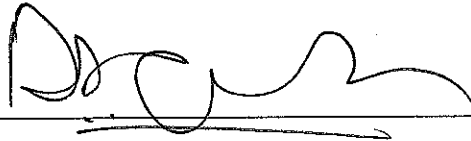
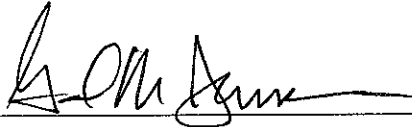


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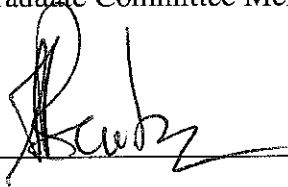


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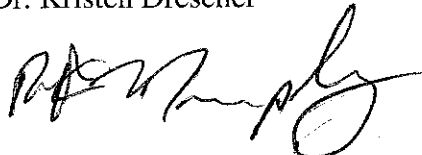
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TGF- β 1-induced Chloride Channel Activity and Migration of Human Eosinophils

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Abstract

Asthma is a chronic disease of the airways characterized by airway hyperresponsiveness, airway inflammation, and airway remodeling. Eosinophils migrate to the airways and play a significant role in the pathogenesis of asthma, particularly through their release of mediators and cytokines. Transforming growth factor (TGF)- β also induces asthmatic features in the lungs, especially airway remodeling. Eosinophils and TGF- β are closely linked to each other in the induction of airway remodeling. TGF- β activity also affects chloride channel activity, which can regulate mediator release and cell migration. Therefore, the role that TGF- β 1 stimulation of eosinophils has on chloride channel activity and migration of eosinophils and the underlying mechanisms involved in this process were examined.

Human blood eosinophils were stimulated with TGF- β 1, the tyrosine kinase inhibitor genistein, the protein kinase C (PKC)- δ/ϵ inhibitor rottlerin, the general PKC inhibitor staurosporine, and the chloride channel inhibitors 4,4'-diisothiocyanatostilbene-2,2'-disulfonic acid (DIDS) and 5-nitro-2-(3-phenylpropylamino)benzoic acid (NPPB). Protein expression of the chloride channel (CLC) family of chloride channels, PKC isozymes, and Smad transcription factors was determined using western blot, and mRNA expression of CLC was examined using RT-PCR. Chloride channel activity was measured by patch clamping, and eosinophil chemotaxis was assessed using a Boyden microchemotaxis chamber. Flow cytometry was used to determine shape change.

The results showed that TGF- β 1 stimulation increased protein expression of CLC-3. There was also more CLC-3 protein expression in asthmatic eosinophils than normal eosinophils. TGF- β 1 increased protein expression of PKC- β 1, PKC- δ , and phospho-Smad3. TGF- β 1 increased the chloride channel activity of the eosinophils, which was blocked by

rottlerin. TGF- β 1 induced chemotaxis of eosinophils, which was blocked by rottlerin, DIDS and NPPB. Eosinophil shape change occurred after TGF- β 1 stimulation, and both DIDS and NPPB inhibited morphological changes of the cells. Rottlerin, as well as staurosporine, decreased TGF- β 1-induced phosphorylation of Smad3. Genistein was more robust at reducing Smad3 phosphorylation than rottlerin or staurosporine.

These results indicate that TGF- β 1 increases the chloride channel activity of eosinophils by upregulating CLC-3 via a pathway dependent on PKC (particularly PKC- δ) and possibly Smad3. TGF- β 1 induced chloride channel activity also induces eosinophil migration and shape change via a PKC- δ -dependent pathway. These data offer new insights into a TGF- β 1-dependent mechanism that may increase eosinophil infiltration and activation, thereby inducing airway remodeling.

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Abbreviations

Ang-1	Angiopoietin-1
AP-1	Activator Protein-1
Arp	Actin-Related Protein
ASM	Airway Smooth Muscle
BAL	Bronchoalveolar Lavage
CCL-11	CXC Chemokine Ligand-11
CCR	C-C Chemokine Receptor
CFTR	Cystic Fibrosis Transmembrane Regulator
CLC	Chloride Channel
CRTH2	Chemoattractant Receptor Homologous Molecule Expressed on TH2 Cells
CXCR-3	CXC Chemokine Receptor-3
DAG	Diacylglycerol
DC	Dendritic Cell
DIDS	4,4'-Diisothiocyanatostilbene-2,2'-Disulfonic Acid
DP1	PGD2 Receptor
EB1	End Binding Protein-1
ECP	Eosinophil Cationic Protein
EGR-1	Early Growth Response-1
ERK	Extracellular Signal-Related Protein
FSC	Forward Scatter
GM-CSF	Granulocyte Macrophage-Colony Stimulating Factor
GSK-3 β	Glycogen Synthase Kinase 3 β

HES-1	Hairy Enhancer of Split 1
HGF	Hepatocyte Growth Factor
HSP-27	Heat shock protein-27
ICAM-1	Intercellular Adhesion Molecule-1
IL	Interleukin
JAK-3	Janus Kinase-3
JNK	Jun N-terminal Kinase
LFA-1	Lymphocyte Function-Associated Antigen-1
LTC4	Leukotriene C4
LPP-1	Lipid Phosphate Phosphatase-1
MAPK	MAP Kinase
MBP	Major Basic Protein
MCP	Monocyte Chemoattractant Protein
MDC	Macrophage-Derived Chemokine
MEK	MAPK/ERK Kinase
MMP	Matrix Metalloproteinase
NF- κ B	Nuclear Factor- κ B
NPPB	5-Nitro-2-(3-Phenylpropylamino)Benzoic Acid
OVA	Ovalbumin
PAF	Platelet Activating Factor
PAI-1	Plasminogen Activator Inhibitor-1
PEA-15	Phosphoprotein Enriched in Astrocytes-15 kDa
PGD2	Prostaglandin D2

PD1	Protectin D1
PDGF	Platelet-Derived Growth Factor
PI3K	Phosphoinositide 3-Kinase
PKC	Protein Kinase C
PLC	Phospholipase C
RANTES	Regulated upon Activation, Normal T Cell Expressed and Secreted
SSC	Side Scatter
SDF-1	Stromal Cell-Derived Factor-1
SK-1	Sphingosine Kinase-1
STAT	Signal Transducer and Activator of Transcription
TARC	Thymus- and Activation-Regulated Chemokine
TGF- β	Transforming Growth Factor- β
TNF- α	Tumor Necrosis Factor- α
Tregs	Regulatory T Cells
VCAM-1	Vascular Cell Adhesion Molecule-1
VEGF	Vascular Endothelial Growth Factor
VLA-4	Very Late Antigen-4

Chapter 1

Introduction

Asthma is a chronic disease of the airways, with those suffering from it exhibiting characteristic features such as inflammation of the lung tissue and airway hyperresponsiveness (1). Airway remodeling, characterized by subepithelial fibrosis, smooth muscle cell and goblet cell hyperplasia, and mucus hypersecretion, is another prominent symptom seen in asthmatic patients (2). Immune cells infiltrate the lung tissue and release and/or respond to mediators and cytokines, thus leading to some of the characteristic symptoms of asthma. The eosinophil, through its release of cytokines and inflammatory mediators, is one of the immune cells which plays a significant role in the pathogenesis of asthma (3, 4). One of the key cytokines involved in the induction of asthmatic symptoms is transforming growth factor (TGF)- β . Increased activity of TGF- β generally leads to increased asthmatic features in the lungs, especially airway remodeling (5). Many cellular activities can control cell migration and activation, thus leading to the exacerbation of asthma. One is the activation of chloride channels on cells, which can regulate mediator release and migration of cells in the airways during an asthmatic response (6-8). Chloride channel activity can be affected by, among other things, the action of TGF- β (9), which itself can also induce cell migration (10).

Many molecular activities, such as those of protein kinases and transcription factors, are involved in signal transduction pathways, immune cell activation and airway remodeling. For example, protein kinase C (PKC) is involved in many cell signaling pathways downstream of TGF- β (11, 12). PKC activity regulates expression and activity of chloride channels (13-16), as well as cell migration (11, 17). Also, Smad family transcription factors are involved in TGF- β intracellular signal transduction pathways, in which they are activated by PKC, inducing such cellular functions as proliferation (18) and mediator release (19). Thus,

PKC and Smad proteins were examined for their potential role in TGF- β 1-induced chloride channel activity in and migration of human blood eosinophils.

1.1 Asthma

Asthma and other allergic diseases are becoming bigger health problems throughout the world. The symptoms associated with asthma, such as bronchial constriction and inflammation of the airway tissue, can be caused by exposure to environmental allergens, as well as by having a genetic predisposition to the disease. While much is known about the pathogenesis of asthma, much less is known about the cells, cytokines, and other mediators which could provide good therapeutic targets for alleviating some of the symptoms of asthma.

The initiation of asthma- which is generally induced by exposure to an allergen- initiate a response by the immune system, leading to symptoms such as inflammation of the lung tissue, constriction of the bronchi, and airway hyperresponsiveness (1, 2). Another common feature of the asthmatic lung is airway tissue remodeling, involving cellular and protein structural changes in the lung tissue, such as smooth muscle cell and goblet cell hyperplasia, mucus hypersecretion, tissue fibrosis, increased collagen deposition, and angiogenesis (2).

This response by the immune system, which leads to asthma, is induced by infiltration of immune cells into the lung tissue, which release and/or are acted upon by mediators and cytokines (1). There are many cells, signaling molecules, and proteins that are involved in the onset of asthma. Many of the mechanisms involved in the pathogenesis of asthma have been studied, providing numerous insights into possible therapeutic treatments for people who suffer from asthma.

1.2 Eosinophils in Asthma

Eosinophils play a significant role in the pathogenesis of asthma, particularly through their migration to the lung tissue and their subsequent induction of airway inflammation and airway remodeling once in the lungs. Eosinophils were shown to be one of the dominant immune cells extracted from bronchoalveolar lavage (BAL) fluid of asthmatic mice (20). In addition, the number of eosinophils in the sputum taken from asthmatic patients correlates with severity of asthma (21). Eotaxin, one of the major chemotactic factors for eosinophils which draws them to the airways, has its release initiated by Th2 lymphocytes and mast cells releasing cytokines such as interleukin (IL)-4, IL-13, and tumor necrosis factor (TNF)-alpha. These cytokines stimulate the airway smooth muscle cells, airway epithelial cells, and fibroblasts to release the eotaxin (22). Eosinophils have been reported to undergo significant shape change in response to eotaxin binding to C-C Chemokine Receptor 3 (CCR3) receptor in vitro. This shape change reflects the in vivo response when eosinophils are stimulated to migrate to an area of inflammation (23). Once in the lungs, more eosinophils from asthmatic subjects are activated as compared to eosinophils from non-asthmatic subjects, thus augmenting asthmatic responses in the airways (24).

Eosinophil numbers in the lungs are strongly correlated with airway inflammation and airway hyperresponsiveness. Administration of the pro-inflammatory cytokine IL-4 led to an increase in eosinophilia and airway hyperresponsiveness (25). Induction of airway inflammation in a mouse model after allergenic challenge coincides with increased numbers of eosinophils migrating to the airways (26). The increase in eosinophil numbers in response to allergen challenge is more delayed than for other inflammatory cells, as shown by the percentage of cells in BAL fluid from mice, as well as BAL fluid and peripheral blood from

humans, after challenge with an allergen to induce airway inflammation (27). IL-3 induces degranulation and activation of eosinophils by increasing expression of CD48 in eosinophils, thus leading to increased airway inflammation. Anti-IL-3 antibody decreases the number of eosinophils infiltrating the airways, as well as inflammation in the lung tissue (28). Asthmatic mice that were given mycobacterial antigens to reduce airway hyperresponsiveness also exhibited reduced eosinophilia (29). When allergen-challenged mice were given the anti-inflammatory drug DA-9201, the number of eosinophils in the BAL fluid was greatly reduced. Inflammation in the lungs was also reduced by this treatment (30). Another anti-inflammatory agent, heme oxygenase-1, diminished the number of eosinophils infiltrating the lung in an asthmatic mouse model. This reduction in eosinophils leads to a decrease in airway inflammation and airway hyperresponsiveness (31).

Some results, however, show that eosinophils may not be important in the pathogenesis of asthma. Anti-IL-5 antibodies decreased eosinophil numbers in the lungs of asthmatic mice, but had no effect on the airway hyperreactivity in the mice associated with the asthma (32). Anti-IL-5 antibodies decreased sputum eosinophil numbers in humans, but had no effect on the airway hyperresponsiveness and late asthmatic response (33). Moreover, IL-5-induced eosinophil infiltration into the lungs has been shown to reduce allergic airway inflammation via a TGF- β -dependent pathway (34). These findings represent a small percentage of the research done on linking eosinophils to asthma, however, since much more research has shown that eosinophils are key mediators in the perpetuation of an asthmatic response.

1.2.1 Chemokines that Affect Eosinophil Migration

Eosinophil recruitment to the airways can involve many different extracellular molecules secreted from various cell types (Table 1). Eosinophils also release chemokines, which act in an autocrine manner to induce chemotaxis. Eotaxin is regarded as the most important chemokine involved in eosinophil chemotaxis, since eotaxin can increase eosinophil migration into the lung tissue, thus amplifying the asthmatic inflammatory response (35). Eotaxin-2 and eotaxin-3 are closely associated with increased numbers of eosinophils infiltrating the lung tissue of asthmatic subjects (36). Eotaxin-1 and eotaxin-2 also play a synergistic role in eosinophil recruitment to the lungs in asthma. While knockout mice of either of these chemokines affected eosinophil recruitment, double knockout of these genes reduced eosinophil recruitment to a greater extent (37) (Figure 1).

