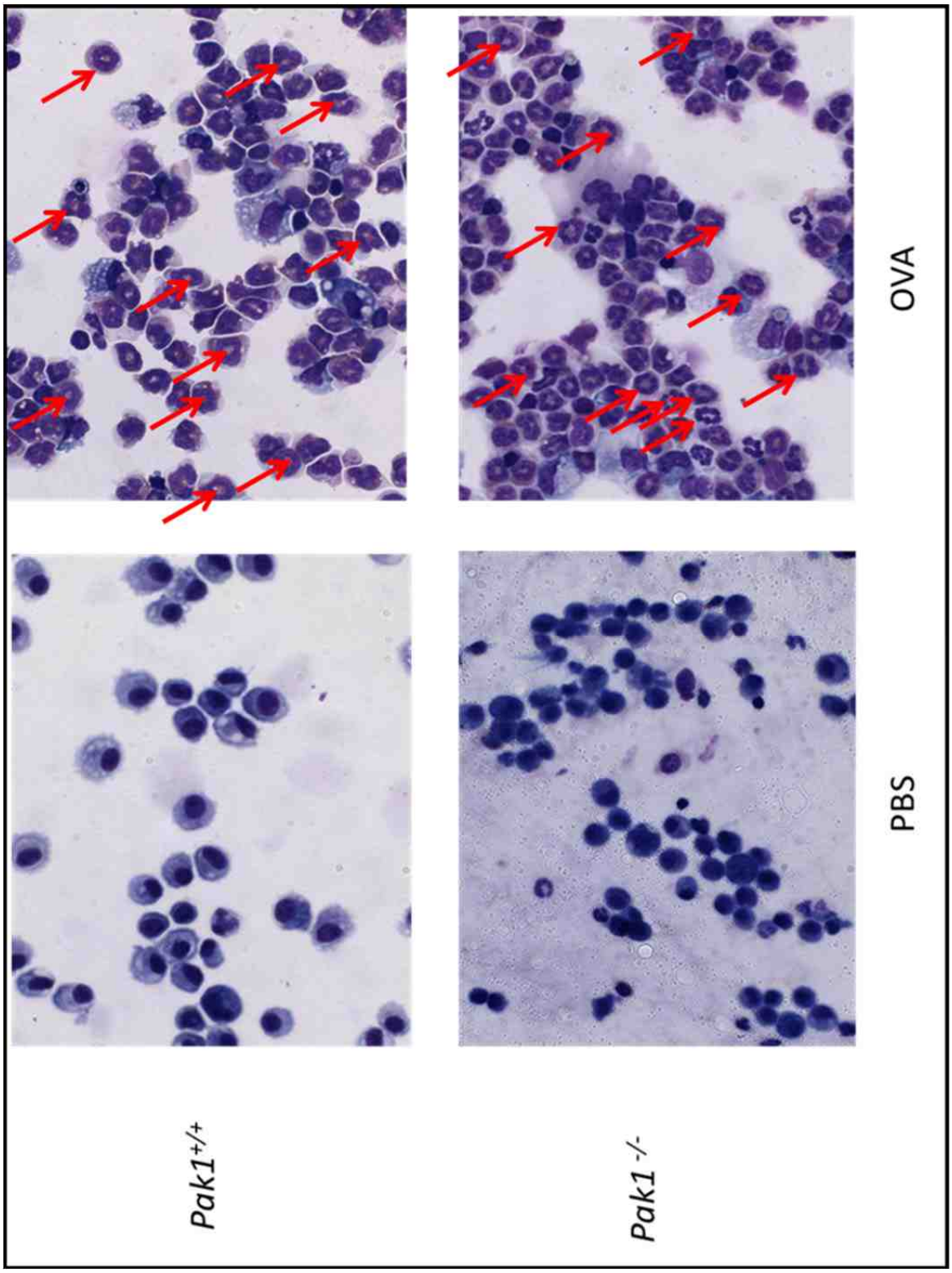


Figure 8a

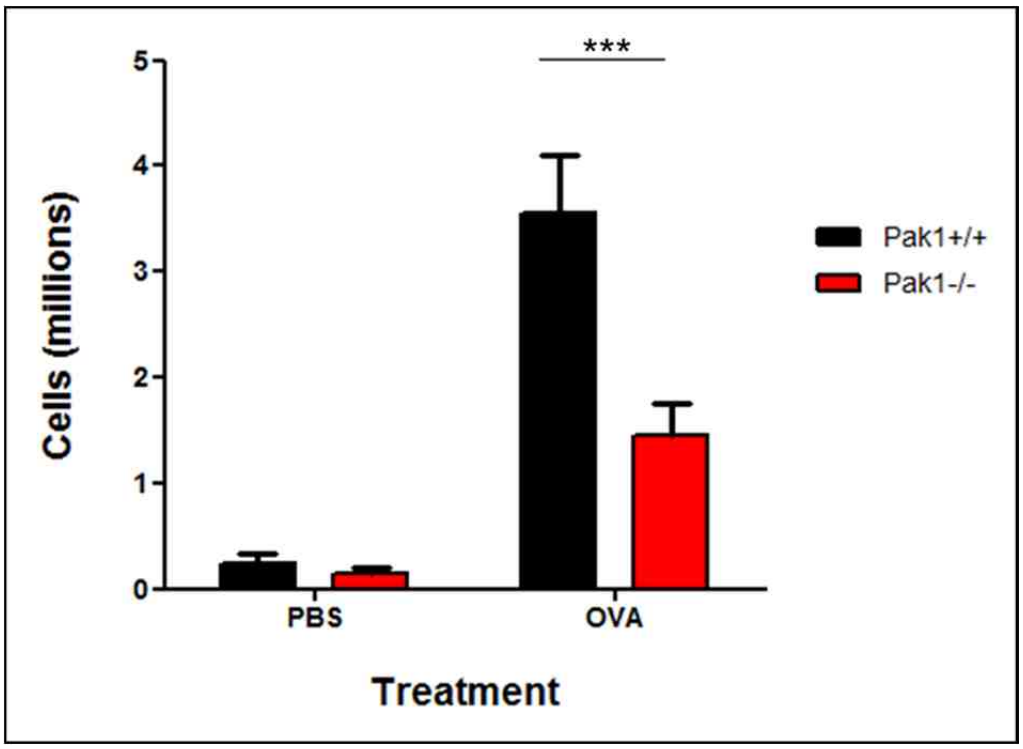


Figure 8b

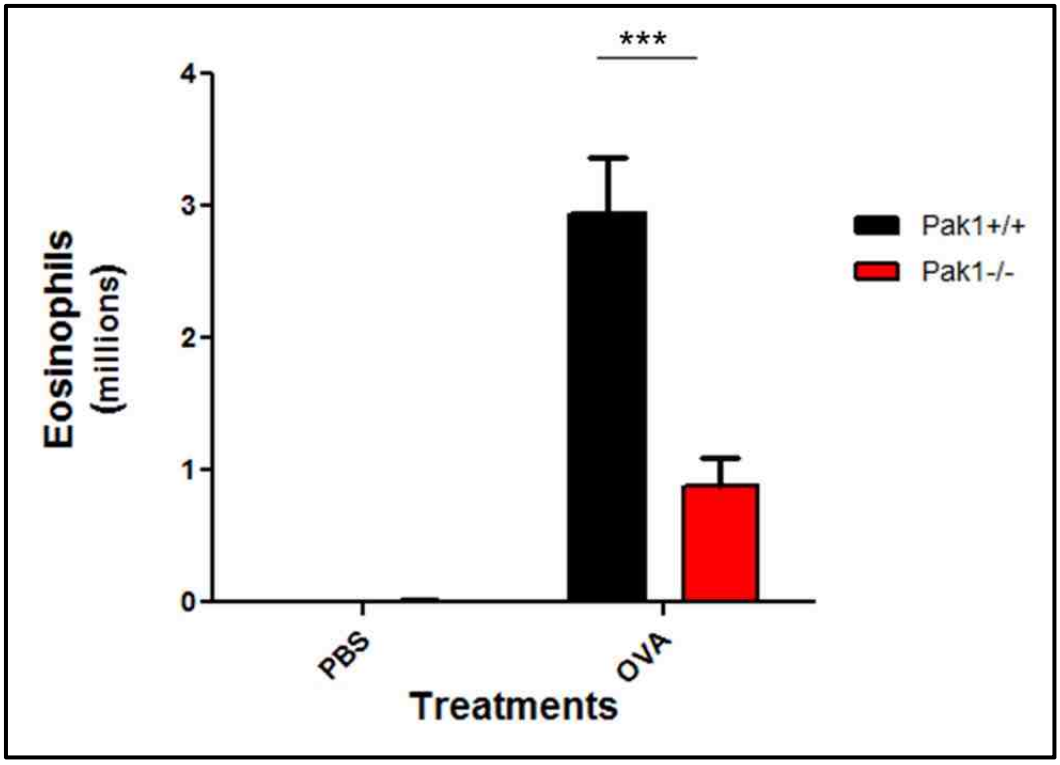


Figure 8c

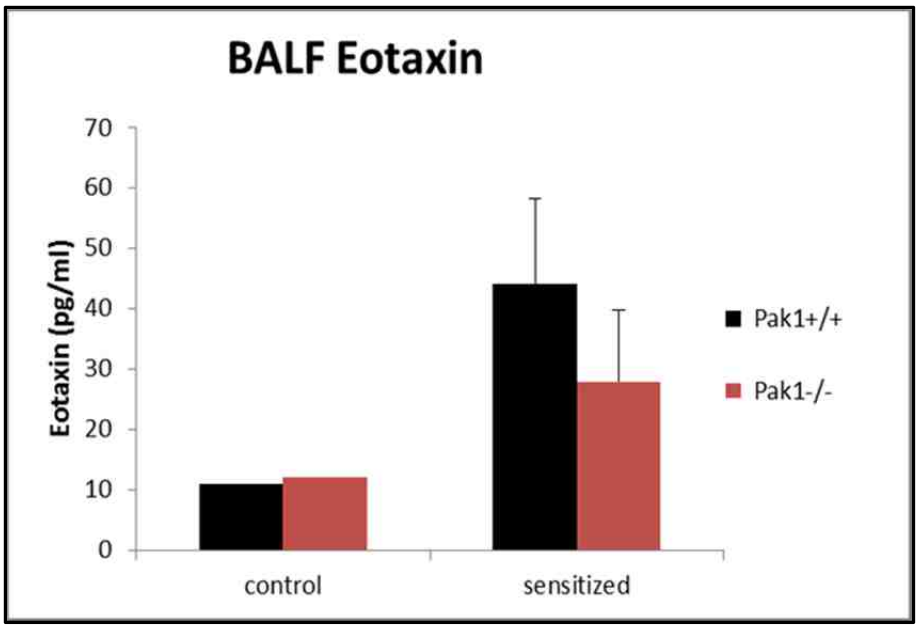
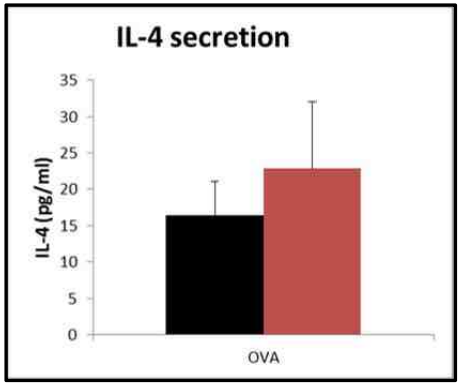

BALF Eotaxin
Eotaxin (pg/ml)
70
60
50
40
30
20
10
0
control
sensitized
Pak1+/+
Pak1-/-

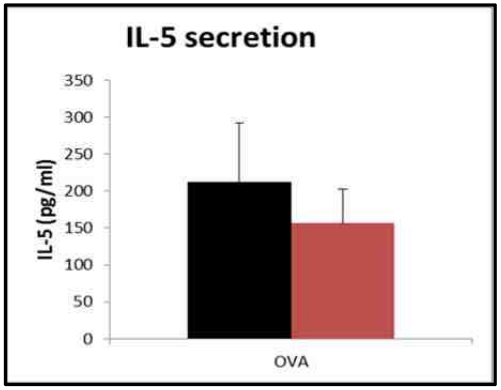
Figure 8d

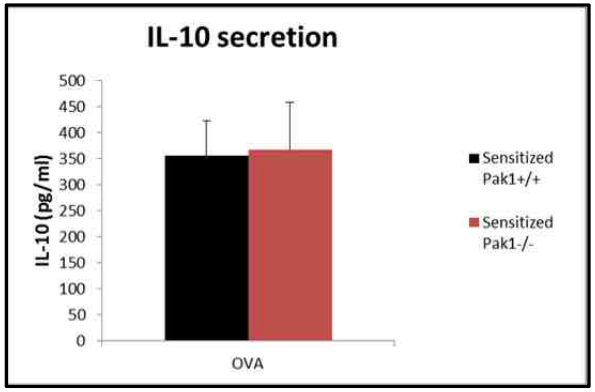
Figure 8: PAK1 promotes eosinophil infiltration into broncho-alveolar lavage fluids (BALF) in a murine model of AAD. *Pak1+/+ and Pak1-/-* mice were sensitized and challenged with OVA to induce allergic airway inflammation or PBS for control. Broncho-alveolar lavage fluid was obtained from these mice following AAD induction, and an aliquot of these cells were cytospun and giesma-stained (Figure 8a), and the total cells enumerated by hemocytometer (Figure 8b). On analysis, leukocyte population gates (all CD45+) were set to distinguish the following populations: Eosinos, FSC_{lo} B220/CD3 (-), CCR3/SiglecF (+), Neutrophils, FSC_{lo} B220/CD3 (-) CCR3/SiglecF(-), Lymphocytes FSC_{lo} B220/CD3 (+), CCR3/SiglecF (-), and alveolar macrophages, $FSC_{hi,}$ CDIIc or CD11b (+), MHCII (+). Eosinophil numbers were computed from fluorescence cytometry percentages (Figure 8c). Accellular fluid assessed for CCL11/eotaxin by ELISA (Figure 8d). Results in b-c are averaged from N=10-12 animals and compared. *** $p<0.001$, ****$p<0.0001$ using a two-tailed student's *t*-test.

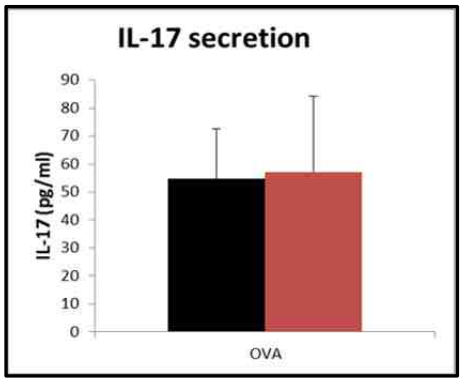
Normal OVA-specific T-helper cell cytokine secretion in *Pak1-/-* OVA-sensitized mice

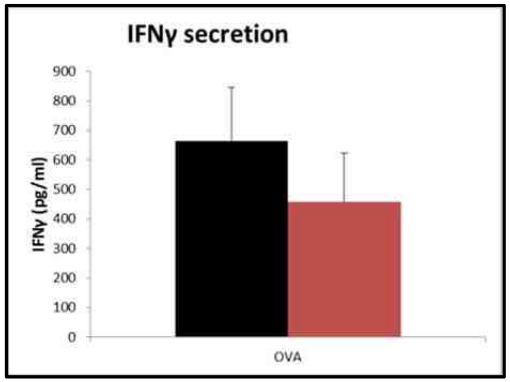
We also harvested spleens from *Pak1+/+ and Pak1-/-* OVA-sensitized and challenged mice. To compare peripheral T-helper cell responses we assessed cytokine secretion from splenocytes of both genotypes, we stimulated these cells *ex vivo* with OVA or plain media, and collected cell supernatants for cytokine analysis after 72 hours of culture. We assessed cytokine levels by multiplex ELISA using specific capture antibody beads. We found similar quantities of the T-helper cell cytokines IL-4, IL-5, IL-10, IL17, IFNγ, and RANTES (Figures 9a-f) in OVA-stimulated splenocyte supernatants from both genotypes. These data suggest preserved OVA-specific T-helper cell cytokine secretion in *Pak1-/-*. Taken together the data from this *Pak1-/-* murine model of AAD suggest a novel role for PAK1 in promoting eosinophil inflammation.











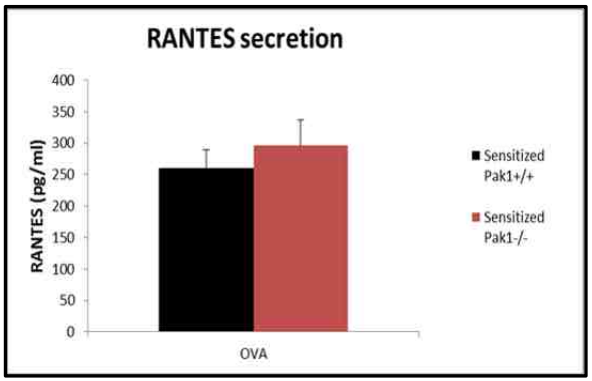


Figure 9a &d

Figure 9b &e

Figure 9c &f

Figure 9: *Pak1* genetic deletion does not affect OVA-specific splenocyte secretion of T-helper cell cytokines. Total splenocytes from OVA-sensitized and challenged *Pak1+/+ and Pak1-/-* mice were stimulated with OVA or plain media and cultured *ex vivo* for 72 hours. Cell-free supernatants were collected and stored for multiplex ELISA cytokine measurement of the following analytes: IL-4, IL-5, IL-10, IL-17, IFNγ, and RANTES (Figures 9a-f). Data from N=7-8 mice from each group were analyzed and compared between genotypes by a student's *t*-test.

Decreased eosinophil inflammation in AAD-induced mice with hematopoeitic *Pak1* deletion in an adoptive bone marrow transplant model

Deregulated interactions of hematopoietic and non-hematopoietic tissues promote the development of allergic airway inflammation. To further dissect the hematopoietic and pulmonary contributions of PAK1 to this eosinophil inflammation, we performed adoptive bone marrow transplantation studies building upon this PAK1 AAD phenotype. We transplanted C56BL/6J CD45.2+ *Pak1+/+* or *Pak1-/-* LDMNCs into lethally-irradiated WT recipient mice that co-express both isoforms of CD45.1 and CD45.2 (CD45.1/2+) common leukocyte antigen (Figure 10a). To complement these transplanted cohorts, we also transplanted CD45.1/2+ Pak1+/+ and CD45.2+ *Pak1-/-* LDMNCs into CD45.2+ *Pak1-/-* bone marrow ablated recipient mice. Following a five-month hematopoietic reconstitution, we assessed peripheral blood chimerism of CD45 antigens and observed comparable and complete reconstitution in hosts from both cohorts (Figures 10 b-c). These transplanted mice were then sensitized and challenged to develop allergic airway inflammation. We observed that *Pak1* deficiency in the bone marrow decreased the eosinophil numbers in BAL retrieved from both *Pak1+/+* (Figure 10d) and *Pak1-/-* (Figure 10e) recipient mice. We additionally observed that while bone marrow graft *Pak1* deficiency in *Pak1+/+* hosts modestly decreased eosinophil numbers in BAL compared to wildtype grafts, this deficiency markedly decreased BAL eosinophil numbers in *Pak1-/-* hosts. These data together suggest hematopoietic PAK1 modulates processes that incite eosinophil inflammation in this murine model of AAD.

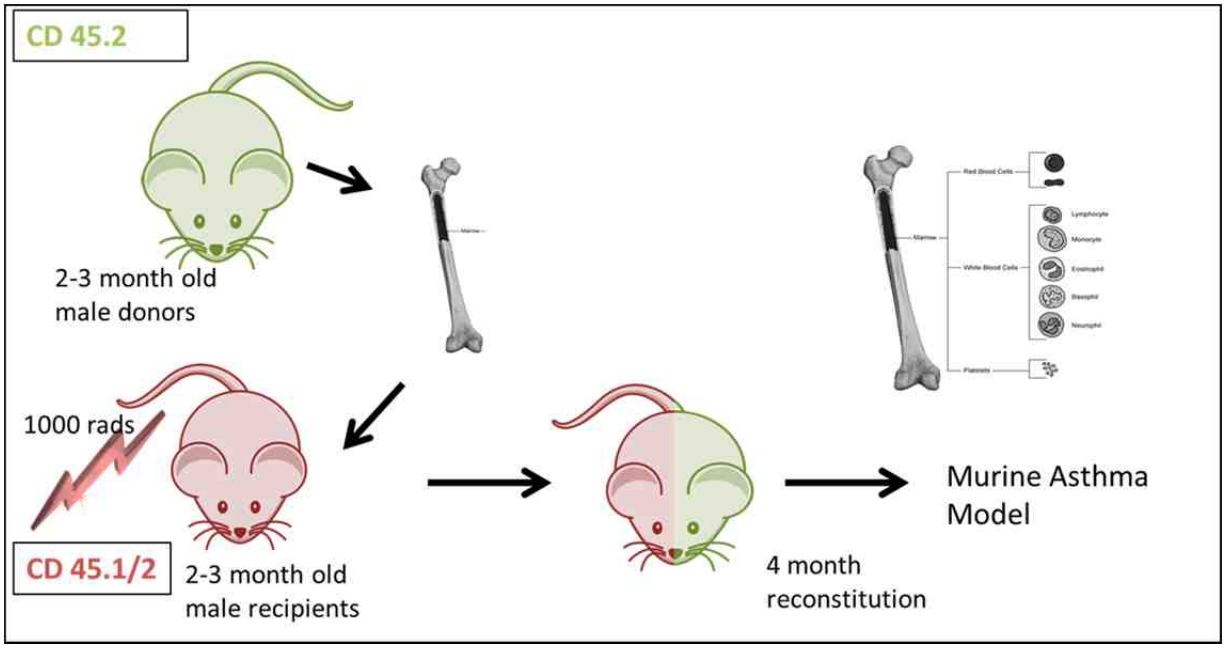

CD 45.2
2-3 month old
male donors
1000 rads
CD 45.1/2
2-3 month old
male recipients
4 month
reconstitution
Murine Asthma
Model

Figure 10a

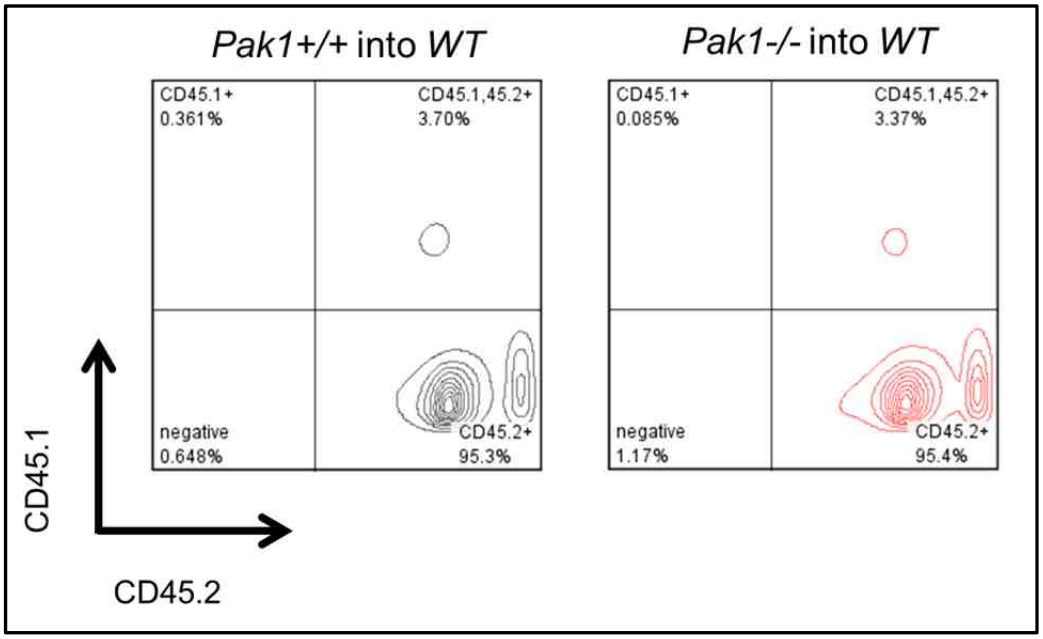

Pak1+/+ into WT
Pak1-/- into WT
CD45.1+
0.361%
CD45.1,45.2+
3.70%
negative
0.648%
CD45.2+
95.3%
CD45.1+
0.085%
CD45.1,45.2+
3.37%
negative
1.17%
CD45.2+
95.4%
CD45.1
CD45.2

Figure 10b

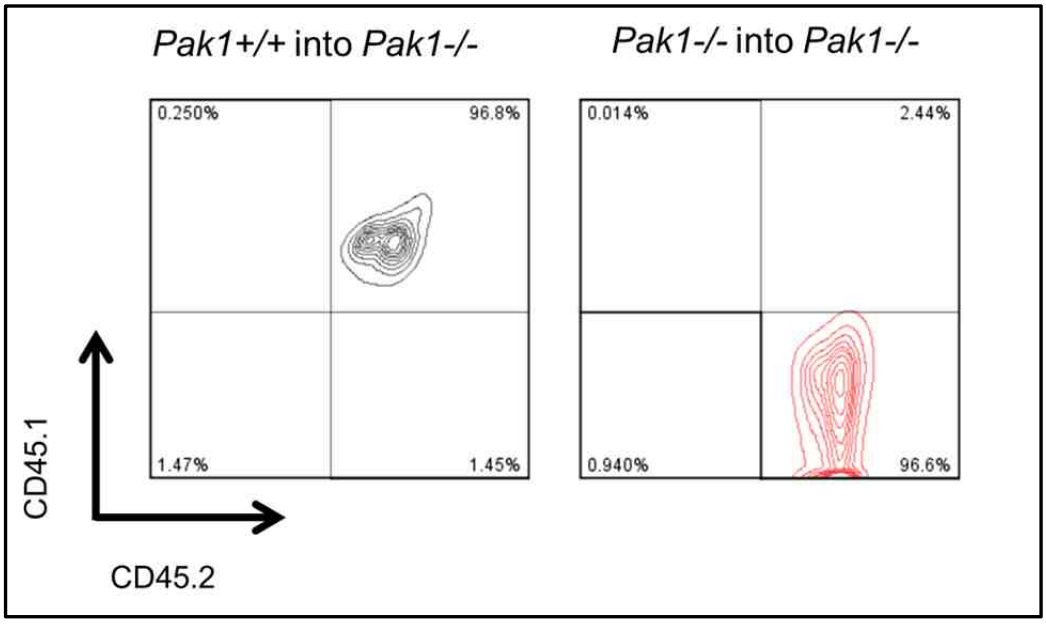

Pak1+/+ into Pak1-/-
Pak1-/- into Pak1-/-
0.250%
96.8%
1.47%
1.45%
0.014%
2.44%
0.940%
96.6%
CD45.1
CD45.2

Figure 10c

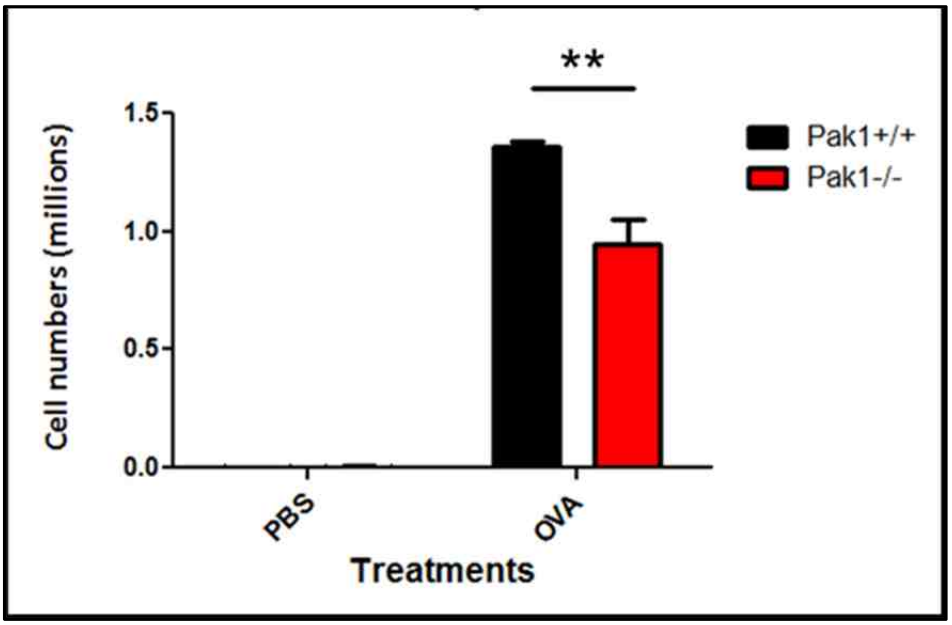


Figure10d

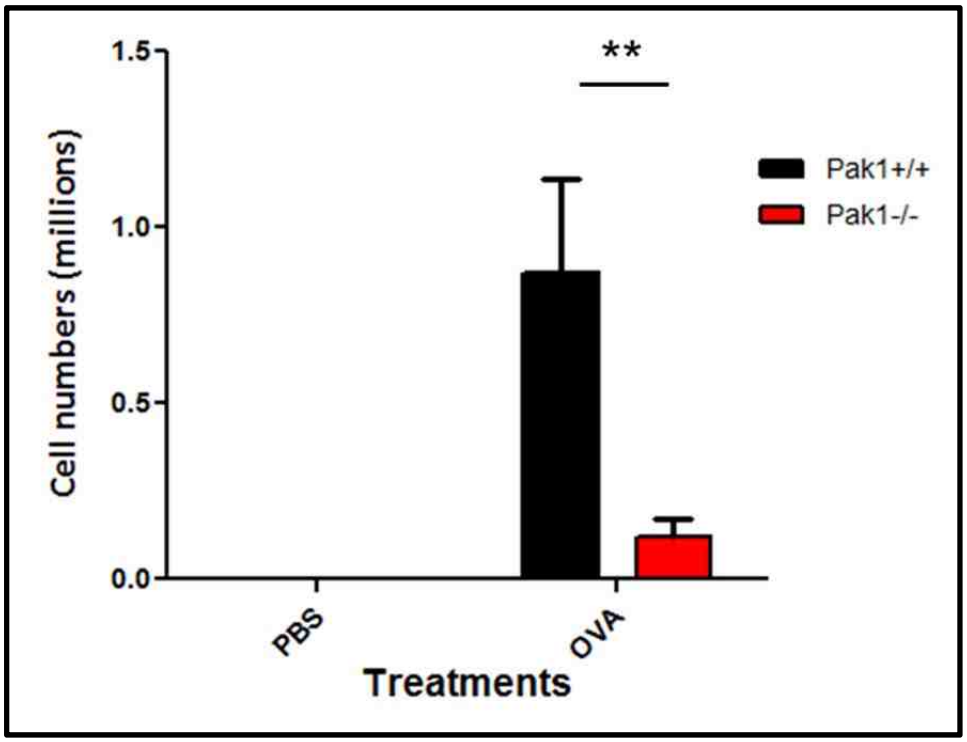


Figure 10e

Figure 10: Hematopoeitic *Pak1* deletion decreases eosinophil inflammation in AAD-induced mice in an adoptive bone marrow transplant model. In an adoptive bone marrow transplant model, CD45.1/2+ WT recipient mice were lethally irradiated and transplanted with either CD45.2+ *Pak1+/+* or *Pak1-/-* LDMNCs. After hematopoietic reconstitution these transplanted mice were utilized in a murine AAD model as shown (Figure 10a). Hematopoietic reconstitution was assessed for *WT* recipients that received *Pak1+/+* versus *Pak1-/-* bone marrow at 6 months by fluorescence cytometry of peripheral blood leukocytes (94.39+/-0.46% versus 94.04+/-0.26%, [Figure 10a]). Bronchoalveolar cells were enumerated and analyzed for eosinophil markers and eosinophil numbers computed (Figure 10d). Conversely, CD45.2+ *Pak1-/-* recipients were lethally irradiated and transplanted with CD45.1/2+ *Pak1+/+* or CD45.2+ *Pak1-/-* LDMNCs. Hematopoietic reconstitution was assessed for *Pak1-/-* recipients that received *Pak1+/+* versus *Pak1-/-* bone marrow at 6 months by fluorescence cytometry of peripheral blood leukocytes (95.75+/-0.63% versus 95.23+/-1.27%, [Figure 10c]). Bronchoalveolar cells from animals in (c) were enumerated and analyzed for eosinophil markers and eosinophil numbers computed (Figure 10e). Graphs from (b) and (c) are representative of two experiments and results from (d) and (e) are averaged from four mice (N=4). * $p<0.05$, ** $p<0.01$, ***$p<0.001$ using a two-tailed student's *t*-test.

Decreased eosinophil inflammation in AAD-induced mice with hematopoietic *Pak1* deletion in an orthotopic lung transplant model

Bone marrow transplants, usually used to study hematopoiesis require ionizing radiation that irreparably disrupts the pulmonary resident cells and architecture in transplant recipient mice. To further investigate the hematopoietic and pulmonary contributions of PAK1 to allergic airway inflammation, we collaborated with Dr. David Wilkes' lab to transplant left lung grafts into *Pak1+/+ and Pak1-/-* mice. We separately transplanted *Pak1-/-* lung*s* into *Pak1+/+* mice and conversely *Pak1+/+* left lungs into *Pak1-/-* recipient mice. Following a recovery period, we OVA-sensitized and challenged these transplanted mice in a murine AAD model (Figure 11a).

We found that *Pak1-/-* transplanted lungs in *Pak1+/+* recipient mice demonstrated more eosinophilic inflammation than *Pak1+/+* transplanted lungs in *Pak1-/-* recipients on H&E stained sections (Figures 11b-e) despite comparable goblet cell metaplastic changes characteristic of AAD between the two groups (Figures 11f-g). In these preliminary studies, three out of four (3/4) transplants in the groups of *Pak1+/+* mice transplanted with *Pak1-/-* lungs had peribronchiolar eosinophil inflammation on histological inspection. Conversely, none of four (0/4) transplants in the group of *Pak1-/-* mice transplanted with *Pak1+/+* lungs had these lesions. Likewise, slides from *Pak1+/+* mice transplanted with *Pak1+/+* lungs, but not *Pak1-/-* mice transplanted with *Pak1-/-* lungs demonstrated eosinophil lesions (Table 1). The *chi*-square association between transplanted

mice with *Pak1+/+* versus *Pak1-/-* hematopoietic cells and peribronchiolar eosinophil lesions was statistically significant.

In subsequent studies with separate cohorts of mice, we transplanted *Pak1-/-* left lung into *Pak1+/+* mice and conversely *Pak1+/+* left lungs into *Pak1-/-* recipient mice. We procured lung grafts from these mice, and enzymatically digested whole lung tissue to quantify eosinophils by fluorescence cytometry. Consistent with results from our AAD model, we saw a trend towards decreased total BAL eosinophil numbers (Figure 11h) and percentages (Figure 11i) in *Pak1-/-* hosts relative to *Pak1+/+* hosts. Leukocytes retrieved from native right lung (Figure 11j) and engrafted left lung (Figure 11k) revealed a trend towards decreased non-lymphocyte, non-monocyte Siglec F+ cells in *Pak1-/-* hosts compared with *Pak1+/+* hosts. Future studies are needed to resolve these trends by increasing the number of animals and the experimental power. However, taken together these histological and quantitative data suggest a role for host/hematopoietic PAK1 modulation in eosinophil inflammation in a murine model of AAD.