Other chemokines can induce eosinophil migration, though not as effectively as eotaxin. For example, the chemokine regulated on activation normal T cell expressed and secreted (RANTES) helps recruit eosinophils to the lungs during an allergen-induced response, but eotaxin does so to a much greater extent in a rat asthma model (38). Another study also showed that both eotaxin-1 and eotaxin-2 had a greater effect on eosinophil shape change (indicating that there is increased migration of the cells) than RANTES (Figure 1). The eotaxins also increased eosinophil shape change to a greater extent than did other chemokines such as monocyte chemoattractant protein (MCP)-1, MCP-3, and MCP-4 (23). Therefore, the effect of eotaxin on the induction of eosinophil recruitment is much greater than that of other molecules known to induce the migration of eosinophils.

Table 1. A List of various chemokines, cytokines, growth factors, and other molecules that increase the recruitment of eosinophils to the airways during an asthmatic response.

Chemokines	Cytokines	Growth Factors	Other Molecules
Eotaxin-1	IL-4	VEGF	MMP-12
Eotaxin-2	IL-5		PGD2
Eotaxin-3	IL-12		
RANTES	IL-13		
MCP-1	IL-25		
MCP-3	TGF- β 1		
MCP-4	TNF- α		

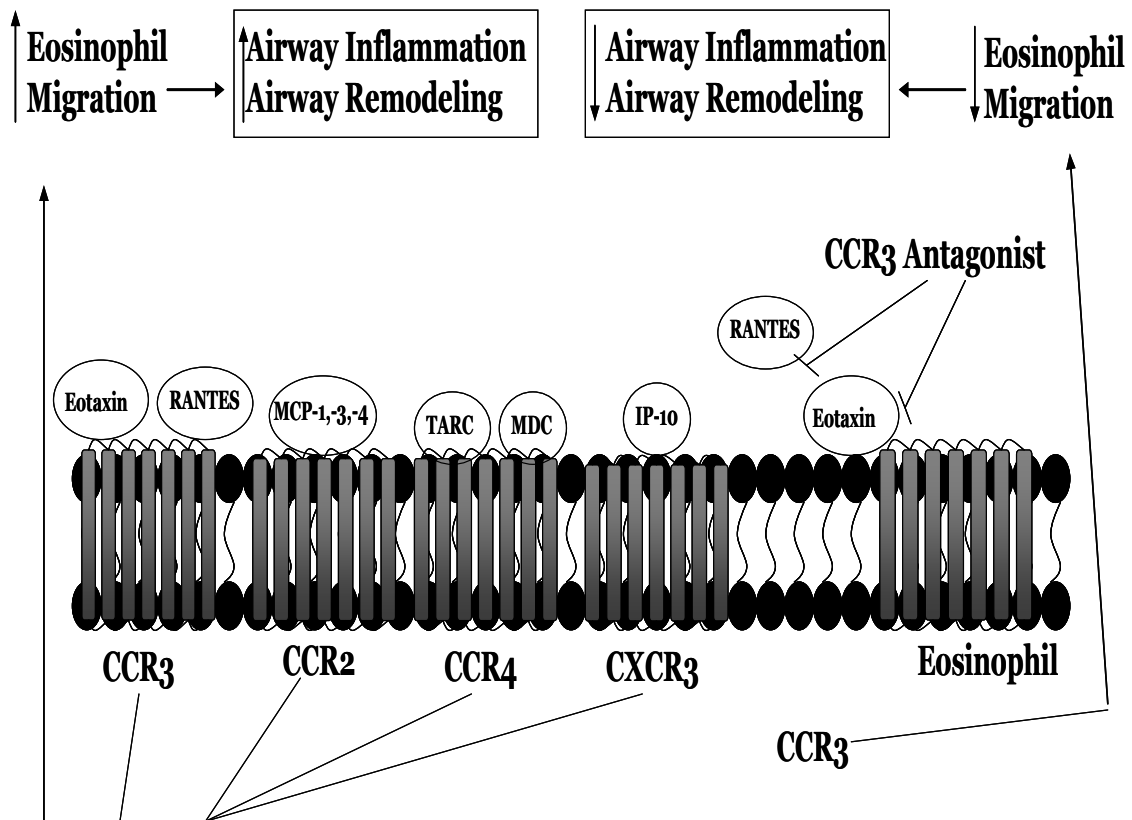


Figure 1. Chemokines and chemokine receptors. Chemokines such as eotaxin, RANTES, and MCP, can bind to CCRs on eosinophils, which induces eosinophil migration. A CCR3 antagonist prevents eotaxin or RANTES from binding to CCR3, thus reducing eosinophil chemotaxis.

1.2.2 Cytokines, Growth Factors, and Other Molecules Involved in Eosinophil Migration

Cytokines are also involved in the recruitment of eosinophils. TH2 cytokines, for example, are widely known to exacerbate inflammation in the lungs of asthmatics. One way they can induce inflammation is through recruitment of eosinophils. IL-5 increases the level of eosinophils that infiltrate the airways during an asthmatic response (35) (Figure 2), as does IL-

13 (39). IL-13, as well as IL-4, can induce eosinophil migration to the lungs by causing airway smooth muscle (ASM) cells to release eotaxin (40). IL-4 secretion from basophils can also increase lung tissue eosinophil numbers, which in turn increases airway inflammation (41). IL-13 increases eotaxin-2-induced eosinophil recruitment to the airways (42). Granulocyte-macrophage colony-stimulating factor (GM-CSF) recruits eosinophils to the lungs, as well (43). At the molecular level, the transcription factor GATA-3, which is known to induce TH2 immune responses, increases eosinophil infiltration, leading to airway inflammation and airway remodeling (44). Another transcription factor involved in the induction of a TH2 response, signal transducer and activator of transcription (STAT)-6, can increase infiltration of eosinophils into the airways (45) (Figure 2). T-bet, a transcription factor involved in TH1 immune responses, reduces eosinophil recruitment to the lungs and subsequent goblet cell hyperplasia, a major feature of airway remodeling (46).

Other cytokines and mediators not associated with eosinophils in asthma also have a significant impact on eosinophil migration. For example, IL-25 increases eosinophil recruitment into the airways, which corresponds to an increase in airway inflammation and goblet cell hyperplasia (47). IL-12 increases eosinophil recruitment to the lungs by increasing levels of TH2 cytokines IL-4, IL-5, and IL-13 (48). TNF- α can also increase eosinophil recruitment to the lungs by inducing IL-5 production and secretion (49). In addition, vascular endothelial growth factor (VEGF) has the ability to induce migration of eosinophils by binding to the receptor flt1 on eosinophils, causing the cells to migrate and release mediators from their granules, such as eosinophil cationic protein (ECP), thus increasing asthmatic symptoms such as airway inflammation (50). Angiopoietin (Ang)-1 can induce eosinophil chemotaxis by binding to the Tie-2 receptor, increasing airway inflammation and remodeling, as well (51).

Prostaglandin D2 (PGD2) also stimulates eosinophils to migrate by binding to PGD2 receptor 1 (DP1) (52). Platelet activating factor (PAF) induces eosinophil chemotaxis, and this leads to an increase of eosinophil activation as shown by release of leukotriene C4 (LTC4) (53) (Figure 2). Several neuropeptides, such as substance P, neurokinin A, calcitonin gene-related peptide, and cholecystinin octapeptide, also induce eosinophil chemotaxis (54).

Certain molecules within the cell, such as the phosphoinositide 3-kinase (PI3K) isoform gp110 δ , can induce eosinophil recruitment, thereby augmenting airway inflammation and airway hyperresponsiveness (55). Janus kinase (JAK)-3 is involved in signal transduction that leads to eosinophil recruitment to the lungs in allergic airway disease (56). p38 mitogen-activated protein kinase (MAPK) activation is also critical for eosinophil recruitment, since its blockage reduced eosinophil infiltration of the lungs in an asthmatic animal model (57) (Figure 2). NADPH oxidase has been shown to be crucial for VCAM-1-mediated adhesion of eosinophils. Its absence from endothelial cells reduces eosinophil infiltration into the lungs, as well as airway hyperresponsiveness (58). Some molecules, such as protectin D1 (PD1), negatively regulate eosinophil recruitment, which reduces eosinophil recruitment, airway inflammation, and airway hyperresponsiveness (59).

Matrix metalloproteinases (MMPs) are involved in recruitment of eosinophils to the airways. MMP-12 deficiency was shown to decrease infiltration of eosinophils into the lungs, as well as airway inflammation and airway remodeling (60). By contrast, MMP-9 is involved in decreasing eosinophils in the lungs by decreasing the TH2 response. Specifically, MMP-9 decreases the levels of IL-4, IL-5, IL-13, and eotaxin (61). This illustrates an internal mechanism by which eosinophil infiltration is regulated. Manipulation of this could potentially reverse the increase in eosinophils seen in the asthmatic lung. The examples given here of pro-

migratory molecules indicate that the classic chemokines and TH2 cytokines are not all that act on the eosinophils. Many cytokines and signaling molecules are involved in inducing or blocking eosinophil recruitment to the airways during the asthmatic response.

1.2.3 Eosinophil Surface Markers that Regulate Eosinophil Recruitment

Particular eosinophil cell surface markers are important for their recruitment to the airways during an asthmatic response. The binding to CCR-3 on eosinophils by eotaxin, eotaxin-2, and MCP-4 induces a shape change response in eosinophils (23). In addition to CCR-3, increased CCR-4 and CXC chemokine receptor (CXCR)-3 expression on eosinophils correlates with increased eosinophil infiltration into the lung tissue during an allergic inflammatory response in the lungs (62). Levels of thymus- and activation-regulated chemokine (TARC) and macrophage-derived chemokine (MDC), ligands for CCR-4, and interferon-inducible protein (IP)-10, a ligand for CXCR3, are increased in the lungs of allergic asthmatic mice with high eosinophil levels in the lungs (63). CCR-2 also induces migration of eosinophils into the airways during asthma, since its blockade reduces eosinophil infiltration, thereby reducing airway hyperresponsiveness (64). The increased expression of multiple chemokine receptors on eosinophils illustrates how crucial some chemokines are in inducing eosinophil migration to the lung tissue.

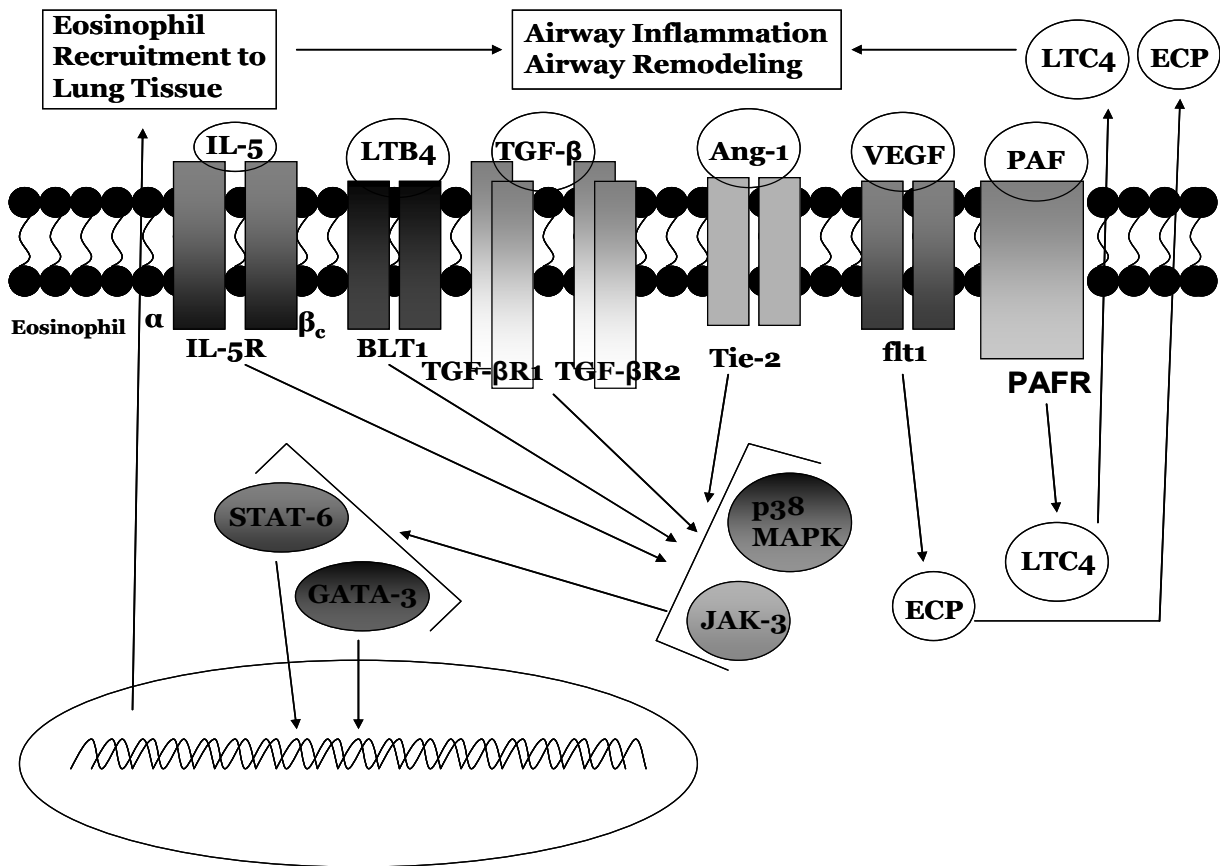


Figure 2. Stimulation of eosinophils inducing migration. Various cytokines, growth factors and other mediators which are involved in inducing eosinophil migration could stimulate eosinophils directly, thus inducing activation of particular kinases and transcription factors. This leads to the migration of eosinophils and eosinophil mediator release, thus perpetuating airway inflammation in asthma.

Other surface molecules on eosinophils are involved in eosinophil adhesion and extravasation (Figure 3). Eosinophils from asthmatics that infiltrate airways exhibit very high expression of very late antigen (VLA)-4 on their surface (65). VLA-4 on eosinophils binds to vascular cell adhesion molecule (VCAM)-1 on the endothelium, which helps promote migration of the eosinophils from the blood to surrounding tissues (66). The P-selectin

adhesion molecule on platelets binds to VLA-4 on the eosinophils, thus facilitating its transendothelial migration (67). Integrin adhesion molecules such as $\alpha 1\beta 1$ and $\alpha 2\beta 1$ aid the attachment of eosinophils to the endothelium, allowing the cells to migrate to the lung tissue. These particular integrins facilitate eosinophil attachment to collagen in the vascular wall (68). Another integrin expressed on eosinophils ($\alpha M\beta 2$) also assists eosinophil binding to the endothelium by binding to the VCAM-1 molecule on the endothelial cells (69). Lymphocyte function-associated antigen (LFA)-1 expression on eosinophils also increases the migration of eosinophils to the lung tissues of asthmatics (70). Intercellular adhesion molecule (ICAM)-1, which is expressed on the endothelial wall, can bind to eosinophils, inducing their migration to the lung tissue (71).

Chemoattractant receptor-homologous molecule expressed on TH2 cells (CRTH2), also known as the DP2 receptor, and expressed on eosinophils, can enhance eosinophil recruitment to the lungs when PGD2 binds to it (52, 72). Another study, however, showed that CRTH2 is important for decreasing eosinophil infiltration into the lung tissue through actions such as decreasing IL-5 production (73). The surface marker BLT1 is also important for eosinophil recruitment. When stimulated by leukotriene B4, BLT1 increases eosinophil recruitment into the airways in conjunction with increased airway inflammation and airway hyperresponsiveness (74). Another molecule expressed on eosinophils, CD147, is important in eosinophil infiltration of the airways, since its blockade significantly reduces eosinophil accumulation in the lungs in an asthma model. This increase in eosinophil migration involves the interaction of cyclophilins with the CD147 surface marker (75).

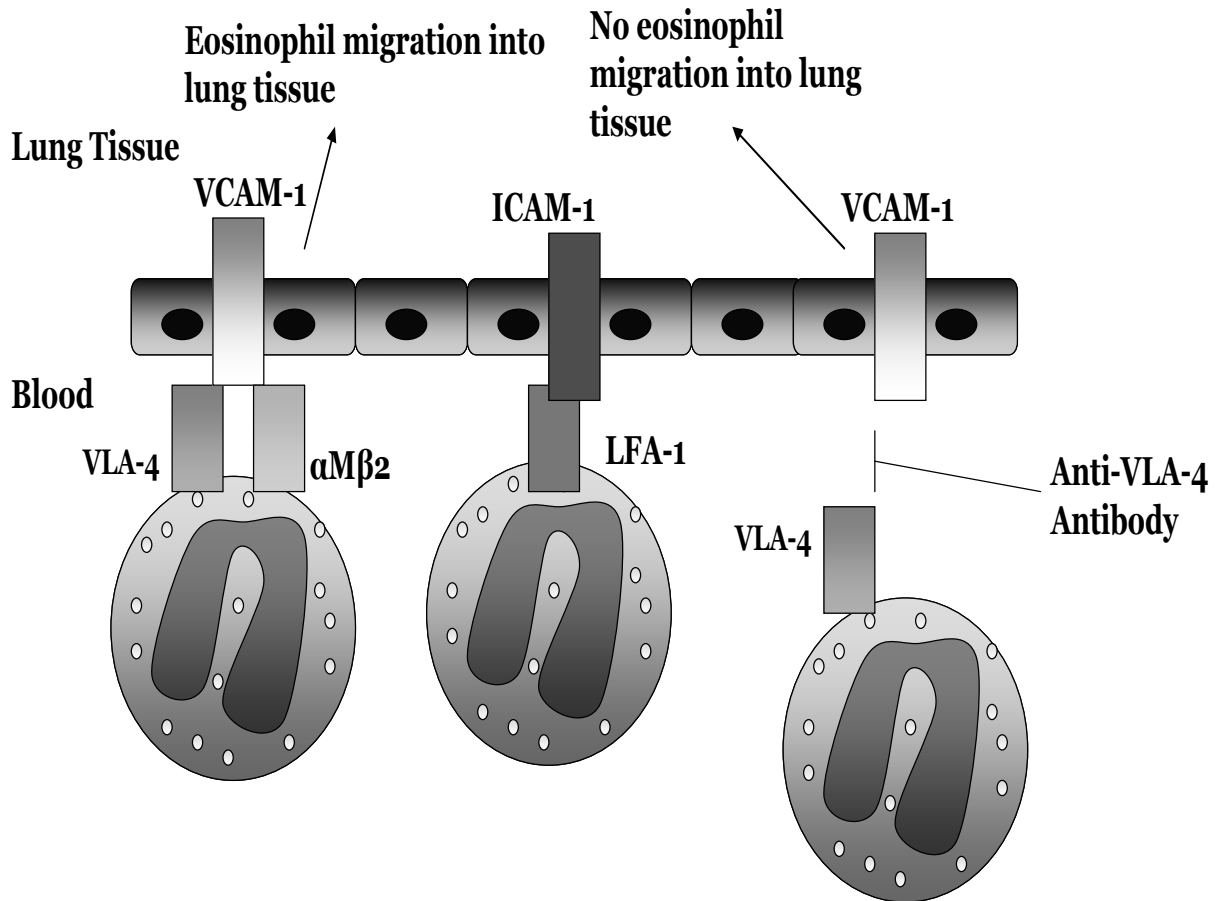


Figure 3. Eosinophil transmigration. Eosinophils express integrins such as VLA-4 or LFA-1, which can bind to VCAM-1 and ICAM-1 on the endothelial cells, thus facilitating eosinophil transmigration from the blood into the tissues. An inhibitor of VLA-4 or an anti-VLA-4 antibody will prevent eosinophils from binding to the endothelial layer, which prevents eosinophil migration into the lung tissue.

1.2.4 Eosinophils and Airway Remodeling

Structural changes in the airways which characterize airway remodeling contribute to the symptoms of asthma, as do airway hyperresponsiveness and airway obstruction. While the role of eosinophils in the asthmatic lung has been well established for many years, the role of

eosinophils in airway remodeling during asthma is not as well known. A galectin-3 gene therapy mouse model demonstrated a connection between airway remodeling and accumulation of airway eosinophils (76). Corticosteroid treatment that decreased goblet cell and airway smooth muscle cell hyperplasia also inhibited the allergen-induced increase in eosinophil numbers in an asthma model (77). Eosinophil accumulation is also associated with subepithelial fibrosis (44), and eosinophils can promote subepithelial fibrosis through the release of mediators and cytokines such as TGF- β 1 (78). Ovalbumin (OVA) challenge of eosinophil-ablated mice causes decreased deposition of extracellular matrix protein in subepithelial tissue and significantly less proliferation of airway smooth muscle cells (79). Anti-IL-13 antibody treatment decreased subepithelial fibrosis and goblet cell hyperplasia in asthmatic mice. Furthermore, this antibody negated the increase in eosinophil numbers in these mice (39). A plasmid encoding the *Gal-3* gene significantly reduced the number of eosinophils, as well as inflammation in the airways, of antigen-challenged mice. In addition, subepithelial fibrosis was significantly decreased (76), suggesting a role for eosinophils and airway remodeling in the asthmatic lung. The role of Jun N-terminal kinase (JNK) in airway remodeling also sheds light on some intracellular mechanisms involved in the pathogenesis of asthma, since its inhibition has been shown to decrease airway smooth muscle cell proliferation and infiltration of eosinophils into the airways (80). JNK may be involved in eosinophil-mediated airway remodeling.

The use of therapeutic drugs in animal models has demonstrated a role for eosinophils in airway remodeling. The anti-inflammatory drugs ciclesonide and fluticasone, when administered to rats challenged with OVA, significantly decreased features of airway remodeling, including goblet cell hyperplasia and increased airway smooth muscle mass. In

addition, there was a decrease in the amount of airway eosinophils (77). The cysteinyl leukotriene receptor antagonist montelukast has been shown to decrease the subepithelial deposition of collagen and increase airway smooth muscle mass in mice challenged with OVA to become asthmatic. The number of eosinophils in the lungs was also decreased (81). The inhibitors of cysteinyl leukotriene receptors provide potential mechanisms by which to inhibit the actions of eosinophils that negatively affect the airways during asthma.

1.3 TGF- β in Asthma

1.3.1 TGF- β

One molecule whose activity plays a pivotal role in asthma, particularly in airway remodeling, is TGF- β . Eosinophils can either release TGF- β or be stimulated by TGF- β , thus contributing to asthmatic features. Three isoforms of TGF- β protein ligand (TGF- β 1, TGF- β 2, and TGF- β 3), and six receptors (TGF- β RI, TGF- β RII, TGF- β RIII, TGF- β BRIV, TGF- β BRV, and TGF- β BRVI) have been identified (82). Thus, TGF- β can affect many cellular pathways to produce many different outcomes. For example, TGF- β 1 induces apoptosis of human fetal lung fibroblasts via Smad3 signaling (83). TGF- β 1 increases the metastasis of gastric carcinoma cells, and this effect is further enhanced by the overexpression of PKC- δ and Smad3 (11). TGF- β 1 treatment of human bone marrow stromal cells increases the expression of VLA-5 and decreases the expression of VLA-4 by human hematopoietic precursor cells and human myeloma cells for attachment to stromal cells (84). TGF- β 1 stimulation also increases the expression of cystic fibrosis transmembrane regulator (CFTR) chloride channels in human nasal polyps (85).

1.3.2 TGF- β and Asthma

TGF- β plays an essential role in asthmatic responses, and is involved with many different cell types when exerting its effects on the lungs during asthma (Table 2). Several studies have shown a genetic linkage between the TGF- β 1 gene and a predisposition to asthma. Gene polymorphisms in the promoter regions of the TGF- β 1 and TGF- β 2 genes in asthmatic subjects are linked with elevated levels of IgE, a key immunoglobulin in allergic reactions (86). The C-509T region of the TGF- β 1 gene is also linked with a higher frequency of asthma (87, 88). Two haplotypes of the TGF- β 1 gene (21GC and 23GT) are associated with a higher frequency of asthma and higher TGF- β 1 levels in the serum, while two different haplotypes (22GC and 24GC) are associated with a lower frequency of asthma and lower TGF- β 1 levels in serum (89).

The linkage between TGF- β and asthma is also shown in studies that do not involve genetic linkages. Asthmatic subjects have more TGF- β in their exhaled breath condensate than non-asthmatic subjects have (90). Asthmatic subjects also have higher TGF- β 1 levels in their serum (91). Some research has shown that TGF- β reduces asthmatic symptoms, however, since a subset of regulatory T cells (Tregs) that suppresses airway hyperresponsiveness and remodeling secretes high levels of TGF- β (92). Although some reports are contradictory, they all illustrate the important role that TGF- β plays in asthma.

Table 2. Major airway disease-associated effects of TGF- β acting on or being secreted from some of the major cells involved in chronic asthma.

Cell Type	Cellular Effects Associated with TGF- β on Augmenting or Alleviating Airway Disease Symptoms	
	<i>Release from Cell</i>	<i>Stimulation of Cell</i>
Eosinophil	ASM cell proliferation Extracellular matrix protein deposition Subepithelial fibrosis Airway tissue inflammation Mucus hypersecretion	Eosinophil apoptosis Eosinophil activation ECP release
Airway Epithelial Cell	Airway inflammation Airway tissue fibrosis Angiogenesis	
Airway Smooth Muscle Cell	Mast cell recruitment	MBP release Extracellular matrix protein deposition ASM cell proliferation ASM cell hypertrophy Airway smooth muscle cell migration
Dendritic Cell	Differentiation/activation of Tregs	Reduction in airway inflammation
Fibroblast	Airway tissue fibrosis	Eosinophil recruitment ECM protein deposition Tissue fibrosis
Monocyte/Macrophage	T lymphocyte apoptosis Airway Inflammation	
Neutrophil	Airway inflammation ASM cell activation & associated symptoms	
Regulatory T Cells	Suppression of TH2 cell responses	Treg differentiation/activation
TH2 cells		TH2 cell recruitment Subepithelial fibrosis Goblet cell hyperplasia Mucus hypersecretion

One facet of TGF- β activity that illustrates its dual role in both exacerbating and alleviating symptoms of asthma is its release from and effect on dendritic cells (DCs) and Tregs. TGF- β release provides an immunoregulatory mechanism that Tregs use to reduce asthmatic inflammation (31). TGF- β released from Tregs suppresses certain antigen-specific TH2 cell responses by inducing antibody class switching to the IgA isotype (93). In addition, expression of membrane-bound TGF- β on Tregs can interact directly with the Notch1-hairy and enhancer of split 1 (Notch1-HES1) mechanism on other CD4⁺ T cells via Notch ligands on their surface to suppress the deleterious immune response exerted by these cells in allergic asthma (94). TGF- β stimulation of a precursor TH cell population is important for cell differentiation into Tregs by inducing expression of CD25 and Foxp3 (95, 96) and DCs are a major source of this TGF- β (97). Moreover, release of TGF- β from DCs can induce differentiation of CD4⁺CD25⁻ T cells into CD4⁺CD25⁺ Tregs, which help regulate the asthmatic inflammatory response (97). Knockout of the transcription factor Runx3 inhibits dendritic cell response to TGF- β 1. This increases dendritic cell maturation and infiltration into the lungs, which causes airway inflammation and mucus hypersecretion (98) (Figure 4).