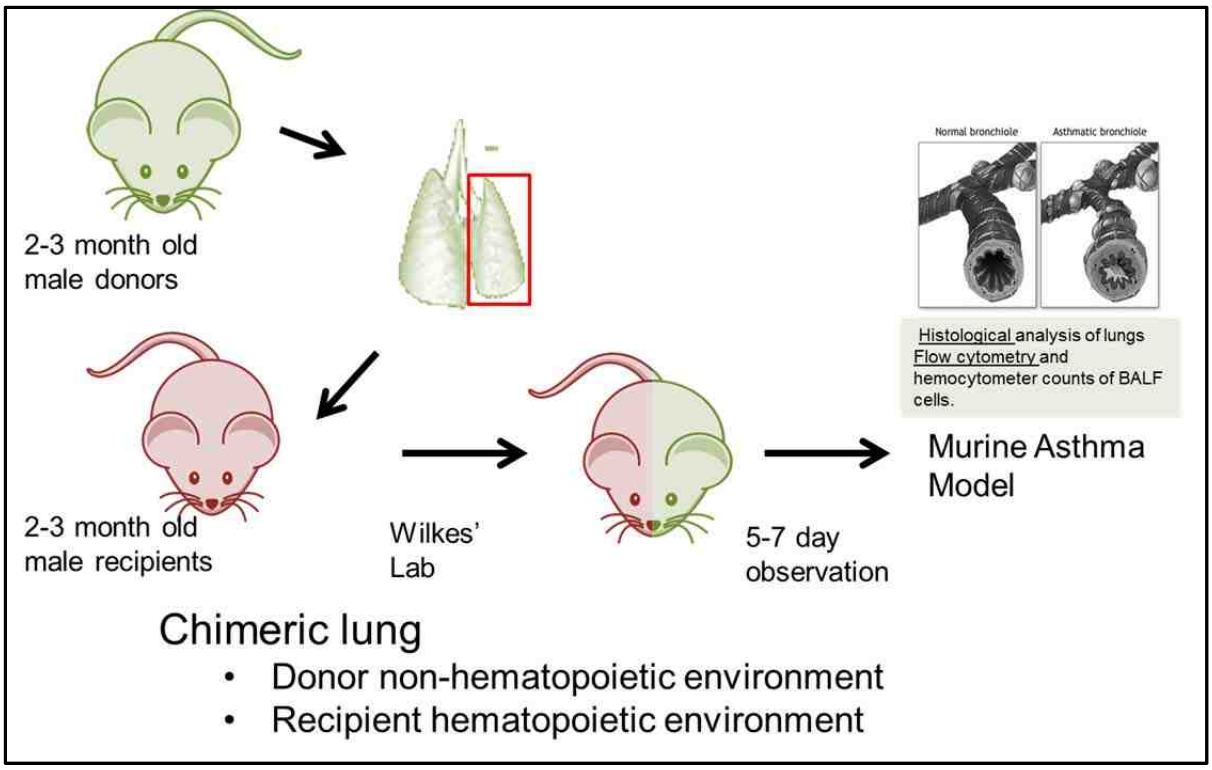
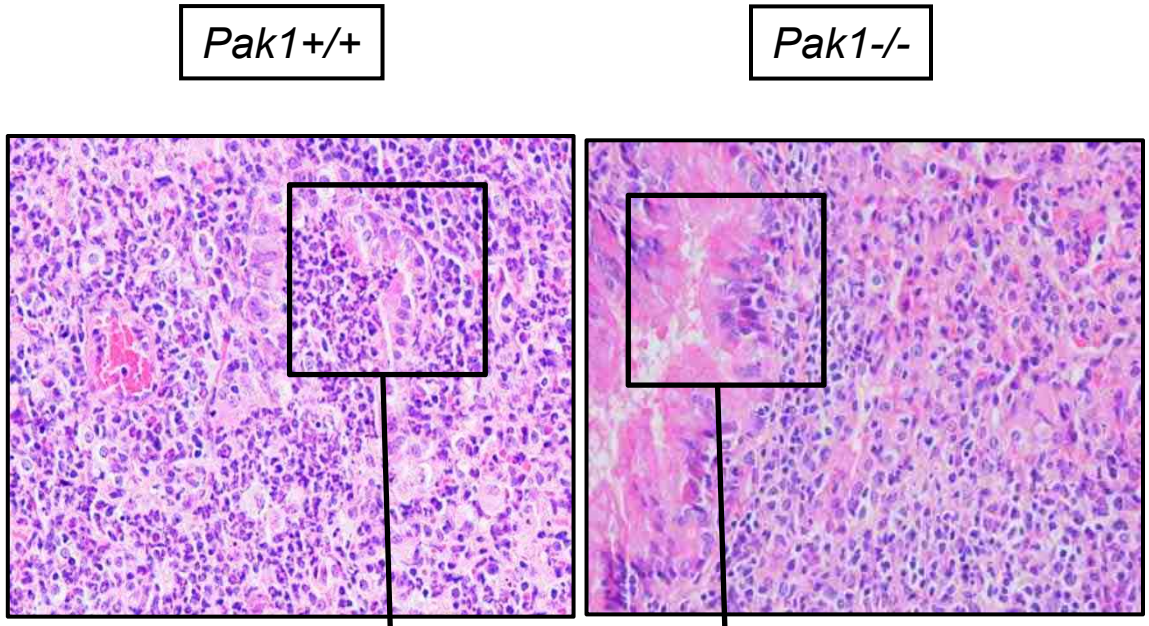
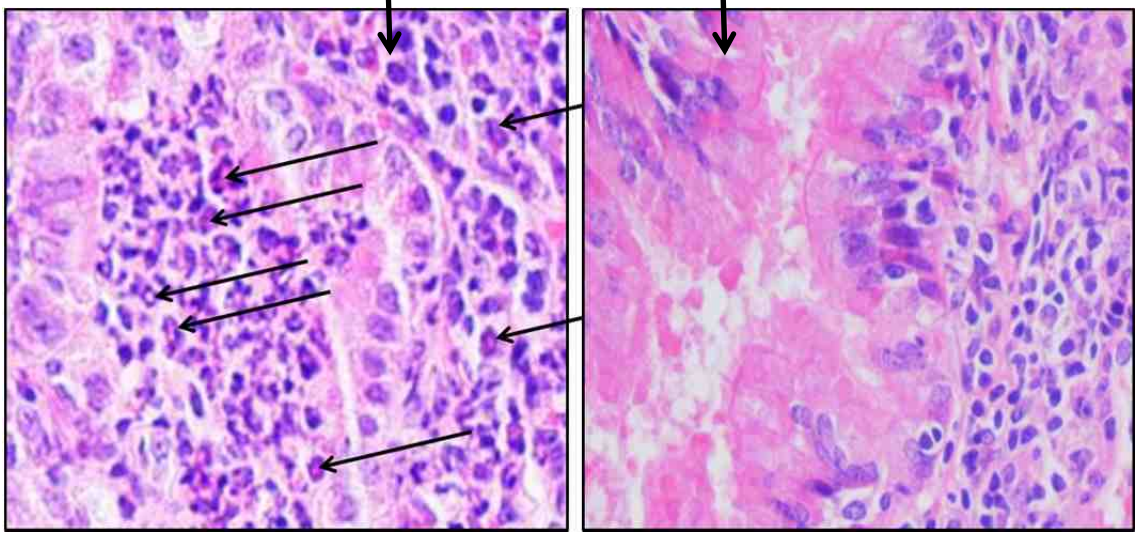

2-3 month old
male donors
Normal bronchiole
Asthmatic bronchiole
Histological analysis of lungs
Flow cytometry and
hemocytometer counts of BALF
cells.
2-3 month old
male recipients
Wilkes'
Lab
5-7 day
observation
Murine Asthma
Model
Chimeric lung
• Donor non-hematopoietic environment
• Recipient hematopoietic environment

Figure 11a



Figures 11b-c



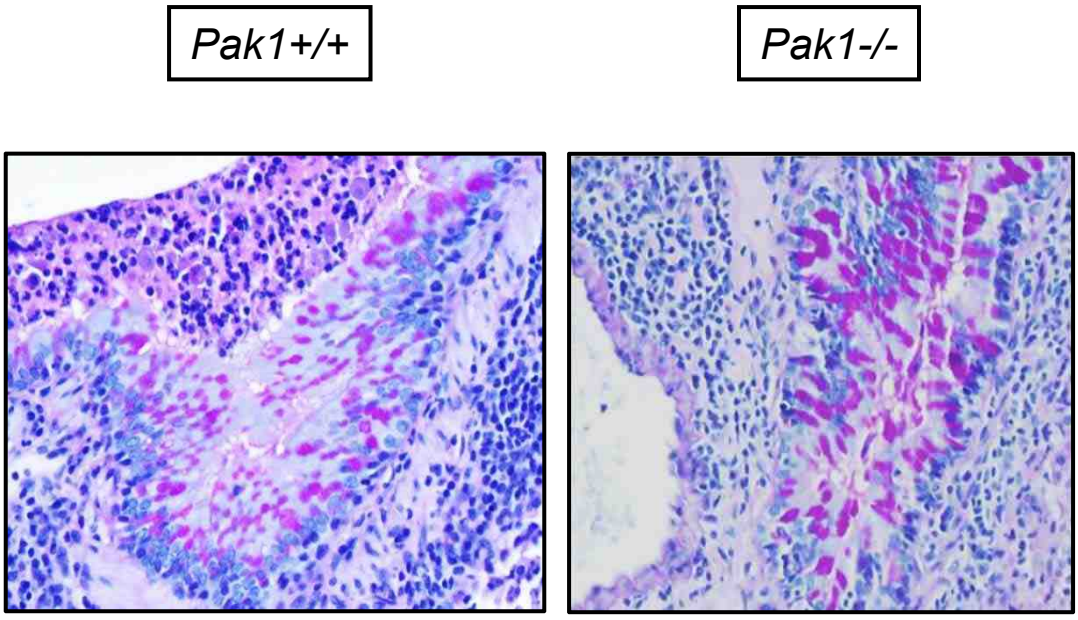
Figures 11d-e

		Grafts	
		Pak1+/+	*Pak1*-/-
Hosts	*Pak1*+/+	1/1	3/4
	Pak1-/-	0/4	0/1

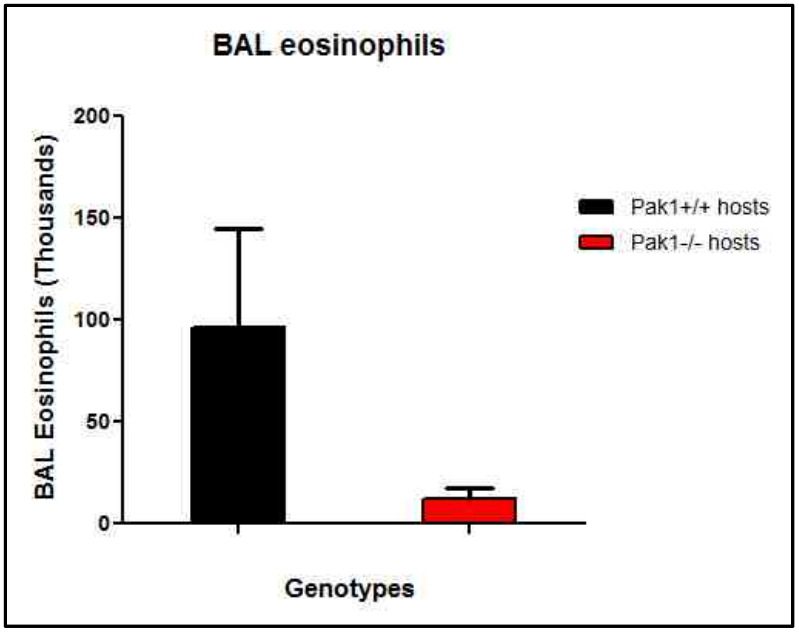
Table 1: Fraction of transplanted mice that demonstrated eosinophil infiltration on histology

	Eosinophils	No eosinophils	Total
Pak1+/+ hosts	4	1	5
Pak1-/- hosts	0	5	5
totals	4	6	10

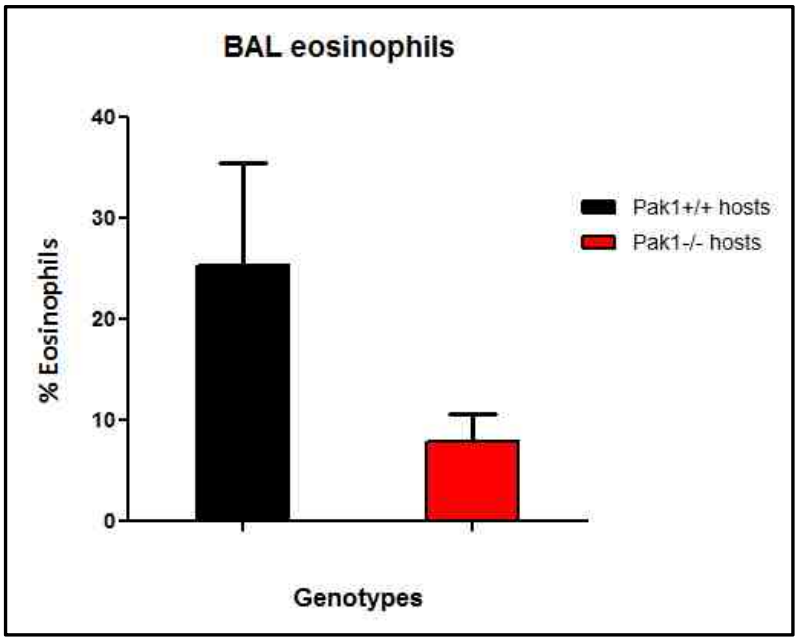
Table 2: Chi square comparison w/o Yates correction **$p<0.01$ comparing all *Pak1*+/+ and *Pak1*-/- hosts percentages that demonstrated eosinophil inflammation on histology



Figures 11f-g

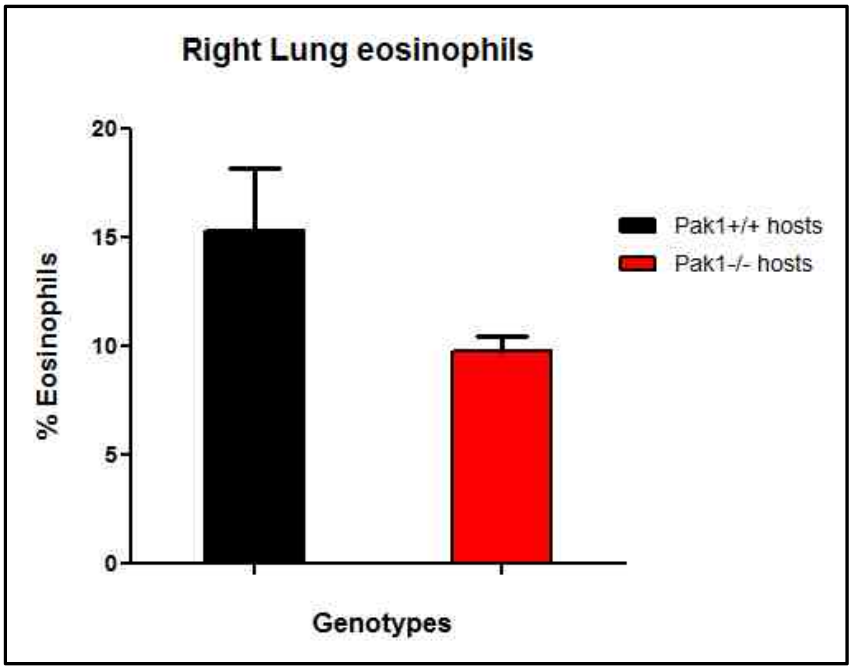


N=3
t-test p=0.16399
MW-U p=0.2

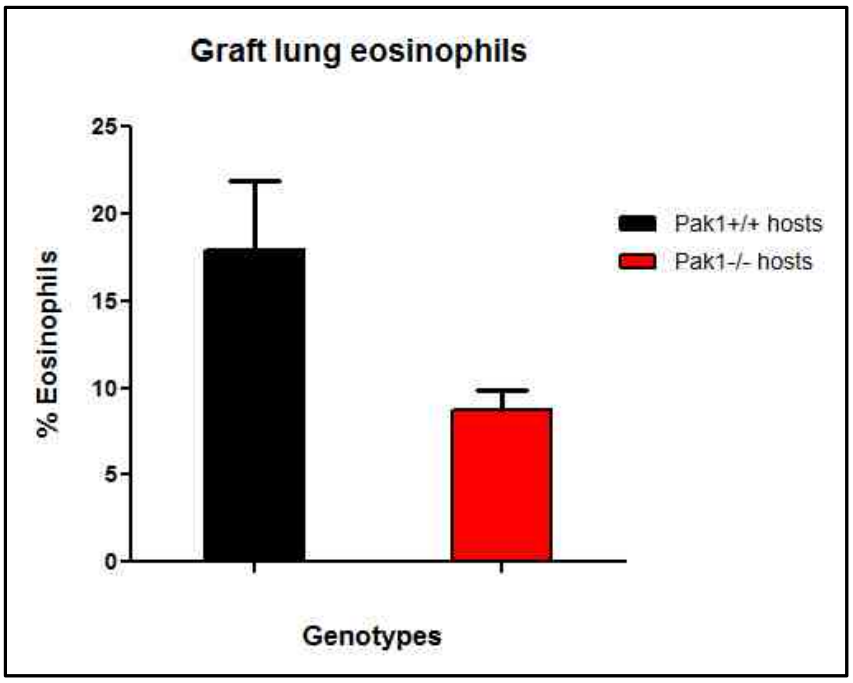


N=3
t-test P=0.16928,
MW-U p=0.2

Figures 11h-i



N=3
t-test p=0.129
MW-U p=0.2



N=3
t-test p=0.08356
MW-U p=0.1

Figures 11j-k

Figure 11: Hematopoeitic *Pak1* deletion may decrease eosinophil inflammation in AAD-induced mice in an orthotopic lung transplant model. In an orthotopic lung transplant model, we transplanted lungs from *Pak1-/-* mice into *Pak1+/+* host mice. Conversely we transplanted *Pak1+/+* left lungs into *Pak1-/-* recipient mice as shown. Following a five day recovery, we utilized these mice in a murine model of allergic airway inflammation. We prepared H&E (Figures 11b-e) and PAS (Figures 11f-g) sections of left lung grafts from these mice. Micrographs from (b-g) are representative of four mice (N=4). On histological inspection of H&E stained micrographs presence of peribronchiolar eosinophilic lesions was scored (Table 1) and chi-square association computed for *Pak1+/+* versus *Pak1-/-* hosts (Table 2) $p<0.01$.

In a separate cohort, we collected, enumerated, and stained BAL cells from test transplanted and OVA-sensitized and challenged *Pak1+/+* and *Pak1-/-* mice for eosinophil markers and obtained eosinophil percentages by fluorescence cytometry (Figures 11h-i). We also obtained total leukocytes from native right lungs and the engrafted left lungs and stained BAL cells from these mice for eosinophil markers and obtained eosinophil percentages by fluorescence cytometry (Figures 11j-k). Results from (h) through (k) are from three mice (N=3) and do not yet show statistical significance.

PAK1 in Eotaxin- mediated Eosinophil function

Building upon our whole animal studies, we sought to determine PAK1's functional and molecular role in regulating eosinophil function in allergic airway inflammation. Allergic sensitization induces expression and secretion of chemokines and cytokines that recruit hematopoietic cells eliciting inflammation in airways. Eotaxin expression in allergic airway inflammation potently recruits eosinophils *en masse* to the lung effecting chronic inflammation. Eotaxins also elicit eosinophil degranulation and cationic protein release mediating damage and remodeling in lung tissue. We hypothesized that PAK1 modulates eotaxin-mediated eosinophil infiltration and degranulation. To investigate the role PAK1 plays in these eotaxin-mediated eosinophil functions, we used two *in vivo* eotaxin infiltration murine models. We further evaluated the mechanism of PAK1-dependent eosinophil recruitment *in vitro* using bone-marrow derived eosinophils.

Eotaxin-mediated Eosinophil infiltration *in vivo* is PAK1-dependent

Given our previous findings in whole and chimeric animal AAD studies, we assessed isolated eotaxin recruitment of eosinophils *in vivo* to explain the apparent defect in eosinophil recruitment to the lungs in AAD *Pak1-/-* mice as compared to their wild-type counterparts. We injected a dose of intraperitoneal or intratracheal eotaxin in sedated *Pak1+/+* and *Pak1-/-* mice and collected peritoneal or broncho-alveolar lavage fluids for cell population evaluation by fluorescent cytometry. We observed from giesma-stained slides of peritoneal lavage cells obtained from eotaxin-treated *Pak1-/-* mice an increase in eosin-staining cells. Similarly, giesma-stained and quantified peritoneal lavages cells (Figures 12c-d) and fluorescence cytometry quantified CD45+, CD3/B220, SiglecF+ broncho-alveolar lavage fluid cells (Figures 12e-f) from *Pak1-/-* mice were increased with eotaxin treatment but decreased compared to cells in fluids retrieved from eotaxin-treated *Pak1+/+* mice (Figures 12a-d).

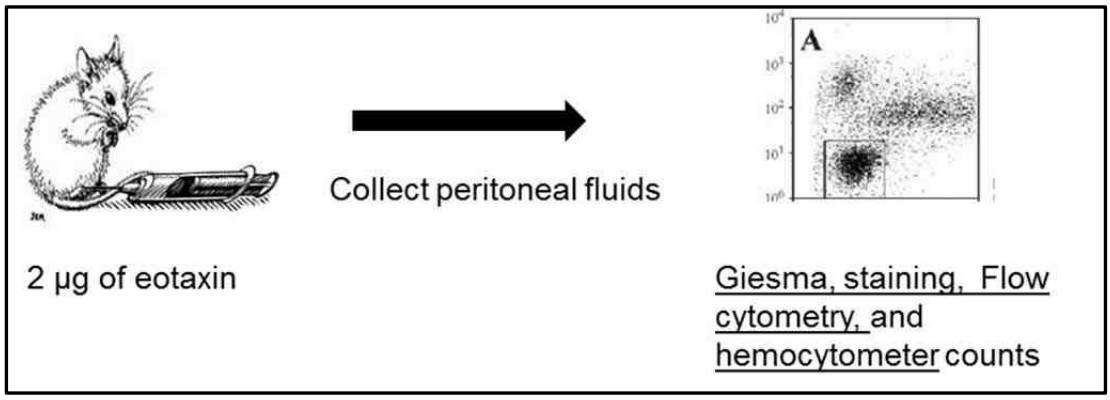


Figure 12a

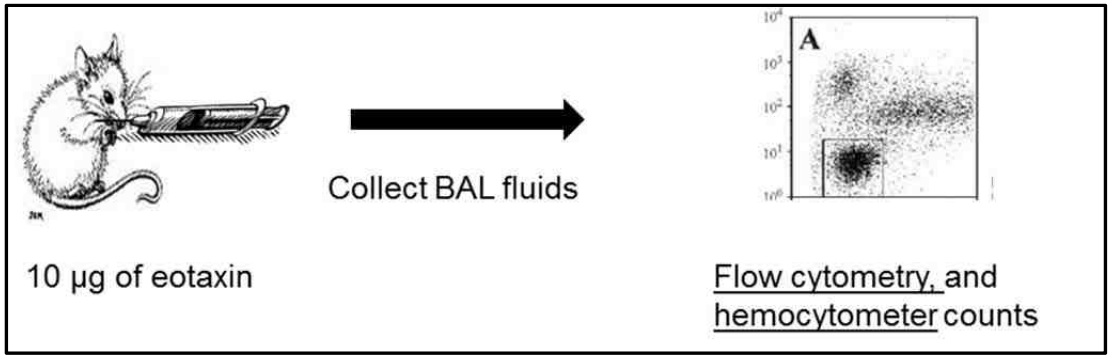


Figure 12b

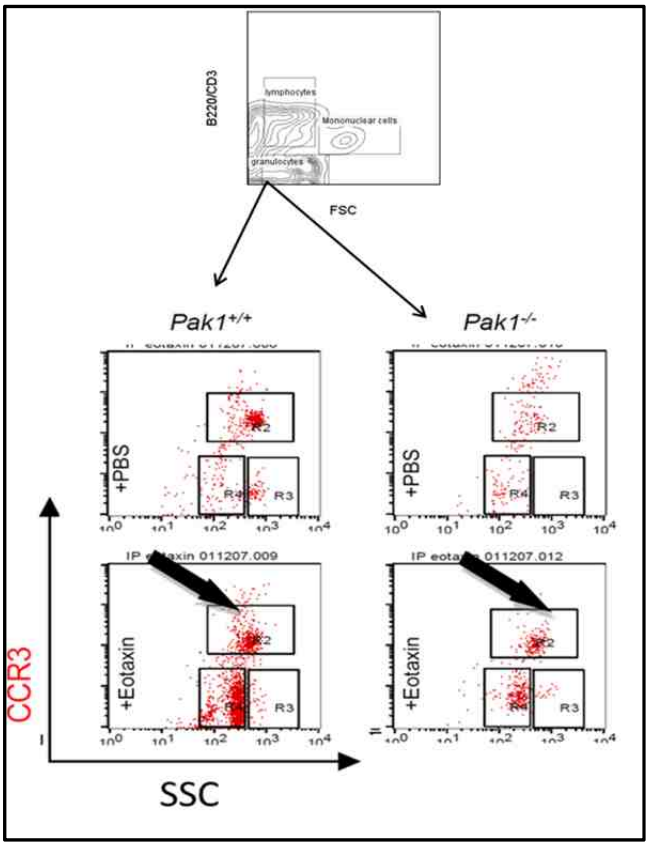


Figure 12c

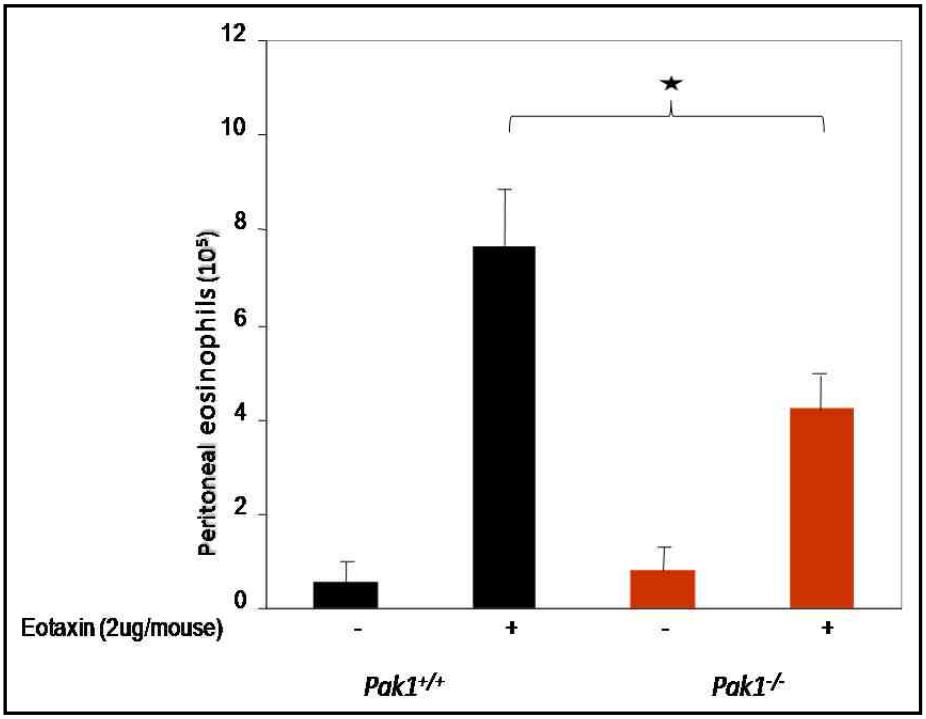


Figure 12d

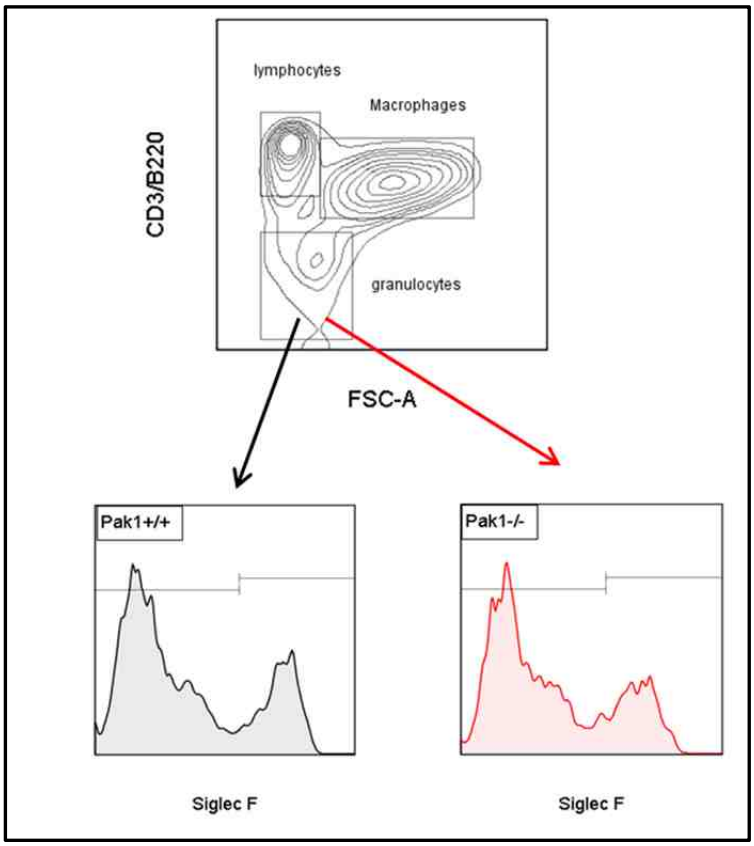


Figure 12e

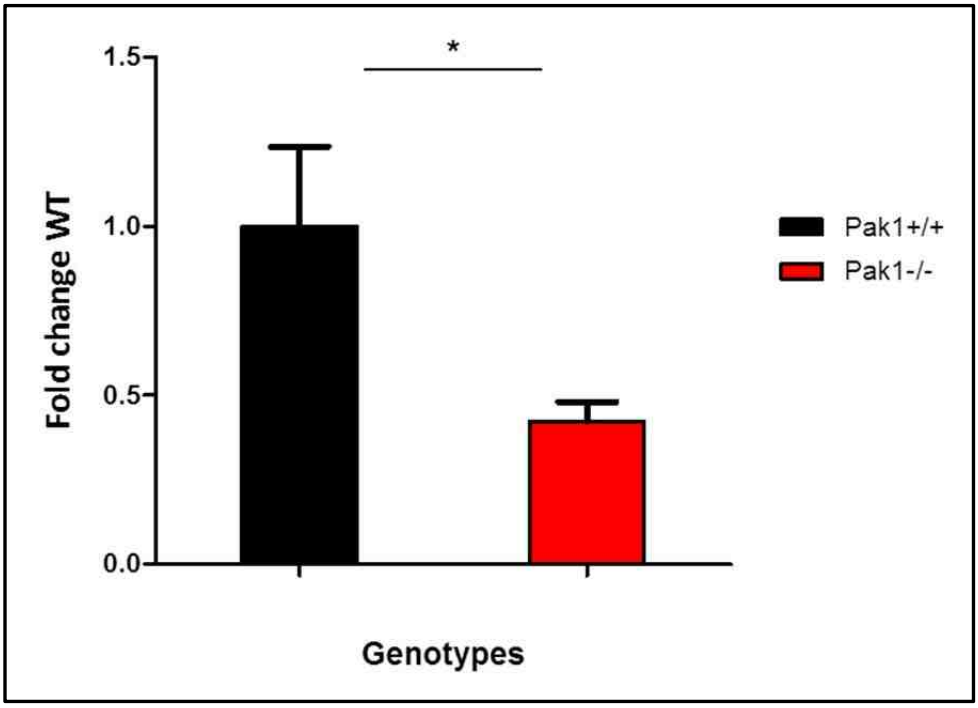


Figure 12f

Figure 12: Eotaxin-mediated infiltration *in vivo* is PAK1-dependent. *Pak1+/+ and Pak1-/-* mice were treated with PBS and eotaxin by intraperitoneal or intratracheal injections. Leukocytes from the respective cavities were lavaged and assessed for eosinophil numbers after 2 and 4 hours according to the schemes above (Figures 12a-b). Eosinophils were identified by giesma-staining and total CCR3+ cells back calculated and compared between genotypes (Figures 12c-d). Similarly, non-lymphocyte and non-monocyte populations of BAL cells were analyzed for SiglecF expression and SiglecF+ cells calculated and compared between genotypes (Figures 12e-f). Results in (d) and (f) are averaged from 4-6 mice and representative of at least three independent experiments. $p<0.05$, ** $p<0.01$, using a two-tailed student's *t*-test.

IL-5 LDMNC colony formation *in vitro* is not PAK1-dependent

In order to study eosinophil function *in vitro*, we chose to differentiate eosinophils from bone marrow stem and progenitor cells as previously published [244, 245]. The impact of *Pak1* deletion on stem and progenitor cell differentiation is incompletely understood and remains unexplored in eosinophil differentiation. IL-5 critically coordinately controls murine eosinophil differentiation *in vivo* and *in vitro* and reproducibly drives the formation of murine eosinophil colonies in methocellulose agar [245, 246]. To assess the impact of *Pak1* deletion on stem and progenitor capacity to form IL-5 colonies we performed an IL-5 colony forming assay using *Pak1* sufficient and deficient bone marrow derived mononuclear cells. We plated mononuclear cells in methocellulose semi solid agar medium enriched with high and low concentrations of IL-5 as the only growth and differentiation factor, incubated these cells, and counted total colonies on the tenth day of culture as previously described [236]. We showed that IL-5 stimulation resulted in growth of both *Pak1*+/+ and *Pak1*-/- colonies. However we also observed no difference in total number of colonies between the two genotypes (Figure 13). These results suggest that IL-5 induced bone marrow mononuclear cell colony formation does not depend on PAK1.

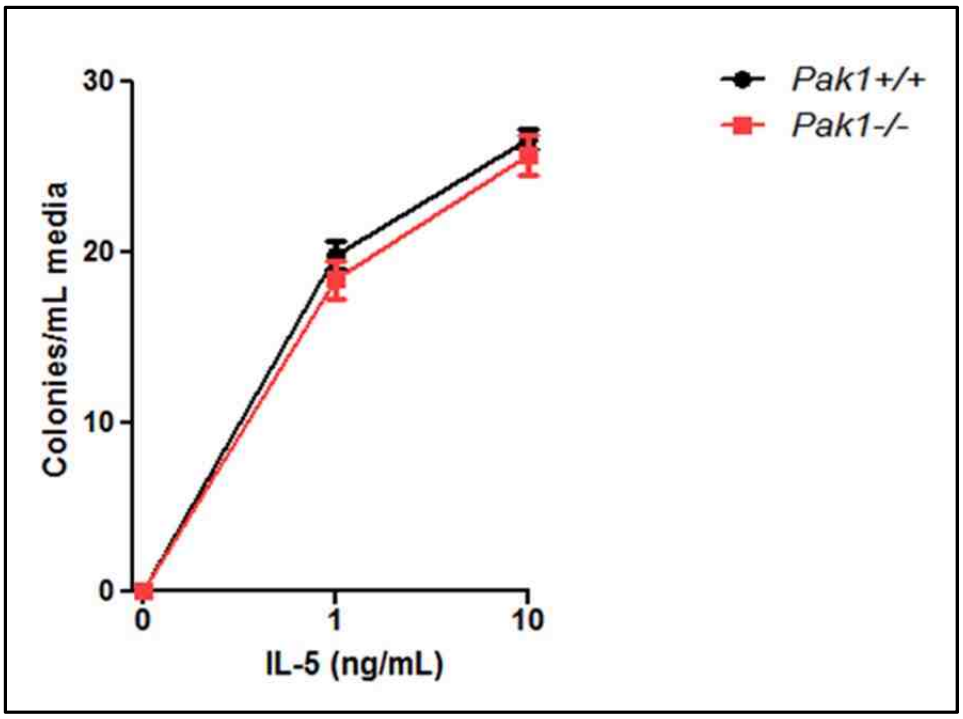


Figure 13

Figure 13: IL-5 BMMC colony formation *in vitro* is not PAK1-dependent. We harvested *Pak1+/+* and *Pak1-/-* bone marrow and isolated LDMNCs on a ficol gradient (N=3). We counted cells by trypan blue exclusion and plated 200,000 LDMNCs of each genotype in methocellulose soft agar medium containing 20% FBS and supplemented with IL-5 as the only growth and differentiation factor. One ml of cell/media was plated on 6cm dishes and incubated at 37°C for 10 days. Each animal for each concentration of IL-5 was assayed in triplicate. We counted the total number of colonies per plate on day 10 of culture, averaged the repeated measurements, and compared numbers of colonies at each IL-5 concentration between genotypes by a student's *t*-test (Figure 13).

Eosinophil expression of transmigration integrins as well as adhesion to fibronectin is not PAK1-dependent

Reorganization of the cell cytoskeleton facilitates rolling, adhesion, and transmigration of leukocytes as they infiltrate sites of tissue inflammation. Effective transmigration requires coordination between adhesive and motile forces, which are controlled by extracellular matrix signals acting on the cell cytoskeleton. CDC42/Racs, activators of PAKs, are implicated in optimally balancing between these processes [247, 248]. Eotaxin acts as a potent chemoattractant for eosinophil infiltration *in vivo* as well as adhesion of eosinophils to fibronectin [249, 250]. We sought to determine the effect of *Pak1* deletion on expression of adhesive proteins and eotaxin-mediated eosinophil adhesion. We cultured *Pak1+/+* and *Pak1-/-* bmEos and assessed the expression of eosinophil adhesive molecules important for endothelial transmigration by fluorescence cytometry. We observed no difference in the frequency of cells expressing these proteins, with greater than 95% of cells expressing CD11a/αL, Mac1/αMβ2, α4β7, and VLA4/α4β1 in both genotypes (Figures 14a-d). This global surface integrin expression was maintained following eotaxin stimulation (data not shown). Likewise, MFIs denoting surface expression of these integrins was comparable between genotypes. We used *Pak1+/+* and *Pak1-/-* bmEos in a microfluidic chamber adhesion assay to fibronectin. We coated microfluidic plates with gelatin or recombinant fibronectin fragments for one hour. Eosinophils showed preferential eotaxin-induced adherence to fibronectin in contrast to gelatin (Figure 14e). A micro-fluid pressure-gradient

maintained steady-flow of *Pak1+/+* and *Pak1-/-* bmEos in media through the fibronectin-coated chambers for 30 minutes. Micrographs (Figure 14f) and hemocytometer enumeration (Figure 14g) of total adherent cells both demonstrate indistinguishable eotaxin-mediated eosinophil adhesion between the two genotypes. These data warrant further functional characterization of PAK1's role in modulating eotaxin-mediated eosinophil adhesion via CD11a/LFA-1, Mac1, α4β7, and VLA4 interactions with recombinant fragments of the endothelium-expressed ICAM, VCAM, and MadCAM adhesive molecules [251-253].