1.3.3 TGF- β and Airway Remodeling

TGF- β has been found to have a major role in airway remodeling that takes place during the asthmatic response. TGF- β 1 enhances proliferation of ASM cells (99), and administration of an anti-TGF- β antibody decreases ASM cell proliferation (100). TGF- β stimulation increases levels of the extracellular matrix proteins collagen and fibronectin in human ASM cells, suggesting that deposition of extracellular matrix proteins is augmented by TGF- β 1 (101). IL-13 stimulation to induce asthmatic inflammation in mice increased levels of

TGF- β 1 and tissue fibrosis, indicating that TGF- β 1 could play a role in inducing fibrosis of airway tissue during airway remodeling (102). TGF- β 1 has also been found to directly regulate airway tissue fibrosis that is induced by IL-13 (103). TGF- β can also increase collagen deposition on the extracellular matrix in airway tissue (100, 104).

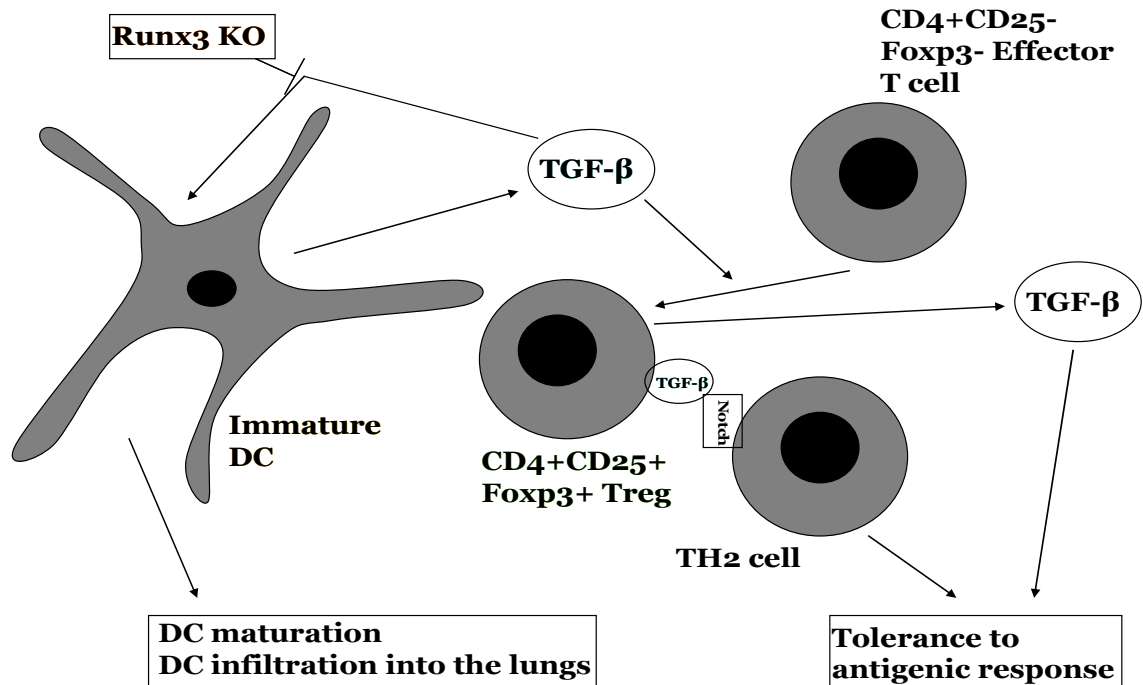


Figure 4. TGF- β action in asthma regulation. Immature DCs are a main source of TGF- β that can cause effector CD4+CD25- T cells to differentiate into CD4+CD25+ Tregs. These Tregs can then exert their immunomodulatory effects by releasing TGF- β to keep the asthmatic response in check. Membrane-bound TGF- β can bind to Notch on TH2 cells to activate the Notch-HES-1 complex, which suppresses the immune response induced by TH2 cells in asthma. When the transcription factor Runx3 is knocked out in immature DCs, however, they can mature and migrate to the lungs, inducing airway inflammation and mucus hypersecretion.

Intracellular mechanisms in signaling pathways downstream of TGF- β stimulation or extracellular mechanisms that induce TGF- β expression and secretion are important for the study of airway remodeling in asthma. Upon stimulation with TGF- β 1, p38 MAPK, extracellular signal-related protein kinase (ERK)-1/2, and JNK are all involved in airway smooth muscle cell signaling pathways which lead to increased airway smooth muscle cell proliferation (99). p38 MAPK phosphorylation of heat shock protein (HSP)-27 is a mechanism by which smooth muscle cell hyperplasia, hypertrophy, and migration are increased. HSP-27 modulates actin polymerization and cytoskeletal remodeling, thus allowing cells to migrate (105). Whether or not TGF- β 1 induces phosphorylation of HSP-27 to induce eosinophil migration requires further study. Nevertheless, activation of p38 MAPK and other kinases such as ERK1/2 and JNK are potential mechanisms by which TGF- β 1 acts to promote airway remodeling features. Angiotensin II upregulates the transcription factors c-Fos, early growth response (EGR)-1, and c-Jun in airway smooth muscle cells, which increases their release of TGF- β 1, thus acting in an autocrine manner and causing hypertrophy of the cells (106). Upon IL-1 β stimulation, c-Jun stimulates TGF- β 1 production and secretion from airway epithelial cells by binding to the TGF- β 1 promoter along with NF- κ B (107). These kinases and transcription factors could be key participants in increased TGF- β expression in and release from eosinophils, as well. TGF- β stimulation of bronchial smooth muscle cells induces release of major basic protein (MBP), which leads to increased bronchial fibrosis (108). TGF- β 1 stimulation of TH2 cells leads to the release IL-5 and IL-13, which causes subepithelial fibrosis, goblet cell hyperplasia, and mucus hypersecretion (109) (Figure 5).

Most of the intracellular mechanisms discussed until now promote characteristics of airway remodeling in asthma, but other intracellular molecules can help to decrease airway

remodeling features. The transcription factor Smad7 decreases the transcription of PAI (plasminogen activator inhibitor)-1 in bronchial epithelial cells induced by TGF- β 1, which leads to a decrease in the thickness of the basement membrane (110). Knockout of the transcription factor Runx3 inhibits dendritic cell response to TGF- β 1, which increases dendritic cell maturation and infiltration into the lungs and causes airway inflammation, mucus hypersecretion, and other aspects of airway remodeling (98). Stimulation or overexpression of these transcription factors within eosinophils may alleviate some of the effects of airway remodeling seen in the asthmatic lung.

1.3.4 TGF- β and Eosinophils

The connection between eosinophils and TGF- β in the airways of asthmatics is well documented, especially when examining the effects of certain intracellular or cell surface molecules on inducing or inhibiting asthmatic symptoms. In mice overexpressing GATA-3, increases in subepithelial fibrosis, eosinophil infiltration of the airways, and TGF- β are all observed (44). The chemokine receptor CXCR6, which is important in decreasing airway inflammation, reduces levels of both eosinophils in the lungs and TGF- β 1 (111). Eosinophils can also be activated directly by TGF- β , since they express TGF- β RI and TGF- β RII receptors. Stimulation by TGF- β 1 increases expression of both TGF- β 1 and TGF- β 2 mRNA within the eosinophils, and involves signaling via Smad2 and Smad3 pathways (112). TGF- β also increases the expression of IL-13R α on human eosinophils, thus allowing cytokines such as IL-4 and IL-13 to activate eosinophils in the airways of asthmatics (113). TGF- β stimulation of eosinophils can lead to the release of the mediator ECP (114) (Figure 5). TGF- β 1 stimulation, in conjunction with IL-17E stimulation, increases human lung fibroblasts' expression of

CXCL-8, which is a strong attractant and activator of eosinophils (115). TGF- β 1 can also be expressed in eosinophils after IL-4 stimulation via a pathway involving the transcription factor STAT-6 (116).

Reports on the relationship between TGF- β and eosinophils have not always shown a positive correlation, however. For example, one study demonstrated that TGF- β induces eosinophils to undergo apoptosis and blocks the pro-survival effects of IL-3, IL-5, and GM-CSF on eosinophils (117). Another report showed that TGF- β reduced airway hyperresponsiveness in allergen-challenged mice by, among other things, reducing the number of eosinophils in the airways (25). Although these studies are the exception, if more research shows that TGF- β can help control the activation of eosinophils, it would provide a different angle to take in using TGF- β to help regulate some of the negative effects caused by eosinophils on the lungs during asthma.

1.3.5 TGF- β and Eosinophils in Airway Remodeling

A strong correlation between TGF- β and eosinophils is observed when studying them in connection with symptoms of airway remodeling. Eosinophil recruitment to the airways and an increase in TGF- β 1 levels in the lungs causes subepithelial fibrosis and mucus hypersecretion (118). Eosinophils from asthmatic subjects show greater expression of TGF- β 1 mRNA than eosinophils from non-asthmatic subjects. This increase in eosinophil expression of TGF- β 1 increased the fibrosis of the airway tissue in asthmatic subjects (78). Similarly, an increase in TGF- β -positive eosinophils in asthmatics enhances the synthesis of procollagen type I in the airways of the asthmatic subjects (119). TGF- β released from eosinophils increases tissue fibrosis in nasal polyp tissue in asthmatic patients (114). Eosinophils can also

induce collagen synthesis in the airway tissue through the release of TGF- β (120). Stimulation of eosinophils with leukotriene D4, one of the cysteinyl leukotrienes that infiltrates the airways during asthma and induces airway smooth muscle cell proliferation, amplifies production of TGF- β by the eosinophils when IL-5 and GM-CSF are also present (121).

Other cytokines can work in conjunction with TGF- β and eosinophils to produce some of the effects of airway remodeling. In OVA-challenged mice, there are increases in eosinophil numbers in the BAL fluid and TGF- β concentrations after challenge, leading to increases in subepithelial and peribronchial fibrosis. However, knocking out the gene for IL-5R α or using an antibody against IL-5 decreases the fibrosis seen in the airways of the mice (122). Asthmatic subjects show higher TGF- β 1 concentrations and greater eosinophil counts than normal subjects, which augments deposition of extracellular matrix proteins. Inhibition of IL-5 reduces all three of these effects (123). TGF- β 1 and eosinophils induce fibroblast secretion of IL-6, producing airway tissue fibrosis (124). The ability of TGF- β 1 and eosinophils to induce remodeling events such as tissue fibrosis is also augmented by IL-13, working alone or synergistically with EGR-1 (125). Some cellular molecules naturally reduce the effects of both TGF- β and eosinophils on airway remodeling. Hepatocyte growth factor (HGF) decreases levels of both eosinophils and TGF- β 1, which leads to a decrease in airway tissue fibrosis and smooth muscle cell hyperplasia (126).

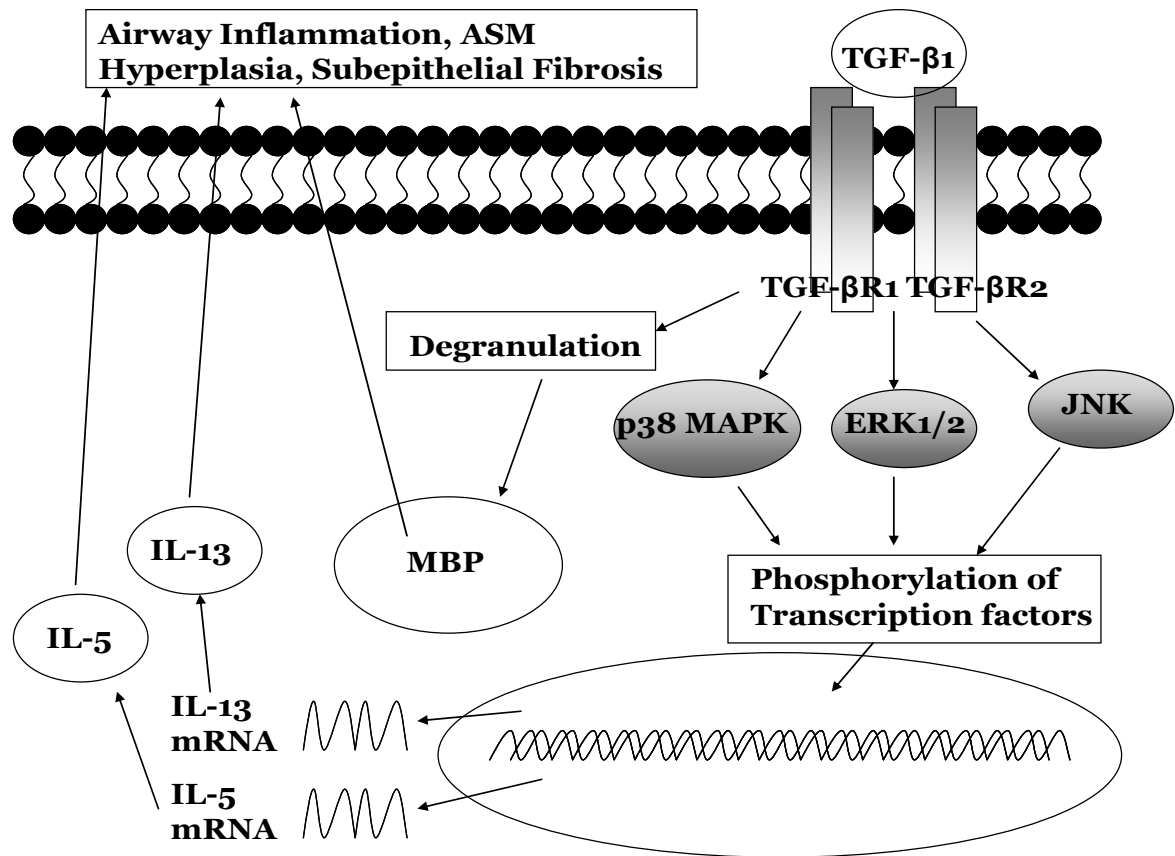


Figure 5. Effect of TGF-β1 on cytokine and mediator release. TGF-β1 stimulation induces activation of kinases, which leads to activation of transcription factors. This causes many different types of cells to release mediators and cytokines, which can augment features of airway remodeling.