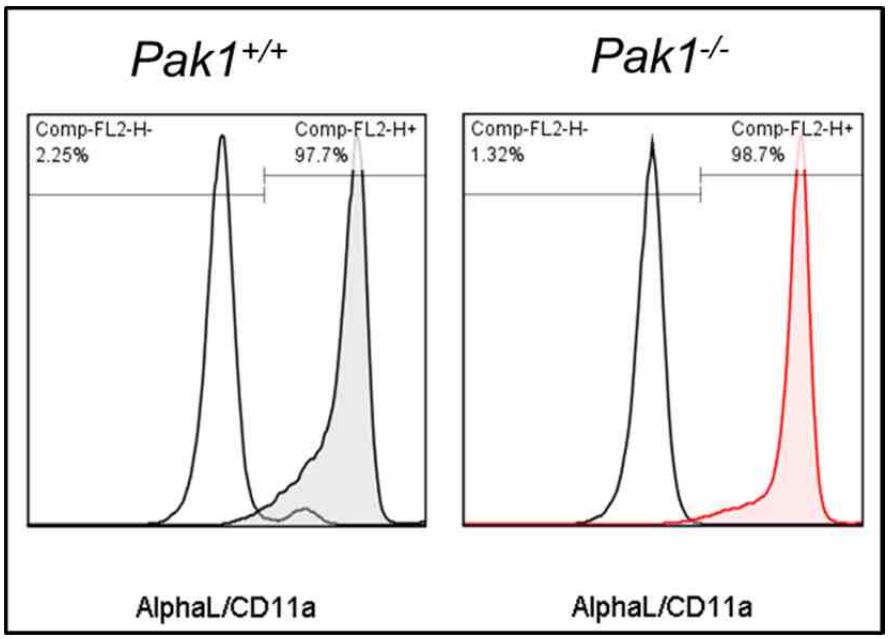

Pak1+/+
Pak1-/-
Comp-FL2-H-
2.25%
Comp-FL2-H+
97.7%
Comp-FL2-H-
1.32%
Comp-FL2-H+
98.7%
AlphaL/CD11a
AlphaL/CD11a

Figure 14a

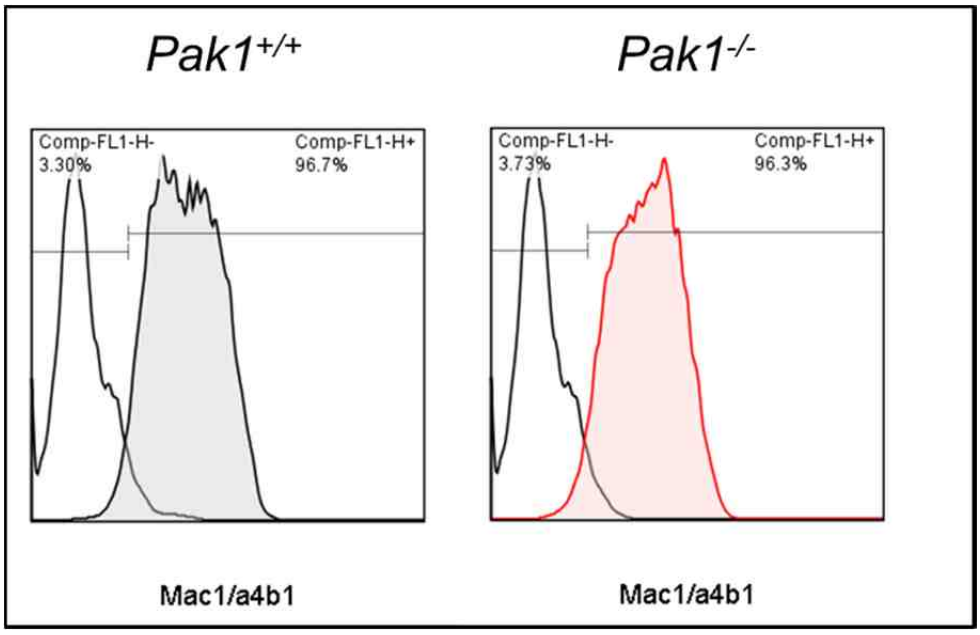

Pak1+/+
Comp-FL1-H-
3.30%
Comp-FL1-H+
96.7%
Mac1/a4b1
Pak1-/-
Comp-FL1-H-
3.73%
Comp-FL1-H+
96.3%
Mac1/a4b1

Figure 14b

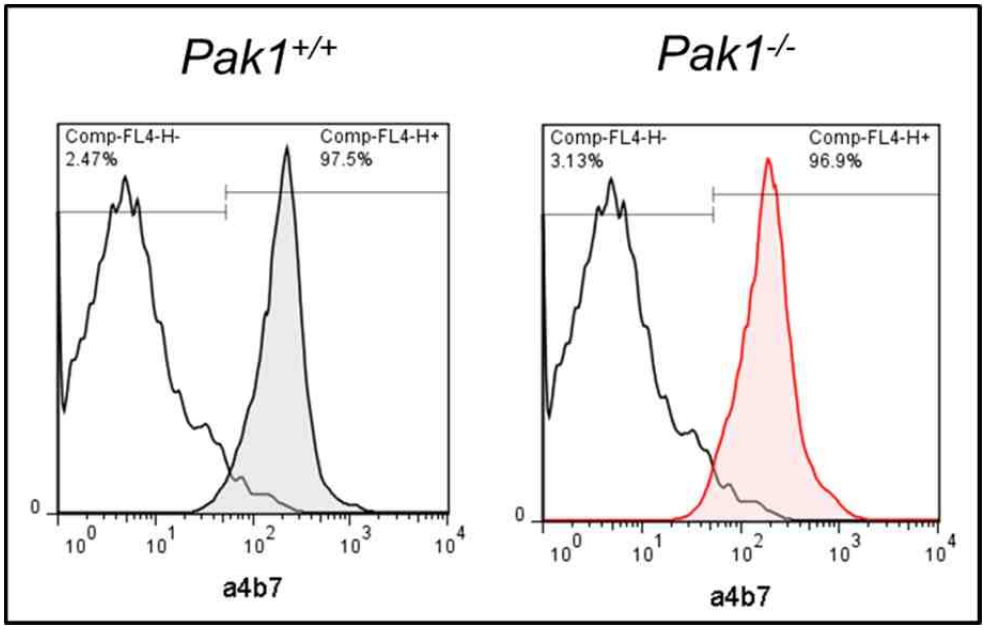

Pak1+/+
Comp-FL4-H-
2.47%
Comp-FL4-H+
97.5%
0
10^0
10^1
10^2
10^3
10^4
a4b7
Pak1-/-
Comp-FL4-H-
3.13%
Comp-FL4-H+
96.9%
0
10^0
10^1
10^2
10^3
10^4
a4b7

Figure 14c

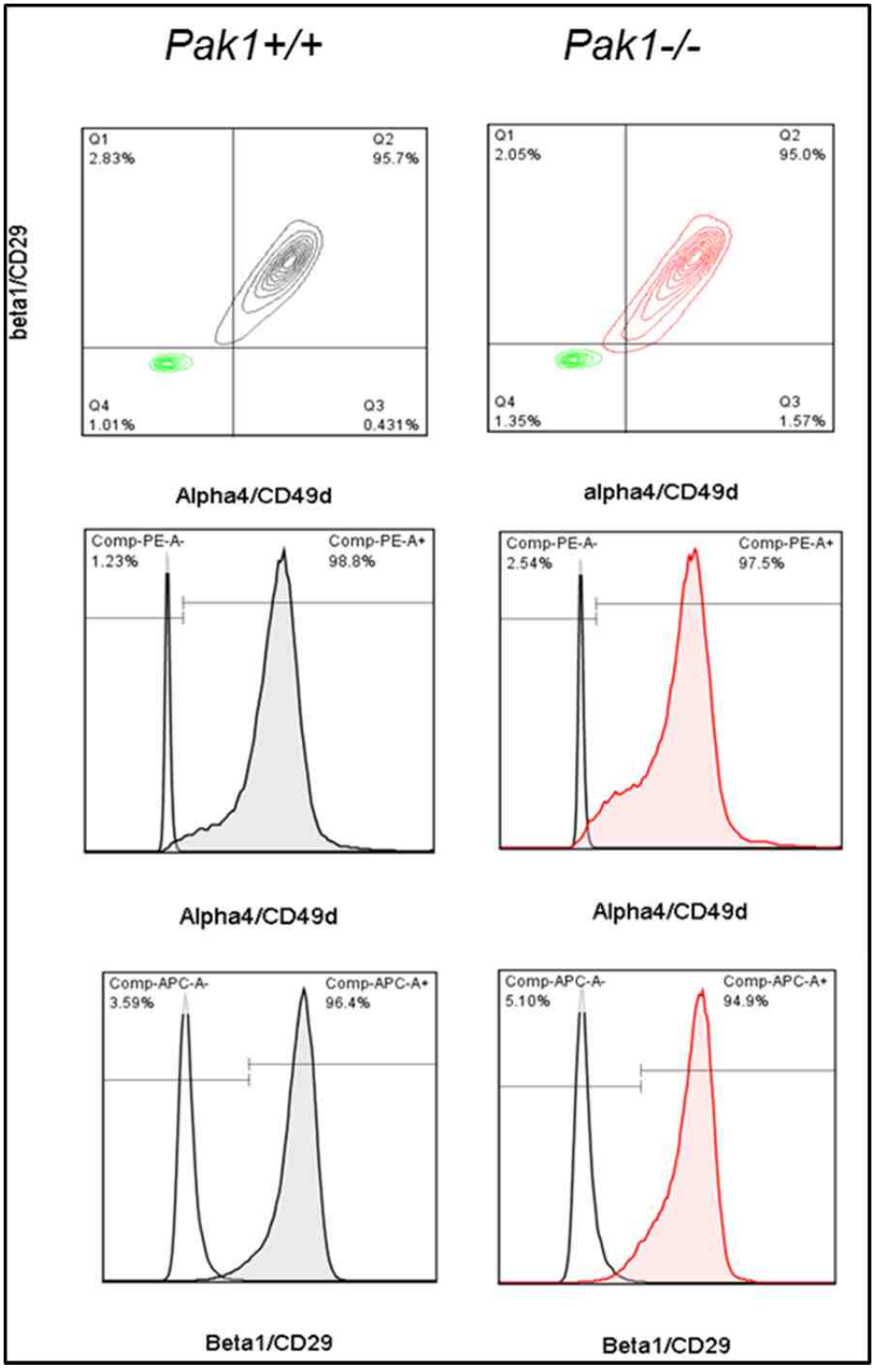

Pak1+/+
Pak1-/-
beta1/CD29
Q1
2.83%
Q2
95.7%
Q4
1.01%
Q3
0.431%
Alpha4/CD49d
Q1
2.05%
Q2
95.0%
Q4
1.35%
Q3
1.57%
alpha4/CD49d
Comp-PE-A-
1.23%
Comp-PE-A+
98.8%
Alpha4/CD49d
Comp-PE-A-
2.54%
Comp-PE-A+
97.5%
Alpha4/CD49d
Comp-APC-A-
3.59%
Comp-APC-A+
96.4%
Beta1/CD29
Comp-APC-A-
5.10%
Comp-APC-A+
94.9%
Beta1/CD29

Figure 14d

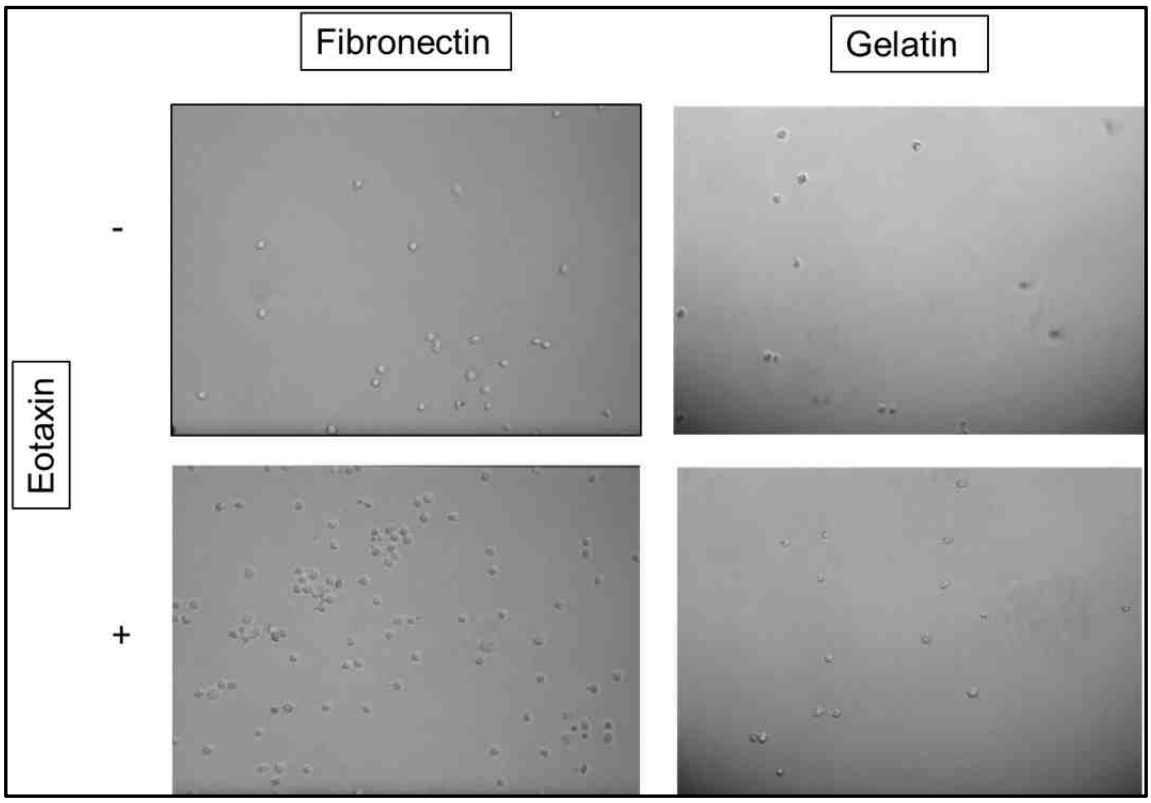


Figure 14e

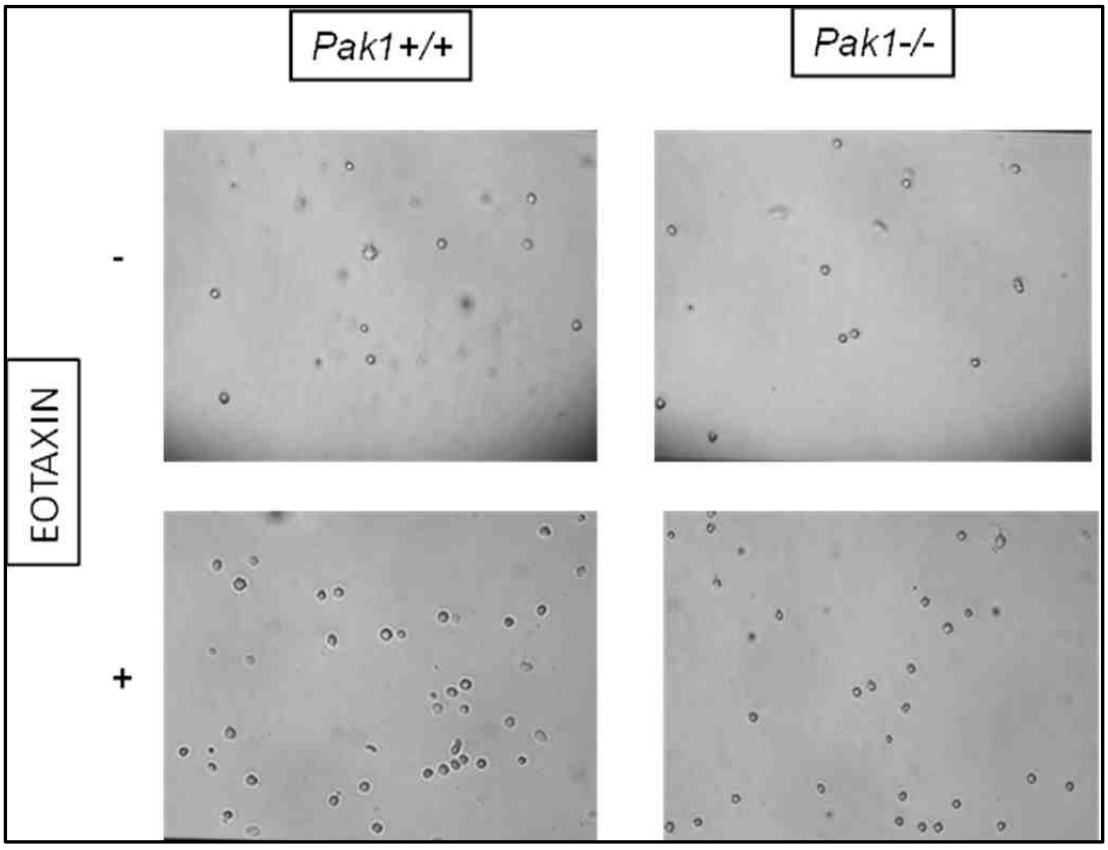


Figure 14f

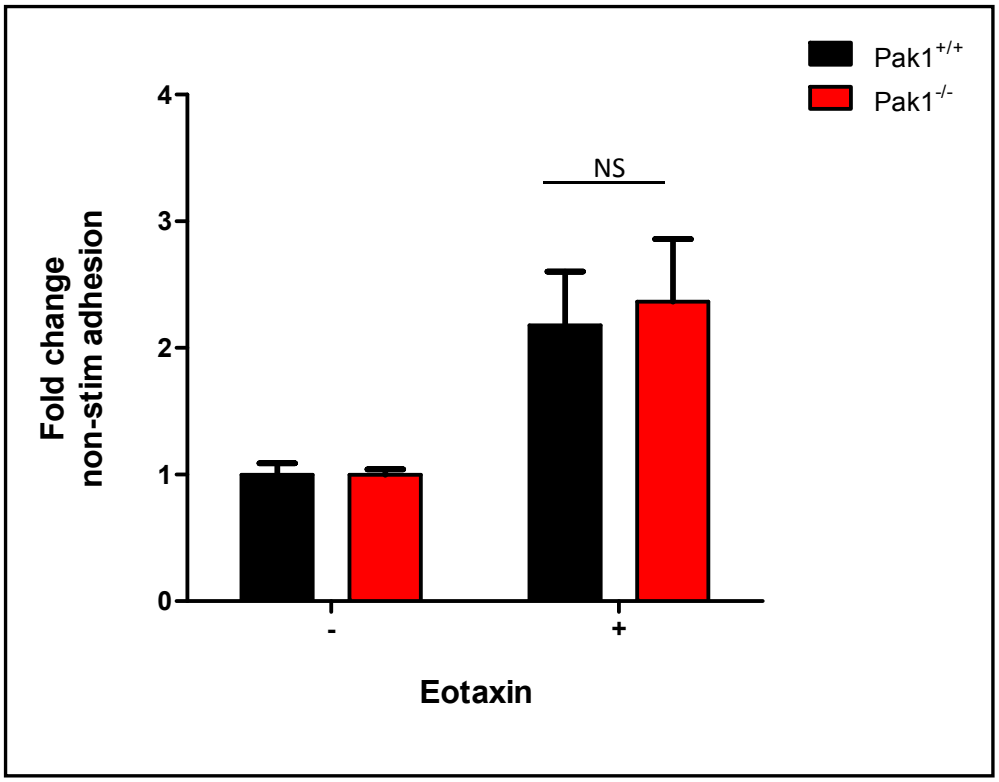


Figure 14g

Figure 14: *Pak1* does not affect eotaxin-induced adhesion of eosinophils to fibronectin or surface expression of adhesion molecules. We cultured *Pak1+/+* and *Pak1-/-* bmEos and utilized these cells in adhesion studies. First, we assessed the expression of αL of LFA-1, Mac1, α4β7, and VLA4 integrins in these bmEos by fluorescence cytometry of (Figures 14a-d). We tested for eotaxin-induced eosinophil adhesion on fibronectin and gelatin coated wells in a microfluidic adhesion assay (Figure 14e). Subsequently, we assessed eotaxin-induced adhesion to recombinant fibronectin fragments in a fluid system. Micrographs that represent microfluidic chambers of adherent eotaxin-stimulated and control *Pak1+/+* and *Pak1-/-* bmEos are shown (Figure 14f). We enumerated the total cells adherent to the microfluidic chambers after 20 minutes of stimulated flow (Figure 14g) and compared numbers from N=4 cultures for each genotype and eotaxin concentration.

Decreased eotaxin-mediated chemotaxis of murine eosinophils *in vitro* with *Pak1* deletion

We additionally evaluated PAK1's role in this eotaxin-mediated chemotaxis by deriving bone marrow eosinophils (bmEos) from *Pak1+/+ and Pak1-/-* mice. We cultured bone marrow stem and progenitor cells harvested from *Pak1+/+* and *Pak1-/-* mice *ex vivo* in eosinophil polarizing conditions for 10-14 days and assessed eosinophil maturity by expression of CCR3, the eotaxin receptor. We cultured eosinophils deficient in PAK1 expression as determined by immunoblotting (Figure 15a). We observed no difference in CCR3 mean fluorescence intensity (MFI) of bmEos between the genotypes. This observation suggests a comparable surface expression density of CCR3 *Pak1+/+* and *Pak1-/-* bmEos (Figures 15b-c). This observation suggests that both *Pak1+/+* and *Pak1-/-* bmEos have a similar opportunity to bind eotaxin ligand in solution. Furthermore cytospin analysis of these cells demonstrated approximately 80% purity red-granule staining cells on Giesma staining. This purity was at least 95% in both genotypes when assessed by Siglec F expression on fluorescence cytometry. (Figure 15d)

In optimization studies that we performed with wild-type bmEos, we observed maximal migration as early as two hours and a significant difference in chemotaxis over control from mreotaxin concentrations of 500ng/mL and greater (data not shown). We then used *Pak1+/+ and Pak1-/-* bmEos in a checkerboard transwell-migration assay in which we separated or combined eotaxin and

bmEos in the top transwell chamber and allowed the cells to migrate for two hours according to the schematic below (Figure 15e). We collected the cells migrating to the bottom well in each condition and expressed migration as a chemotactic index of background. We only observed a significant difference in migration over background levels when we applied an eotaxin-gradient to the bmEos of both genotypes. Remarkably, *Pak1-/-* bmEos demonstrated 40% decreased chemotaxis compared with *Pak1+/+* cells (Figure 15d). These data taken together implicate eosinophil PAK1 in regulating infiltration by intrinsically promoting eotaxin- mediated eosinophil chemotaxis.

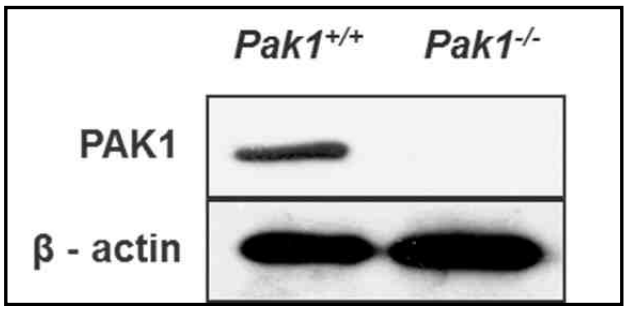


Figure 15a

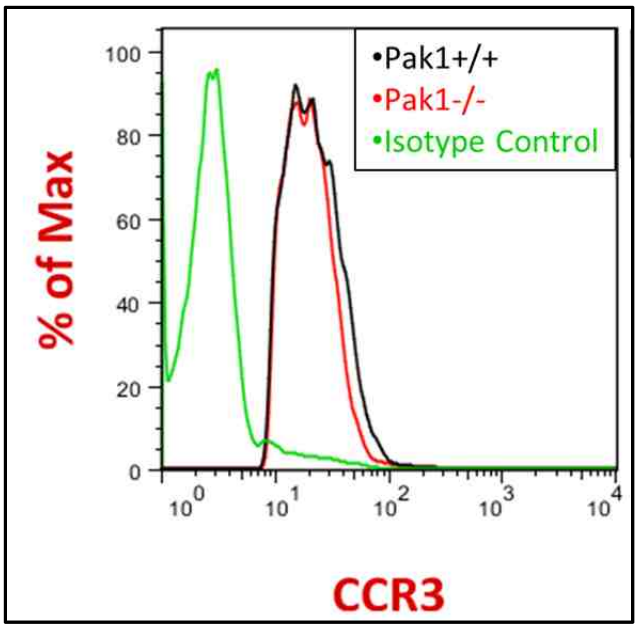

•Pak1+/+
•Pak1-/-
•Isotype Control
% of Max
100
80
60
40
20
0
10^0
10^1
10^2
10^3
10^4
CCR3

Figure 15b

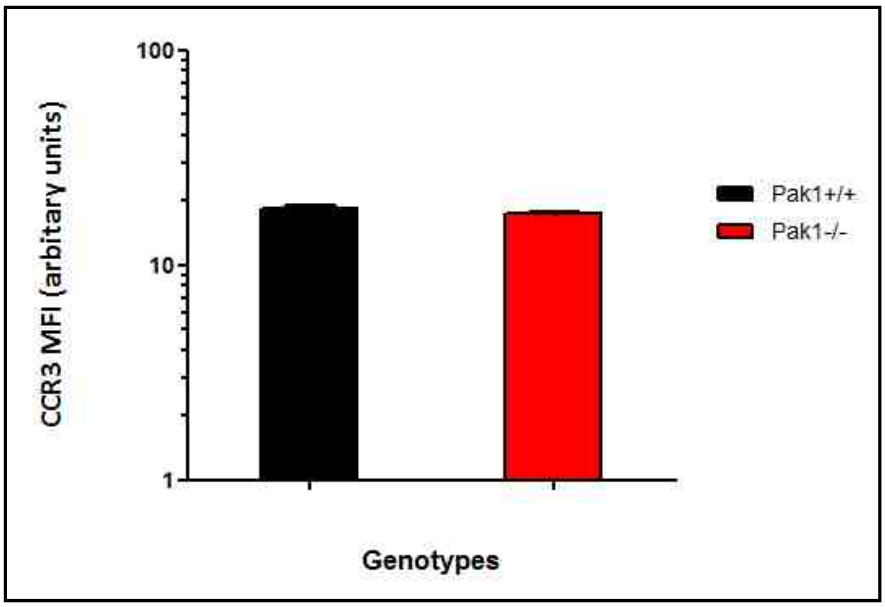


Figure 15c

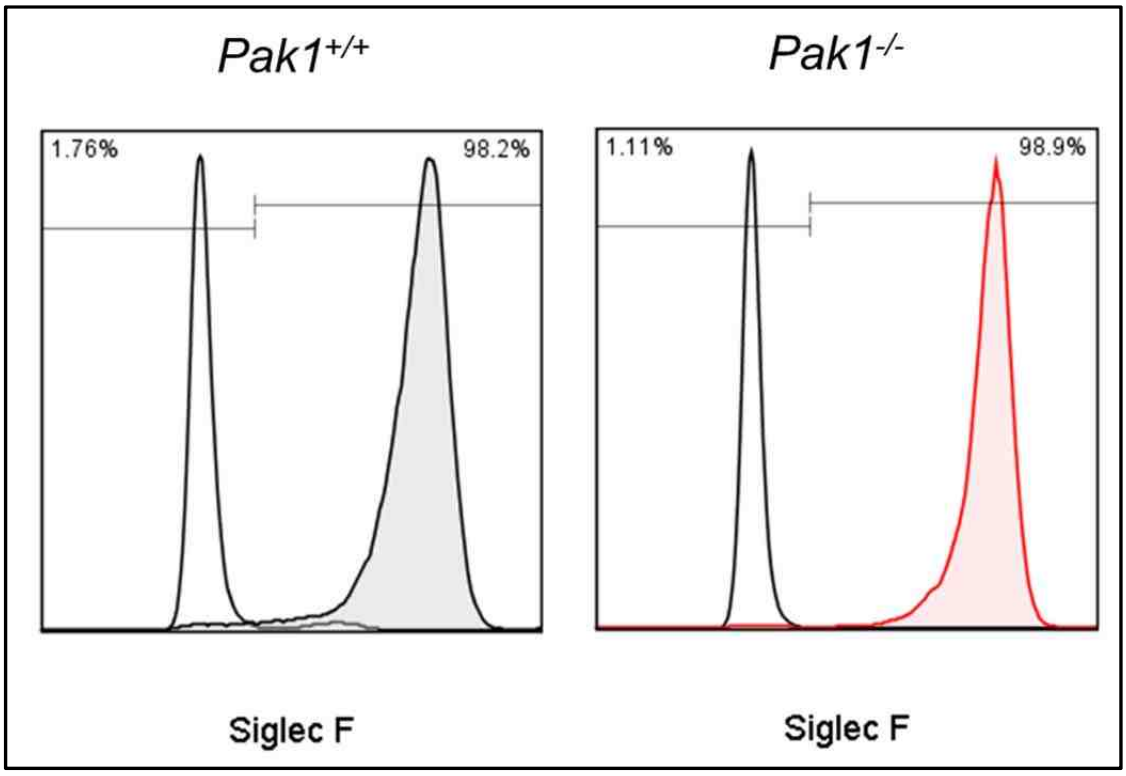


Figure 15d

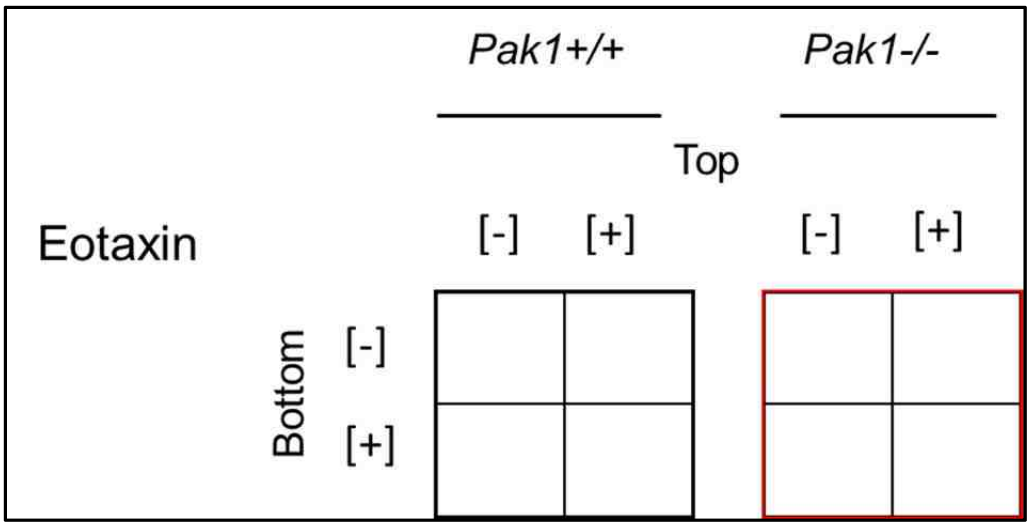


Figure 15e

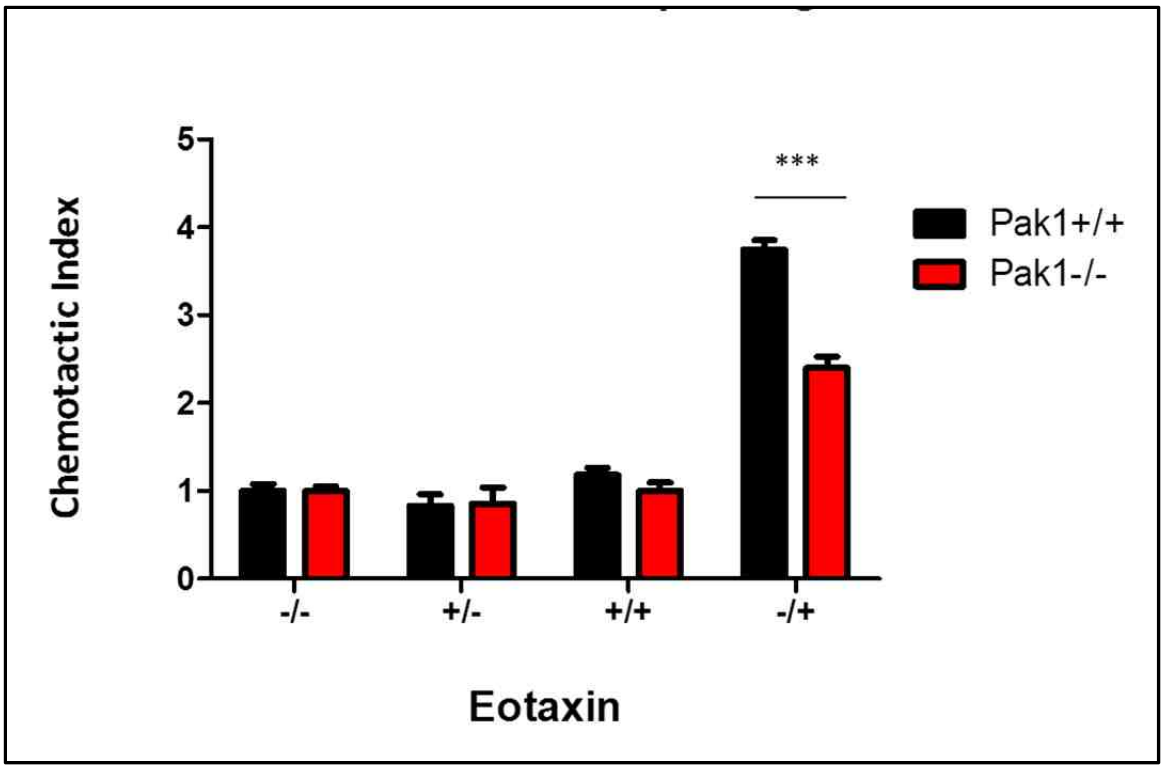


Figure 15f

Figure 15: PAK1 promotes eotaxin-mediated chemotaxis *in vitro*. *Pak1+/+/* Wild-type and *Pak1-/-* mice bmEos were cultured and assessed for PAK1 expression by immunoblot (Figure 15a) and surface expression of CCR3 by fluorescent cytometry (Figure 15b). This surface CCR3 bmEos expression was compared between genotypes (Figure 15c). The percentage of bmEos expressing Siglec F was assessed after 10 days of culture by fluorescence cytometry (Figure 15d) Eotaxin-mediated chemotaxis was evaluated using a transwell system *Pak1+/+* and *Pak1-/-* BmEos were compared in a checker-board migration format. Checkerboard migration assays were conducted with no stimulus, with eotaxin in the top chamber only, both chambers as shown in the schematic (Figure 15e). The cells migrating to the bottom chamber only were enumerated by hemocytometer (Figure 15f). Results in (c and f) are an average of at least three experiments (N=3-4) while results in (d) are representative of at least five independent experiments. ***$p<0.001$ using a two-tailed student's *t*-test.

Decreased eotaxin-mediated chemotaxis of HL-60 derived human eosinophils (HL-60 Eos) with PAK1 RNA Interference

To assess the importance of PAK1 in a human eosinophil migration, we derived human eosinophils from a promyelocytic leukemia cell-line, HL-60. These cells acquire eosinophil characteristics when cultured in alkaline conditions in the presence of *n*-butyrate. We differentiated human eosinophils from this cell line and assessed for purity by giesma staining. We transfected cultured HL-60 human eosinophils with siRNA against human PAK1 or scrambled siRNA and compared their chemotaxis. We successfully delivered siRNA into HL-60 derived eosinophils as assessed by color-tagged siRNA control experiments with greater than 95% transfection efficiency (Figure 16a).

In preliminary experiments we tested three siRNA constructs specific for human PAK1 and selected the construct showing greatest PAK1 knockdown. We selected construct #3 for subsequent transfection experiments (Figure 16b). After a 72 hour transfection with this siRNA construct, we demonstrated efficacious knockdown of PAK1 protein expression in these cells (Figures 16c-d). We compared the eotaxin-mediated chemotaxis of PAK1 siRNA treated eosinophils with scramble siRNA-treated eosinophils in a transwell chemotaxis assay for two hours. We found that PAK1 RNA silencing decreased eotaxin-mediated migration of human eosinophils compared to control (Figure 16e).

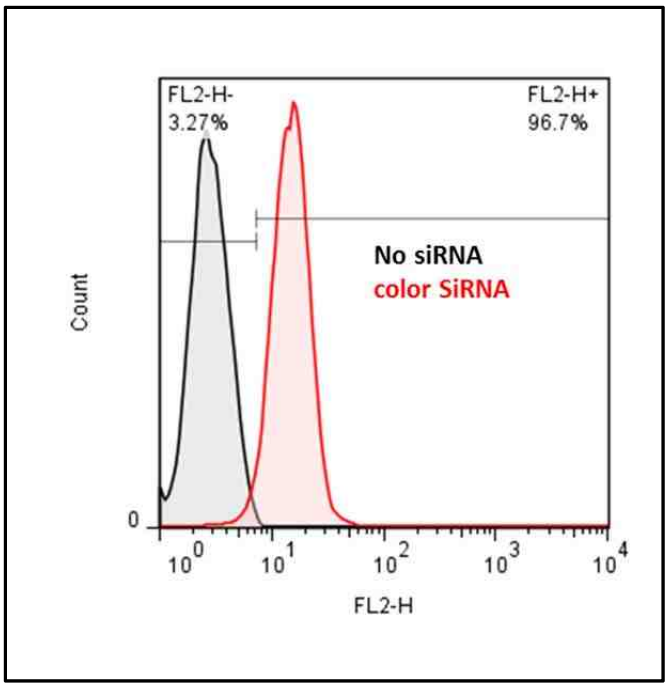

FL2-H-
3.27%
FL2-H+
96.7%
No siRNA
color SiRNA
Count
0
10^0
10^1
10^2
10^3
10^4
FL2-H

Figure 16a

Figure 16b

Figure 16c

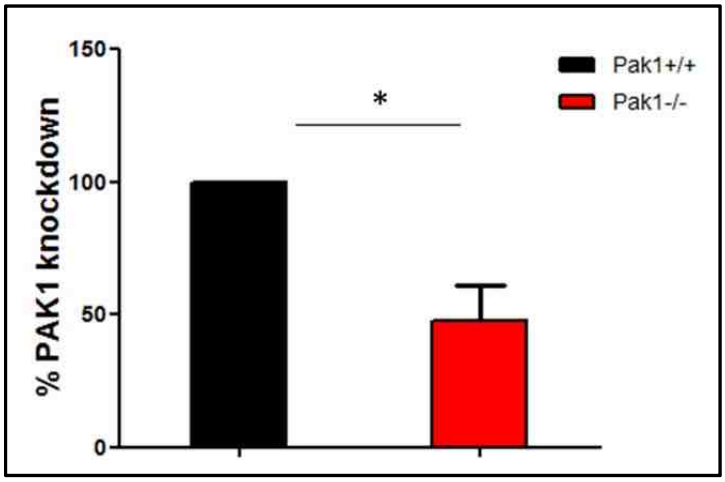


Figure 16d

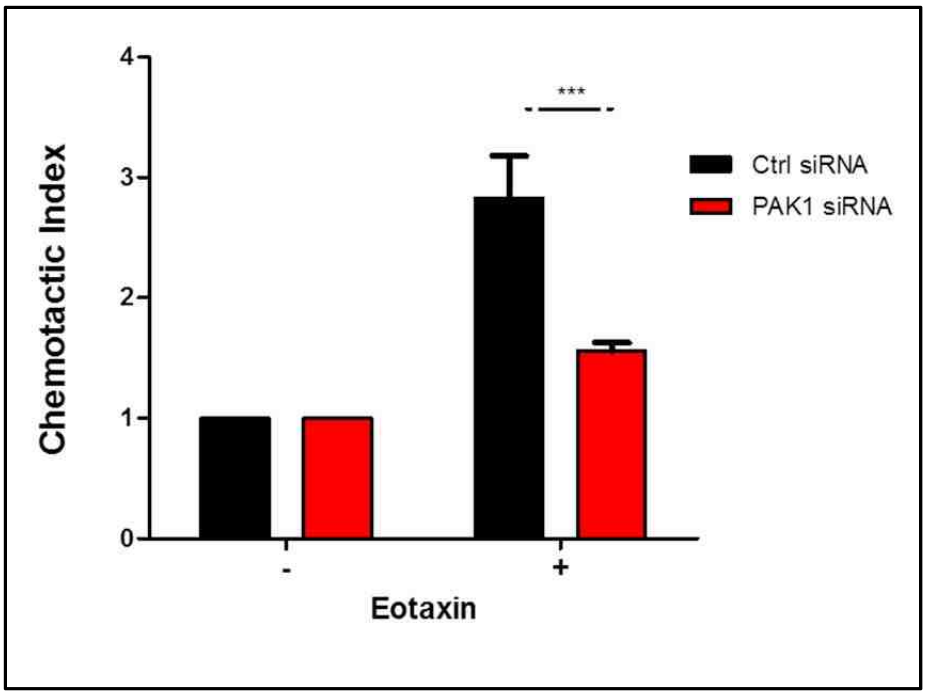


Figure 16e

Figure 16: PAK1 promotes decreases eotaxin-mediated chemotaxis in HL-60 derived human eosinophils. We cultured HL-60 clone 15 cells in eosinophil differentiating conditions for three days and tranfected them with color-tagged sequence as a positive control, scramble siRNA, or three siRNA constructs against human PAK1. Transfection efficiency was assessed using color-tagged siRNA (Figure 16a). Three PAK1 siRNA constructs were screened for PAK1 knockdown by western blot and construct marked #3 selected for subsequent studies (Figure 16b). PAK1 siRNA (#3) transfected cells used in migration studies were assessed for PAK1 expression 72 hours after siRNA treatment by western blot (Figure 16c) and percentage knockdown quantified by densitometry (Figure 16d) for N=3 independent cultures. Migration of human eosinophil derived from HL-60 clone 15 cell line transfected with scramble or PAK1 siRNA were compared from N=5 independent cultures for each siRNA construct (Figure 16e). Results in (d) are representative of at least three experiments. ** $p<0.01$, ***$p<0.001$ using a two-tailed student's *t*-test.

Restored and enhanced eotaxin-mediated eosinophil migration with ectopic expression of full length PAK1 but not kinase-dead mutant PAK1 in *Pak1-/-* eosinophils

Gene ablation by homologous recombination can occasionally result in the disruption of surrounding genes. Since preliminary studies demonstrate that *Pak1*-/- eosinophils have impaired migration, we validated the specificity of this knockout by ectopically expressing PAK1 to restore eosinophil migration. We utilized a state-of-the-art lentiviral-mediated transgene expression system to overexpress PAK1, and a kinase-dead PAK1 mutant construct, K299R. We adapted and ligated PAK1 cDNA into the pCL1eGFPGwo lentiviral backbone developed, optimized and kindly given to us by our collaborator Dr. Helmut Hanenberg as described [223].

Likewise, we obtained the pCMV6M-*Pak1* K299R deposited at Addgene as a generous gift by Dr. Jonathan Chernoff (Figure 17a). Using molecular techniques we NOTI-adapted and PCR-amplified *Pak1* K299R yielding approximately a 1620 base pair insert by gel analysis (Figure 17c). We used the PCL11eGwo lentiviral vector for this construct, another generous gift from Dr. Hanenberg. This vector utilizes an HPGK promoter that expresses both PAK1K299R and EGFP efficiently in myeloid cells. Following a NotI restriction enzyme digestion of the PCL11eGwo lentiviral vector, we blunted this vector and NotI-pak1K299R-NotI insert by Klenow treatment. We ligated vector and insert, transformed competent cells, and picked colonies for plasmid restriction digests. To confirm the 5'->3'

orientation after ligation, we amplified these plasmids and subjected them to a restriction digest with AgeI and BstbI that yielding a DNA fragment spanning the eGFPPAK1 constructs. We obtained a DNA fragment of about 2000bp (Figure 17d). To confirm translation of our construct in mammalian cells, we transfected HEK293T cells with these constructs and demonstrated the expression of eGFP fusion proteins approximately 85kDA (Figure 17e). We sequenced the lentiviral plasmid to confirm that *Pak1* K299R cDNA is encoded, in frame and without further mutations.

We generated viral particles in a HEK293T packaging cell line by co-transfecting our *Pak1* plasmid constructs and a recombinant foamy virus envelope plasmid that enhances entry into both quiescent stem cells and proliferating progenitor cells as described [15]. We filtered and concentrated viral supernatant fifty times to yield viral titers of between 5 $X10^7$ to 5 $X10^8$ particles per milliliter.

We magnetically sorted for *c-Kit*+ cells from LDMNCs for *Pak1+/+ and Pak1-/-* bone marrow to enrich for stem and progenitor cells. We transduced these cells with PCL11eGwo, PCL1Pak1, and PCL11Pak1K299R at multiplicities of infection (MOIs) of between 50 and 100 infectious particles per cell. The transduced cells were cultured in eosinophil polarizing conditions and allowed to express these transgenes. Seven days post-transduction, these cells were collected and sorted according to GFP or GFP-fusion protein expression. We obtained yields of approximately 10 to 20% GFP+ cells (Figure 17f). We continued culturing these

eosinophil precursors for three to five days post-FACS after which they were utilized in transwell migration assays. These cells variably expressed Gr-1/Ly-6, but expressed CCR3 and almost invariably, SiglecF, surface markers of eosinophil maturity (Figure 17g).

We noted that eGFPPAK1 ectopic expression in *Pak1-/-* eosinophils enhanced their eotaxin-induced chemotaxis (Figure 17h). This chemotactic index was greater than *Pak1+/+* eosinophils transduced with PCL11eGFP. However, eGFPPAK1K299R ectopic expression did not change the chemotactic potential of *Pak1-/-* eosinophils (Figure 17h). These data complement and validate our findings in *Pak1-/-* eosinophils. Furthermore, these studies suggest that eotaxin-mediated eosinophil migration is at least partly PAK1 kinase-dependent.

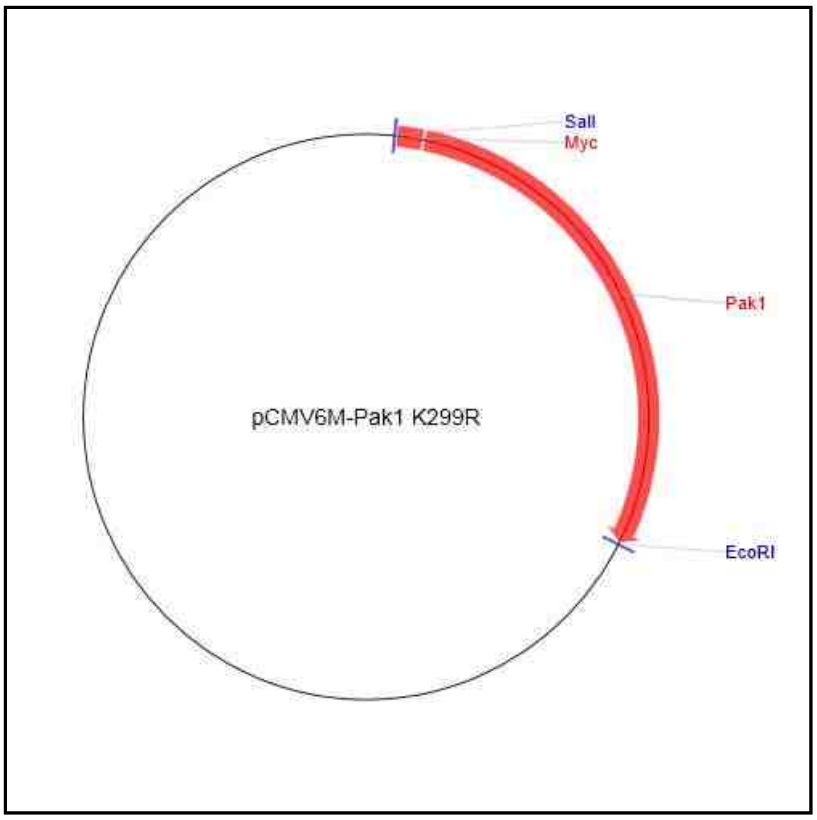


Figure 17a

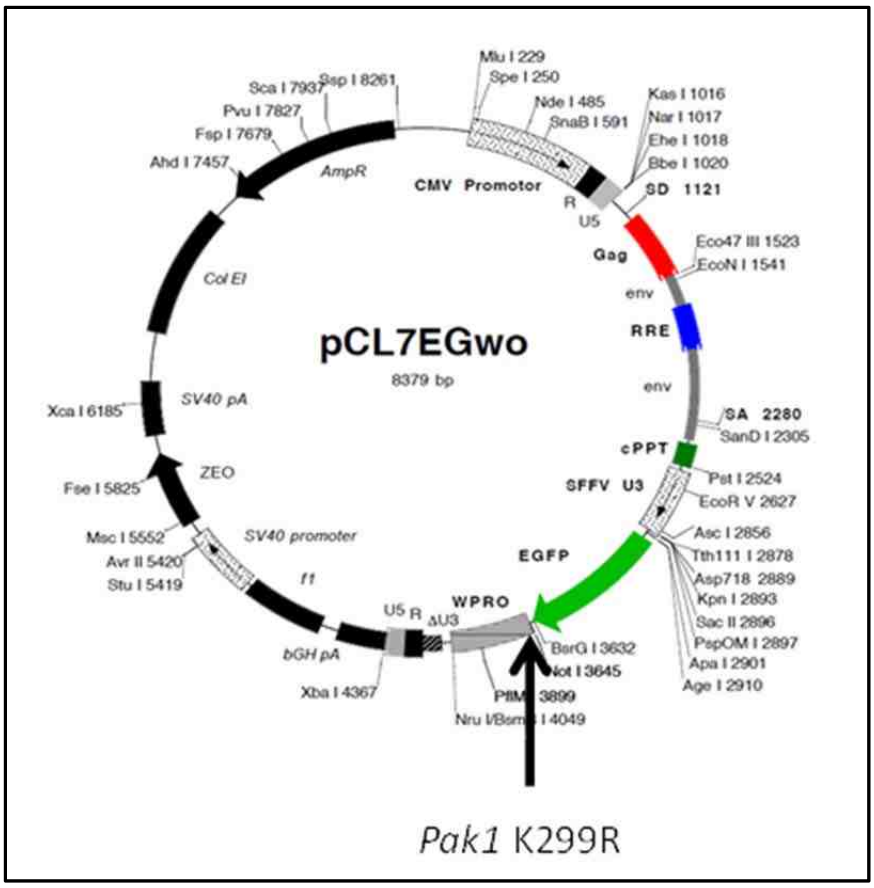

pCL7EGwo
8379 bp
Mlu I 229
Spe I 250
Nde I 485
SnaB I 591
Kas I 1016
Nar I 1017
Ehe I 1018
Bbe I 1020
SD 1121
Eco47 III 1523
EcoN I 1541
RRE
SA 2280
SanD I 2305
cPPT
Pst I 2524
EcoR V 2627
Asc I 2856
Tth111 I 2878
Asp718 2889
Kpn I 2893
Sac II 2896
PspOM I 2897
Apa I 2901
Age I 2910
BsrG I 3632
Not I 3645
Nru I/BsmB I 4049
Xba I 4367
Sca I 7937
Ssp I 8261
Pvu I 7827
Fsp I 7679
Ahd I 7457
Xca I 6185
Fse I 5825
Msc I 5552
Avr II 5420
Stu I 5419
AmpR
CMV Promotor
R
U5
Gag
env
env
SFFV U3
EGFP
WPRO
ΔU3
U5 R
bGH pA
f1
SV40 promoter
ZEO
SV40 pA
Col EI
Pak1 K299R

Figure 17b

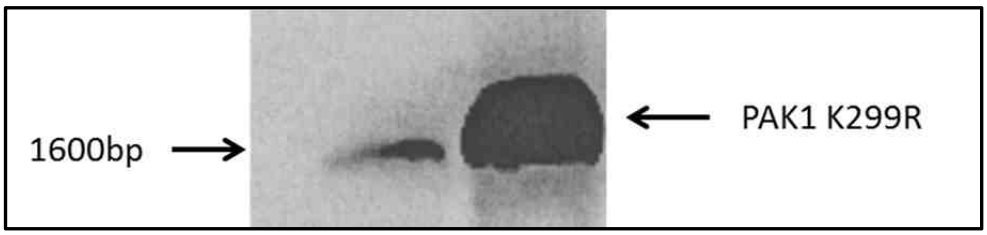


Figure 17c

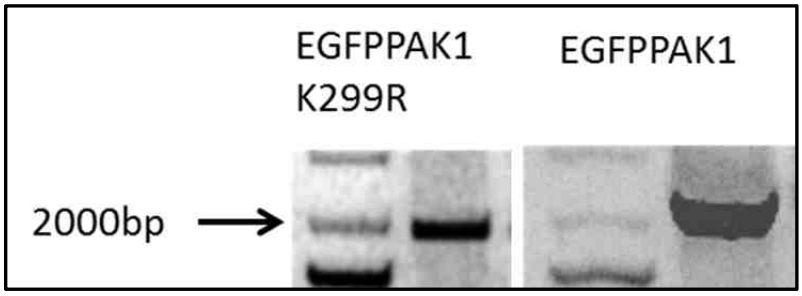


Figure 17d

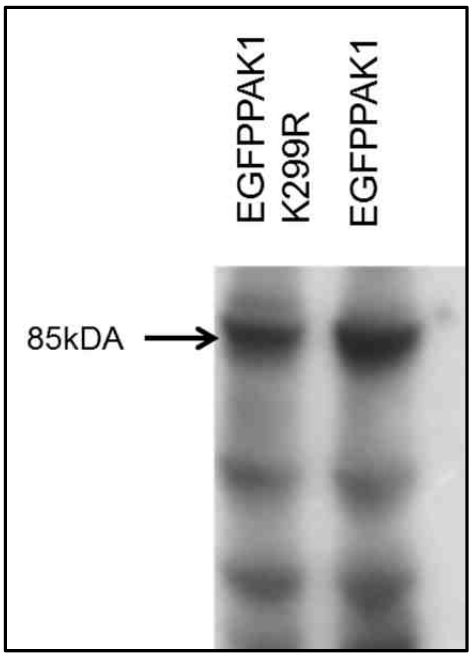


Figure 17e

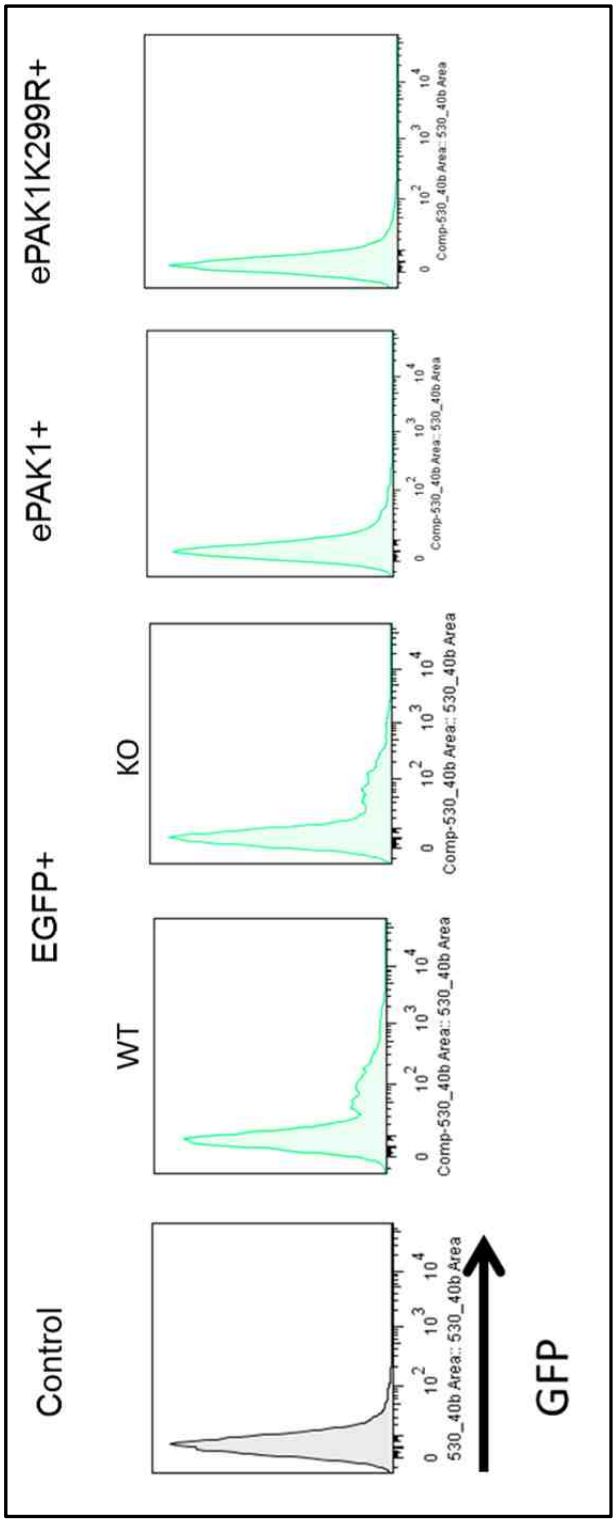

Control
EGFP+
WT
KO
ePAK1+
ePAK1K299R+
GFP
530_40b Area:: 530_40b Area
Comp-530_40b Area:: 530_40b Area

Figure 17f

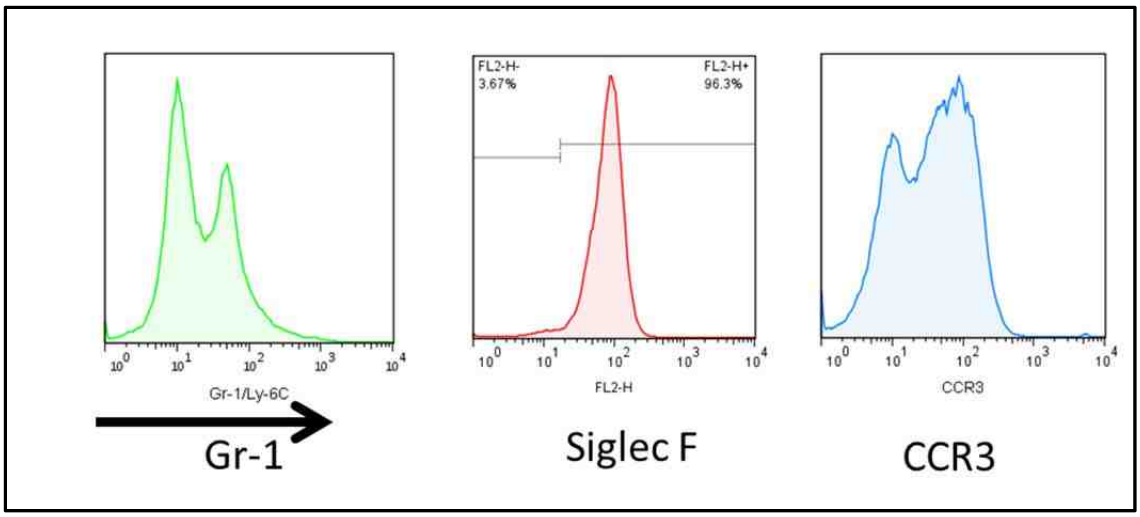


Figure 17g

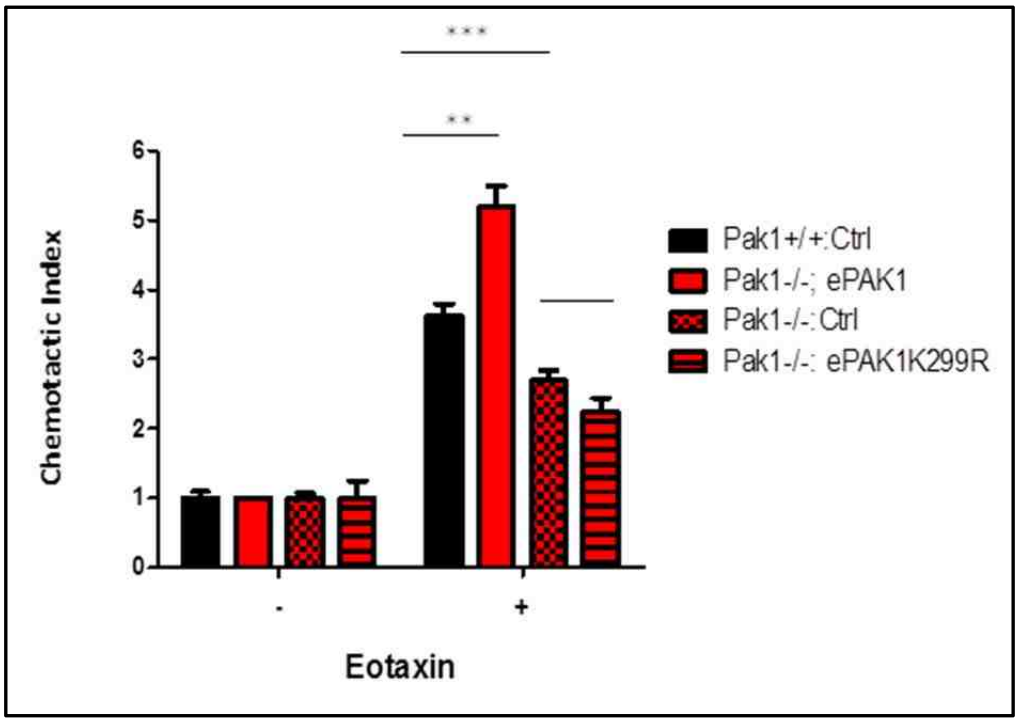

***
**
Chemotactic Index
6
5
4
3
2
1
0
-
+
Eotaxin
Pak1+/+:Ctrl
Pak1-/-; ePAK1
Pak1-/-:Ctrl
Pak1-/-: ePAK1K299R

Figure 17h

Figure 17: Ectopic expression of full length PAK1 but not kinase-dead PAK1 mutant in *Pak1-/-* eosinophils restores enhanced eotaxin-mediated migration. We Not1-adapted and amplified ~1620bp *Pak1* K299R insert (Figure 17c) from the PCMV6M-Pak1K229R vector (Figure 17a) and blunted it by klenow-fragment treatment. We digested PCL11 vector with Not1 and similarly blunted and CIAP treated it to prevent self-ligation. We ligated the *Pak1* K299R insert into the cut PCL11eGwo vector to form an eGFPPak1K299R construct as shown (Figure 17b). We confirmed orientation of inserts in both PCL1eGFPPAK1 and PCL11eGFPPAK1K299R by restriction enzyme digestion that would generate ~2000bp eGFPPAK1 and eGFPPAK1K299R DNA fragments (Figure 17d). As expected, these plasmid constructs ectopically expressed ~85kDA eGFPPAK1 and eGFPPAK1K299R fusion proteins when transfected into HEK293T packaging cells. *Pak1+/+* and *Pak1-/- c-kit*+ LDMNCs were transduced with PCL11eGwo only, PCL1eGFPPAK1 and PCL11eGFPPAK1K299R, fluorescently sorted (Figure 17f), cultured in eosinophil conditions and assessed for Gr-1, Siglec F and CCR3 surface expression (Figure 17g). We used cells transduced with these viral expressed constructs for eotaxin-mediated transwell chemotaxis studies (Figure 17h).

Eosinophil F-actin reorganization by Eotaxin: CCR3 signaling through PAK1

Eotaxin signaling pathways through PAK1 in eosinophils have not been studied to date. Since our data indicate *Pak1* deletion decreases eotaxin-mediated eosinophil F-actin polymerization and consequent migration, we sought to determine PAK1's downstream molecular targets responsible for these changes. To elucidate F-actin dynamics in the eotaxin-stimulated eosinophil, we examined the phosphorylation of putative PAK1-dependent actin-regulating proteins by immunoblot from total cell extracts obtained from eotaxin-stimulated *Pak1-/-* and *Pak1+/+* bmEos. First to show that eotaxin ligation of CCR3 induced PAK1 activation, we used an *in vitro* kinase assay where we immunoprecipitated PAK1 from HL-60 derived human eosinophils. Eotaxin-stimulation increased PAK1's phosphorylation of MEK1 at its serine 298 residue (Figure 18a). PAK1's kinase activation is a complex process that critically requires autophosphorylation of its threonine 423 (T423) residue [139]. Eotaxin-treatment of *Pak1+/+* murine bmEos phosphorylated PAKs at threonine 423, their signal activation residue (Figure 18b). Although work has not yet been done in eosinophils, PAK1 is known to regulate cell migration via the cytoskeleton. [155, 202, 205]. To test the role of PAK1 in cytoskeletal rearrangement, we cultured *Pak1-/-* and *Pak1+/+* bmEos and examined F-actin dynamics by fluorescence cytometry. Eotaxin stimulation in bmEos of both genotypes caused initial polymerization and reorganization of F-actin, but this eotaxin-induced F-actin polymerization at 30 seconds was significantly diminished in *Pak1-/-* bmEos compared to *Pak1+/+* (Figure 18c).

Furthermore eotaxin-stimulated *Pak1-/-* bmEos had altered cortical F-actin arrangement compared to WT bmEos (Figure 18f). The F-actin and the tubulin cytoskeleton are coordinately controlled in migration. We examined tubulin and F-actin polymerization in eotaxin-treated bmEos of both genotypes and likewise observed perturbed tubulin polymerization. These data together suggest that eotaxin-mediated F-actin polymerization and organization in eosinophils is PAK1-dependent. We hypothesized that these cytoskeletal dynamics perturbed in *Pak1-/-* eosinophils were associated with eotaxin-induced migration (Figure 18g).

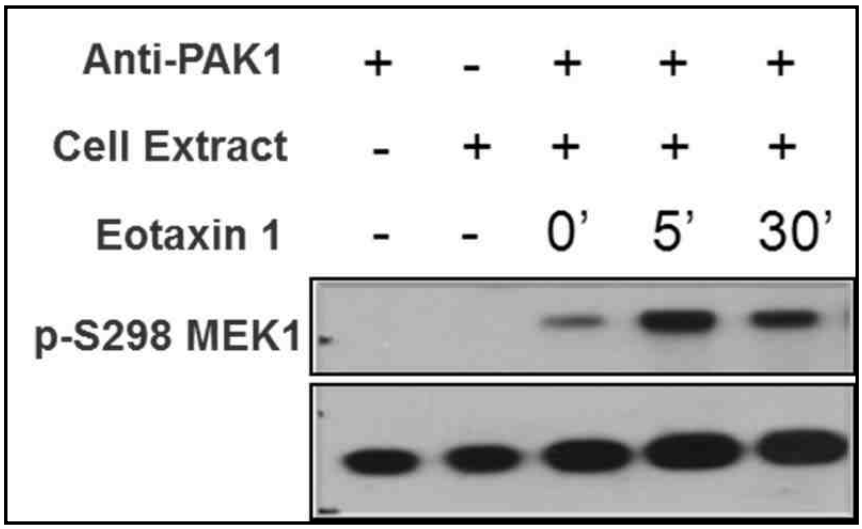


Figure 18a

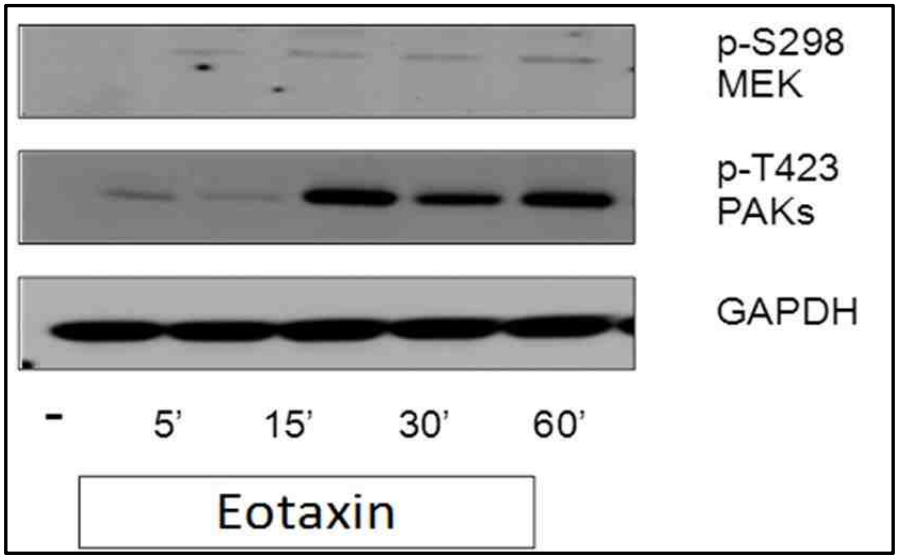


Figure 18b

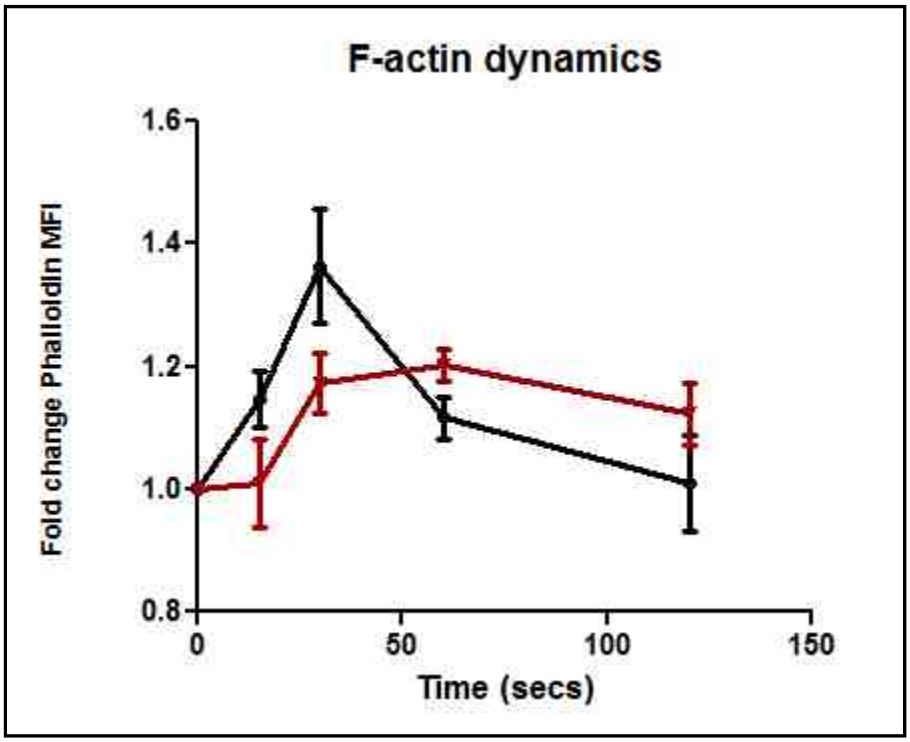


Figure 18c

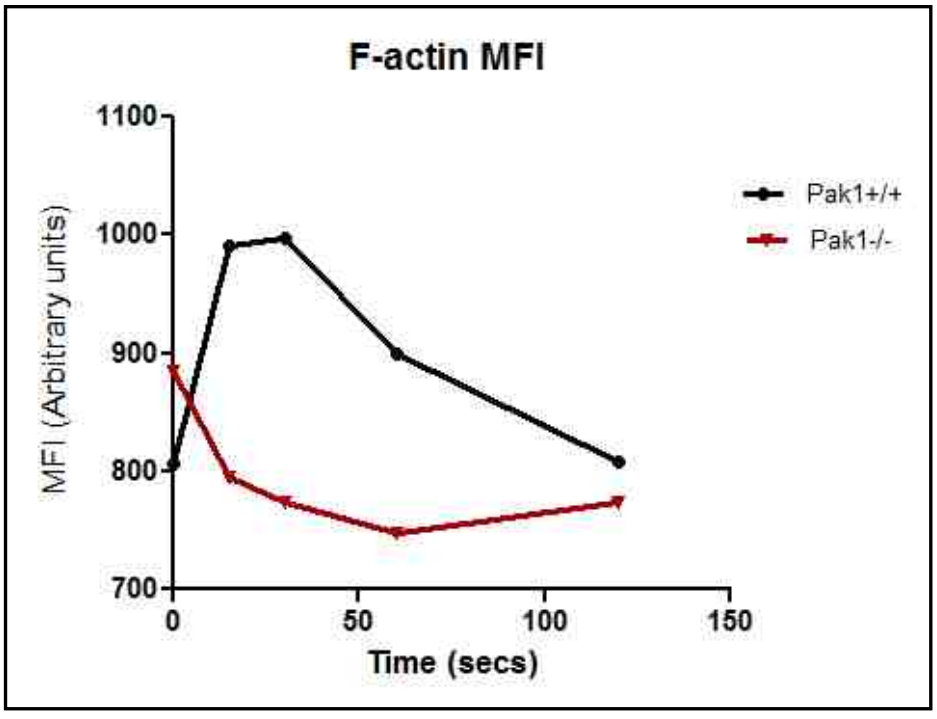


Figure 18d

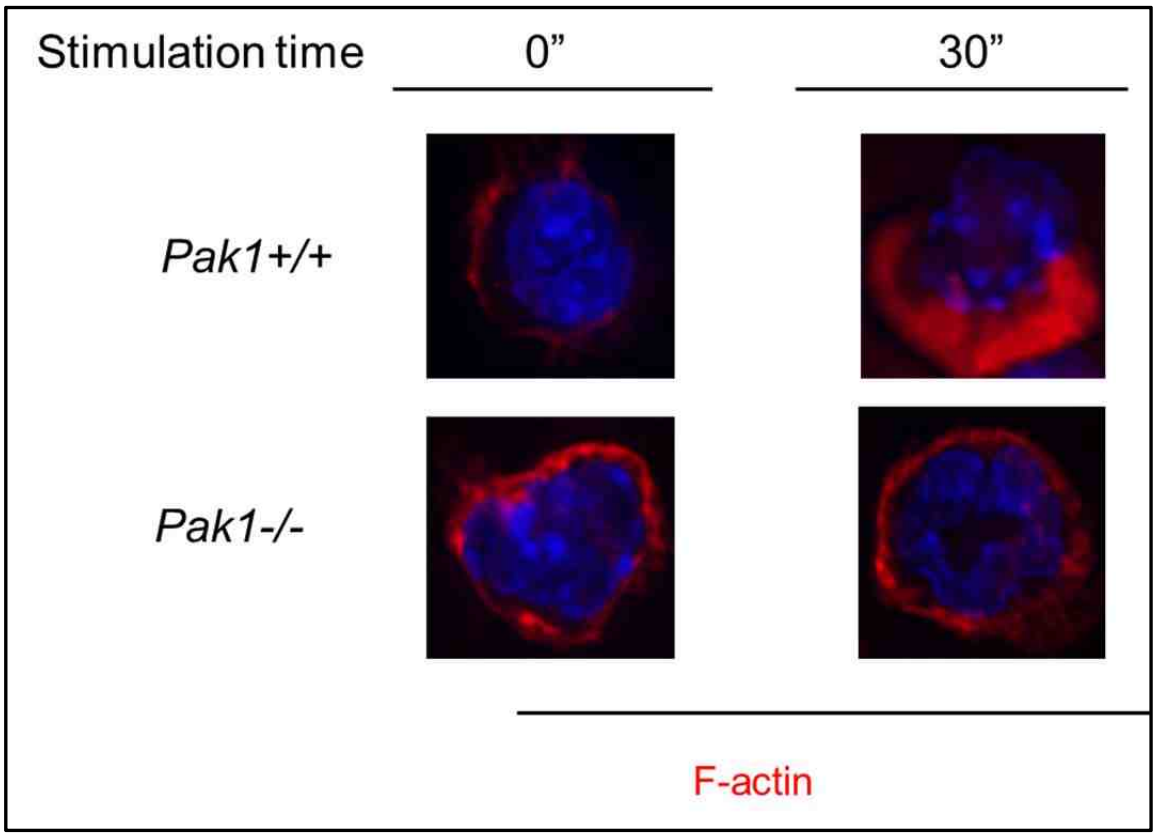


Figure 18e

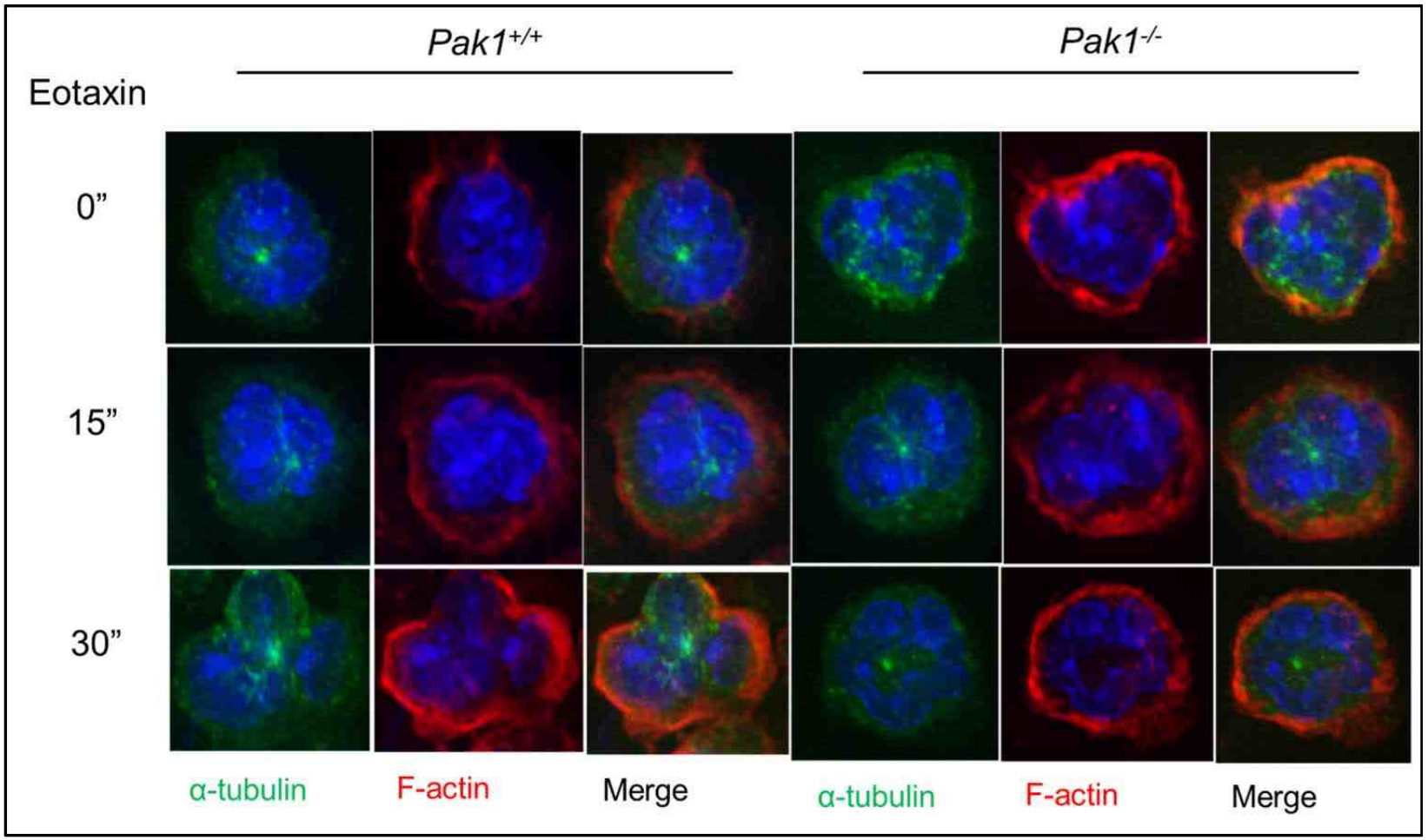

Pak1+/+
Pak1-/-
Eotaxin
0”
15”
30”
α-tubulin
F-actin
Merge
α-tubulin
F-actin
Merge

Figure 18f

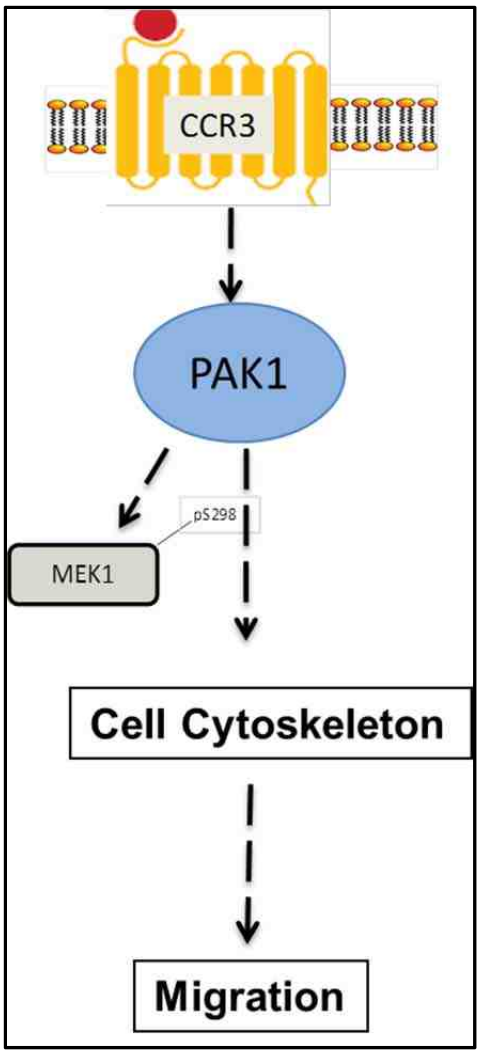


Figure 18g

Figure 18: PAK1 promotes F-actin reorganization induced by eotaxin signaling in eosinophils. HL-60 derived human eosinophils were generated *in vitro* and human PAK1 immunoprecipitated from eotaxin treated HL-60 Eos. PAK1's kinase activity was assessed to its substrated purified MEK1 (Figure 18a). *Pak1+/+* and *Pak1-/-* bmEos were cultured *in vitro,* treated with eotaxin, total cell extracts prepared and immunoprobed for PAKs and MEK1 phosphorylation (Figure 18b). Results in (a-b) are representative of at least three independent experiments. *Pak1+/+* and *Pak1-/-* BmEos were also cultured and stimulated with eotaxin over a two-minute duration for cytoskeletal studies. Eotaxin treated *Pak1+/+ and Pak1-/-* bmEos were fixed and relative (Figure 18c) and representative arbitrary (Figure 18d) mean fluorescent intensities (MFI) of phalloidin-tagged F-actin polymerization quantified by fluorescence cytometry, visualized by confocal microscopy (Figure 18e) and compared. Eotaxin-treated or untreated bmEos of both genotypes were also stained for tubulin and F-actin and visualized by deconvolution microscopy (Figure 18f). Results in (c) are averaged from five experiments (N=5) while results in (d-f) are representative from at least five experiments. A schematic summarizing conclusions from this study is illustrated (Figure 18g) * $p<0.05$ using a two-tailed student's *t*-test.