Earlier, it was postulated that TGF-β may enhance the expression and activation of certain intracellular molecules, such as various transcription factors, in order to induce some of the features of airway remodeling. By showing the connection that both increased TGF-β levels and eosinophil numbers have with airway remodeling, it is reasonable to suggest that TGF-β stimulation of eosinophils may produce some of the remodeling effects through the release of various mediators and cytokines from the eosinophils. TGF-β could even act in an

autocrine manner or act on other cell types. Therefore, if models involving TGF- β stimulation of eosinophils are studied and mechanisms worked out, it may provide additional potential treatment targets for asthma, particularly with regard to airway remodeling in asthma.

Potential treatments for asthma have already been used experimentally, and these have also demonstrated the major role that eosinophils and TGF- β play in airway remodeling. Many treatments to alleviate asthmatic symptoms focus on immunotherapies that reduce the TH2 response, which includes high levels of cytokines such as IL-5 that induce eosinophil proliferation and activation (127). Immunostimulatory DNA used in a mouse model decreases bronchial tissue fibrosis, while also decreasing eosinophil counts and levels of TGF- β 1 (128). In addition, use of flunisolide on sputum cells from asthmatic subjects decreases factors such as MMP-9 and fibronectin that promote tissue remodeling, along with decreasing the release of TGF- β from the sputum cells and increasing apoptosis of eosinophils (129). The steroid fluticasone decreases collagen synthesis and airway inflammation by reducing eosinophil numbers and TGF- β -positive cells (130). Another asthma treatment, pirfenidone, decreases eosinophil numbers and TGF- β concentration in the lungs of a murine asthma model. Decreases in airway tissue fibrosis and smooth muscle cell proliferation are also present in the lungs after this treatment (131). Administration of edaravone to rabbits with pulmonary fibrosis, an important facet of airway remodeling in asthma, reduces fibrosis by reducing numbers of eosinophils and the amount of TGF- β -expressing cells, many of which could be eosinophils (132). Another anti-allergic drug, suplatast tosilate, attenuates airway inflammation by reducing eosinophil chemotaxis and extravasation (133). Not much work has been done on isoforms of TGF- β besides TGF- β 1 in relation to asthma and airway remodeling. However, one report showed increased expression of TGF- β 2 by eosinophils in asthmatic

subjects, as well as an increase in the thickness of the subepithelial basement membrane (134). Similarly, an increase in TGF- β 2-positive eosinophils in the lungs is seen in asthmatic patients after allergen challenge (135). This demonstrates a correlation between a different isoform of TGF- β and eosinophils that is connected with airway remodeling.

1.4. Intracellular Kinases and Transcription Factors

1.4.1 PKC Isozymes

PKC isozymes can be divided into three groups: conventional, novel, and atypical. The conventional isozymes consist of PKC- α , PKC- β 1, PKC- β 2, and PKC- γ . These isozymes can be activated by diacylglycerol (DAG) through their DAG-sensitive C1 domain, or by calcium through their calcium-sensitive C2 domain. The novel isozymes, which include PKC- δ , PKC- ϵ , PKC- η , and PKC- θ , have a DAG-sensitive C1 domain, but they also have a C2-related domain that is insensitive to calcium. The atypical isozymes, PKC- ζ and PKC- τ/λ , have a C1 domain that is not DAG-sensitive, and have not C2 or C2-related domain. Regulation of activation of this group of isozymes occurs via a PB1 domain on the N-terminus of the protein (136, 137).

DAG recruits PKC isozymes to the membrane, thus increasing PKC's binding affinity to the membrane. DAG also stabilizes the active configuration of PKC (138). Phorbol esters also increase the binding affinity of PKC to the membrane (139), and they do so to a greater extent than DAG (138). Activation occurs only after the pseudosubstrate that inhibits the PKC isozymes is exposed for proteolysis (140). Upon activation of PKC, its isozymes generally phosphorylate other intracellular molecules, causing many different responses in the cell to

take place, such as cell differentiation and cell proliferation (141). PKC can also cleave molecules, as shown by its ability to act as an ATPase (142).

1.4.2 PKC and Smad

PKC isozymes interact with many downstream molecules when exerting their various effects on cells. For example, PKC molecules can be involved in signaling pathways with the Smad proteins. PKC- δ induces increased expression of collagen I in human mesangial cells after TGF- β 1 stimulation, since inhibition of PKC- δ diminished the effect of TGF- β 1 on increasing collagen I expression. Inhibition of Smad3 also attenuated increased collagen I expression, showing how Smad3 is involved in this signaling pathway with PKC- δ (12). PKC can also work with other Smad proteins. In human pancreatic stellate cells, overexpression of Smad7 increased cellular proliferation induced by angiotensin II. The inhibition of PKC blocked the increased expression of Smad7 induced by angiotensin II that leads to proliferation of these cells, and the activation of PKC increased Smad7 expression (18). In endothelial cells, overexpression of the short form of Smad6 (Smad6s) increases the TGF- β 1-induced production and secretion of PAI-1, and depletion of Smad6s decreases PAI production. This also involves PKC- β , since inhibition of PKC- β reduces PAI production that is stimulated by Smad6s (143).

1.5 Chloride Channels

Chloride channels are transmembrane regulators which can induce or prevent a wide variety of cellular functions. There are many different groups of chloride channels which are separated based upon structure and method of activation. Some well-known examples include the CFTR, ligand-gated channels and Ca²⁺-activated channels (144). Another major family of

chloride channels is the CLC family. The mammalian homologues of the CLC family can be divided into three groups based upon genetic similarity: (CLC-0, -1, -2, -K1, -K2), (CLC-3, -4, -5), and (CLC-6, -7) (145). Structurally, they are all homodimers, thus giving them two pores for anions to pass through (145, 146). All of the CLC family members are voltage-gated, but they can also respond to other stimuli, such as changes in pH (CLC-0, -1, -4, and -5), or changes in cell volume (CLC-2, -3). CLC-3 can also be activated by phosphorylation. Although most of the CLC family proteins are expressed on the plasma membrane, CLC-4 and CLC-5 are mostly expressed on intracellular organelles, such as mitochondria (145).

1.6 Cell Migration

Many different signaling networks and molecules underlie the process of cell migration. Actin and myosin II are molecules that play a pivotal role in cell migration. The actin-myosin II system induces polarization of the cytoplasm, which leads to the formation of an asymmetrical shape. This shape change induced by self-polarization facilitates migration of the cell (147). Another protein that binds to actin, coronin, increases the ability of cells to migrate (148). Coronin has also shown an ability to inhibit cell migration, however, by binding to and inhibiting the action of actin-related protein (Arp) 2/3 (149). Microtubules also function in cell motility through their involvement in formation of the shape of the cytoskeleton. Microtubules are stabilized by a cap at their plus-end. This cap contains an ATPase that allows for the stabilization of the microtubules, thus allowing them to take part in cell functions such as inducing shape change to lead to cell migration (150). Cell motility is also dependent on the flexibility of the substrate to which the cell is attached or along which the cell moves. A substrate that is more flexible allows for increased motility of the cell by

allowing the cell to make more adjustments to the surrounding environment to increase its capability of migrating and/or adhering for migration on or through the substrate (151).

1.6.1 PKC and Cell Migration

One major cell functions that PKC can affect is cell migration. Various PKC isozymes play a major role in fibroblast migration. Microtubule stabilization in fibroblasts induced by the molecules Rho and mDia leading to cell migration requires the activity of PKC, which is upstream of glycogen synthase kinase 3 β (GSK3 β) and the microtubule stabilization protein end binding protein-1 (EB1) (152). PDGF-induced migration of fibroblasts via phosphorylation of p42/44 MAPK is dependent on PKC, and PKC activity in this pathway is negatively regulated by lipid phosphate phosphatase (LPP)-1 (153). Specifically, PKC- δ is part of a pathway in dermal fibroblasts that induces their migration upon stimulation with platelet-derived growth factor (PDGF)-BB. In this signaling cascade, PKC- δ is upstream of STAT-3 (154). PKC phosphorylation of coronin 1B in fibroblasts allows coronin 1B to interact with Arp 2/3, which leads to migration of the fibroblasts (155).

PKC isozymes play a significant role in the migration of endothelial cells. Endothelin-1 induction of endothelial cell migration involves PKC, with p38 MAPK, ERK, and JNK being downstream of PKC in this endothelial cell signaling cascade (156). PKC- α induces migration of endothelial cells that is necessary for wound healing (157). Sphingosine 1-phosphate (S1P)-mediated endothelial cell migration is dependent on PKC- α (158). PKC- α is also involved in histamine-induced migration of endothelial cells. Activation of PKC- α upregulates sphingosine kinase (SK)-1 to increase the cell migration (159). In contrast, PKC- δ negatively regulates endothelial cell migration (160).

Intracellular signaling pathways in other cell types demonstrate key roles for PKC isozymes in cell migration and chemotaxis. Stress-induced migration of vascular smooth muscle cells via phospholipase D activation is dependent on PKC- α activation (17). Vascular smooth muscle cell chemotaxis in response to PDGF-BB stimulation is dependent on PKC- β II activation, which leads to activation of PI3K and the ERK/MAPK pathway (161). PKC- β 1 is involved in neutrophil chemotaxis (162). Neutrophil chemotaxis is also indirectly affected by PKC- α activity in keratinocytes, since PKC- α promotes nuclear factor (NF)- κ B-induced transcription of inflammatory cytokines and chemokines that draw neutrophils to the sites of allergic inflammation (163). PKC- α activation is also necessary for migration of osteoclasts and Chinese hamster ovary cells (164). T cell migration induced by LFA-1 is dependent on PKC- β , and hepatitis C envelope protein E2 can inhibit T cell migration by targeting this pathway (165). In a T cell line, PKC was shown to have a significant effect on increasing cell migration through upregulation of CXCR-3. This receptor can then be bound by the CXC chemokine ligand (CCL)-11 to induce the migration of the cells (166). Progenitor CD34+ cells in the bone marrow demonstrate increased chemotaxis in response to stromal cell-derived factor (SDF)-1, and this is dependent on PKC- ζ (167). Phosphoprotein enriched in astrocytes-15 kDa (PEA-15) stimulates astrocyte migration via a PKC- δ -dependent pathway (168). Mesangial cell migration is dependent on PKC which, in conjunction with MAPK/ERK kinase (MEK), leads to transcription of SK-1, thus inducing migration of the cell (169).

1.6.2 Chloride Channels and Cell Migration

Chloride channel activity is one functional aspect within eosinophils that may play a role in eosinophil activation. TGF- β 1 could be involved in the induction of chloride channel

activity in eosinophils, since it increases chloride channel expression in fibroblasts (9). Increased chloride channel activity may play a role in eosinophil migration, since it is key for the migration of other cell types, such as neutrophils and monocytes (7, 8). Chloride channels, specifically chloride channel (CLC)-3 channel, are involved in production and release of superoxide ions from eosinophils (6). In addition, TGF- β induces chemotaxis in eosinophils (10).

Shape change of the cell is another important cell process which allows cell migration across the endothelium and into the lung tissue to occur. This is true for eosinophils, as their shape change has been shown to be critical to the cells' migration (170). When a cell changes shape, its cytoskeleton must reorganize in order to facilitate the new cell shape. Changes in chloride ion concentration can activate the Rho molecular pathway, which reorganizes the cytoskeleton. Chloride channel activation can induce changes in cell volume, which causes activation of molecules such as Src. Src activates Vav, which activates Rho GTPases such as RhoA or Rac1 to reorganize the cytoskeleton (171). Chloride channel activation can also lead to activation of myosin light chain proteins by PI3 kinase or phospholipase C (PLC), which produces tension on the cytoskeleton (172). If PLC activity produces changes in the cytoskeleton which could induce migration of the cell, it is also possible that PLC activates PKC molecules to produce tension on the cytoskeleton independently of chloride channel transcription and/or activation, but this has yet to be investigated.

1.6.3 PKC, Smad and Cell Migration

The interactions between PKC, Smad, and oxide can affect the cell in many ways, one of which is by causing increased cell migration. Overexpression of PKC in gastric

carcinoma cells increases the spreading of the cells that is induced by TGF- β 1, and this increase in cell spreading correlates with an increase in cell migration. The PKC- δ isozyme showed the greatest increase in activity in response to this pathway that is activated. In addition, the increased activity of PKC- δ that is induced by TGF- β 1 leading to greater migration and spreading of the cells is further enhanced by the overexpression of Smad3 (11).

1.7 Hypothesis and Specific Aims

Hypothesis

TGF- β 1 induces migration of human blood eosinophils by increasing the activity of chloride channels, and this chloride channel-dependent migration is enhanced in allergic asthmatic eosinophils.

Specific Aims

1. Examine the effect of TGF- β 1 on eosinophil chloride channel expression, chloride current and migration, and determine if chloride channel activity is necessary for TGF- β 1-induced migration
2. Determine the intracellular molecules (kinases, transcription factors) that play a role in inducing chloride channel transcription and activity.
3. Compare eosinophils from non-asthmatic and allergic asthmatic subjects to determine if differences exist between chloride channel expression and activity, migration, and PKC

isozyme and Smad transcription factor activities, which are similar to differences observed between unstimulated and TGF- β 1-stimulated normal eosinophils.