Eotaxin-induced eosinophil phosphorylation changes of cofilin as well as F-actin colocalization with p-34 Arc are PAK1-dependent

Eotaxin signaling pathways through PAK1 in eosinophils have not been studied to date. PAK1 in many cell systems has been known to regulate several F-actin regulating proteins [150, 176, 254]. To elucidate F-actin dynamics in the eotaxin-stimulated eosinophil, we examined the phosphorylation of putative PAK1-dependent actin-regulating proteins by immunoblot from total cell extracts obtained from eotaxin-stimulated *Pak1-/-* and *Pak1+/+* bmEos. We showed that eotaxin induced dephosphorylation of cofilin in bmEos occurs in a PAK1-dependent manner (Figures 19a-b). Remarkably, *Pak1-/-* bmEos demonstrated impaired dephosphorylation of cofilin at serine 3 relative to *Pak1+/+* bmEos (Figures 19a-b). PAK1 regulates cofilin in signaling events essential for F-actin cytoskeletal dynamics in cell migration [176, 195].

Pak1 deficiency perturbed spatial eosinophil F-actin localization/reorganization 30 seconds after eotaxin stimulation (Figure 18c-d). To further examine F-actin cytoskeletal events at this time point, we fluorescently tagged p34-ARC a subunit of the ARP2/3 protein complex. ARP2/3 is a known substrate of PAK1 that initiates new F-actin polymerization sites [177]. We found decreased colocalization of F-actin with this PAK1-regulated component of ARP2/3 in *Pak1-/-* bmEos although p34ARC expression is comparable between the two genotypes (Figures 19c-d). These data taken together suggest decreased *de novo* polymerization of actin-filaments in eotaxin-stimulated *Pak1-/-* bmEos.

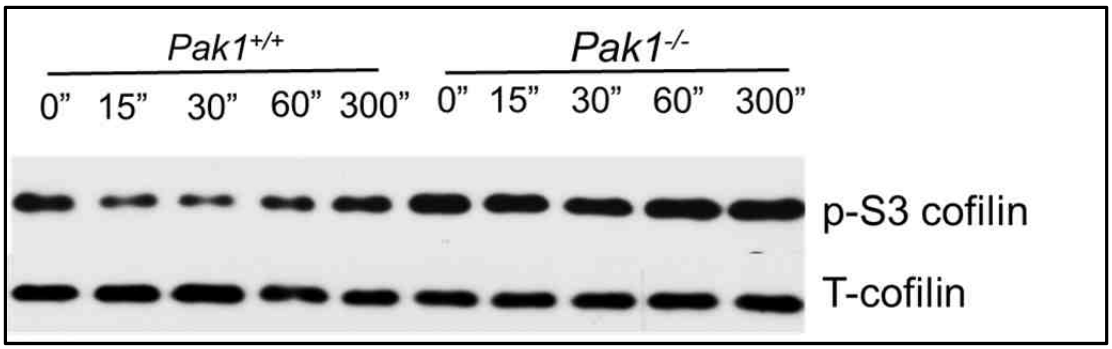


Figure 19a

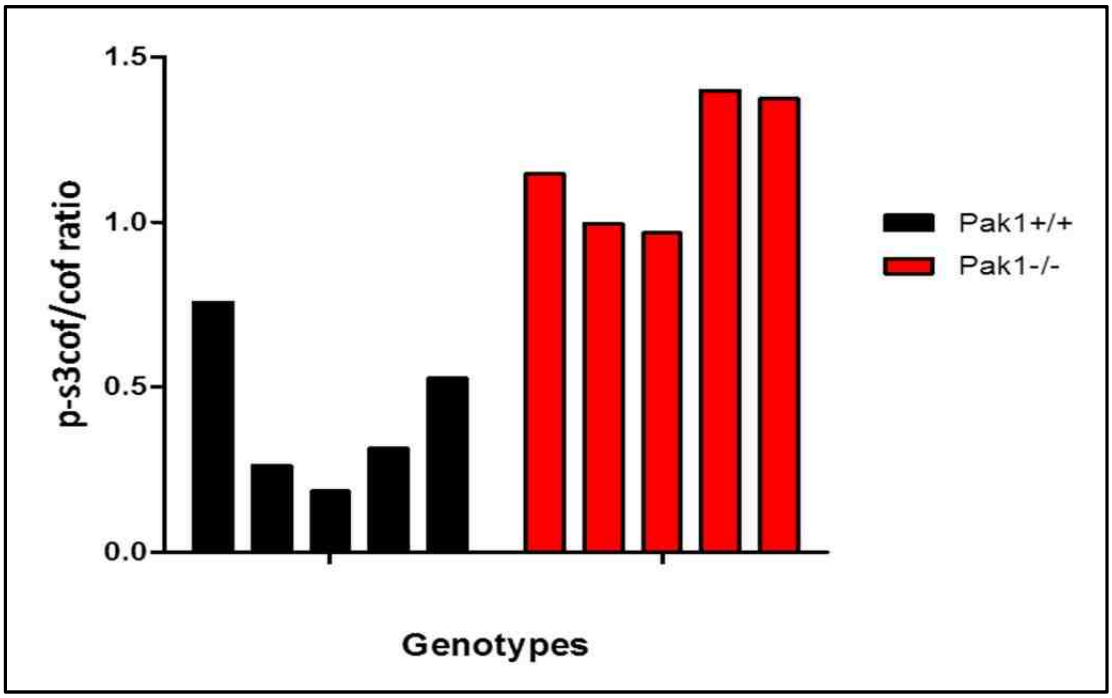
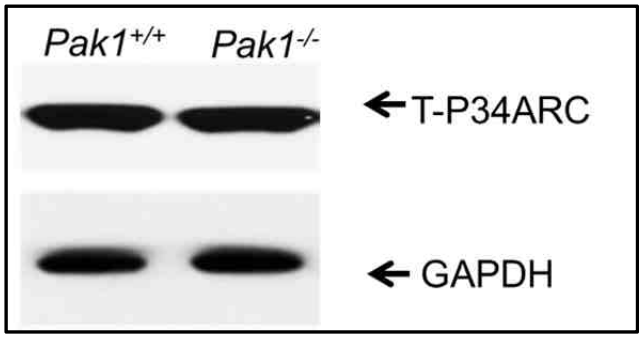


Figure 19b


Pak1+/+
Pak1-/-
T-P34ARC
GAPDH

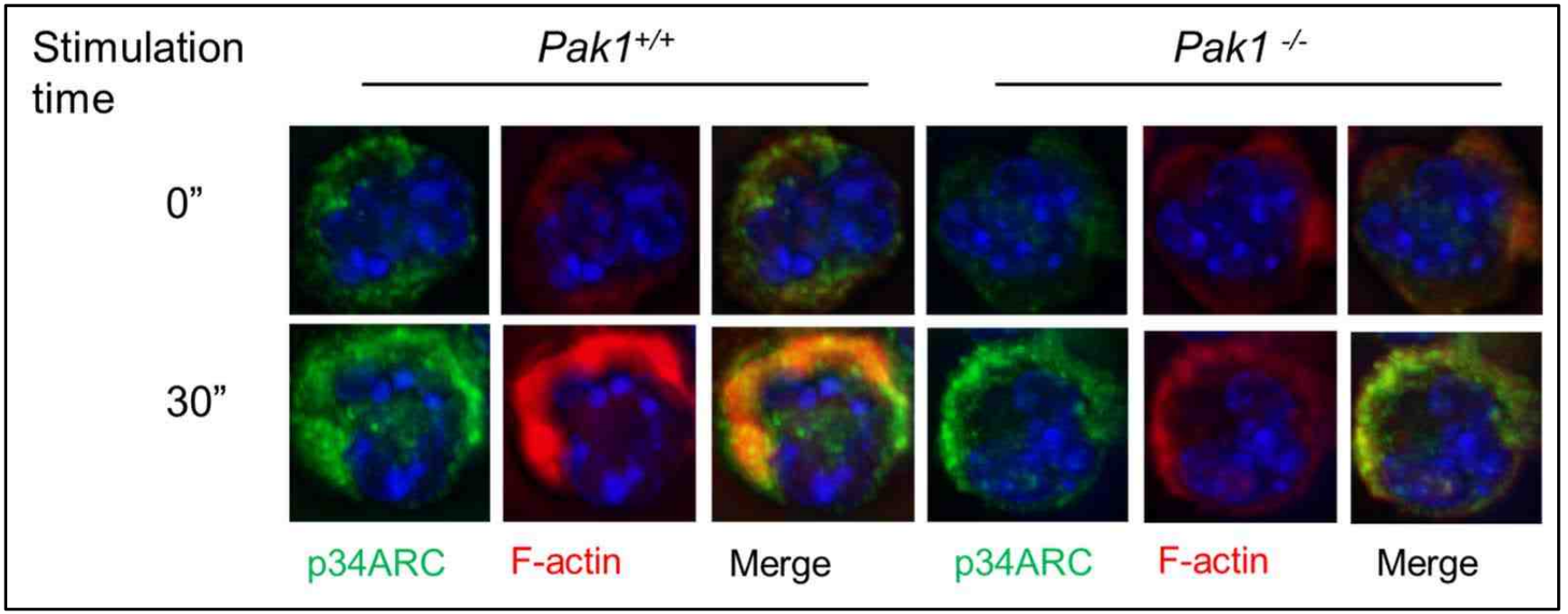

Stimulation time
Pak1+/+
Pak1 -/-
0”
30”
p34ARC
F-actin
Merge
p34ARC
F-actin
Merge

Figure 19c-d

Figure 19: Eotaxin induces PAK1-dependent phosphorylation changes of cofilin, filamin A and F-actin localization with p-34 ARC. *Pak1+/+* and *Pak1-/-* bmEos were treated with eotaxin and fixed for immunocytochemical analysis or total cell extracts prepared for western blot. Cell extracts were immunoprobed for pS3-cofilin and total cofilin (Figure 19a) and levels of these proteins compared by densitometry (Figure 19b). Cell extracts from bmEos were immunoprobed for p-34 ARC levels (Figure 19c). Fixed and permeabilized cells were probed for total p-34 ARC and F-actin (Figure 19d). Results in (a-c) are representative from at least three experiments. * $p<0.05$ using a two-tailed student's *t*-test.

Eotaxin-induced phosphorylation of PAK2, Ezrin/ moeisin/ Radixin, Op18/ Stathmin, and p38 MAPK in the eosinophil is PAK1-independent

To expand upon and explain PAK1-dependent F-actin polymerization, we also immunoblotted for phosphorylated PAK2, Ezrin/moeisin/Radixin, Op18/Stathmin, and p38 MAPK in total cell extracts from eotaxin-treated *Pak1+/+* and *Pak1-/-* bmEos. PAK2, an isoform of PAK1, has been shown to have some redundant substrate specificities and function *in vitro* and *in vivo* [150]. PAK1 and PAK2 are both expressed in hematopoietic tissues although their redundant and disparate role in eosinophil function remain to be explored. We show that eotaxin stimulation induced PAK2 phosphorylation at the threonine 423 residue that is independent of PAK1 expression (Figures 20a-b). To further investigate the role of these two isoforms of PAKs in eosinophil migration we used, IPA3, an allosteric inhibitor of PAK1 that also inhibits PAK2. We pretreated *Pak1+/+* eosinophils with IPA3 for 20 minutes prior to using these cells in a transwell migration assay. We observed that PAKs inhibition prevented eotaxin-mediated eosinophil migration (Figure 20c). Furthermore, IPA3 treatment significantly decreased spontaneous non-chemokine driven chemokinesis of eosinophils (Figure 20d) despite no profound effect on cell membrane compromise as assessed by trypan blue exclusion counts two hours after IPA3-incubation (not depicted). These findings suggest both PAK1 and PAK2 may have additive or synergistic effects on eotaxin-mediated eosinophil migration.

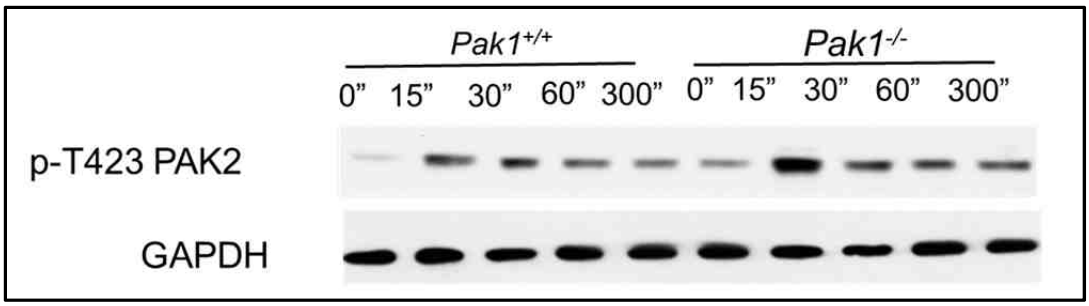
ERM, p38 MAPK, Filamin A, and Op18/Stathmin have been shown in other cell systems to regulate cytoskeletal remodeling downstream of PAK1 [121, 195, 196, 200, 255]. We have previously observed that *Pak1* modulates F-actin depolymerization and mast cell degranulation through Ezrin [192]. We show for the first time that eotaxin-treatment induced phosphorylation changes of p-T567 ERM (Figures 20i-j). Phospho-T567 ERM also colocalized with F-actin in the eotaxin-polarized *Pak1+/+* eosinophil (Figure 20e) so we hypothesized that a PAK1-ERM signaling axis is involved in eotaxin-induced F-actin changes and migration. To test this hypothesis, we generated mice with conditional hematopoietic *Ezrin* deficiency. We crossed Ezrin *floxed* mice with mice expressing the *Mx-1 Cre* transgene that is expressed in hematopoietic and liver tissues in an inducible fashion (Figure 20f). Approximately 25% of the second generation of pups yielded the desired *Ezrin flox/flox Mx1Cre-* (Ez-WT) and *Ezrinflox/flox Mx1Cre*$^{+}$ *(*Ez-KO). We induced Mx-1 Cre expression by intraperitoneal administration of a tapered dose of PolyIC on alternative days standardized by mouse weight. We demonstrated hematopoietic ablation of Ezrin expression by western blot analysis of Ez-KO LDMNCs (Figure 20g). We cultured Ez-WT and Ez-KO bmEos and used them in eotaxin migration assays. We observed no difference between migration of Ez-WT and Ez-KO eosinophils (Figure 20h). These data together suggest eotaxin-induced phosphorylation of Ezrin is neither necessary for eotaxin-mediated eosinophil F-actin polymerization nor migration. Furthermore, these Ezrin changes are not PAK1-dependent.

p38 MAPK is known to regulate the cell cytoskeleton and cell migration via its downstream effector HSP27 [122, 245]. In hematopoietic cells, SCF-mediated mast-cell migration is in part PAK1-dependent [121]. We postulated that PAK1-p38-dependent signaling may also be important in eotaxin-mediated eosinophil migration. To examine p38 MAPK's role in PAK1-dependent eotaxin-mediated eosinophil migration, we used chemical inhibition of p38 MAPK activity in transwell migration assays as well as western blot analysis of eotaxin-treated bmEos. p38 MAPK inhibition by SB 203580 decreased eotaxin-mediated wild-type eosinophil migration by greater than 40% in a similar manner as PAK1 (Figure 20k). We also measured and quantified eotaxin-induced phosphorylation of p38 MAPK and observed phosphorylation of T180/Y182 p38 MAPK. However, the eotaxin-mediated phosphorylation of p38 MAPK did not appear to be PAK1-dependent in the initial eotaxin-mediated PAK1 signaling (Figures 20i-j).

PAK1 also phosphorylates Filamin A at its serine 2152 residue. Filamin A anchors integrins and transmembrane receptor complexes to the cell cytoskeleton modulating cell shape. Moreover, studies in FLNA knockout systems demonstrate multiple defects with migrational etiology [193, 194]. We show that eotaxin-induces phosphorylation of Filamin A comparably in both *Pak1+/+* and *Pak1-/-* bmEos (Figures 20l-m). Additionally, PAK1 is known to phosphorylate Op18/stathmin at its serine 16 residue mediating tubulin and consequently F-actin reorganization. In our studies, while we show a novel

eotaxin-induced phosphorylation of Op18/Stathmin in eosinophils, this phosphorylation does not appear to be PAK1-dependent (Figures 20i-j).

From the biochemical and microscopic data we report in Figures 19 and 20, we propose a model of eotaxin signaling in the eosinophil (Figure 20n). We show that eotaxin:CCR3 independently phosphorylates PAK1 and PAK2. We also show that PAK1 in the eosinophil mediates eotaxin-induced depolymerization of cofilin in modulating F-actin temporal-spatial polymerization. In so doing, PAK1 may increase *de novo* polymerization of F-actin at new sites that are ARP2/3-mediated. However, the intital eotaxin-induced phosphorylation of ERM, p38 MAPK, filamin A, and Op18/Stathmin is PAK1-independent.


Pak1+/+
Pak1-/-
0” 15” 30” 60” 300”
0” 15” 30” 60” 300”
p-T423 PAK2
GAPDH

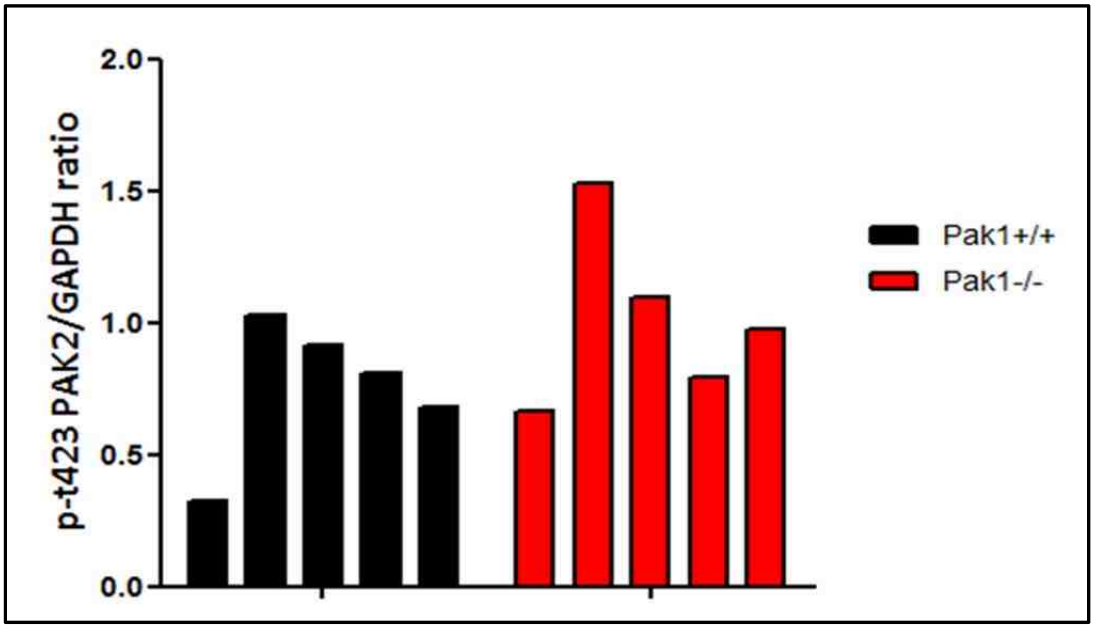

p-t423 PAK2/GAPDH ratio
2.0
1.5
1.0
0.5
0.0
Pak1+/+
Pak1-/-

Figure 20a-b

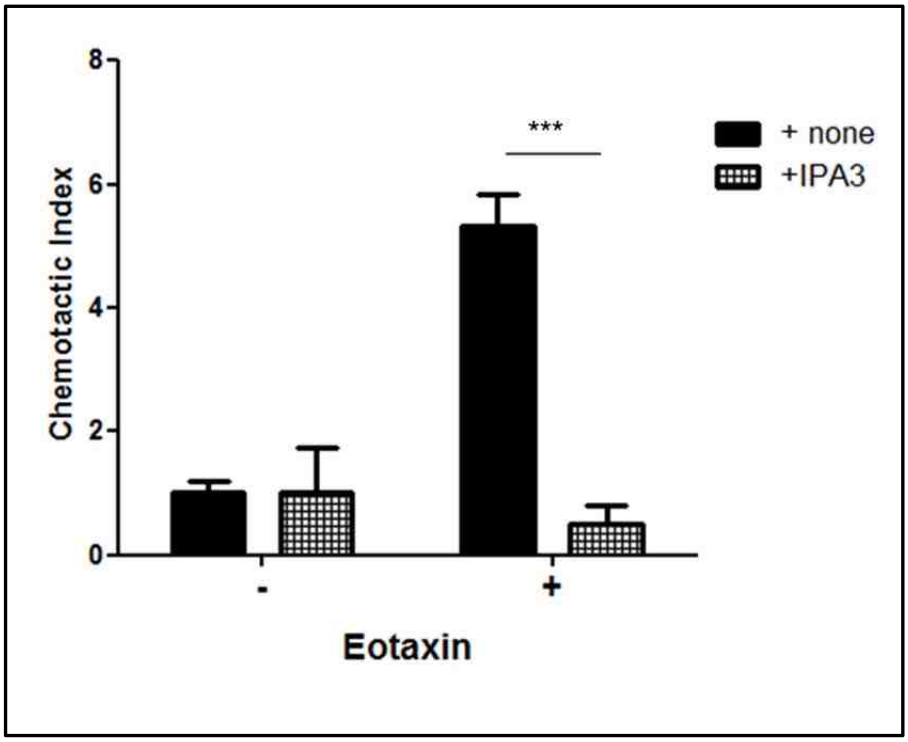


Figure 20c

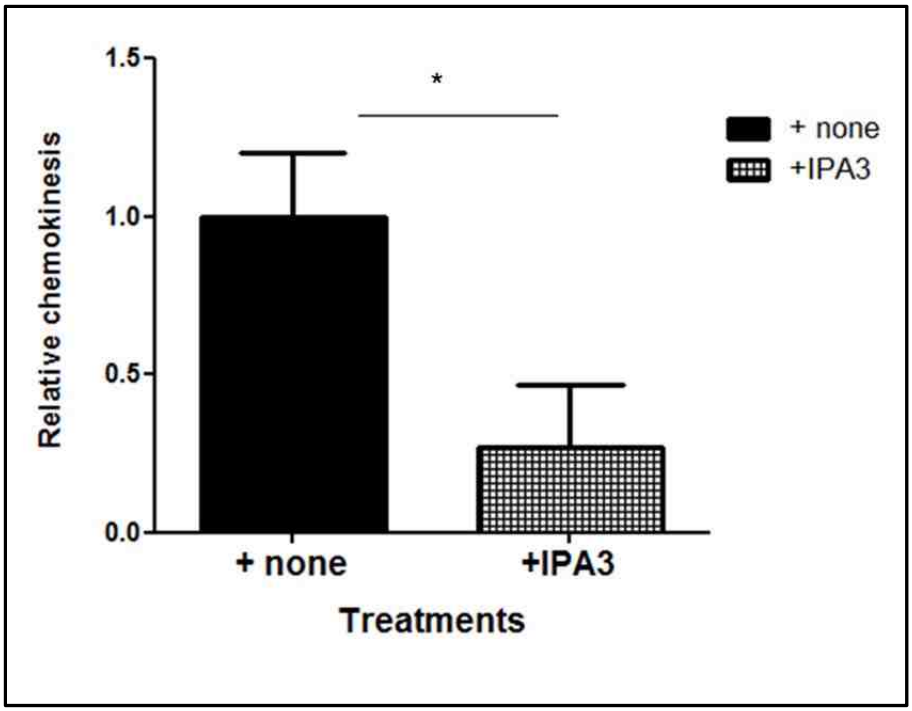


Figure 20d

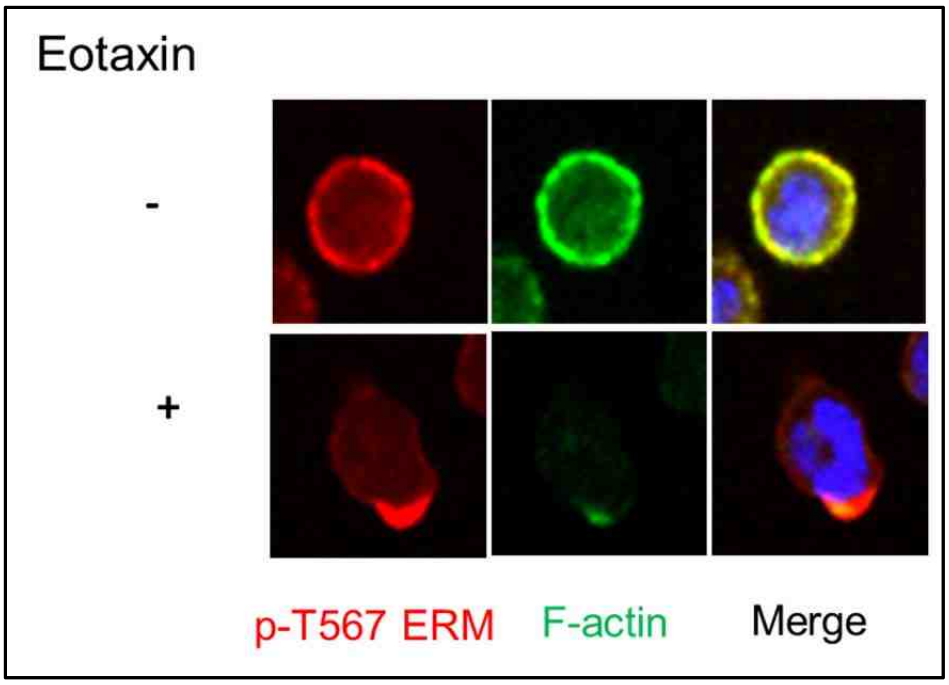


Figure 20e

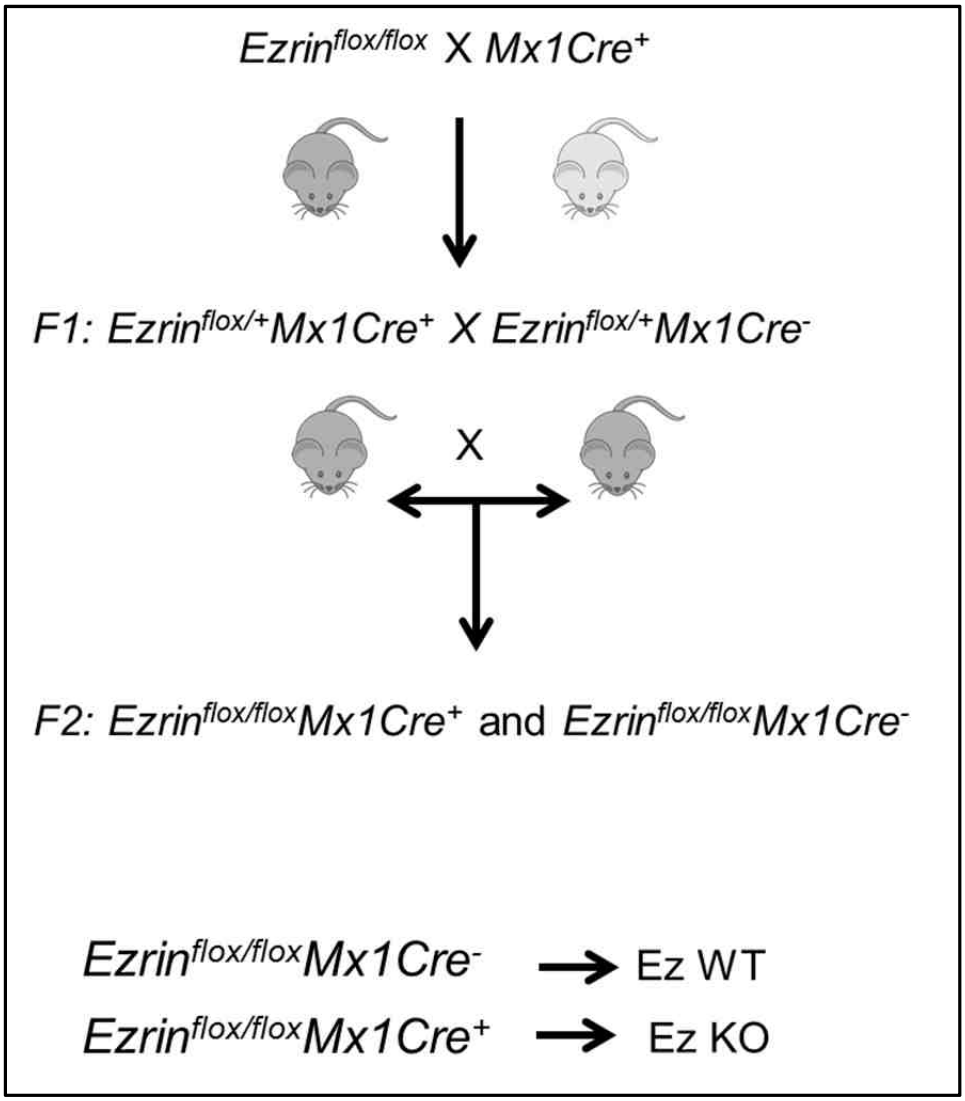

Ezrin flox/flox X Mx1Cre+
F1: Ezrin flox/+ Mx1Cre+ X Ezrin flox/+ Mx1Cre-
X
F2: Ezrin flox/flox Mx1Cre+ and Ezrin flox/flox Mx1Cre-
Ezrin flox/flox Mx1Cre- → Ez WT
Ezrin flox/flox Mx1Cre+ → Ez KO

Figure 20f

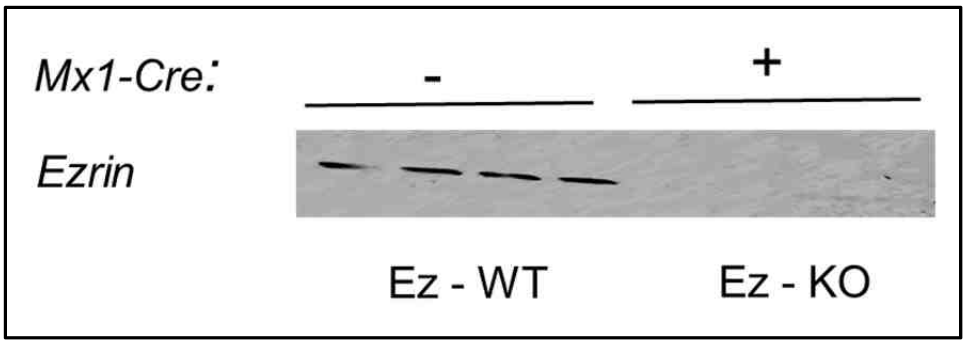


Figure 20g

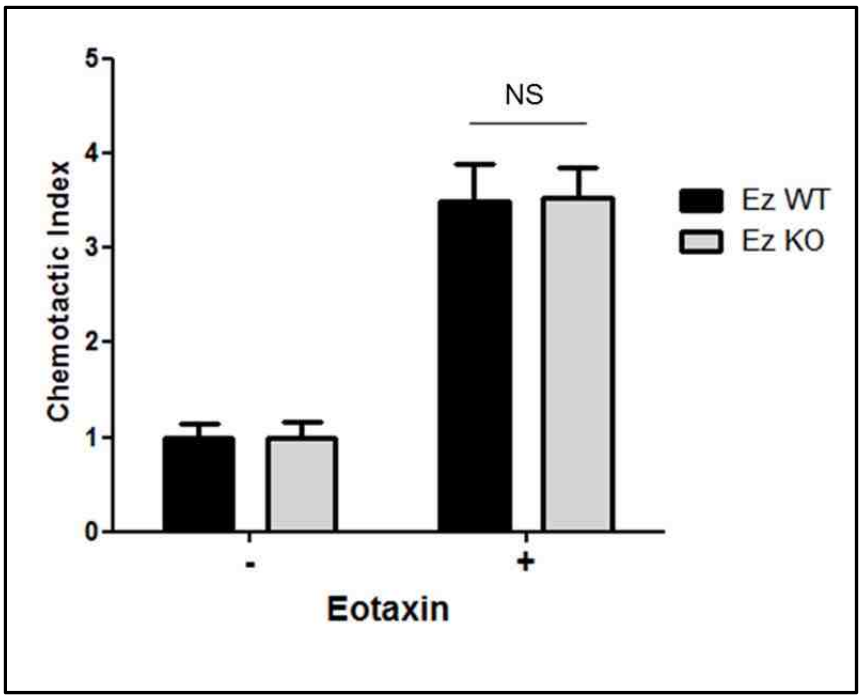


Figure 20h

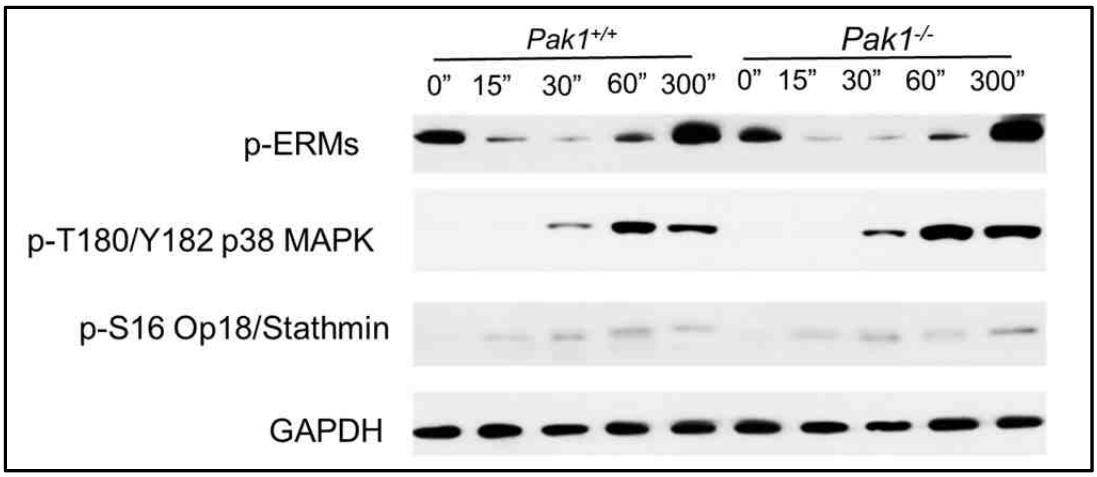

Pak1+/+
Pak1-/-
0" 15" 30" 60" 300"
0" 15" 30" 60" 300"
p-ERMs
p-T180/Y182 p38 MAPK
p-S16 Op18/Stathmin
GAPDH

Figure 20i

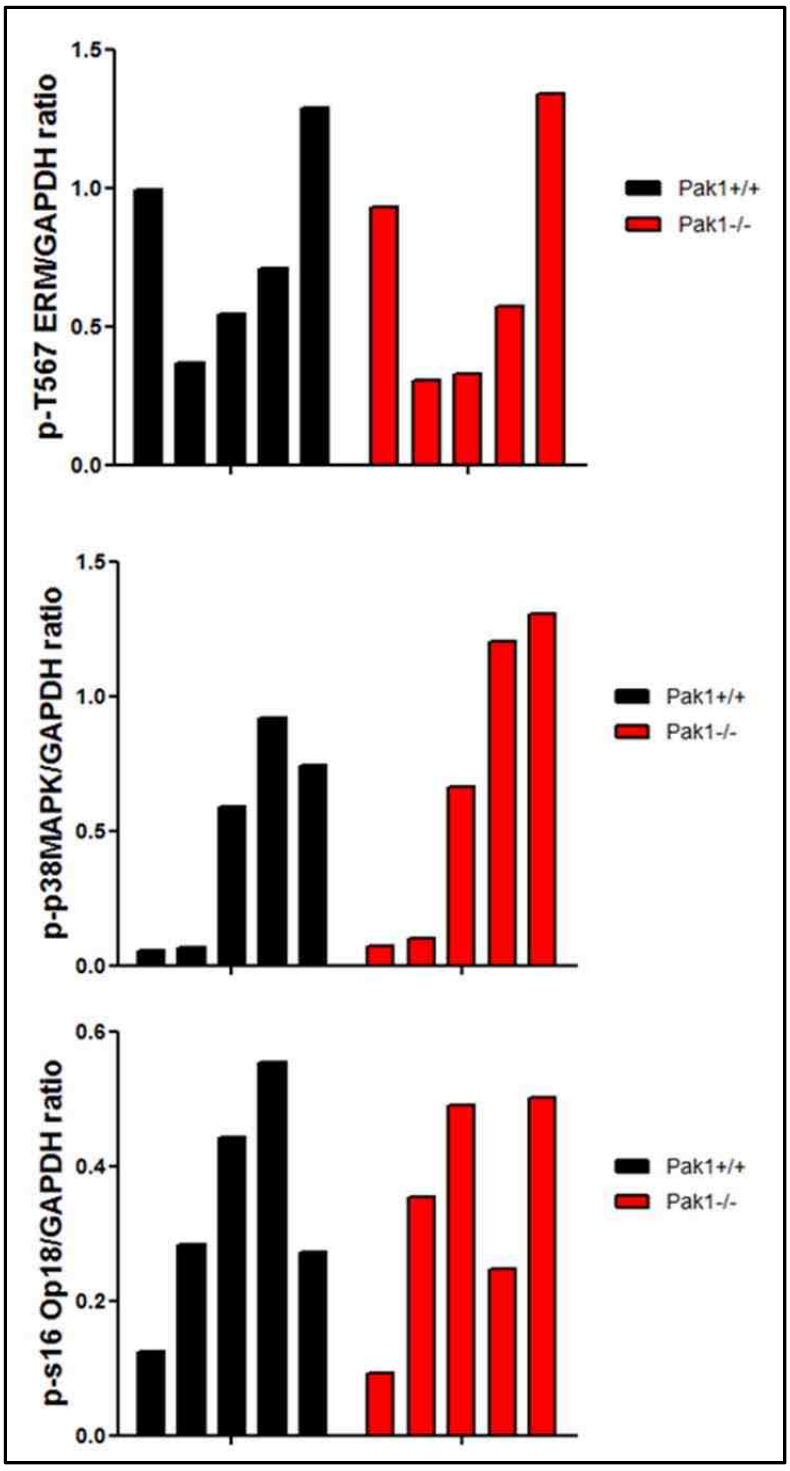

p-T567 ERM/GAPDH ratio
1.5
1.0
0.5
0.0
Pak1+/+
Pak1-/-
p-p38MAPK/GAPDH ratio
1.5
1.0
0.5
0.0
Pak1+/+
Pak1-/-
p-s16 Op18/GAPDH ratio
0.6
0.4
0.2
0.0
Pak1+/+
Pak1-/-