Chapter 2

Materials and Methods

2.1 Isolation of Human Blood Eosinophils

Eosinophils were isolated from peripheral blood of normal human donors or allergic asthmatic donors with the approval of the Internal Review Board of Creighton University. All subjects gave informed written consent, as required by the IRB. 120 ml of blood was drawn from each person using EDTA as an anti-coagulant. Blood was diluted 1:1 with 0.9% saline, layered over Fico/Lite LymphoH solution (Atlanta Biologicals, Lawrenceville, GA), and centrifuged to separate mononuclear cells from RBCs and granulocytes. Dextran sedimentation and hypotonic lysis were used to remove red blood cells. Eosinophils were then isolated by negative selection using a cocktail of antibodies with magnetic beads in an Eosinophil Isolation Kit (Miltenyi Biotec, Auburn, CA) and passing the cells through an autoMACS (Miltenyi Biotec, Auburn, CA). Purity (98-99%) and viability (95%) of eosinophils was determined by staining with Hema-Diff (StatLab, Lewisville, TX).

2.2 Eosinophil Culture and RT-PCR

Eosinophils were stimulated with 10 ng/ml of TGF- β 1 (Peprotech, Rocky Hill, NJ) in RPMI-1640 (Sigma-Aldrich, St. Louis, MO) with 10% FBS for 24 h at 37°C and 5% CO₂. RNA was extracted using TRI Reagent (Sigma-Aldrich, St. Louis, MO). RNA was reverse transcribed and the cDNA product was amplified by PCR using the ImProm-II Reverse Transcription System (Promega, Madison, WI). Primers specific for CLC-2 (F:5'-TGGTTCCCAGATGGGATTCACACA-3', R:5'-TGGCCAGAATGACGGCGATCATAA-3'), CLC-3 (F:5'-CATGTCAATGGGGAGG-3', R:5'-GCAAGAAAGGCAAAACT-3'), CLC-4 (F:5'-AAATAACGCCAGACAGAGGCAGGA-3', R:5'-TCACTGTAAACGGGCTGAGGTTCA-3'), CLC-5 (F:5'-

AACTGACTGGTGGCCTGGAATACA-3', R:5'-ATCACGTCCATTGCCAGGGTCTTA-3), and β -actin (F:5'-TGACCGGCTTGTATGCTATC-3', R:5'-CAGTGTGAGCCAGGATATAG-3') (IDT, Coralville, IA) were used for the PCR. Electrophoresis of the amplified PCR product was performed on a 1% agarose gel, and the mRNA expression was examined under UV light in the UVP Bioimaging System (Upland, CA).

Primers specific for real-time PCR for CLC-2 (F:5'-GGGAGTGGTGCTGAAAGAATACCT-3', R:5'-CAAAGAGGGAGAGGAACTTGCTGA-3'), CLC-3 (F:5'-ACAAGAATGACTGTCTCCCTGGTG-3', R:5'-CATAAATGCCTTCCCTGCCAAAGG-3'), CLC-4 (F:5'-GATGAGTGGCTCTGGAAACCTGAT-3', R:5'-TCTTGCTGGTGATCTTCCTGTGTC-3'), CLC-5 (F:5'-CCATCAATCCATTTGGGAACAGCC-3', R:5'-GTTTGTGCGGATAAACAGTGCTCC-3'), and β -actin (F:5'-CTGGCACCCAGCACAATG-3', R:5'-GCCGATCCACACGGAGTACT-3') (Invitrogen, Carlsbad, CA) were used to amplify and quantify the cDNA product and determine statistical differences between groups. Amount of mRNA from the gene of interest that was present was determined by the number of PCR cycles that were needed for the sample to begin doubling the amount of cDNA each cycle. One less cycle corresponds to twice as much mRNA. β -actin mRNA was also measured in the same manner for each sample as a housekeeping gene.

2.3 Western Blotting

Isolated eosinophils were stimulated with 1, 10, or 25 ng/ml of TGF- β 1 (Peprotech) in RPMI-1640 with 10% FBS for 15 minutes, 30 minutes, 1 hour, 2 hours, 3 hours, 4 hours, or 24

hours in the presence and absence of the PKC- δ /PKC- ϵ inhibitor rottlerin (10 μ M), the general PKC inhibitor staurosporine (1 μ M), or the tyrosine kinase inhibitor genistein (10 μ M). Protein was extracted from the human blood eosinophils using RIPA Buffer (Sigma-Aldrich, St. Louis, MO). The protein samples were run on an electrophoresis gradient gel (4-20%) using SDS-PAGE and then transferred to a nitrocellulose membrane. After incubating the membranes with 5% milk in PBS (with 0.05% Tween-20) to block any nonspecific binding, they were incubated with primary antibodies for CLC-2, CLC-3, CLC-4, CLC-5 and PKC- ζ (Alpha Diagnostic, San Antonio, TX), PKC- β 1, PKC- δ , PKC- γ , PKC- ϵ and phospho-Smad3 (Santa Cruz Biotechnology, Santa Cruz, CA) at 1:500 dilution overnight, then with the secondary antibody conjugated to horseradish peroxidase for 1 h. The intensity of the protein expression was determined using the UVP Bioimaging System.

2.4 Electrophysiology

The isolated human blood eosinophils were patch clamped in the whole-cell configuration. Eosinophils were soaked in RPMI-1640 medium and were untreated or treated with 10 ng/ml TGF- β 1, 10 ng/ml eotaxin, or 25 ng/ml interleukin (IL)-5 at 37°C for 30 minutes, or with TGF- β 1 (10ng/ml) for 30 minutes in the presence and absence of the PKC- δ and PKC- ϵ inhibitor rottlerin (10 μ M) or the general PKC inhibitor staurosporine (1 μ M). The patch pipettes (resistance of 4-7 M Ω) were fabricated from 1.5 O.D. borosilicate glass capillaries (World Precision Instruments, New Haven, CT) on a micropipette puller (Sutter Instrument Company, Novato, CA). The pipettes were filled with an internal solution (pH 7.4) containing 140 mM CsCl, 1 mM CaCl₂, 1 mM MgCl₂, and 10 mM HEPES. The extracellular solution (pH 7.4) contained 140 mM CsCl, 1 mM EGTA, 0.05 mM CaCl₂, 1 mM MgCl₂, and

10 mM HEPES. The osmolarity of the solutions was 300 mOsm. All recordings were performed at room temperature (20-25 °C) using Digidata 1322A data acquisition system, Axopatch 200B patch-clamp amplifier, and pClamp 8.2 software (Axon Instruments, Foster City, CA, USA). Currents were low-pass filtered at 5 kHz using Axopatch amplifier. Data analysis was performed using Clampfit (version 8.2 of pClamp). Pipette offset current was zeroed immediately before contacting the cell membrane. Only standard whole-cell experiments with access resistance less than 10 M Ω and membrane resistance greater than 500 M Ω were included in this study. Chloride channel activity was indicated by the magnitude of the chloride current obtained.

2.5 Chemotaxis Assay

Eosinophil chemotaxis was measured with a 48-well Boyden microchemotaxis chamber (Neuroprobe, Cabin John, MD). TGF- β 1 (1, 10, or 100 ng/ml) in the presence and absence of the chloride channel inhibitors 4,4'-diisothiocyanatostilbene-2,2'-disulfonic acid (DIDS) (100 μ M) or 5-nitro-2-(3-phenylpropylamino)benzoic acid (NPPB) (100 μ M), in the presence and absence of the PKC- δ and PKC- ϵ inhibitor rottlerin (10 μ M) or the tyrosine kinase inhibitor genistein (10 μ M), or chemotaxis buffer (26.5 μ l) alone, was warmed to 37°C and added to the lower compartment. A Nucleopore Track-etch membrane with a pore width of 5 μ m (Corning, Acton, MA) was placed between the lower and upper compartments. The filters were pre-soaked in chemotaxis buffer. Purified eosinophils were added to the upper compartment. There were approximately 100,000 cells in each well. The chamber was subsequently incubated at 37°C with 5% CO₂ for 3 h. The filter was removed, fixed in ethanol for 1 minute

and stained with Diff Quick (Baxter, Miami, FL). Migrated eosinophils were counted in 10 randomly selected fields at a magnification of 400x.

2.6 Cell Shape Change Assay

Eosinophil shape change was assayed as previously described (23), to determine if cells are simulating what they would do in vivo when eosinophils migrate out of the peripheral blood and into the lung tissue. Purified eosinophils were suspended in assay buffer for 30 minutes at 37°C, and were unstimulated, stimulated with 1, 10, or 100 ng/ml TGF- β 1, 100 μ M DIDS or NPPB, or 100 μ M DIDS or NPPB and 10 ng/ml TGF- β 1. To stop the reaction, samples were transferred to ice and fixed with 250 μ l of fixative solution. Samples were immediately analyzed on a FACS Calibur flow cytometer (BD Biosciences, Franklin Lakes, NJ) and eosinophils were identified by their forward scatter/side scatter (FSC/SSC) characteristics. As cells were passed through the tube of the flow cytometer, pressure was maintained as nearly identical between the solution that the cells were in and the sheath saline solution that encircles the cell suspension, thus keeping the cells flowing in a very narrow stream. This allows changes in cell shape to better correlate with the amount of forward scatter. Higher forward scatter was considered to represent a change in the cell shape. TGF- β 1-induced eosinophil shape change was compared with unstimulated eosinophils, and a percent change in FSC was used to estimate the extent of the shape change from unstimulated cells. Ten thousand eosinophil events were counted for each sample.

2.7 Assessment of Differences between Normal and Asthmatic Eosinophils

Eosinophils were isolated from the peripheral blood of normal and asthmatic subjects. Protein was extracted for use in western blotting. The protein expression was assessed using antibodies specific for CLC-2, CLC-3, CLC-4 and CLC-5.

2.8 Statistical Analysis

All values are expressed as means \pm SEM. Data were calculated and statistical differences between two groups were analyzed using a *t* test. Statistical differences between more than two groups were analyzed using a standard one-way ANOVA test followed by Bonferroni's multiple comparison test. Differences were considered significant at $p < 0.05$.

Chapter 3

Results

3.1 Chloride Channel Expression

Using quantitative RT-PCR, we found that the expression of chloride channel CLC-3 mRNA significantly increased ($p < .05$) in eosinophils after TGF- β 1 stimulation at 10 ng/ml for 24 hours (Figure 6A). There was also an increase in CLC-3 mRNA (Figure 6B) and protein (Figure 6C) expression as shown by conventional PCR and western blot, respectively, after TGF- β 1 stimulation at 10 ng/ml for 24 hours. CLC-2, CLC-4, and CLC-5 showed no protein expression in eosinophils with or without stimulation, and mRNA expression of these three isozymes was inconsistent.

3.2 Chloride Channel Activity

Chloride channel activity of the eosinophils increased upon stimulation with TGF- β 1 at 10 ng/ml for 30 minutes, as demonstrated by an increase in the chloride current (Figure 7A). IL-5 (25 ng/ml) and eotaxin (10 ng/ml) stimulation of eosinophils had no effect on the chloride current (Figure 7B, 7C).

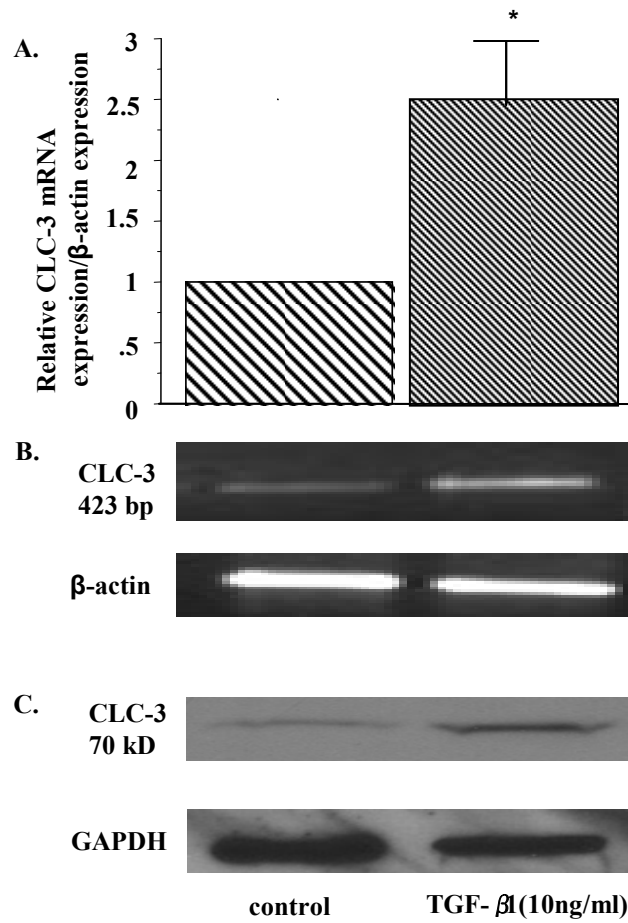


Figure 6. TGF-β1 induction of CLC expression in eosinophils. A) Quantitative PCR analysis of CLC-3 after stimulation with TGF-β1 (10 ng/ml) for 24 hours, * $p < .05$, $N = 3$; B) mRNA expression of CLC-3 after stimulation with TGF-β1 (10 ng/ml) for 24 hours as shown by semi-quantitative PCR; C) Western blot showing the expression of CLC-3 after treatment with TGF-β1 (10 ng/ml) for 24 hours.

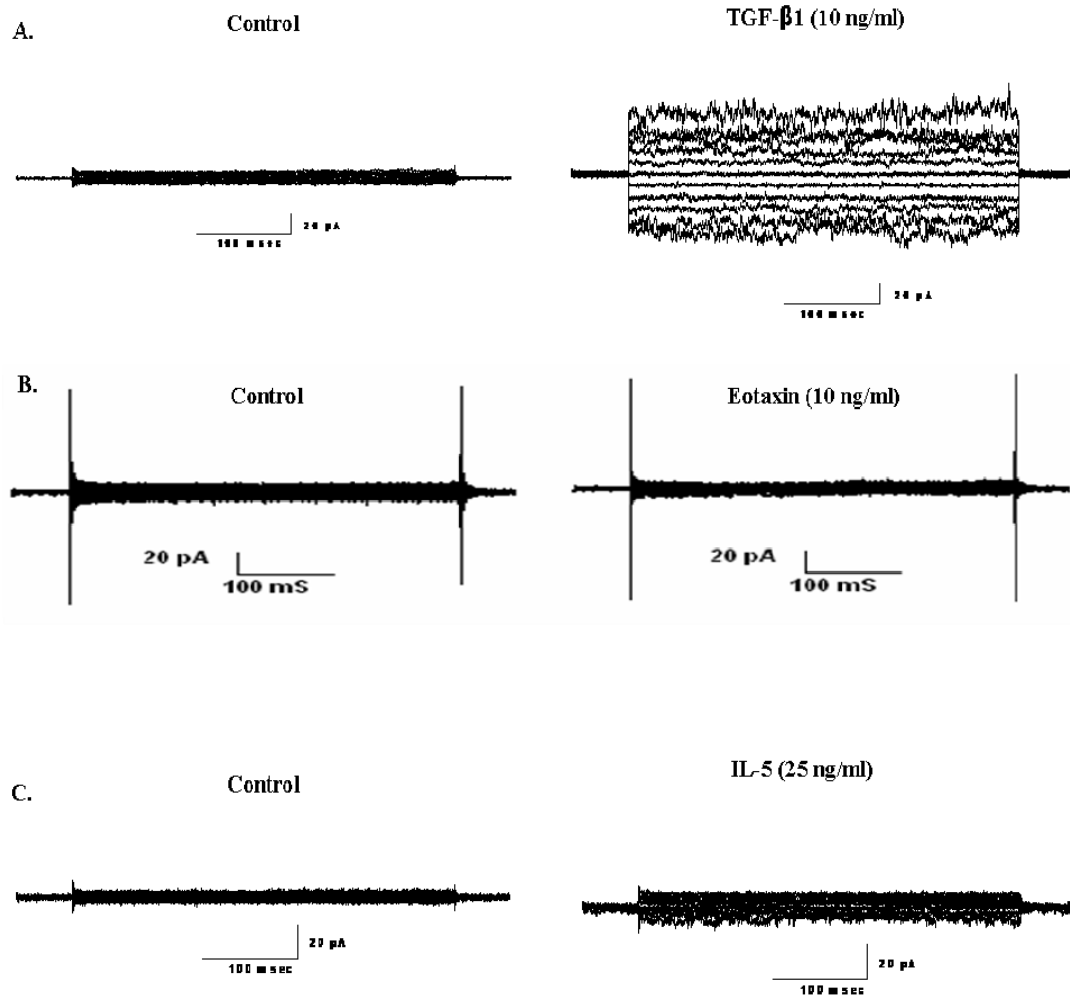


Figure 7. TGF- β 1 induction of chloride channel activity in eosinophils. Chloride channel activity of human blood eosinophils as measured by whole-cell current obtained by patch clamping. Comparisons are made between unstimulated control cells and cells after: **A.** TGF- β 1 (10 ng/ml) N=10; **B.** eotaxin (10 ng/ml) N=3; or **C.** IL-5 (25 ng/ml) stimulation for 30 minutes; N=3.

3.3 Chemotaxis

Using the Boyden microchemotaxis chamber, an increase in chemotaxis of eosinophils was observed upon stimulation with TGF- β 1 at 1, 10, and 100 ng/ml, when compared to the unstimulated control ($p < 0.05$) (Figure 8A). In addition, the chloride channel inhibitors DIDS and NPPB both decreased the chemotaxis of eosinophils induced by TGF- β 1 when compared to the TGF- β 1-stimulated cells ($p < 0.05$) (Figure 8B).

3.4 Cell Shape Change

Use of flow cytometry showed a significant change in shape of eosinophils after stimulation with both 10 ng/ml and 100 ng/ml ($p < 0.05$) (Figure 9A). Both chloride channel blockers, DIDS and NPPB, significantly decreased the amount of shape change induced by TGF- β 1 ($p < 0.05$) (Figure 9B). Cell viability of each sample was approximately 90-95%.

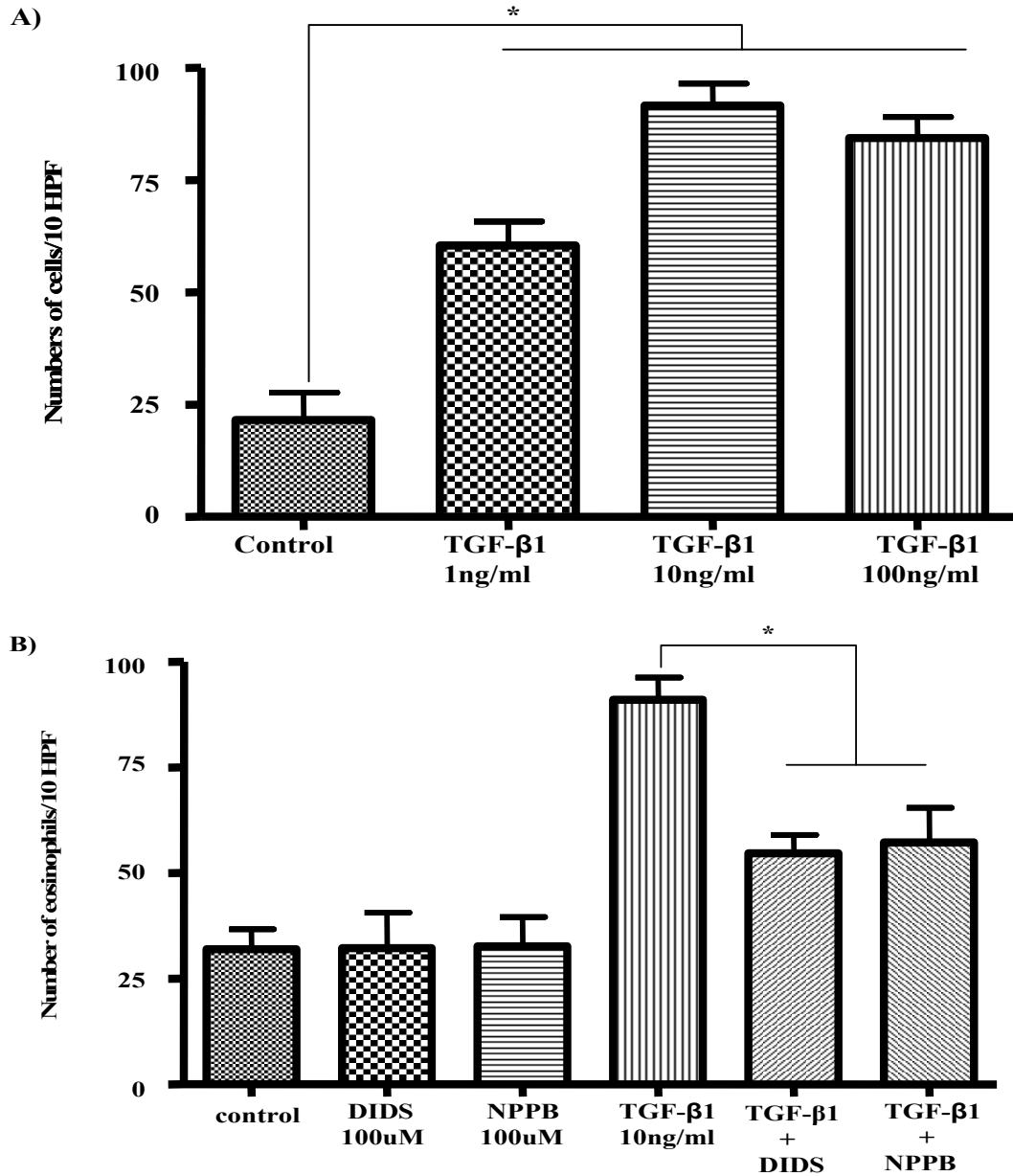


Figure 8. TGF-β1 induction of chemotaxis in eosinophils. A) Dose response of TGF-β1 on eosinophil chemotaxis. * $p < 0.05$ for all three doses as compared to control. **B)** Effect of the chloride channel blockers DIDS (100 μ M) and NPPB (100 μ M) on eosinophil chemotaxis induced by TGF-β1 (10 ng/ml). * $p < 0.05$. Results for each group are expressed as mean \pm SEM of the number of eosinophils in 10 randomly selected high power fields (HPF) from four experiments.