Figure 20j

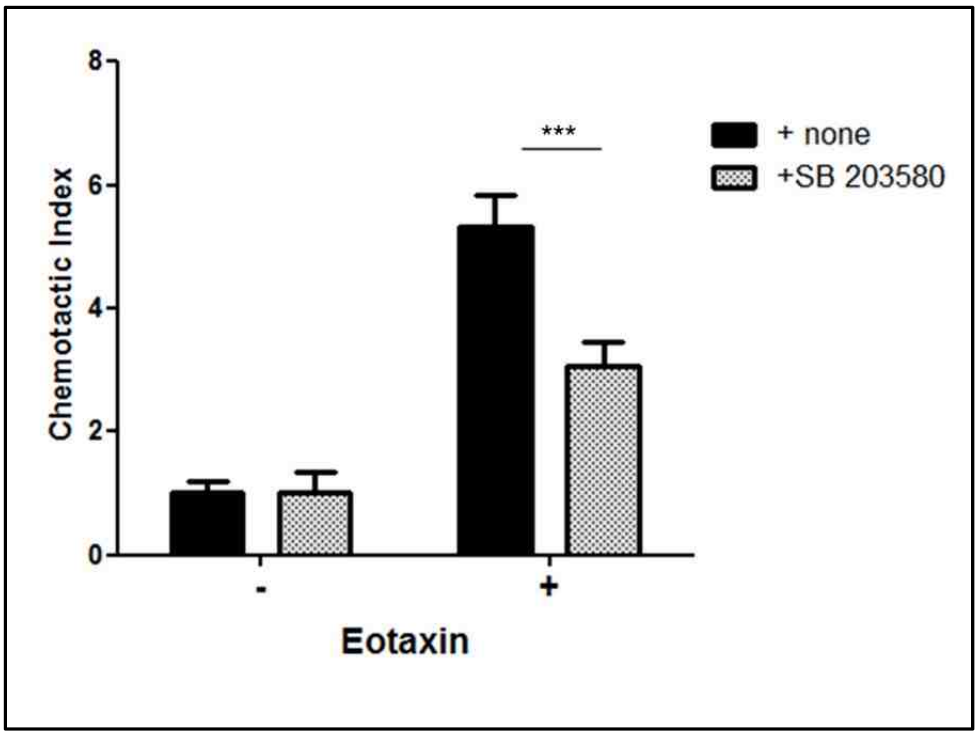
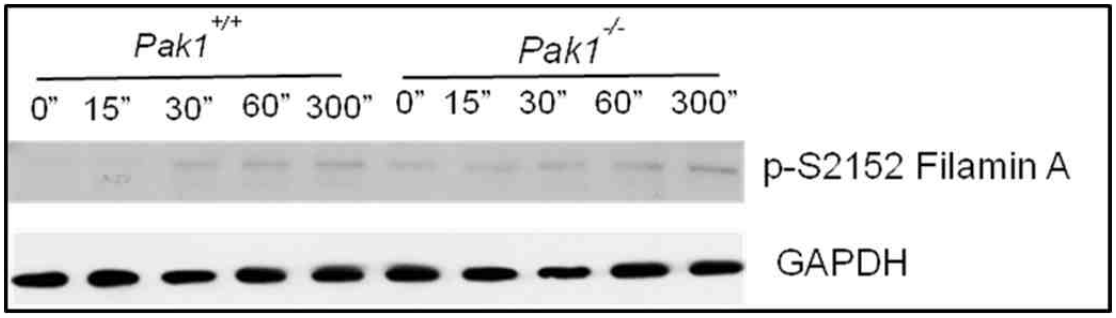
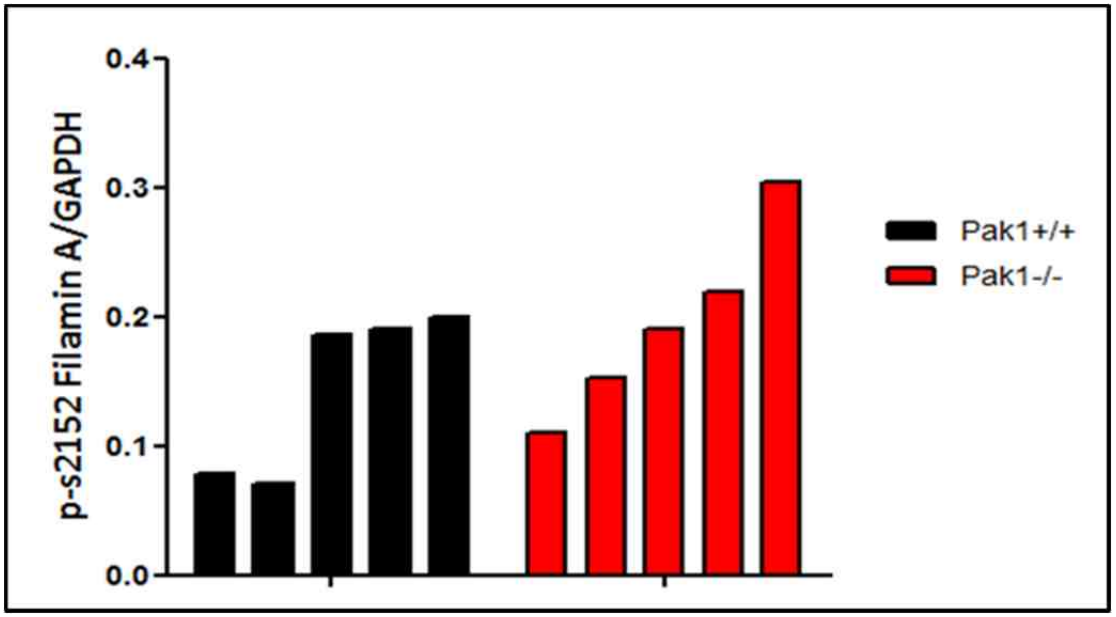


Figure 20k





Figures 20l-m

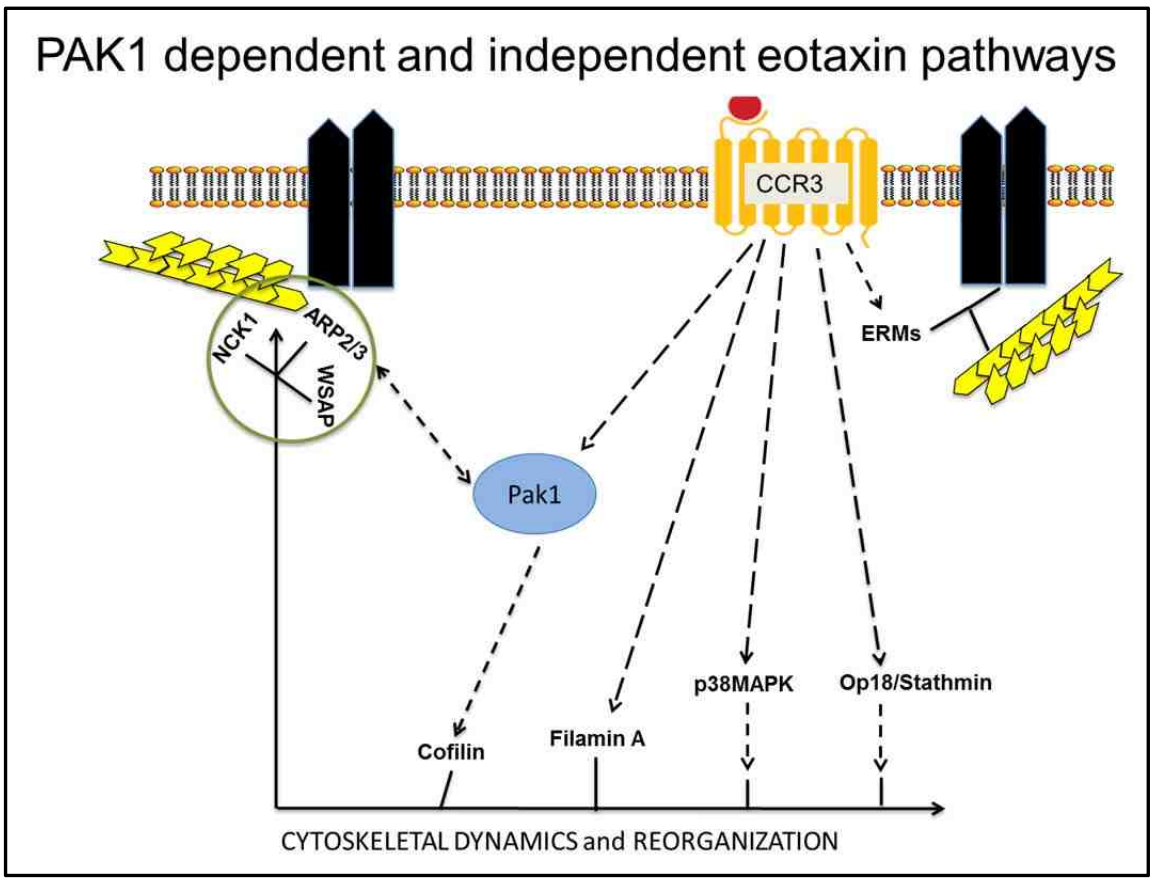

PAK1 dependent and independent eotaxin pathways
CCR3
NCK1
ARP2/3
WSAP
ERMs
Pak1
Cofilin
Filamin A
p38MAPK
Op18/Stathmin
CYTOSKELETAL DYNAMICS and REORGANIZATION

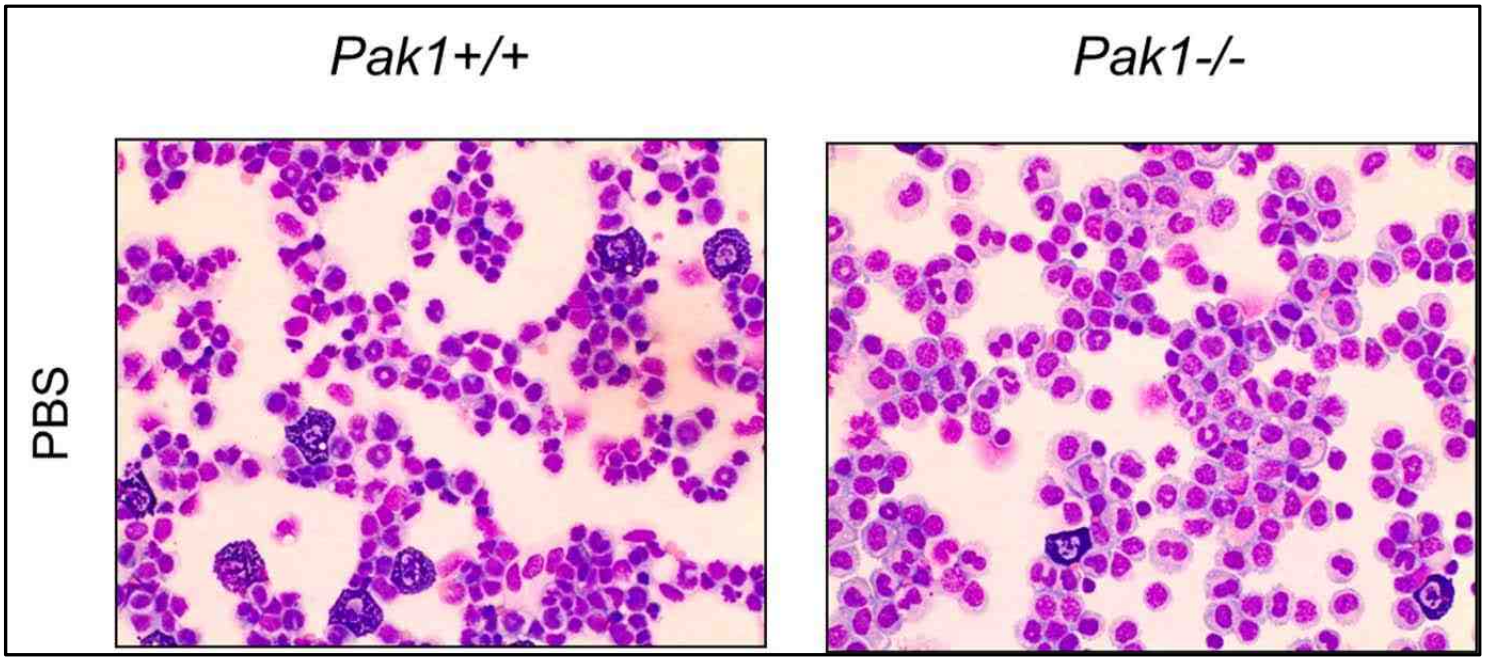
Figure 20n

Figure 20: PAK1-independent Eotaxin-induced phosphorylation of PAK2, Ezrin/moeisin/Radixin, Op18/Stathmin, and p38 MAPK in the eosinophil. *Pak1+/+* and *Pak1-/-* bmEos were treated with eotaxin and total cell extracts prepared for western blot or fixed for immunocytochemical analysis. Cell extracts were blotted for p-T423 PAKs (Figures 20a-b), p-T567 ERM, p-T180/Y182 p38 MAPK, Fillamin A, p-S16 Op18/Stathmin as well as GAPDH as a loading control (Figures 20i-m). Paraformaldehyde fixed bmEos were permeabilized and immunoprobed for p-T567 Ezrin and phalloidin-treated for F-actin visualization (Figure 20e). To assess migration inhibitor treated cells and cell deficient in Ezrin were utilized in transwell migration assays where bmEos migrating to the bottom chamber were enumerated by hemocytometer. *Pak1+/+* bmEos were pretreated with inhibitors against PAKs, IPA3, and p38 MAPK, SB 203580, before being utilized in an eotaxin transwell migration assays, N=3-6 (Figures 20c-d, k). *Ezrin floxed* mice were crossed with mice expressing the inducible Mx-1 Cre transgene, to yield the desired F2 descendants according to this schematic (Figure 20f). Ez WT and Ez KO mice were injected with five doses of PolyIC on alternate days to induce expression of Mx-1 Cre in Ez KO bone marrow. Cell extracts from LDMNCs from four Ez WT and Ez KO mice, post-PolyIC treatment was immunoblotted for Ezrin (Figure 20g). Ez WT and Ez KO eotaxin-mediated chemotaxis was evaluated using a transwell system, N=5 (Figure 20h). Our eosinophil migration studies, suggest a generalized schematic of PAK1-dependent and -independent eotaxin-induced pathways (Figure 20n). *p<0.05, ** p<0.01, ***p<0.001 using a two-way ANOVA with Bonferroni post hoc.

Eotaxin-induced eosinophil degranulation *in vitro* is PAK1- independent

Eosinophil degranulation and mediator release promote airway remodeling [90]. Based on the results from our murine asthma model that demonstrated decreased eosinophils in the BAL and lung tissue of *Pak1-/-* mice, we wanted to examine Pak1's role in eotaxin-mediated murine eosinophil degranulation. Eotaxin is known to induce degranulation and granule protein release from human and murine eosinophils [117, 256, 257]. In a peritoneal eotaxin-mediated eosinophil infiltration model, we show eotaxin induces potent eosinophil recruitment and degranulation *in vivo*. Furthermore, this degranulation seems to be dependent on PAK1 with fewer degranulated eosinophils in *Pak1-/-* mouse peritoneal lavage Giesma-stained cytospins compared with *Pak1+/+* (Figures 21a-b). We sought to characterize the peritoneal lavage cell populations by FACS. We found consistent with previous studies that whereas granulated eosinophils were predominantly CCR3+, prolonged exposure to eotaxins cause degranulation and receptor internalization [236, 258]. Hence degranulated eosinophils were predominantly CCR3- (like neutrophils) and appeared to have without red granules on giesma staining (Figure 21c). This ablated CCR3 expression makes fluorescence cytometric CCR3 assessment of eosinophils from peritoneal lavage fluid variable and unreliable. Our previous work supports a role for PAK1 in mast cell degranulation. *Pak1-/-* mast cell degranulation is decreased measured by β-hexoaminidase enzymatic activity [223]. Furthermore, transgenic lentiviral expression of PAK1 restored IgE/DNP-mediated mast cell degranulation *in vitro* (Figure 21d). Moreover, we observed that this PAK1-

dependent degranulation is mediated in part by Ezrin [192]. Similar to *Pak1* deletion, *Ezrin* deficiency in mast cells decreased IgE/DNP-mediated degranulation (Figure 21e) and F-actin depolymerization (Figure 21f). We consequently hypothesized that PAK1 regulates eotaxin-mediated eosinophil degranulation and used a well-documented colorimetric assay quantifying supernatant EPO activity *in lieu* of granule release. We found that we could not elicit eotaxin-induced EPO degranulation over baseline despite strong *in vivo* degranulation. Furthermore, we could not distinguish between relative degranulation levels of *Pak1-/-* versus *Pak1+/+* bmEos (Figure 21g). Similarly, further degranulation studies using Ez-KO bmEos revealed no difference in degranulation compared with Ez-WT (Figure 21h). Our inconclusive degranulation results suggest a need for a more sensitive system to study eotaxin-mediated degranulation *in vitro*.



Figures 21a

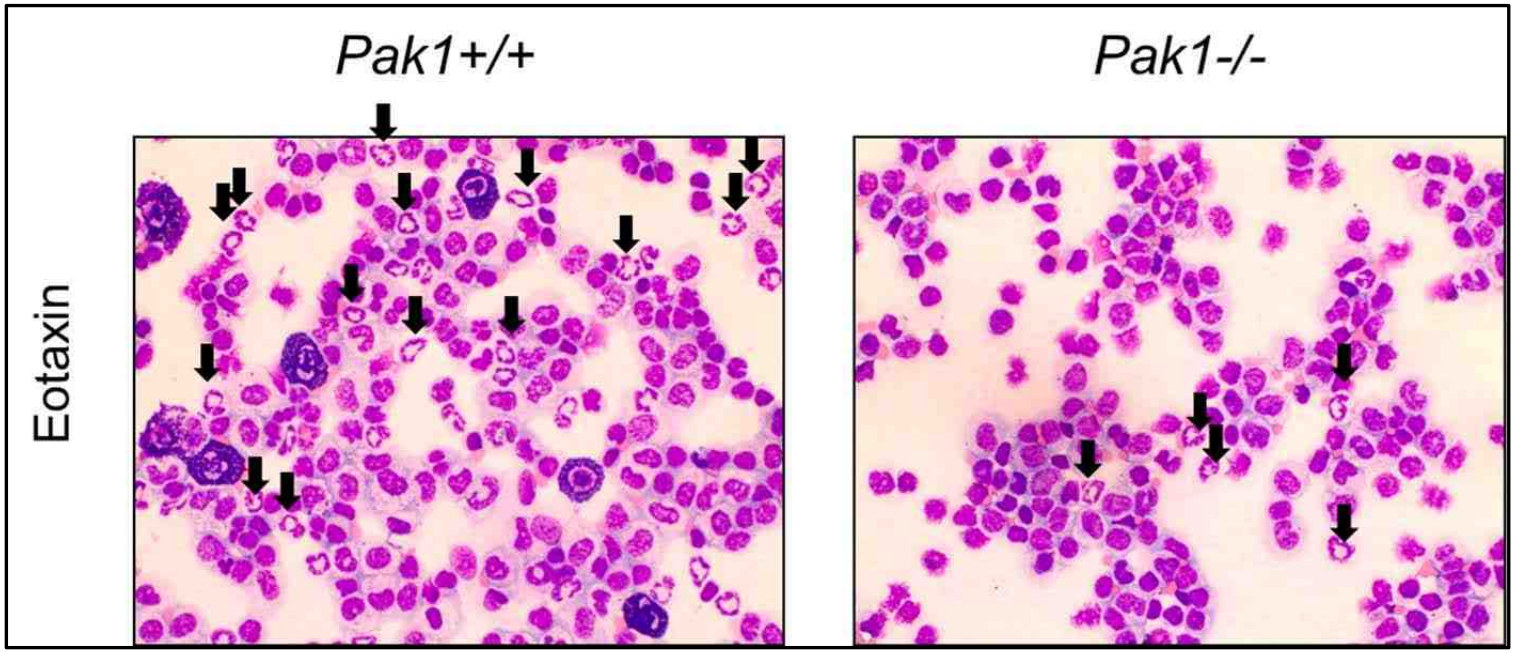


Figure 21b

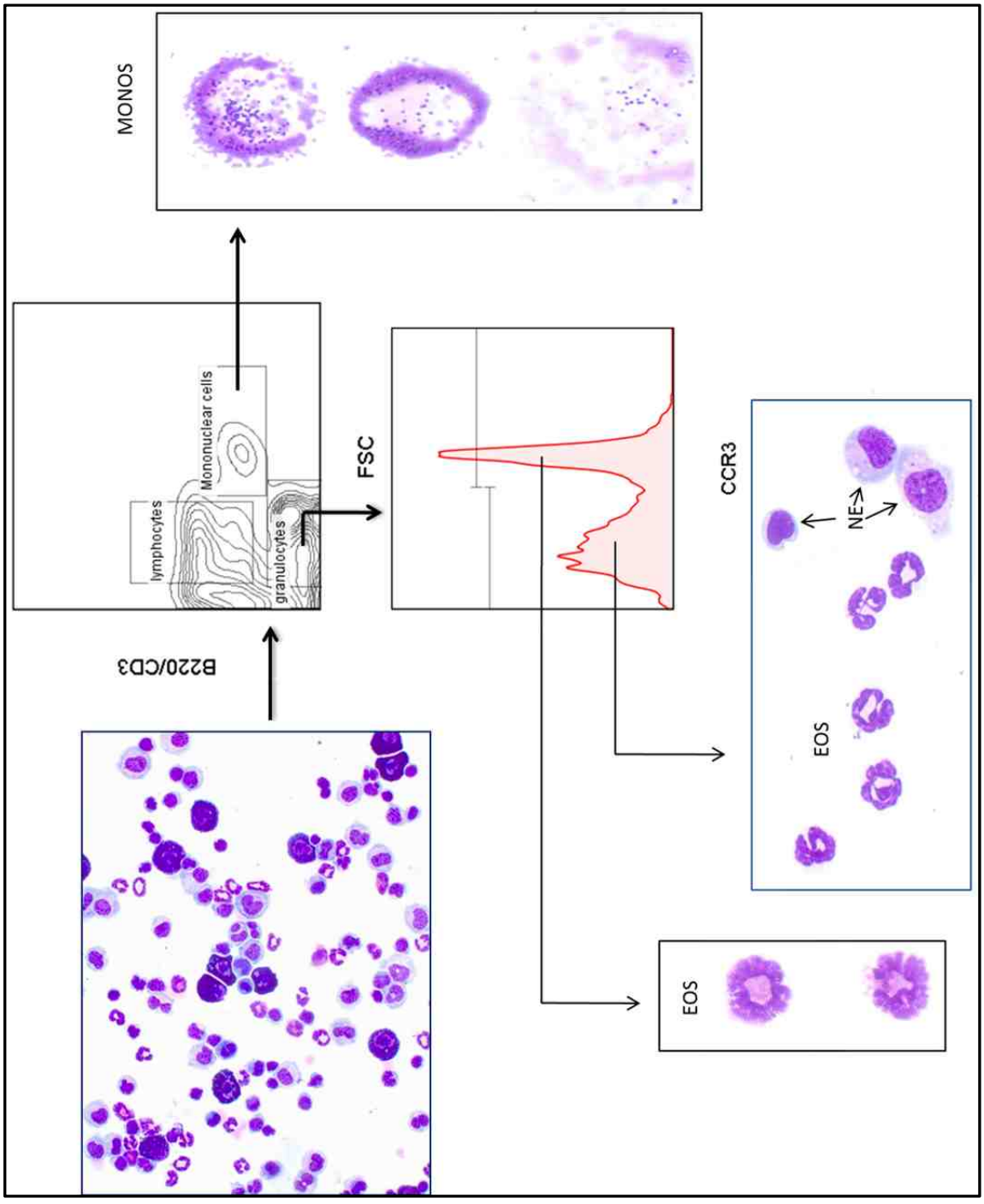

MONOS
Mononuclear cells
lymphocytes
granulocytes
B220/CD3
FSC
CCR3
NE
EOS
EOS

Figure 21c

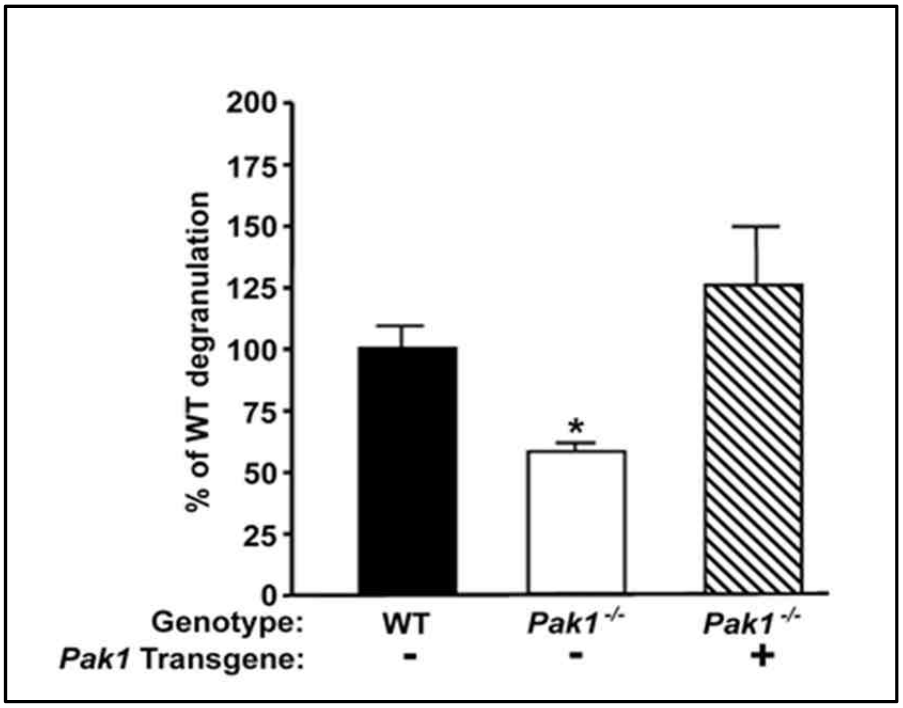

200
175
150
125
100
75
50
25
0
% of WT degranulation
*
Genotype:
WT
Pak1 -/-
Pak1 -/-
Pak1 Transgene:
-
-
+

Figure 21d

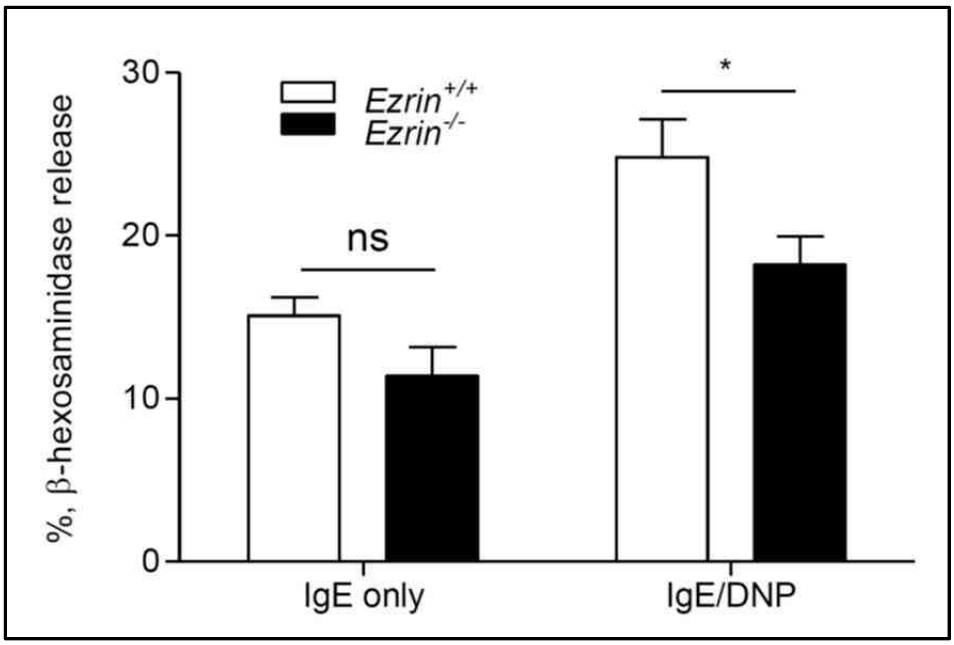


Figure 21e

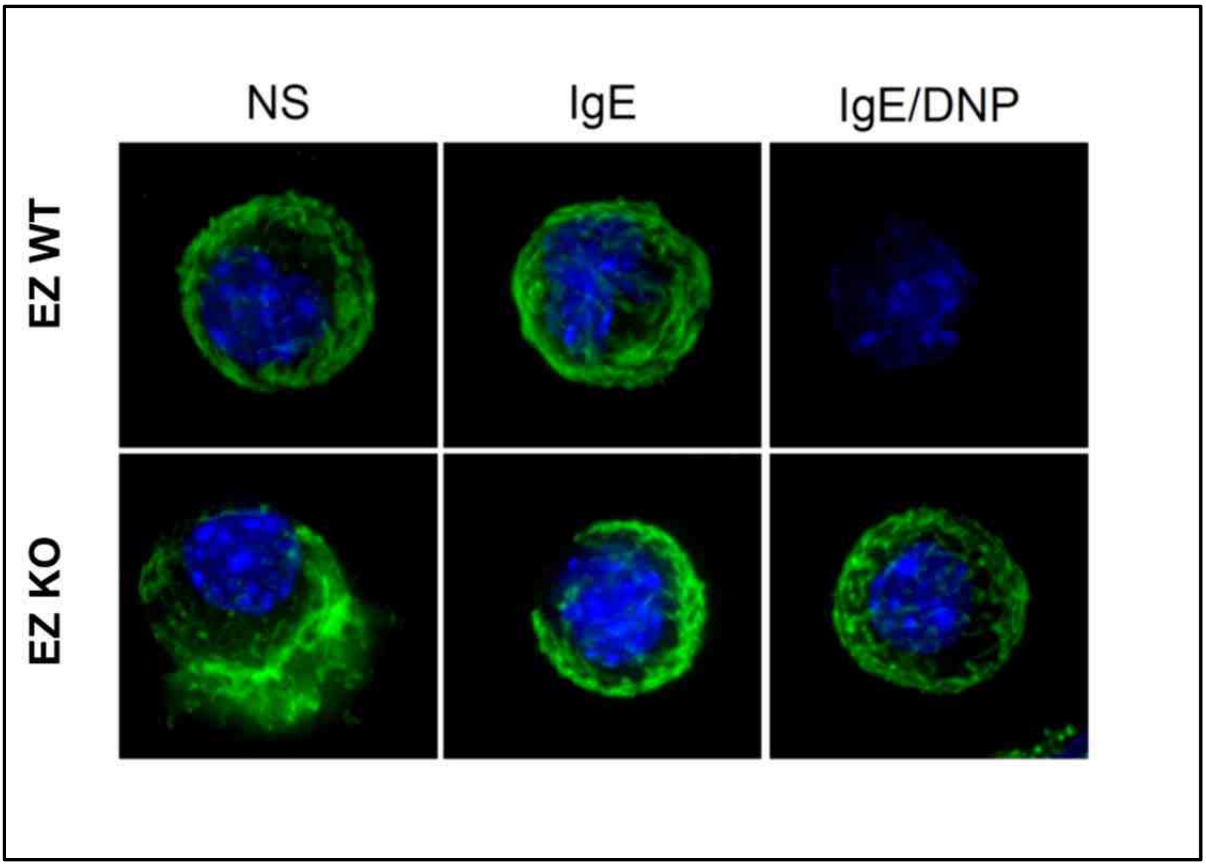


Figure 20f

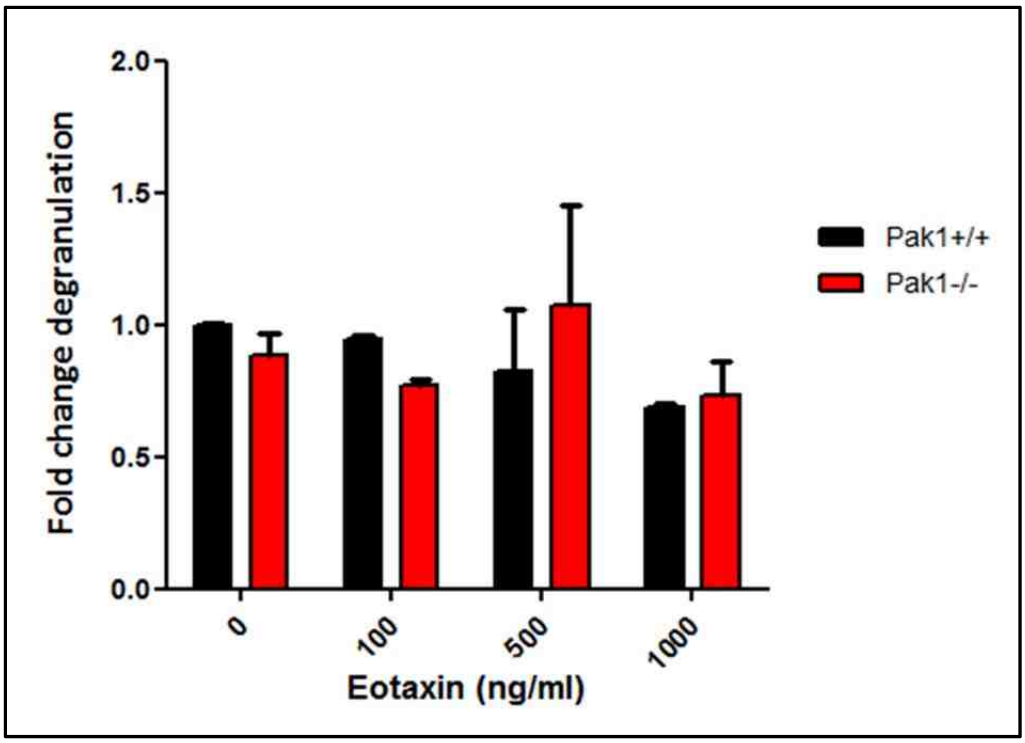

Fold change degranulation
2.0
1.5
1.0
0.5
0.0
0
100
500
1000
Eotaxin (ng/ml)
Pak1+/+
Pak1-/-

Figure 21g

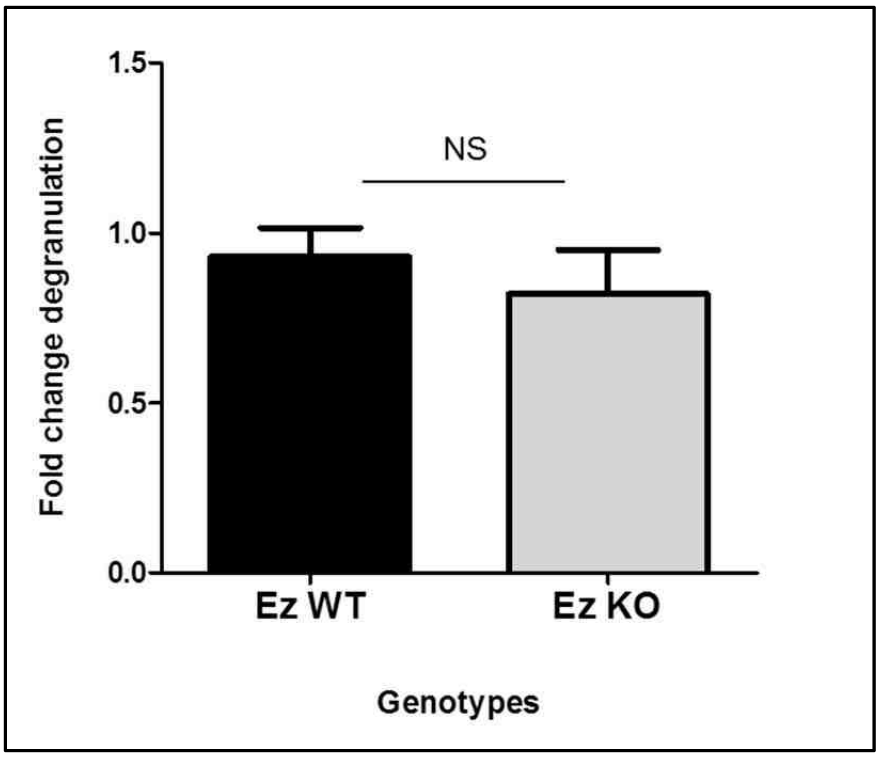

1.5
1.0
0.5
0.0
Fold change degranulation
NS
Ez WT
Ez KO
Genotypes

Figure 21h

Figure 21: Eotaxin-induced eosinophil degranulation *in vitro.* Cytospin slides from whole animal eotaxin-infiltration studies showed degranulation of eosinophils *in vivo* following PBS (Figure 21a) or eotaxin (Figure 21b) treatment. Black arrows indicate degranulated eosinophils (Figure 21b). FACS of *Pak1+/+* peritoneal lavage cell populations followed by giesma-staining of different cell populations (EOS=eosinophil, NE=neutrophil, MONOS=mononuclear cells [Figure 21c]). Our previous work showed the effect of *Pak1* deletion and ectopic transgene expression in bone-marrow mast cells on IgE/DNP-mediated degranulation and β-hexosaminadase activity, N=6 (Figure 21d). Follow-up studies demonstrated the result of genetic deletion of a PAK1 downstream effector, *Ezrin*, on this mast cell degranulation, N=5 (Figure 21e) and F-actin depolymerization (Figure 21f). A colorimetric degranulation assay measuring released EPO activity measures eotaxin-mediated degranulation of *Pak1+/+* and *Pak1-/-* (Figure 21g) and *Ez WT and Ez KO* (Figure 21h) eosinophils *in vitro*. Experiments in (b-h) are an average of at least three experiments, N=3-6.

DISCUSSION AND FUTURE EXPERIMENTS

Eosinophils whose major function had largely been regarded as T-helper 2 cell effectors, are increasingly gaining recognition for their integral role in modulating airway hyperresponsiveness, inflammation, and remodeling through toxic degranulation products, lipid mediators, growth factors, and cytokines. In an OVA-albumin murine AAD model, eosinophil T-helper cell airway epithelial signaling networks establish self-perpetuating chronic inflammation in the airway that recapitulates human asthma. Identifying the mechanisms through which eosinophils infiltrate the lung in asthma would offer novel specific targets for therapeutic intervention. In these studies we have identified PAK1 as a key regulator of allergic airway eosinophil inflammation *in vivo* as well as eotaxin-mediated eosinophil migration *in vitro*.

PAK1 in murine allergic airway inflammation

We have previously demonstrated that genetic ablation of *Pak1* does not alter murine survival or *in vivo* hematopoietic lineage differentiation as assessed by peripheral blood differential leukocyte assessment [223]. Studies done with these *Pak1-/-* mice implicate PAK1 in migration and degranulation of mast cells [121, 192, 218]. These pro-inflammatory processes drive allergic airway inflammation and depend heavily on stimulus-induced F-actin dynamics [192, 223]. While several studies propose a role for PAK1's in force generation in airway smooth muscle hyperresponsiveness in AAD, no reports discuss PAK1's potential role in allergic airway inflammation [218].

The data presented in our studies support a PAK1 kinase-dependent mechanism for eosinophil infiltration *in vivo*. Several Rho-family GTPase pathways have been associated with promoting allergic airway inflammation [125, 259]. Our data implicate PAK1, a known Rho-GTPase molecular substrate in eosinophil airway inflammation characteristic of AAD. We show for the first time an attenuated inflammatory cell response in a *Pak1-/-* OVA-albumin murine AAD model that recapitulates human eosinophil-rich airway inflammation in tissue (Figure 7) and BALF (Figure 8). The levels of eotaxin in the BAL and other T-helper cell derived cytokines from OVA-stimulated splenocytes of *Pak1+/+* and *Pak1-/-* sensitized mice were indistinguishable (Figures 8d and 9) implying a preservation of T-cell responses in this *Pak1-/-* AAD model.

This markedly depressed airway inflammation seems primarily hematopoietically-derived and intrinsic to the eosinophil as both *Pak1+/+* and *Pak1-/-* recipient mice transplanted with *Pak1-/-* bone marrow showed decreased eosinophil inflammation compared to their counterpart recipients transplanted with *Pak1+/+* bone marrow (Figure 10). However, our data cannot rule out the possible role of other factors in the inflammatory environment in recruiting eosinophils to the lung in these sensitized mice as may be suggested by the adoptive bone marrow transplant results. We saw markedly suppressed eosinophil recruitment in BAL fluids from *Pak1-/-* mice transplanted with *Pak1-/-* bone marrow compared to BAL fluids from *Pak1+/+* mice transplanted with *Pak1-/-* bone marrow (Figure 10d-e). Similar to our bone marrow transplantation studies, orthotopic lung transplants demonstrated a trend towards greater lung eosinophil inflammation in host mice

with a *Pak1+/+* hematopoietic system as compared to *Pak1-/-* hosts (Figure 11), however, this trend needs to be clarified by increasing the number in subsequent cohorts of mice that are transplanted.

The exact contribution of PAK1 to allergic airway inflammation remains largely unexplored. Our studies demonstrate PAK1's integral role in eosinophil infiltration of the lungs following allergen-sensitization and challenge. However, PAK1 role in other cells in this allergic process remains understudied. Mast cell degranulation and SCF-mediated migration are impaired in *Pak1-/-* mice [121, 223]. A role of PAK1 in neutrophil chemotaxis is also reported however studies in *Pak1-/-* mice are indicated to clarify its mechanism [260, 261]. One possibility is that PAK1 is selectively effective in promoting allergen-induced infiltration of inflammatory cells. The other possibility is that PAK1 promotes the motility of cells of multiple hematopoietic cell lineages in which case further studies are critical to fully characterizing the immune responses of *Pak1-/-* mice.

Our data in this section are obtained from an OVA-Alum sensitization model that elicits a potent Th2-driven eosinophil response *in vivo*. While this model is a very useful tool in studying eosinophil infiltration, the model does not fully recapitulate human disease. In human bronchial asthma, mast cells inflammation plays a major role in eosinophil infiltration as well as the other hallmarks of asthma while the adjuvant Alum potentiates the Th2 response to override the mast cell effect on development of this allergic airway inflammation [54, 262]. Similarly, here we only examine acute eosinophil inflammation recruitment [21]. Future studies utilizing a chronic sensitization and challenge protocol for *Pak1-/-* mice would

complement and supplement our findings in a model that mimics the mast-cell involvement and chronic accumulation of eosinophils *in vivo*. Since the exact effect of ionizing-radiation on the lung parenchyma from bone marrow transplanted mice is also unknown [259, 263] we also utilized complementary orthotopic lung transplantation studies. Our results from these studies indicate a need for increased numbers in cohorts used in future studies. Likewise, these prospective studies should include groups of isogeneic transplant animals as controls for the surgical procedure. Ultimately, AAD studies in genetic crosses between *Pak1-/-* and $Kit^{W\text{-}sh/W\text{-}sh}$ mice are warranted to study eosinophil inflammation in a mast-cell deficient context. These studies would clarify the distinct roles of PAK1 in mast cells, T-helper cell and eosinophils in AAD.

The process of eosinophil migration and infiltration is critical to the development of chronic airway inflammation in asthma. Eosinophils take up residence in the lungs and modulate chronic inflammatory processes that cause airway tissue injury and remodeling. Our studies have identified PAK1 as a potential transducer of allergen-induced chemotactic signals in the migrating eosinophil. Although PAK1 is likely not the exclusive regulator of eotaxin-mediated migration, it is a promising targetable molecular switch for therapeutic intervention. Our work is a basis for future studies testing PAK1's role in allergen recruitment of eosinophils.

PAK1 in Eotaxin- mediated Eosinophil function

No published reports have examined the role of PAK1 in eosinophil function to date. Building upon our robust *in vivo* AAD model of eosinophil recruitment, we sought to understand the mechanism by which PAK1 modulated eosinophil function. We hypothesized based on our transplant data, that PAK1 plays a role in intrinsic eosinophil activity. We test this question in two *in vivo* murine infiltration models where we standardized the concentration of eotaxin, a potent eosinophil chemoattractant. Our results suggest a role for PAK1 in eotaxin-mediated infiltration of eosinophils *in vivo* (Figure 12). In previous eotaxin infiltration studies, eotaxin preferentially recruited eosinophils relative to other cell lineages to the peritoneum [227, 228]. Both studies identify the importance of a readily available pool of circulating or tissue eosinophils to respond to this eotaxin signal, consequently the degree of eosinophil accumulation *in vivo* was dependent on allergen-sensitization or IL-5 in transgenic mice. Our studies in the non-sensitized animal could therefore be complemented by repeating these studies in the OVA-sensitized mice.

We obtained further evidence on PAK1's role in eotaxin-mediated eosinophil function from bmEos and HL-60 Eos. We demonstrated for the first time that eotaxin phosphorylated and activated PAK1 in murine and HL-60 derived eosinophils (Figures 19a-b). We subsequently sought to generate Pak1 deficient eosinophils and were able to demonstrate a comparable potential of *Pak1-/-* stem and progenitor cells to generate colonies from IL-5 the major eosinophilopoietic cytokine (Figure 14). *Pak1-/-* bmEos generated using IL-5 also demonstrated

indistinguishable expression of CCR3, siglec F as well as many adhesive molecules compared to Pak1+/+ bmEos. Since eosinophil infiltration involves margination, adhesion, and transmigration we assessed PAK1's role in adhesive and motive eosinophil processes. First we assessed PAK1's effect on eotaxin-mediated eosinophil adhesion. Previous studies have shown the importance of eotaxin-mediated adhesion to eosinophil migration to sites of inflammation. These studies have frequently used recombinant fibronectin to mimic adhesion of eosinophils to the endothelial wall [192, 250, 264]. Adhesion in eotaxin-stimulated eosinophils is mediated by integrin association with neighboring cell integrins. We examined the effect *Pak1* deletion had on surface expression of CD11a/αL, Mac1/αMβ2, α4β7, and VLA4/α4β1, integrins well-documented to play important roles in eosinophil trafficking in various body organs [265]. We found comparable percentages of cells expressing similar densities of these integrins (Figures 16a-d). These data suggest that *Pak1* deletion does not affect the surface expression of the important adhesive eosinophil integrins. We subsequently performed functional adhesive studies on fibronectin coated plates in a microfluidic system that recapitulates capillary flow. We confirmed in our studies, that eosinophil preferentially adhered to fibronectin compared to gelatin coated plates, and this adhesion was eotaxin-mediated (Figures 16e-f). However, the adhesion was not different between *Pak1+/+* and *Pak1-/-* eosinophils (Figure 14g). Our studies in adhesion are limited in that they are largely correlative in that functional adhesion of each integrin is not tested. In future studies, we will expand upon these adhesion studies utilizing recombinant ICAM, VCAM, and

MadCAM, the interacting integrins on the endothelium that eosinophils can bind [265, 266].

Our studies also propose a model of eotaxin-mediated eosinophil chemotaxis that depends on PAK1's modulation of F-actin polymerization. Our data indicate that eotaxin: CCR3 signaling phosphorylates and activates PAK1 driving eosinophil chemotaxis (Figure 14). While *Pak1-/-* eosinophils ectopically expressing a full length PAK1 construct had enhanced chemotaxis, ectopic expression of catalytically inactive PAK1, K299R, did not affect *Pak1-/-* eosinophil migration (Figure 16). PAK1 regulates F-actin dynamics via distinct mechanisms [133, 167]. These results suggest an eotaxin-mediated PAK1 kinase-dependent mechanism as opposed to a kinase-independent interaction with downstream proteins in the F-actin changes needed for eosinophil chemotaxis (Figure 16).

Chemokine-induced hematopoietic cell movement depends on cycles of F-actin polymerization, depolymerization and reorganization [267-270]. Our results demonstrate that PAK1 regulates eotaxin-induced eosinophil F-actin polymerization. PAK1 can catalytically control cell motility via cofilin-dependent actin cytoskeletal changes as well as ARP2/3-mediated nucleation and branching of F-actin in different cell types. PAK1's regulation of cofilin in migrating cells is complex [174, 176] and cofilin phosphorylation in eotaxin-stimulated murine eosinophils appears to be spatial-temporally determined. Our data indicate that PAK1 facilitates dephosphorylation of cofilin which may be necessary for F-actin remodeling around the cell (Figure 19). PAK1 directly regulates the p41-Arc

subunit of the ARP2/3 F-actin nucleating complex independent of cofilin [177, 271]. We show that *Pak1* deletion decreases the co-localization of F-actin and the p-34-Arc subunit of this complex suggesting a role for PAK1 in ARP2/3-mediated *de novo* F-actin polymerization. In contrast to these two F-actin binding protein partners of PAK1, p38MAPK, Ezrin/Radixin/Moesin, and Op18/Stathmin do not seem critical to PAK1-dependent eotaxin-mediated eosinophil chemotaxis despite their phosphorylation in the eotaxin-treated eosinophil (Figure 20). Furthermore, migration studies using *Ezrin-/-* eosinophils that showed no attenuated migration make Ezrin an unlikely downstream PAK1 effector critical to eotaxin-mediated eosinophil migration (Figure 20). These data taken together provide initial evidence for a model where eotaxin-induced activation of PAK1's kinase activity results in the catalytic downstream regulation of cofilin and ARP2/3-mediated F-actin changes and eosinophil chemotaxis.

One implication our studies have on the field is the use of small molecule inhibitors to target PAK1 kinase activity to attenuate eosinophil inflammation. The disparate roles of PAK1 and PAK2 in hematopoietic cells, however, remains incompletely understood. PAK1 and PAK2 are highly homologous and of similar substrate specificity *in vitro* [150]. We show that similar to PAK1, PAK2 is phosphorylated at its activation residue with eotaxin-stimulation (Figure 10). However, this activation does not seem critical to eotaxin-mediated eosinophil migration as is shown by our migration studies in *Pak1-/-* ectopically expressing K299R, a dominant negative form of PAKs (Figure 17). Nonetheless, we show in migration studies using IPA3 a pan-PAKs inhibitor severely hampered migration

(Figure 20). These data suggest that loss of *Pak2* may potentiate the loss of eotaxin-mediated migration potential exhibited by *Pak1-/-* eosinophils. However, studies using ectopic expression of K299R and IPA3 are hindered by uncertainties of *in vitro* manipulation and non-selectivity. Other current PAK1 selective inhibitors developed to target cell invasion and metastasis in cancer treatment are unable to select for PAK1 over its group I PAK isoforms PAK2 and PAK3 [244, 272]. Our studies identify a need to evaluate the differential versus redundant roles of PAK1 and its isoform PAK2 in eotaxin-mediated eosinophil migration and infiltration. To adequately address this issue in future studies, we would utilize *Pak1-/-* and *Pak2 floxed* mice crossed with *Mx1Cre* + mice we can begin to assess the differential role of these isoforms in eosinophil inflammation *in vivo*. The results of this study warrant further exploration of the anti-inflammatory attributes of PAK1 inhibition on eosinophil inflammation in a mouse model of AAD.

Lastly, we tested the effect PAK1 has on eotaxin-mediated eosinophil degranulation and mediator release, based on our definitive data in mast-cell degranulation [223]. *In vivo*, in our eotaxin mediated eosinophil peritoneal infiltration model we demonstrate an increased number of degranulated *Pak1+/+* eosinophils compared to *Pak1-/-* eosinophils (Figures 21b-c) [273]. To build upon this *in vivo* phenotype, we used a colorimetric EPO quantifying assay. Surprisingly, we could not elicit increased degranulation over baseline using increasing doses of eotaxin alone (Figure 21g) neither could we demonstrate a difference in degranulation between *Pak1+/+* and *Pak1-/-* nor EZ-WT and EZ-KO

eosinophils (Figures 21g-h). The *in vivo* system is significantly different for eosinophils in contact with other cells and the extracellular matrix when responding to eotaxin [250]. EPO is extremely adhesive and may not readily solubilize during the colorimetric reaction [274, 275]. Consequently, newer studies on eotaxin-mediated eosinophil degranulation utilize multiple degranulation assays [256]. In future degranulation studies we will utilize multiple end-point analysis including MBP, EPO, and β-hexosaminidase to assess degranulation. We will also include adhesive recombinant integrins like ICAM and VCAM or cytokines like IL-5 to potentiate eotaxin-mediated degranulation response.

PAK1 acts as a molecular switch in many physiological cellular processes. In eosinophils our studies demonstrate that PAK1 modulates eosinophil function by transducing the eotaxin:CCR3 signal. We provide novel data implicating PAK1 in regulating actin-mediated inflammatory processes in eosinophil inflammation characteristic of AAD. Our findings have opened up a novel front exploring PAK1 as a potential asthmatic therapeutic. These studies broaden the understanding of the molecular mechanisms underpinning eosinophil inflammation in AAD. Due to relatively innocuous consequences of *Pak1* deletion coupled with its critical role in eosinophil migration, our findings warranted broader investigative exploration of PAK1's role in allergic disease.

REFERENCES

1. CDC/P. *Asthma: A Presentation of Asthma Management and Prevention (Slide Presentation and Speaker Notes)*. 2009 [cited 2012 01/01]; Available from: http://www.cdc.gov/asthma/healthcare.html.
2. WHO. *Asthma: Factsheet No. 307*. 2011; Available from: http://www.who.int/mediacentre/factsheets/fs307/en/index.html.
3. CDC/P. *Flu and People with Asthma*. [Webpage] 2011 [cited 2012 01/01]; Available from: http://www.cdc.gov/flu/asthma/index.htm.
4. Murphy, K.R., et al., *Asthma management and control in the United States: results of the 2009 Asthma Insight and Management survey.* Allergy and asthma proceedings : the official journal of regional and state allergy societies, 2012. **33**(1): p. 54-64.
5. Nathan, R.A., et al., *Comparison of the Asthma in America and Asthma Insight and Management surveys: did asthma burden and care improve in the United States between 1998 and 2009?* Allergy and asthma proceedings : the official journal of regional and state allergy societies, 2012. **33**(1): p. 65-76.
6. Robbins, S.L., V. Kumar, and R.S. Cotran, *Robbins and Cotran pathologic basis of disease*. 8th ed. 2010, Philadelphia, PA: Saunders/Elsevier. xiv, 1450 p.
7. Buc, M., et al., *Immunopathogenesis of bronchial asthma.* Arch Immunol Ther Exp (Warsz), 2009. **57**(5): p. 331-44.
8. Galli, S.J., M. Tsai, and A.M. Piliponsky, *The development of allergic inflammation.* Nature, 2008. **454**(7203): p. 445-54.
9. Costa, J.J., P.F. Weller, and S.J. Galli, *The cells of the allergic response: mast cells, basophils, and eosinophils.* JAMA, 1997. **278**(22): p. 1815-22.
10. Bloemen, K., et al., *The allergic cascade: review of the most important molecules in the asthmatic lung.* Immunol Lett, 2007. **113**(1): p. 6-18.
11. Corry, D.B. and C.G. Irvin, *Promise and pitfalls in animal-based asthma research: building a better mousetrap.* Immunol Res, 2006. **35**(3): p. 279-94.
12. Zuyderduyn, S., et al., *Treating asthma means treating airway smooth muscle cells.* Eur Respir J, 2008. **32**(2): p. 265-74.
13. Bates, J.H., M. Rincon, and C.G. Irvin, *Animal models of asthma.* Am J Physiol Lung Cell Mol Physiol, 2009. **297**(3): p. L401-10.
14. Kroegel, C. and H. Wirtz, *History of guidelines for the diagnosis and management of asthma: from opinion to control.* Drugs, 2009. **69**(9): p. 1189-204.
15. *Expert Panel Report 3 (EPR-3): Guidelines for the Diagnosis and Management of Asthma-Summary Report 2007.* J Allergy Clin Immunol, 2007. **120**(5 Suppl): p. S94-138.
16. Barnes, P.J. and I.M. Adcock, *How do corticosteroids work in asthma?* Ann Intern Med, 2003. **139**(5 Pt 1): p. 359-70.

17. McIvor, R.A., et al., *Potential masking effects of salmeterol on airway inflammation in asthma.* Am J Respir Crit Care Med, 1998. **158**(3): p. 924-30.
18. Weldon, D., *The effects of corticosteroids on bone growth and bone density.* Ann Allergy Asthma Immunol, 2009. **103**(1): p. 3-11; quiz 11-3, 50.
19. Guilbert, T.W., et al., *Long-term inhaled corticosteroids in preschool children at high risk for asthma.* N Engl J Med, 2006. **354**(19): p. 1985-97.
20. Yiamouyiannis, C.A., et al., *Shifts in lung lymphocyte profiles correlate with the sequential development of acute allergic and chronic tolerant stages in a murine asthma model.* Am J Pathol, 1999. **154**(6): p. 1911-21.
21. Bates, J.H., et al., *The synergistic interactions of allergic lung inflammation and intratracheal cationic protein.* Am J Respir Crit Care Med, 2008. **177**(3): p. 261-8.
22. Nygaard, U.C., A. Aase, and M. Lovik, *The allergy adjuvant effect of particles - genetic factors influence antibody and cytokine responses.* Bmc Immunology, 2005. **6**: p. -.
23. Ng, N., et al., *House dust extracts have both T(H)2 adjuvant and tolerogenic activities.* Journal of Allergy and Clinical Immunology, 2006. **117**(5): p. 1074-1081.
24. Horner, A.A., et al., *House dust extracts elicit toll like receptor dependent dendritic cell responses.* Journal of Allergy and Clinical Immunology, 2005. **115**(4): p. 893-893.
25. Barrett, N.A. and K.F. Austen, *Innate cells and T helper 2 cell immunity in airway inflammation.* Immunity, 2009. **31**(3): p. 425-37.
26. Melgert, B.N., et al., *Female mice are more susceptible to the development of allergic airway inflammation than male mice.* Clinical and Experimental Allergy, 2005. **35**(11): p. 1496-1503.
27. Hamada K, G.C., Goldman A, Kobzik L, *Resistance of very young mice to inhaled allergen sensitization is overcome by coexposure to an air-pollutant aerosol.* Am J Respir Crit Care Med, 2000 **Apr**: p. 1285-93.
28. Sakai, K., et al., *Effect of different sensitizing doses of antigen in a murine model of atopic asthma.* Clin Exp Immunol, 1999. **118**(1): p. 9-15.
29. Morokata, T., et al., *C57BL/6 mice are more susceptible to antigen-induced pulmonary eosinophilia than BALB/c mice, irrespective of systemic T helper 1/T helper 2 responses.* Immunology, 1999. **98**(3): p. 345-51.
30. Jeong, S.K., et al., *Mite and cockroach allergens activate protease-activated receptor 2 and delay epidermal permeability barrier recovery.* J Invest Dermatol, 2008. **128**(8): p. 1930-9.
31. Shakib, F., A.M. Ghaemmaghami, and H.F. Sewell, *The molecular basis of allergenicity.* Trends Immunol, 2008. **29**(12): p. 633-42.
32. Antony, A.B., R.S. Tepper, and K.A. Mohammed, *Cockroach extract antigen increases bronchial airway epithelial permeability.* J Allergy Clin Immunol, 2002. **110**(4): p. 589-95.

33. Bhalla, D.K., et al., *Cigarette smoke, inflammation, and lung injury: a mechanistic perspective.* J Toxicol Environ Health B Crit Rev, 2009. **12**(1): p. 45-64.
34. Samuelsen, M., U.C. Nygaard, and M. Lovik, *Allergy adjuvant effect of particles from wood smoke and road traffic.* Toxicology, 2008. **246**(2-3): p. 124-31.
35. Vercelli, D., *Discovering susceptibility genes for asthma and allergy.* Nat Rev Immunol, 2008. **8**(3): p. 169-82.
36. Suarez, C.J., N.J. Parker, and P.W. Finn, *Innate immune mechanism in allergic asthma.* Curr Allergy Asthma Rep, 2008. **8**(5): p. 451-9.
37. Sandor, F. and M. Buc, *Toll-like receptors. III. Biological significance and impact for human medicine.* Folia Biol (Praha), 2005. **51**(6): p. 198-203.
38. Kiss, A., et al., *A new mechanism regulating the initiation of allergic airway inflammation.* J Allergy Clin Immunol, 2007. **120**(2): p. 334-42.
39. Barnes, P.J., *Role of GATA-3 in allergic diseases.* Curr Mol Med, 2008. **8**(5): p. 330-4.
40. Perros, F., et al., *Blockade of CCR4 in a humanized model of asthma reveals a critical role for DC-derived CCL17 and CCL22 in attracting Th2 cells and inducing airway inflammation.* Allergy, 2009. **64**(7): p. 995-1002.
41. Holgate, S.T. and R. Polosa, *Treatment strategies for allergy and asthma.* Nat Rev Immunol, 2008. **8**(3): p. 218-30.
42. Robinson, D.S., et al., *Predominant TH2-like bronchoalveolar T-lymphocyte population in atopic asthma.* N Engl J Med, 1992. **326**(5): p. 298-304.
43. Cousins, D.J., T.H. Lee, and D.Z. Staynov, *Cytokine coexpression during human Th1/Th2 cell differentiation: direct evidence for coordinated expression of Th2 cytokines.* J Immunol, 2002. **169**(5): p. 2498-506.
44. Cohn, L., J.S. Tepper, and K. Bottomly, *IL-4-independent induction of airway hyperresponsiveness by Th2, but not Th1, cells.* J Immunol, 1998. **161**(8): p. 3813-6.
45. Gavett, S.H., et al., *Depletion of murine CD4+ T lymphocytes prevents antigen-induced airway hyperreactivity and pulmonary eosinophilia.* Am J Respir Cell Mol Biol, 1994. **10**(6): p. 587-93.
46. Cohn, L., et al., *IL-4 promotes airway eosinophilia by suppressing IFN-gamma production: defining a novel role for IFN-gamma in the regulation of allergic airway inflammation.* J Immunol, 2001. **166**(4): p. 2760-7.
47. Cohn, L., et al., *Induction of airway mucus production By T helper 2 (Th2) cells: a critical role for interleukin 4 in cell recruitment but not mucus production.* J Exp Med, 1997. **186**(10): p. 1737-47.
48. Ishizaka, K., T. Ishizaka, and M.M. Hornbrook, *Physicochemical properties of reaginic antibody. V. Correlation of reaginic activity wth gamma-E-globulin antibody.* J Immunol, 1966. **97**(6): p. 840-53.
49. Aubry, J.P., et al., *CD23 interacts with a new functional extracytoplasmic domain involving N-linked oligosaccharides on CD21.* J Immunol, 1994. **152**(12): p. 5806-13.

50. Gould, H.J. and B.J. Sutton, *IgE in allergy and asthma today.* Nat Rev Immunol, 2008. **8**(3): p. 205-17.
51. Yu, P., et al., *Negative feedback regulation of IgE synthesis by murine CD23.* Nature, 1994. **369**(6483): p. 753-6.
52. Tsitoura, D.C., et al., *Critical role of B cells in the development of T cell tolerance to aeroallergens.* International Immunology, 2002. **14**(6): p. 659-667.
53. Coyle, A.J., et al., *Central role of immunoglobulin (Ig) E in the induction of lung eosinophil infiltration and T helper 2 cell cytokine production: inhibition by a non-anaphylactogenic anti-IgE antibody.* The Journal of experimental medicine, 1996. **183**(4): p. 1303-10.
54. Yu, M., et al., *Mast cells can promote the development of multiple features of chronic asthma in mice.* J Clin Invest, 2006. **116**(6): p. 1633-41.
55. Wu, W., et al., *Silencing of c-kit with small interference RNA attenuates inflammation in a murine model of allergic asthma.* International journal of molecular medicine, 2012. **30**(1): p. 63-8.
56. Skowron-zwarg, M., et al., *Interleukin-13 interferes with CFTR and AQP5 expression and localization during human airway epithelial cell differentiation.* Exp Cell Res, 2007. **313**(12): p. 2695-702.
57. Gomperts, B.N., et al., *IL-13 regulates cilia loss and foxj1 expression in human airway epithelium.* Am J Respir Cell Mol Biol, 2007. **37**(3): p. 339-46.
58. Laoukili, J., et al., *IL-13 alters mucociliary differentiation and ciliary beating of human respiratory epithelial cells.* J Clin Invest, 2001. **108**(12): p. 1817-24.
59. Moore, P.E., et al., *IL-13 and IL-4 cause eotaxin release in human airway smooth muscle cells: a role for ERK.* Am J Physiol Lung Cell Mol Physiol, 2002. **282**(4): p. L847-53.
60. Grunstein, M.M., et al., *IL-13-dependent autocrine signaling mediates altered responsiveness of IgE-sensitized airway smooth muscle.* Am J Physiol Lung Cell Mol Physiol, 2002. **282**(3): p. L520-8.
61. Soumelis, V., et al., *Human epithelial cells trigger dendritic cell mediated allergic inflammation by producing TSLP.* Nat Immunol, 2002. **3**(7): p. 673-80.
62. Seshasayee, D., et al., *In vivo blockade of OX40 ligand inhibits thymic stromal lymphopoietin driven atopic inflammation.* J Clin Invest, 2007. **117**(12): p. 3868-78.
63. Allakhverdi, Z., et al., *Thymic stromal lymphopoietin is released by human epithelial cells in response to microbes, trauma, or inflammation and potently activates mast cells.* J Exp Med, 2007. **204**(2): p. 253-8.
64. Zhou, B., et al., *Thymic stromal lymphopoietin as a key initiator of allergic airway inflammation in mice.* Nat Immunol, 2005. **6**(10): p. 1047-53.
65. Fort, M.M., et al., *IL-25 induces IL-4, IL-5, and IL-13 and Th2-associated pathologies in vivo.* Immunity, 2001. **15**(6): p. 985-95.

66. Swaidani, S., et al., *The critical role of epithelial-derived Act1 in IL-17- and IL-25-mediated pulmonary inflammation.* J Immunol, 2009. **182**(3): p. 1631-40.
67. Tamachi, T., et al., *IL-25 enhances allergic airway inflammation by amplifying a TH2 cell-dependent pathway in mice.* J Allergy Clin Immunol, 2006. **118**(3): p. 606-14.
68. Hurst, S.D., et al., *New IL-17 family members promote Th1 or Th2 responses in the lung: in vivo function of the novel cytokine IL-25.* J Immunol, 2002. **169**(1): p. 443-53.
69. Kurowska-Stolarska, M., et al., *IL-33 induces antigen-specific IL-5+ T cells and promotes allergic-induced airway inflammation independent of IL-4.* J Immunol, 2008. **181**(7): p. 4780-90.
70. Pecaric-Petkovic, T., et al., *Human basophils and eosinophils are the direct target leukocytes of the novel IL-1 family member IL-33.* Blood, 2009. **113**(7): p. 1526-34.
71. Suzukawa, M., et al., *An IL-1 cytokine member, IL-33, induces human basophil activation via its ST2 receptor.* J Immunol, 2008. **181**(9): p. 5981-9.
72. Korsgren, M., et al., *Natural killer cells determine development of allergen-induced eosinophilic airway inflammation in mice.* J Exp Med, 1999. **189**(3): p. 553-62.
73. Yoshizumi, M., et al., *Relationships between Cytokine Profiles and Signaling Pathways (IkappaB Kinase and p38 MAPK) in Parainfluenza Virus-Infected Lung Fibroblasts.* Frontiers in microbiology, 2010. **1**: p. 124.
74. Radinger, M., et al., *Local proliferation and mobilization of CCR3(+) CD34(+) eosinophil-lineage-committed cells in the lung.* Immunology, 2011. **132**(1): p. 144-54.
75. Panopoulos, A.D. and S.S. Watowich, *Granulocyte colony-stimulating factor: Molecular mechanisms of action during steady state and 'emergency' hematopoiesis.* Cytokine, 2008. **42**(3): p. 277-288.
76. Ponath, P.D., et al., *Cloning of the human eosinophil chemoattractant, eotaxin. Expression, receptor binding, and functional properties suggest a mechanism for the selective recruitment of eosinophils.* J Clin Invest, 1996. **97**(3): p. 604-12.
77. Sanderson, C.J., *Interleukin-5, eosinophils, and disease.* Blood, 1992. **79**(12): p. 3101-9.
78. Collins, P.D., et al., *Cooperation between interleukin-5 and the chemokine eotaxin to induce eosinophil accumulation in vivo.* J Exp Med, 1995. **182**(4): p. 1169-74.
79. Gauvreau, G.M., A.K. Ellis, and J.A. Denburg, *Haemopoietic processes in allergic disease: eosinophil/basophil development.* Clinical and experimental allergy : journal of the British Society for Allergy and Clinical Immunology, 2009. **39**(9): p. 1297-306.

80. Du, J., et al., *Novel combinatorial interactions of GATA-1, PU.1, and C/EBPepsilon isoforms regulate transcription of the gene encoding eosinophil granule major basic protein.* J Biol Chem, 2002. **277**(45): p. 43481-94.
81. Zimmermann, N., et al., *Analysis of the CC chemokine receptor 3 gene reveals a complex 5' exon organization, a functional role for untranslated exon 1, and a broadly active promoter with eosinophil-selective elements.* Blood, 2000. **96**(7): p. 2346-54.
82. Lee, J.J., et al., *Defining a link with asthma in mice congenitally deficient in eosinophils.* Science, 2004. **305**(5691): p. 1773-6.
83. Walsh, E.R., et al., *Strain-specific requirement for eosinophils in the recruitment of T cells to the lung during the development of allergic asthma.* J Exp Med, 2008. **205**(6): p. 1285-92.
84. Gombart, A.F., et al., *Regulation of neutrophil and eosinophil secondary granule gene expression by transcription factors C/EBP epsilon and PU.1.* Blood, 2003. **101**(8): p. 3265-73.
85. Rankin, S.M., D.M. Conroy, and T.J. Williams, *Eotaxin and eosinophil recruitment: implications for human disease.* Mol Med Today, 2000. **6**(1): p. 20-7.
86. Zimmermann, N., et al., *Chemokines in asthma: cooperative interaction between chemokines and IL-13.* J Allergy Clin Immunol, 2003. **111**(2): p. 227-42; quiz 243.
87. Rothenberg, M.E., et al., *Characterization of a human eosinophil proteoglycan, and augmentation of its biosynthesis and size by interleukin 3, interleukin 5, and granulocyte/macrophage colony stimulating factor.* J Biol Chem, 1988. **263**(27): p. 13901-8.
88. Nakamura, H., et al., *Variant eotaxin: its effects on the asthma phenotype.* J Allergy Clin Immunol, 2001. **108**(6): p. 946-53.
89. Fulkerson, P.C., et al., *CXCL9 inhibits eosinophil responses by a CCR3- and Rac2-dependent mechanism.* Blood, 2005. **106**(2): p. 436-43.
90. Kay, A.B., S. Phipps, and D.S. Robinson, *A role for eosinophils in airway remodelling in asthma.* Trends Immunol, 2004. **25**(9): p. 477-82.
91. Shinkai, K., M. Mohrs, and R.M. Locksley, *Helper T cells regulate type-2 innate immunity in vivo.* Nature, 2002. **420**(6917): p. 825-9.
92. Mattes, J., et al., *Intrinsic defect in T cell production of interleukin (IL)-13 in the absence of both IL-5 and eotaxin precludes the development of eosinophilia and airways hyperreactivity in experimental asthma.* The Journal of experimental medicine, 2002. **195**(11): p. 1433-44.
93. Wardlaw, A.J., *Molecular basis for selective eosinophil trafficking in asthma: A multistep paradigm.* The Journal of allergy and clinical immunology, 1999. **104**(5): p. 917-26.
94. Knol, E.F., et al., *Comparison of human eosinophil and neutrophil adhesion to endothelial cells under nonstatic conditions. Role of L-selectin.* Journal of immunology, 1994. **153**(5): p. 2161-7.

95. Bochner, B.S., *Road signs guiding leukocytes along the inflammation superhighway.* The Journal of allergy and clinical immunology, 2000. **106**(5): p. 817-28.
96. Zhang, M., et al., *Defining the in vivo function of Siglec-F, a CD33-related Siglec expressed on mouse eosinophils.* Blood, 2007. **109**(10): p. 4280-7.
97. Bandeira-Melo, C. and P.F. Weller, *Eosinophils and cysteinyl leukotrienes.* Prostaglandins Leukot Essent Fatty Acids, 2003. **69**(2-3): p. 135-43.
98. Flood-Page, P.T., et al., *Eosinophil's role remains uncertain as anti-interleukin-5 only partially depletes numbers in asthmatic airway.* Am J Respir Crit Care Med, 2003. **167**(2): p. 199-204.
99. Pantanowitz, L. and K. Balogh, *Charcot-Leyden crystals: pathology and diagnostic utility.* Ear Nose Throat J, 2004. **83**(7): p. 489-90.
100. Hisamatsu, K., et al., *Cytotoxicity of human eosinophil granule major basic protein to human nasal sinus mucosa in vitro.* J Allergy Clin Immunol, 1990. **86**(1): p. 52-63.
101. Ackerman, S.J., et al., *Comparative toxicity of purified human eosinophil granule cationic proteins for schistosomula of Schistosoma mansoni.* Am J Trop Med Hyg, 1985. **34**(4): p. 735-45.
102. Wang, J. and A. Slungaard, *Role of eosinophil peroxidase in host defense and disease pathology.* Arch Biochem Biophys, 2006. **445**(2): p. 256-60.
103. Hogan, S.P., et al., *Eosinophils: biological properties and role in health and disease.* Clin Exp Allergy, 2008. **38**(5): p. 709-50.
104. Pegorier, S., et al., *Eosinophil-derived cationic proteins activate the synthesis of remodeling factors by airway epithelial cells.* J Immunol, 2006. **177**(7): p. 4861-9.
105. Costello, R.W., et al., *Localization of eosinophils to airway nerves and effect on neuronal M2 muscarinic receptor function.* The American journal of physiology, 1997. **273**(1 Pt 1): p. L93-103.
106. Lacy, P., et al., *Fusion protein vesicle-associated membrane protein 2 is implicated in IFN-gamma-induced piecemeal degranulation in human eosinophils from atopic individuals.* The Journal of allergy and clinical immunology, 2001. **107**(4): p. 671-8.
107. Karawajczyk, M., et al., *Piecemeal degranulation of peripheral blood eosinophils: a study of allergic subjects during and out of the pollen season.* American Journal of Respiratory Cell and Molecular Biology, 2000. **23**(4): p. 521-9.
108. Tanaka, H., et al., *Role of interleukin-5 and eosinophils in allergen-induced airway remodeling in mice.* Am J Respir Cell Mol Biol, 2004. **31**(1): p. 62-8.
109. Moqbel, R. and P. Lacy, *New concepts in effector functions of eosinophil cytokines.* Clin Exp Allergy, 2000. **30**(12): p. 1667-71.
110. Lacy, P. and R. Moqbel, *Eosinophil cytokines.* Chem Immunol, 2000. **76**: p. 134-55.
111. Burgel, P.R., et al., *Human eosinophils induce mucin production in airway epithelial cells via epidermal growth factor receptor activation.* Journal of immunology, 2001. **167**(10): p. 5948-54.

112. Puxeddu, I., et al., *Human peripheral blood eosinophils induce angiogenesis.* The international journal of biochemistry & cell biology, 2005. **37**(3): p. 628-36.
113. Rothenberg, M.E. and S.P. Hogan, *The eosinophil.* Annu Rev Immunol, 2006. **24**: p. 147-74.
114. Duan, W., et al., *Inhaled p38alpha mitogen-activated protein kinase antisense oligonucleotide attenuates asthma in mice.* Am J Respir Crit Care Med, 2005. **171**(6): p. 571-8.
115. Chopra, P., et al., *Therapeutic potential of inhaled p38 mitogen-activated protein kinase inhibitors for inflammatory pulmonary diseases.* Expert Opin Investig Drugs, 2008. **17**(10): p. 1411-25.
116. Eynott, P.R., et al., *Allergen-induced inflammation and airway epithelial and smooth muscle cell proliferation: role of Jun N-terminal kinase.* British journal of pharmacology, 2003. **140**(8): p. 1373-80.
117. Kampen, G.T., et al., *Eotaxin induces degranulation and chemotaxis of eosinophils through the activation of ERK2 and p38 mitogen-activated protein kinases.* Blood, 2000. **95**(6): p. 1911-7.
118. Underwood, D.C., et al., *SB 239063, a potent p38 MAP kinase inhibitor, reduces inflammatory cytokine production, airways eosinophil infiltration, and persistence.* The Journal of pharmacology and experimental therapeutics, 2000. **293**(1): p. 281-8.
119. Gamell, C., et al., *The p38/MK2/Hsp25 pathway is required for BMP-2-induced cell migration.* PLoS One, 2011. **6**(1): p. e16477.
120. Birukov, K.G., et al., *Shear stress-mediated cytoskeletal remodeling and cortactin translocation in pulmonary endothelial cells.* American Journal of Respiratory Cell and Molecular Biology, 2002. **26**(4): p. 453-64.
121. McDaniel, A.S., et al., *Pak1 regulates multiple c-Kit mediated Ras-MAPK gain-in-function phenotypes in Nf1+/- mast cells.* Blood, 2008. **112**(12): p. 4646-54.
122. Rousseau, S., et al., *CXCL12 and C5a trigger cell migration via a PAK1/2-p38alpha MAPK-MAPKAP-K2-HSP27 pathway.* Cellular signalling, 2006. **18**(11): p. 1897-905.
123. Chiba, Y. and M. Misawa, *The role of RhoA-mediated Ca2+ sensitization of bronchial smooth muscle contraction in airway hyperresponsiveness.* J Smooth Muscle Res, 2004. **40**(4-5): p. 155-67.
124. Schaafsma, D., et al., *Allergic sensitization enhances the contribution of Rho-kinase to airway smooth muscle contraction.* Br J Pharmacol, 2004. **143**(4): p. 477-84.
125. Taki, F., et al., *Effects of Rho-kinase inactivation on eosinophilia and hyper-reactivity in murine airways by allergen challenges.* Clin Exp Allergy, 2007. **37**(4): p. 599-607.
126. Takeda, N., et al., *Role of RhoA inactivation in reduced cell proliferation of human airway smooth muscle by simvastatin.* Am J Respir Cell Mol Biol, 2006. **35**(6): p. 722-9.

127. Henry, P.J., T.S. Mann, and R.G. Goldie, *A rho kinase inhibitor, Y-27632 inhibits pulmonary eosinophilia, bronchoconstriction and airways hyperresponsiveness in allergic mice.* Pulm Pharmacol Ther, 2005. **18**(1): p. 67-74.
128. Tan, B.L., et al., *Genetic evidence for convergence of c-Kit- and alpha4 integrin-mediated signals on class IA PI-3kinase and the Rac pathway in regulating integrin-directed migration in mast cells.* Blood, 2003. **101**(12): p. 4725-32.
129. Yang, F.C., et al., *Rac2 stimulates Akt activation affecting BAD/Bcl-XL expression while mediating survival and actin function in primary mast cells.* Immunity, 2000. **12**(5): p. 557-568.
130. Cho, S.H., et al., *Rac and protein kinase C-delta regulate ERKs and cytosolic phospholipase A2 in FcepsilonRI signaling to cysteinyl leukotriene synthesis in mast cells.* J Immunol, 2004. **173**(1): p. 624-31.
131. Jaffer, Z.M. and J. Chernoff, *p21-activated kinases: three more join the Pak.* Int J Biochem Cell Biol, 2002. **34**(7): p. 713-7.
132. Zhao, Z.S., et al., *A conserved negative regulatory region in alphaPAK: inhibition of PAK kinases reveals their morphological roles downstream of Cdc42 and Rac1.* Mol Cell Biol, 1998. **18**(4): p. 2153-63.
133. Frost, J.A., et al., *Differential effects of PAK1-activating mutations reveal activity-dependent and -independent effects on cytoskeletal regulation.* J Biol Chem, 1998. **273**(43): p. 28191-8.
134. Manser, E., et al., *A brain serine/threonine protein kinase activated by Cdc42 and Rac1.* Nature, 1994. **367**(6458): p. 40-6.
135. Bagrodia, S. and R.A. Cerione, *Pak to the future.* Trends Cell Biol, 1999. **9**(9): p. 350-5.
136. Lei, M., et al., *Structure of PAK1 in an autoinhibited conformation reveals a multistage activation switch.* Cell, 2000. **102**(3): p. 387-97.
137. Buchwald, G., et al., *Conformational switch and role of phosphorylation in PAK activation.* Mol Cell Biol, 2001. **21**(15): p. 5179-89.
138. Bokoch, G.M., et al., *A GTPase-independent mechanism of p21-activated kinase activation. Regulation by sphingosine and other biologically active lipids.* J Biol Chem, 1998. **273**(14): p. 8137-44.
139. King, C.C., et al., *p21-activated kinase (PAK1) is phosphorylated and activated by 3-phosphoinositide-dependent kinase-1 (PDK1).* J Biol Chem, 2000. **275**(52): p. 41201-9.
140. Morreale, A., et al., *Structure of Cdc42 bound to the GTPase binding domain of PAK.* Nat Struct Biol, 2000. **7**(5): p. 384-8.
141. Gatti, A., et al., *Multisite autophosphorylation of p21-activated protein kinase gamma-PAK as a function of activation.* J Biol Chem, 1999. **274**(12): p. 8022-8.
142. Chong, C., et al., *The mechanism of PAK activation. Autophosphorylation events in both regulatory and kinase domains control activity.* J Biol Chem, 2001. **276**(20): p. 17347-53.

143. Oskeritzian, C.A., S. Milstien, and S. Spiegel, *Sphingosine-1-phosphate in allergic responses, asthma and anaphylaxis.* Pharmacol Ther, 2007. **115**(3): p. 390-9.
144. Rivera, J., R.L. Proia, and A. Olivera, *The alliance of sphingosine-1-phosphate and its receptors in immunity.* Nat Rev Immunol, 2008. **8**(10): p. 753-63.
145. Buday, L., L. Wunderlich, and P. Tamas, *The Nck family of adapter proteins: regulators of actin cytoskeleton.* Cell Signal, 2002. **14**(9): p. 723-31.
146. Puto, L.A., et al., *p21-activated kinase 1 (PAK1) interacts with the Grb2 adapter protein to couple to growth factor signaling.* J Biol Chem, 2003. **278**(11): p. 9388-93.
147. Lu, W., et al., *Activation of Pak by membrane localization mediated by an SH3 domain from the adaptor protein Nck.* Curr Biol, 1997. **7**(2): p. 85-94.
148. Mayhew, M.W., et al., *Identification of phosphorylation sites in betaPIX and PAK1.* J Cell Sci, 2007. **120**(Pt 22): p. 3911-8.
149. Rudel, T. and G.M. Bokoch, *Membrane and morphological changes in apoptotic cells regulated by caspase-mediated activation of PAK2.* Science, 1997. **276**(5318): p. 1571-4.
150. Bokoch, G.M., *Biology of the p21-activated kinases.* Annu Rev Biochem, 2003. **72**: p. 743-81.
151. Eswaran, J., et al., *UnPAKing the class differences among p21-activated kinases.* Trends Biochem Sci, 2008. **33**(8): p. 394-403.
152. Sells, M.A., A. Pfaff, and J. Chernoff, *Temporal and spatial distribution of activated Pak1 in fibroblasts.* J Cell Biol, 2000. **151**(7): p. 1449-58.
153. Dharmawardhane, S., et al., *Localization of p21-activated kinase 1 (PAK1) to pinocytic vesicles and cortical actin structures in stimulated cells.* J Cell Biol, 1997. **138**(6): p. 1265-78.
154. Dharmawardhane, S., et al., *Localization of p21-activated kinase 1 (PAK1) to pseudopodia, membrane ruffles, and phagocytic cups in activated human neutrophils.* J Leukoc Biol, 1999. **66**(3): p. 521-7.
155. Sells, M.A., J.T. Boyd, and J. Chernoff, *p21-activated kinase 1 (Pak1) regulates cell motility in mammalian fibroblasts.* J Cell Biol, 1999. **145**(4): p. 837-49.
156. Sells, M.A., et al., *Human p21-activated kinase (Pak1) regulates actin organization in mammalian cells.* Curr Biol, 1997. **7**(3): p. 202-10.
157. Dharmawardhane, S., et al., *Regulation of macropinocytosis by p21-activated kinase-1.* Mol Biol Cell, 2000. **11**(10): p. 3341-52.
158. Manser, E., et al., *Expression of constitutively active alpha-PAK reveals effects of the kinase on actin and focal complexes.* Mol Cell Biol, 1997. **17**(3): p. 1129-43.
159. Hing, H., et al., *Pak functions downstream of Dock to regulate photoreceptor axon guidance in Drosophila.* Cell, 1999. **97**(7): p. 853-63.

160. Chen, W., et al., *The Caenorhabditis elegans p21-activated kinase (CePAK) colocalizes with CeRac1 and CDC42Ce at hypodermal cell boundaries during embryo elongation.* J Biol Chem, 1996. **271**(42): p. 26362-8.
161. Leberer, E., et al., *Virulence and hyphal formation of Candida albicans require the Ste20p-like protein kinase CaCla4p.* Curr Biol, 1997. **7**(8): p. 539-46.
162. Weiss, E.L., et al., *Chemical genetic analysis of the budding-yeast p21-activated kinase Cla4p.* Nat Cell Biol, 2000. **2**(10): p. 677-85.
163. Arias-Romero, L.E., et al., *EhPAK2, a novel p21-activated kinase, is required for collagen invasion and capping in Entamoeba histolytica.* Mol Biochem Parasitol, 2006. **149**(1): p. 17-26.
164. Klopocka, W., J. Moraczewska, and M.J. Redowicz, *Characterisation of the Rac/PAK pathway in Amoeba proteus.* Protoplasma, 2005. **225**(1-2): p. 77-84.
165. Nagel, M., et al., *Role of p21-activated kinase in cell polarity and directional mesendoderm migration in the Xenopus gastrula.* Dev Dyn, 2009. **238**(7): p. 1709-26.
166. Manser, E., et al., *PAK kinases are directly coupled to the PIX family of nucleotide exchange factors.* Molecular cell, 1998. **1**(2): p. 183-92.
167. Adam, L., et al., *Regulation of microfilament reorganization and invasiveness of breast cancer cells by kinase dead p21-activated kinase-1.* J Biol Chem, 2000. **275**(16): p. 12041-50.
168. Ito, M., et al., *P21-activated kinase 1: a new molecular marker for intravesical recurrence after transurethral resection of bladder cancer.* J Urol, 2007. **178**(3 Pt 1): p. 1073-9.
169. Delorme, V., et al., *Cofilin activity downstream of Pak1 regulates cell protrusion efficiency by organizing lamellipodium and lamella actin networks.* Dev Cell, 2007. **13**(5): p. 646-62.
170. Chan, A.Y., et al., *Role of cofilin in epidermal growth factor-stimulated actin polymerization and lamellipod protrusion.* The Journal of cell biology, 2000. **148**(3): p. 531-42.
171. Kumar, G., et al., *Over-expression of S4D mutant of Leishmania ADF/cofilin impairs flagellum assembly by affecting actin dynamics.* Eukaryotic cell, 2012.
172. Tang, W., et al., *A PLCbeta/PI3Kgamma-GSK3 signaling pathway regulates cofilin phosphatase slingshot2 and neutrophil polarization and chemotaxis.* Developmental cell, 2011. **21**(6): p. 1038-50.
173. Vartiainen, M.K., et al., *The three mouse actin-depolymerizing factor/cofilins evolved to fulfill cell-type-specific requirements for actin dynamics.* Molecular biology of the cell, 2002. **13**(1): p. 183-94.
174. Coniglio, S.J., S. Zavarella, and M.H. Symons, *Pak1 and Pak2 mediate tumor cell invasion through distinct signaling mechanisms.* Molecular and cellular biology, 2008. **28**(12): p. 4162-72.

175. Starinsky-Elbaz, S., et al., *The pre-GAP-related domain of neurofibromin regulates cell migration through the LIM kinase/cofilin pathway.* Molecular and cellular neurosciences, 2009. **42**(4): p. 278-87.
176. Edwards, D.C., et al., *Activation of LIM-kinase by Pak1 couples Rac/Cdc42 GTPase signalling to actin cytoskeletal dynamics.* Nature cell biology, 1999. **1**(5): p. 253-9.
177. Vadlamudi, R.K., et al., *p41-Arc subunit of human Arp2/3 complex is a p21-activated kinase-1-interacting substrate.* EMBO Rep, 2004. **5**(2): p. 154-60.
178. Pantaloni, D., et al., *The Arp2/3 complex branches filament barbed ends: functional antagonism with capping proteins.* Nature cell biology, 2000. **2**(7): p. 385-91.
179. Machesky, L.M., et al., *Scar, a WASp-related protein, activates nucleation of actin filaments by the Arp2/3 complex.* Proceedings of the National Academy of Sciences of the United States of America, 1999. **96**(7): p. 3739-44.
180. Wu, C., et al., *Arp2/3 is critical for lamellipodia and response to extracellular matrix cues but is dispensable for chemotaxis.* Cell, 2012. **148**(5): p. 973-87.
181. Mullins, R.D., J.A. Heuser, and T.D. Pollard, *The interaction of Arp2/3 complex with actin: nucleation, high affinity pointed end capping, and formation of branching networks of filaments.* Proceedings of the National Academy of Sciences of the United States of America, 1998. **95**(11): p. 6181-6.
182. Fehon, R.G., A.I. McClatchey, and A. Bretscher, *Organizing the cell cortex: the role of ERM proteins.* Nat Rev Mol Cell Biol, 2010. **11**(4): p. 276-87.
183. McClatchey, A.I. and R.G. Fehon, *Merlin and the ERM proteins--regulators of receptor distribution and signaling at the cell cortex.* Trends Cell Biol, 2009. **19**(5): p. 198-206.
184. Shaffer, M.H., et al., *Ezrin and moesin function together to promote T cell activation.* J Immunol, 2009. **182**(2): p. 1021-32.
185. Shaffer, M.H., et al., *Ezrin is highly expressed in early thymocytes, but dispensable for T cell development in mice.* PLoS One, 2010. **5**(8): p. e12404.
186. Belkina, N.V., et al., *LOK is a major ERM kinase in resting lymphocytes and regulates cytoskeletal rearrangement through ERM phosphorylation.* Proceedings of the National Academy of Sciences of the United States of America, 2009. **106**(12): p. 4707-12.
187. Brown, K.L., et al., *Regulation of hyaluronan binding by F-actin and colocalization of CD44 and phosphorylated ezrin/radixin/moesin (ERM) proteins in myeloid cells.* Experimental cell research, 2005. **303**(2): p. 400-14.
188. Saotome, I., M. Curto, and A.I. McClatchey, *Ezrin is essential for epithelial organization and villus morphogenesis in the developing intestine.* Dev Cell, 2004. **6**(6): p. 855-64.

189. Bonilha, V.L., et al., *Microvilli defects in retinas of ezrin knockout mice.* Exp Eye Res, 2006. **82**(4): p. 720-9.
190. Curto, M. and A.I. McClatchey, *Ezrin...a metastatic detERMinant?* Cancer Cell, 2004. **5**(2): p. 113-4.
191. Monni, R., et al., *Ezrin is a target for oncogenic Kit mutants in murine erythroleukemia.* Blood, 2008. **111**(6): p. 3163-72.
192. Staser, K., et al., *A Pak1-PP2A-ERM signaling axis mediates F-actin rearrangement and degranulation in mast cells.* Exp Hematol, 2012.
193. Feng, Y., et al., *Filamin A (FLNA) is required for cell-cell contact in vascular development and cardiac morphogenesis.* Proceedings of the National Academy of Sciences of the United States of America, 2006. **103**(52): p. 19836-41.
194. Cunningham, C.C., et al., *Actin-binding protein requirement for cortical stability and efficient locomotion.* Science, 1992. **255**(5042): p. 325-7.
195. Vadlamudi, R.K., et al., *Filamin is essential in actin cytoskeletal assembly mediated by p21-activated kinase 1.* Nature cell biology, 2002. **4**(9): p. 681-90.
196. Maceyka, M., et al., *Filamin A links sphingosine kinase 1 and sphingosine-1-phosphate receptor 1 at lamellipodia to orchestrate cell migration.* Molecular and cellular biology, 2008. **28**(18): p. 5687-97.
197. Rider, L. and M. Diakonova, *Adapter protein SH2B1beta binds filamin A to regulate prolactin-dependent cytoskeletal reorganization and cell motility.* Molecular endocrinology, 2011. **25**(7): p. 1231-43.
198. Dummler, B., et al., *Pak protein kinases and their role in cancer.* Cancer Metastasis Rev, 2009. **28**(1-2): p. 51-63.
199. Sanders, L.C., et al., *Inhibition of myosin light chain kinase by p21-activated kinase.* Science, 1999. **283**(5410): p. 2083-5.
200. Takahashi, K. and K. Suzuki, *Membrane transport of WAVE2 and lamellipodia formation require Pak1 that mediates phosphorylation and recruitment of stathmin/Op18 to Pak1-WAVE2-kinesin complex.* Cellular signalling, 2009. **21**(5): p. 695-703.
201. Wittmann, T., G.M. Bokoch, and C.M. Waterman-Storer, *Regulation of microtubule destabilizing activity of Op18/stathmin downstream of Rac1.* The Journal of biological chemistry, 2004. **279**(7): p. 6196-203.
202. Somanath, P.R. and T.V. Byzova, *14-3-3beta-Rac1-p21 activated kinase signaling regulates Akt1-mediated cytoskeletal organization, lamellipodia formation and fibronectin matrix assembly.* J Cell Physiol, 2009. **218**(2): p. 394-404.
203. Kiosses, W.B., et al., *A role for p21-activated kinase in endothelial cell migration.* J Cell Biol, 1999. **147**(4): p. 831-44.
204. Smith, S.D., et al., *PAK1-mediated activation of ERK1/2 regulates lamellipodial dynamics.* J Cell Sci, 2008. **121**(Pt 22): p. 3729-36.
205. Parrini, M.C., et al., *Dissecting activation of the PAK1 kinase at protrusions in living cells.* J Biol Chem, 2009. **284**(36): p. 24133-43.

206. Balasenthil, S., et al., *p21-activated kinase-1 signaling mediates cyclin D1 expression in mammary epithelial and cancer cells.* J Biol Chem, 2004. **279**(2): p. 1422-8.
207. Holm, C., et al., *Association between Pak1 expression and subcellular localization and tamoxifen resistance in breast cancer patients.* J Natl Cancer Inst, 2006. **98**(10): p. 671-80.
208. Mira, J.P., et al., *Endogenous, hyperactive Rac3 controls proliferation of breast cancer cells by a p21-activated kinase-dependent pathway.* Proc Natl Acad Sci U S A, 2000. **97**(1): p. 185-9.
209. Carter, J.H., et al., *Pak-1 expression increases with progression of colorectal carcinomas to metastasis.* Clin Cancer Res, 2004. **10**(10): p. 3448-56.
210. Davidson, B., M. Shih Ie, and T.L. Wang, *Different clinical roles for p21-activated kinase-1 in primary and recurrent ovarian carcinoma.* Hum Pathol, 2008. **39**(11): p. 1630-6.
211. Schraml, P., et al., *Combined array comparative genomic hybridization and tissue microarray analysis suggest PAK1 at 11q13.5-q14 as a critical oncogene target in ovarian carcinoma.* Am J Pathol, 2003. **163**(3): p. 985-92.
212. O'Sullivan, G.C., et al., *Modulation of p21-activated kinase 1 alters the behavior of renal cell carcinoma.* Int J Cancer, 2007. **121**(9): p. 1930-40.
213. Brown, L.A., et al., *Amplification of EMSY, a novel oncogene on 11q13, in high grade ovarian surface epithelial carcinomas.* Gynecol Oncol, 2006. **100**(2): p. 264-70.
214. Brown, L.A., et al., *Amplification of 11q13 in ovarian carcinoma.* Genes Chromosomes Cancer, 2008. **47**(6): p. 481-9.
215. Mao, X., et al., *Genomic alterations in blastic natural killer/extranodal natural killer-like T cell lymphoma with cutaneous involvement.* J Invest Dermatol, 2003. **121**(3): p. 618-27.
216. Mao, X., et al., *Amplification and overexpression of JUNB is associated with primary cutaneous T-cell lymphomas.* Blood, 2003. **101**(4): p. 1513-9.
217. Gerthoffer, W.T., *Actin cytoskeletal dynamics in smooth muscle contraction.* Can J Physiol Pharmacol, 2005. **83**(10): p. 851-6.
218. McFawn, P.K., et al., *Calcium-independent contraction and sensitization of airway smooth muscle by p21-activated protein kinase.* Am J Physiol Lung Cell Mol Physiol, 2003. **284**(5): p. L863-70.
219. Gunst, S.J. and W. Zhang, *Actin cytoskeletal dynamics in smooth muscle: a new paradigm for the regulation of smooth muscle contraction.* Am J Physiol Cell Physiol, 2008. **295**(3): p. C576-87.
220. Tang, D.D. and S.J. Gunst, *The small GTPase Cdc42 regulates actin polymerization and tension development during contractile stimulation of smooth muscle.* J Biol Chem, 2004. **279**(50): p. 51722-8.
221. Tang, D.D., Y. Bai, and S.J. Gunst, *Silencing of p21-activated kinase attenuates vimentin phosphorylation on Ser-56 and reorientation of the vimentin network during stimulation of smooth muscle cells by 5-hydroxytryptamine.* Biochem J, 2005. **388**(Pt 3): p. 773-83.

222. Wu, Y., et al., *Integrin-linked kinase regulates smooth muscle differentiation marker gene expression in airway tissue.* Am J Physiol Lung Cell Mol Physiol, 2008. **295**(6): p. L988-97.
223. Allen, J.D., et al., *p21-activated kinase regulates mast cell degranulation via effects on calcium mobilization and cytoskeletal dynamics.* Blood, 2009. **113**(12): p. 2695-705.
224. Sauer, B., *Inducible gene targeting in mice using the Cre/lox system.* Methods, 1998. **14**(4): p. 381-92.
225. Kuhn, R., et al., *Inducible gene targeting in mice.* Science, 1995. **269**(5229): p. 1427-9.
226. Kiel, M.J., et al., *Hematopoietic stem cells do not depend on N-cadherin to regulate their maintenance.* Cell Stem Cell, 2009. **4**(2): p. 170-9.
227. Das, A.M., R.J. Flower, and M. Perretti, *Eotaxin-induced eosinophil migration in the peritoneal cavity of ovalbumin-sensitized mice: mechanism of action.* Journal of immunology, 1997. **159**(3): p. 1466-73.
228. Rothenberg, M.E., et al., *Eotaxin triggers eosinophil-selective chemotaxis and calcium flux via a distinct receptor and induces pulmonary eosinophilia in the presence of interleukin 5 in mice.* Molecular medicine, 1996. **2**(3): p. 334-48.
229. Fukuyama, S., et al., *Effect of eotaxin and platelet-activating factor on airway inflammation and hyperresponsiveness in guinea pigs in vivo.* American journal of respiratory and critical care medicine, 2000. **161**(6): p. 1844-9.
230. Chang, H.C., et al., *The transcription factor PU.1 is required for the development of IL-9-producing T cells and allergic inflammation.* Nat Immunol, 2010. **11**(6): p. 527-34.
231. van Rijt, L.S., et al., *A rapid flow cytometric method for determining the cellular composition of bronchoalveolar lavage fluid cells in mouse models of asthma.* J Immunol Methods, 2004. **288**(1-2): p. 111-21.
232. Yang, F.C., et al., *Nf1-dependent tumors require a microenvironment containing Nf1+/-- and c-kit-dependent bone marrow.* Cell, 2008. **135**(3): p. 437-48.
233. Jungraithmayr, W.M., et al., *A mouse model of orthotopic, single-lung transplantation.* J Thorac Cardiovasc Surg, 2009. **137**(2): p. 486-91.
234. Krupnick, A.S., et al., *Orthotopic mouse lung transplantation as experimental methodology to study transplant and tumor biology.* Nat Protoc, 2009. **4**(1): p. 86-93.
235. Okazaki, M., et al., *A mouse model of orthotopic vascularized aerated lung transplantation.* American journal of transplantation : official journal of the American Society of Transplantation and the American Society of Transplant Surgeons, 2007. **7**(6): p. 1672-9.
236. Saito, H., et al., *Pathogenesis of murine experimental allergic rhinitis: a study of local and systemic consequences of IL-5 deficiency.* J Immunol, 2002. **168**(6): p. 3017-23.

237. Sanderson, C.J., D.J. Warren, and M. Strath, *Identification of a lymphokine that stimulates eosinophil differentiation in vitro. Its relationship to interleukin 3, and functional properties of eosinophils produced in cultures.* J Exp Med, 1985. **162**(1): p. 60-74.
238. Dyer, K.D., et al., *Functionally competent eosinophils differentiated ex vivo in high purity from normal mouse bone marrow.* J Immunol, 2008. **181**(6): p. 4004-9.
239. Iellem, A., et al., *Unique chemotactic response profile and specific expression of chemokine receptors CCR4 and CCR8 by CD4(+)CD25(+) regulatory T cells.* J Exp Med, 2001. **194**(6): p. 847-53.
240. Tiffany, H.L., F. Li, and H.F. Rosenberg, *Hyperglycosylation of eosinophil ribonucleases in a promyelocytic leukemia cell line and in differentiated peripheral blood progenitor cells.* Journal of leukocyte biology, 1995. **58**(1): p. 49-54.
241. Fischkoff, S.A., et al., *Eosinophilic differentiation of the human promyelocytic leukemia cell line, HL-60.* The Journal of experimental medicine, 1984. **160**(1): p. 179-96.
242. Ingram, D.A., et al., *Hyperactivation of p21(ras) and the hematopoietic-specific Rho GTPase, Rac2, cooperate to alter the proliferation of neurofibromin-deficient mast cells in vivo and in vitro.* The Journal of experimental medicine, 2001. **194**(1): p. 57-69.
243. Hiatt, K.K., et al., *Neurofibromin GTPase-activating protein-related domains restore normal growth in Nf1-/- cells.* The Journal of biological chemistry, 2001. **276**(10): p. 7240-5.
244. Lu, L., et al., *Detection of luxol-fast-blue positive cells in human promyelocytic leukemia cell line HL-60.* Exp Hematol, 1981. **9**(9): p. 887-92.
245. Johnson, G.R. and D. Metcalf, *Detection of a new type of mouse eosinophil colony by Luxol-fast-blue staining.* Exp Hematol, 1980. **8**(5): p. 549-61.
246. Clutterbuck, E.J., E.M. Hirst, and C.J. Sanderson, *Human interleukin-5 (IL-5) regulates the production of eosinophils in human bone marrow cultures: comparison and interaction with IL-1, IL-3, IL-6, and GMCSF.* Blood, 1989. **73**(6): p. 1504-12.
247. Lozano, E., et al., *PAK is required for the disruption of E-cadherin adhesion by the small GTPase Rac.* J Cell Sci, 2008. **121**(Pt 7): p. 933-8.
248. Delorme-Walker, V.D., et al., *Pak1 regulates focal adhesion strength, myosin IIA distribution, and actin dynamics to optimize cell migration.* The Journal of cell biology, 2011. **193**(7): p. 1289-303.
249. Bates, M.E., et al., *Human airway eosinophils respond to chemoattractants with greater eosinophil-derived neurotoxin release, adherence to fibronectin, and activation of the Ras-ERK pathway when compared with blood eosinophils.* Journal of immunology, 2010. **184**(12): p. 7125-33.

250. Lintomen, L., et al., *Human eosinophil adhesion and degranulation stimulated with eotaxin and RANTES in vitro: lack of interaction with nitric oxide.* BMC pulmonary medicine, 2008. **8**: p. 13.
251. Jia, G.Q., et al., *Selective eosinophil transendothelial migration triggered by eotaxin via modulation of Mac-1/ICAM-1 and VLA-4/VCAM-1 interactions.* International Immunology, 1999. **11**(1): p. 1-10.
252. Nakajima, H., et al., *Role of vascular cell adhesion molecule 1/very late activation antigen 4 and intercellular adhesion molecule 1/lymphocyte function-associated antigen 1 interactions in antigen-induced eosinophil and T cell recruitment into the tissue.* J Exp Med, 1994. **179**(4): p. 1145-54.
253. Walsh, G.M., et al., *Integrin alpha 4 beta 7 mediates human eosinophil interaction with MAdCAM-1, VCAM-1 and fibronectin.* Immunology, 1996. **89**(1): p. 112-9.
254. Wittmann, T., G.M. Bokoch, and C.M. Waterman-Storer, *Regulation of leading edge microtubule and actin dynamics downstream of Rac1.* The Journal of cell biology, 2003. **161**(5): p. 845-51.
255. Adyshev, D.M., et al., *Differential involvement of ezrin/radixin/moesin proteins in sphingosine 1-phosphate-induced human pulmonary endothelial cell barrier enhancement.* Cellular signalling, 2011. **23**(12): p. 2086-96.
256. Shamri, R., et al., *CCL11 elicits secretion of RNases from mouse eosinophils and their cell-free granules.* FASEB J, 2012. **26**(5): p. 2084-93.
257. El-Shazly, A., et al., *Human eotaxin induces eosinophil-derived neurotoxin release from normal human eosinophils.* International Archives of Allergy and Immunology, 1998. **117 Suppl 1**: p. 55-8.
258. Zimmermann, N., J.J. Conkright, and M.E. Rothenberg, *CC chemokine receptor-3 undergoes prolonged ligand-induced internalization.* The Journal of biological chemistry, 1999. **274**(18): p. 12611-8.
259. Ro, J.Y., et al., *Effects of 7,8-dihydro-8-oxo-deoxyguanosine on antigen challenge in ovalbumin-sensitized mice may be mediated by suppression of Rac.* British journal of pharmacology, 2009. **158**(7): p. 1743-52.
260. Carstanjen, D., et al., *Rac2 regulates neutrophil chemotaxis, superoxide production, and myeloid colony formation through multiple distinct effector pathways.* Journal of immunology, 2005. **174**(8): p. 4613-20.
261. Reutershan, J., et al., *Blocking p21-activated kinase reduces lipopolysaccharide-induced acute lung injury by preventing polymorphonuclear leukocyte infiltration.* American journal of respiratory and critical care medicine, 2007. **175**(10): p. 1027-35.
262. Nakae, S., et al., *Mast cell-derived TNF contributes to airway hyperreactivity, inflammation, and TH2 cytokine production in an asthma model in mice.* J Allergy Clin Immunol, 2007. **120**(1): p. 48-55.

263. McGlade, J.P., et al., *Suppression of the asthmatic phenotype by ultraviolet B-induced, antigen-specific regulatory cells.* Clinical and experimental allergy : journal of the British Society for Allergy and Clinical Immunology, 2007. **37**(9): p. 1267-76.
264. Sung, K.P., et al., *Eotaxin induces a sustained reduction in the functional adhesive state of very late antigen 4 for the connecting segment 1 region of fibronectin.* The Journal of allergy and clinical immunology, 2000. **106**(5): p. 933-40.
265. Barthel, S.R., et al., *Roles of integrin activation in eosinophil function and the eosinophilic inflammation of asthma.* Journal of leukocyte biology, 2008. **83**(1): p. 1-12.
266. Croker, B.A., et al., *Rac2-deficient mice display perturbed T-cell distribution and chemotaxis, but only minor abnormalities in T(H)1 responses.* Immunology and cell biology, 2002. **80**(3): p. 231-40.
267. Insall, R.H. and L.M. Machesky, *Actin dynamics at the leading edge: from simple machinery to complex networks.* Developmental cell, 2009. **17**(3): p. 310-22.
268. Voermans, C., et al., *SDF-1-induced actin polymerization and migration in human hematopoietic progenitor cells.* Exp Hematol, 2001. **29**(12): p. 1456-64.
269. Kim, C.H. and H.E. Broxmeyer, *SLC/exodus2/6Ckine/TCA4 induces chemotaxis of hematopoietic progenitor cells: differential activity of ligands of CCR7, CXCR3, or CXCR4 in chemotaxis vs. suppression of progenitor proliferation.* J Leukoc Biol, 1999. **66**(3): p. 455-61.
270. Weiner, O.D., et al., *Spatial control of actin polymerization during neutrophil chemotaxis.* Nat Cell Biol, 1999. **1**(2): p. 75-81.
271. Ichetovkin, I., W. Grant, and J. Condeelis, *Cofilin produces newly polymerized actin filaments that are preferred for dendritic nucleation by the Arp2/3 complex.* Current biology : CB, 2002. **12**(1): p. 79-84.
272. Yi, C., et al., *Development of small-molecule inhibitors of the group I p21-activated kinases, emerging therapeutic targets in cancer.* Biochemical pharmacology, 2010. **80**(5): p. 683-9.
273. Fujisawa, T., et al., *Chemokines induce eosinophil degranulation through CCR-3.* The Journal of allergy and clinical immunology, 2000. **106**(3): p. 507-13.
274. Ochkur, S.I., et al., *The development of a sensitive and specific ELISA for mouse eosinophil peroxidase: assessment of eosinophil degranulation ex vivo and in models of human disease.* Journal of Immunological Methods, 2012. **375**(1-2): p. 138-47.
275. Adamko, D.J., et al., *The induction of eosinophil peroxidase release: improved methods of measurement and stimulation.* Journal of Immunological Methods, 2004. **291**(1-2): p. 101-8.

CURRICULUM VITAE

Muithi Mwanthi

Current Position:

2006 Student, NIH Medical Scientist Training Program Indiana University School of Medicine (IUSOM) Department of Microbiology and Immunology Mentor/Advisor - D. Wade Clapp, MD

Education:

2005 B.S. - Pre-medicine *Summa cum laude*, Clearwater Christian College, Clearwater FL

2013 Ph.D. - Microbiology and Immunology, Indiana University, Indianapolis IN

2014 M.D. - Indiana University School of Medicine, expected

Academic and Professional Honors and Fellowships:

Undergraduate

2002 Presidents' Academic Fellowship, 2002-04

2003 *Alpha Chi* academic chapter, National College Honor Scholarship Society Membership, 2003-04

2003 Bailey Family Foundation Scholarship, 2003-04

Graduate

2006 University Fellowship, 2006-07

2009 Edwin T. Harper Scholarship (R25 GM079657), 2009-10

2010 Joint Best first-time presenter, Research in Progress, Department of Microbiology and Immunology,

2010 1st place, Sigma Xi Graduate Biomedical Research Competition

2010 Harold Raidt Department of Microbiology and Immunology Graduate Student Teaching Award

2011 Ruth L. Kirschstein NRSA for Individual Predoctoral Fellowship to Promote Diversity in Health-Related Research - F31HL108569 PI: “Pak1's Role in Eotaxin-Mediated Murine Eosinophil Migration” National Heart Lung and Blood Institute, National Institutes of Health (NHLBI/NIH), 2011-13

2011 Southern Regional Education Board (SREB) Doctoral Scholarship, Institute of Teaching and Mentoring, 2011-12

2011 Abstract achievement travel award (MGSAAA), American Society of Hematology (ASH)

Professional Experience:

2004 Kaplan Test Prep and Admissions, Class Instructor and Tutor Indianapolis IN, and Tampa, Fl, 2004-09

2005 Florida Department of Citrus, Research assistant, Lake Alfred, FL

2005 Sun Orchards of Florida, Quality Assurance Technician Haines City, FL

2008 IU School of Medicine MSMS MCAT Summer Prep Program, Tutor/Facilitator, Indianapolis, IN

2009 IU School of Medicine Teaching Assistant & Lecturer J210, Principles in Microbiology, 2009-11

Publications:

1. Mwanthi, M., S. Chen, E. Michaels, K. Staser, Z. Sun, S. Sehra, D. Wilkes, M. Kaplan, S.Park, and D. W. Clapp. 2012. PAK1 regulates eosinophil migration *in vivo* and *in vitro* (In submission)

2. Staser, KW, M. Shew, E. Michaels, M. Mwanthi**,** D. Clapp, and S. Park. 2011. A Pak1-PP2A-Ezrin signaling axis mediates IgE-dependent F-actin rearrangement and degranulation in mast cells. *Exp Hematology,* 2012

3. Zeng, Y., K. Staser, K. Menon, S. Park, M. Mwanthi, L. Jiang, and D. W. Clapp. 2012. Ezrin Regulates Hematopoietic Stem/Progenitor Cell Motility (In preparation)

Abstracts and Presentations:

1. Mwanthi MM, Micheals GE, Park SJ, Clapp DW. "PAK1's regulation of eotaxin-mediated murine eosinophil migration" IUSM Medical Scientist Training Program (MSTP) retreat, July 2009, Lafayette, IN (Abstract and Poster)

2. Mwanthi MM, Micheals GE, Yongqi Y, Tepper RS, Park SJ, Clapp DW. "PAK1 Modulates F-Actin Dynamics in Murine Eosinophil Migration and Tissue Infiltration". Annual Biomedical Research Conference for Minority Students (ABRCMS), Nov 4-7 2009, Phoenix, AZ (Abstract and Poster)

3. Mwanthi MM, Micheals GE, Yongqi Y, Tepper RS, Park SJ, Clapp DW. "PAK1 regulates eotaxin-mediated eosinophil migration and inflammation" American Society of Clinical Investigation (ASCI/AAP) 2010: April 17, 2010, Chicago, IL (Abstract and Poster)

4. Mwanthi MM, Micheals GE, Yongqi Y, Tepper RS, Park SJ, Clapp DW. "PAK1 Regulates Eotaxin-Mediated Murine Eosinophil Migration and Tissue Infiltration". Sigma Xi Research Day May 10, 2010, Indianapolis, IN (Abstract and Oral Presentation)

5. Mwanthi MM, Micheals GE, Staser KW, Yongqi Y, Tepper RS, Park SJ, Clapp DW. "PAK1 regulates eotaxin-mediated eosinophil migration" Regional conference, National Organization for the Professional Advancement of Black Chemists and Chemical Engineers (NOBCCHE), November 4, 2011, Indianapolis, IN (Abstract and Poster)

6. Mwanthi MM, Micheals GE, Staser KW, Sehra S, Jander M, Chen S, Wilkes DS, Kaplan, MH, Park SJ, Clapp DW. Annual conference for American Society of Hematology: "PAK1 regulates eotaxin-mediated eosinophil migration *in vivo* and *in vitro*" December 9, 2011, San Diego, CA (Abstract and Oral Presentation)

7. Mwanthi MM, Chen S, Micheals GE, Staser KW, Yongqi Y, Sehra S, Tepper RS, Kaplan, MH, Park SJ, Clapp DW. "PAK1 regulates eotaxin-mediated eosinophil migration" 10th Annual Midwest Blood Club Symposium, March 15, 2012, Indianapolis, IN (Abstract and Poster)

8. Walline C, Benson H, Sehra S, Mwanthi M, Fisher A, Clapp DW, Brutkiewicz R, Kaplan M, Wilkes DS, and Blum, J. “Alterations in pulmonary immunity in response to vaccinia virus” Immunology 98th Annual Meeting May 14-15, 2011, San Francisco, CA (Abstract, Poster and Oral Presentation)

9. Zeng, Yi, Staser KW, Menon KM, Park SJ, Mwanthi MM, Jiang L, and Clapp DW. “Ezrin Regulates Hematopoietic Stem/Progenitor Cell Motility” Annual conference for American Society of Hematology, December 10, 2011, San Diego, CA (Abstract and Poster)