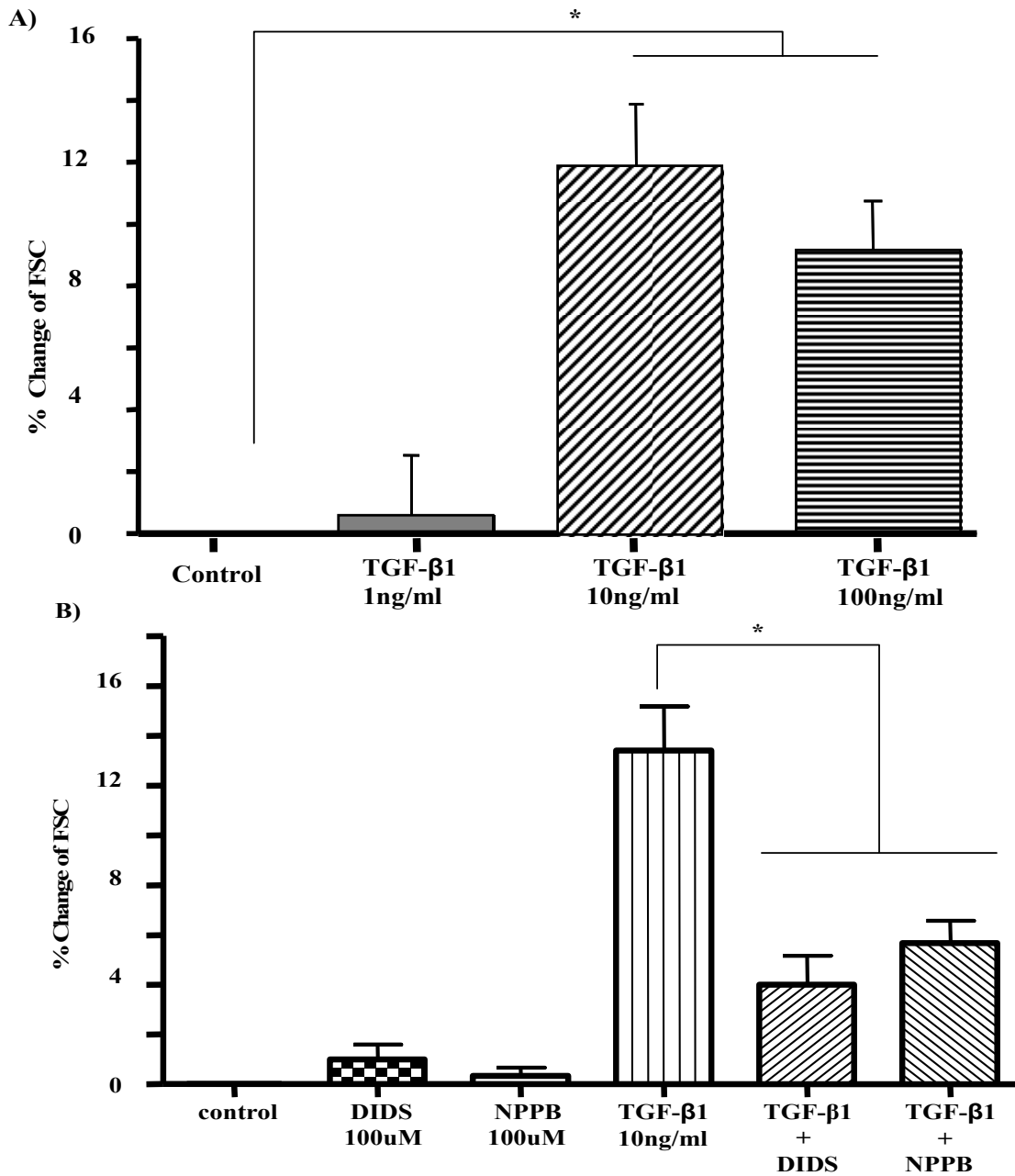


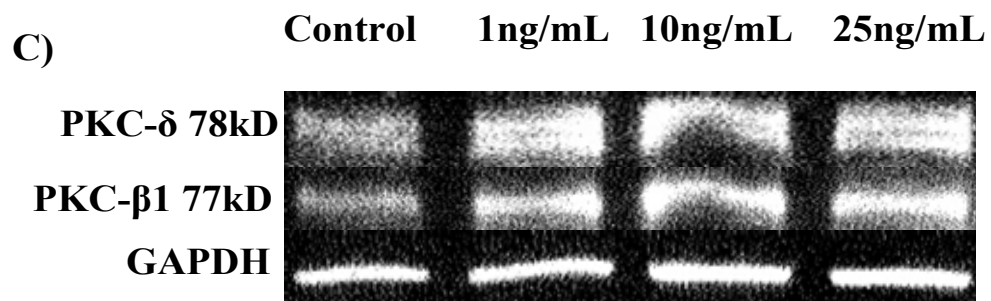
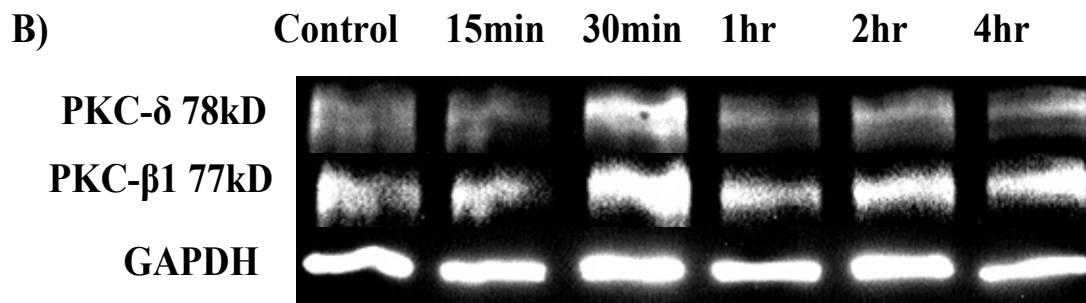
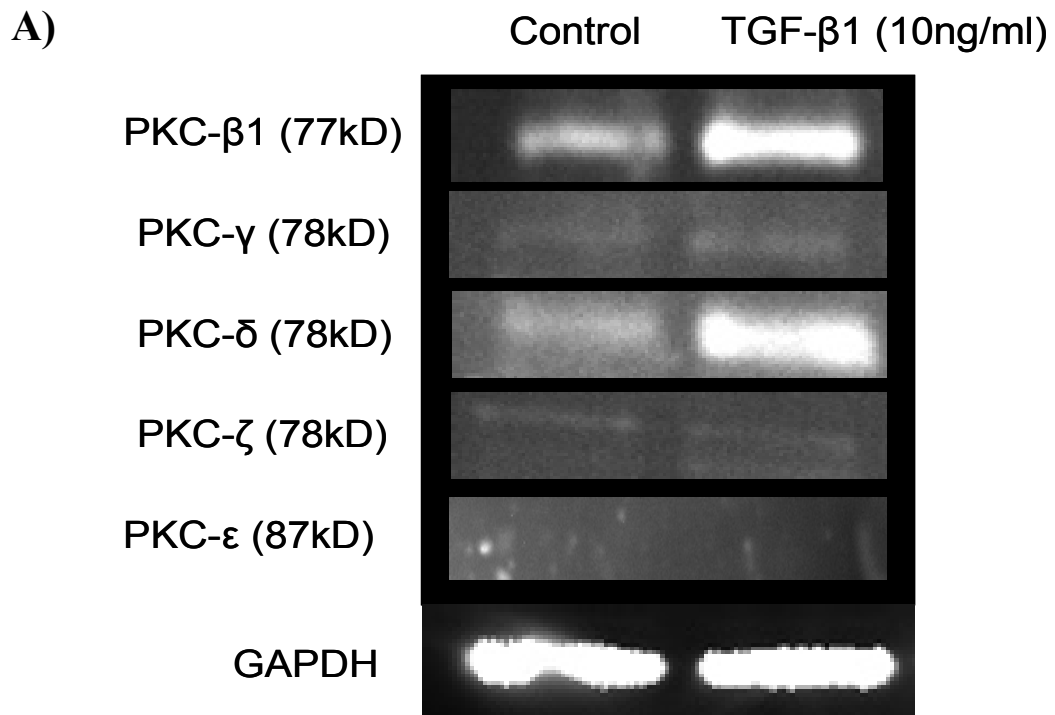
Figure 9. Effect of TGF-β1 and chloride channel blockers on eosinophil shape change. Shape change in eosinophils as measured by the percentage change in forward scatter (FSC). **A)** Results are expressed as percent increase in FSC induced by TGF-β compared with that of unstimulated cells. **B)** Effect of chloride channel blockers DIDS and NPPB (100 μM) on eosinophil shape change by TGF-β1 (10ng/ml). *p<0.05. The data represent the mean ± SEM (n = 4).

3.5 PKC Expression

Western blot analysis showed that eosinophils had increased expression of PKC- β 1 and PKC- δ after stimulation with TGF- β 1 at 10 ng/ml for three hours (Figure 10A). TGF- β 1 stimulation did not increase the already low basal expression of PKC- γ , PKC- ζ or PKC- ϵ (Figure 10A). The time-dependent stimulation of eosinophils with TGF- β 1 showed that both PKC- β 1 and PKC- δ were upregulated after 30 minutes of stimulation, and PKC- β 1 was also upregulated after both two and four hours of stimulation (Figure 10B). For the dose-dependent stimulation with TGF- β 1, both PKC- β 1 and PKC- δ were upregulated after stimulations of 1, 10, and 25 ng/ml (Figure 10C). In eosinophils stimulated with TGF- β 1 for 3 hours, protein expression of both PKC- β 1 (Figure 10D) and PKC- δ (Figure 10E) showed significant increases ($p < .05$) when compared to control eosinophils.

3.6 Role of PKC in Eosinophil Chloride Channel Activity and Chemotaxis

Earlier, it was shown that TGF- β 1 treatment increased chloride channel activity of eosinophils (Figure 7A). Treatment with the PKC- δ /PKC- ϵ inhibitor rottlerin, however, blocked the TGF- β 1-induced increase in chloride channel activity (Figure 11). Earlier data also demonstrated that TGF- β 1 stimulation induced chemotaxis of eosinophils (Figure 8A). The TGF- β 1-induced increase in chemotaxis is also blocked by rottlerin (Figure 12).



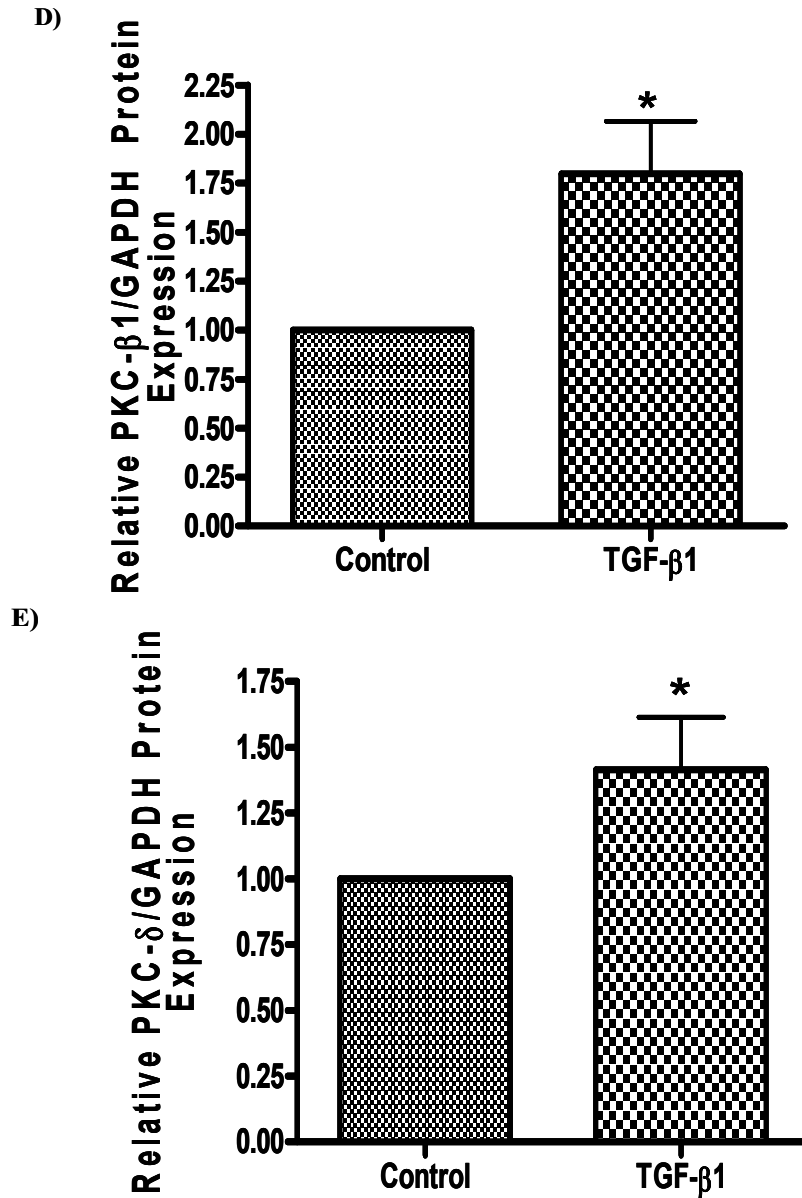


Figure 10. TGF-β1 induction of PKC expression in eosinophils. Western blots in human blood eosinophils showing **A)** Expression of PKC-β1, PKC-γ, PKC-δ, PKC-ζ and PKC-ε after treatment with TGF-β1 (10 ng/ml) for 3 hours; N=2-6 **B)** Time course for PKC-δ and PKC-β1 after treatment with TGF-β1 (10 ng/ml); N=2 **C)** Dose response for PKC-δ and PKC-β1 after treatment with TGF-β1 for 3 hours; N=2; **D)** Densitometry for PKC-β1 from **A**, *p<.05, N=4; **E)** Densitometry for PKC-δ from **A**, *p<.05, N=6. Data represent mean ± SEM.

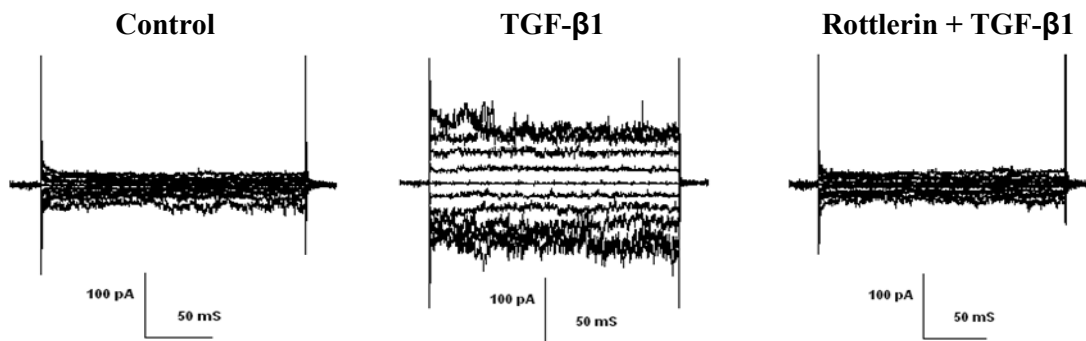


Figure 11. Effect of PKC- δ inhibitor on chloride channel activity in eosinophils. Eosinophils after incubation with TGF- β 1 (10 ng/ml) for 30 minutes or with rottlerin (10 μ M) for 30 minutes followed by TGF- β 1 (10 ng/ml) stimulation for 30 minutes. N=3

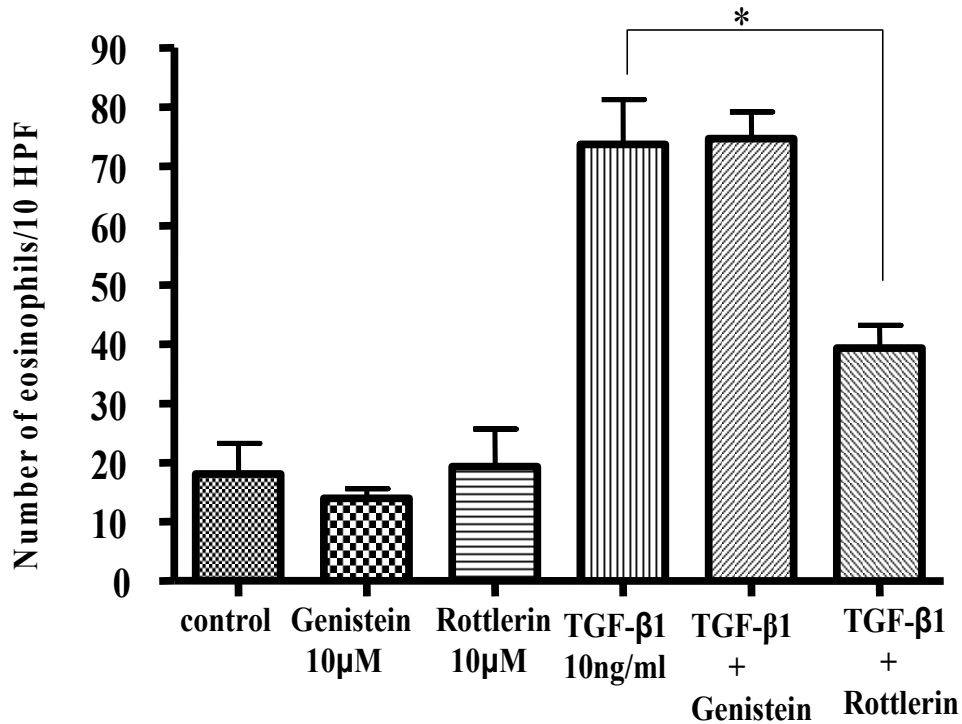


Figure 12. Effect of PKC- δ inhibitor on chemotaxis in eosinophils. Eosinophils incubated with rottlerin (10 μ M) and genistein (10 μ M), and their effects on TGF- β 1 (10 ng/ml) induced eosinophil chemotaxis. * p <0.05. N=4. Data represent mean \pm SEM.

3.7 Transcription Factor Expression and Dependence on PKC Pathway

Upon stimulation of eosinophils with TGF- β 1 for 30 minutes, phosphorylation of Smad3 is induced. This TGF- β 1-induced Smad3 phosphorylation is reduced when the cells are incubated with the general PKC inhibitor staurosporine (Figure 13A). Incubation of the eosinophils with rottlerin for 30 minutes prior to TGF- β 1 stimulation, however, does not reduce phosphorylation of Smad3 as compared to the phosphorylation seen when the cells are stimulated with TGF- β 1 alone (Figure 13B). The tyrosine kinase inhibitor genistein also reduced the TGF- β 1-induced phosphorylation of Smad3 in eosinophils (Figure 13B).

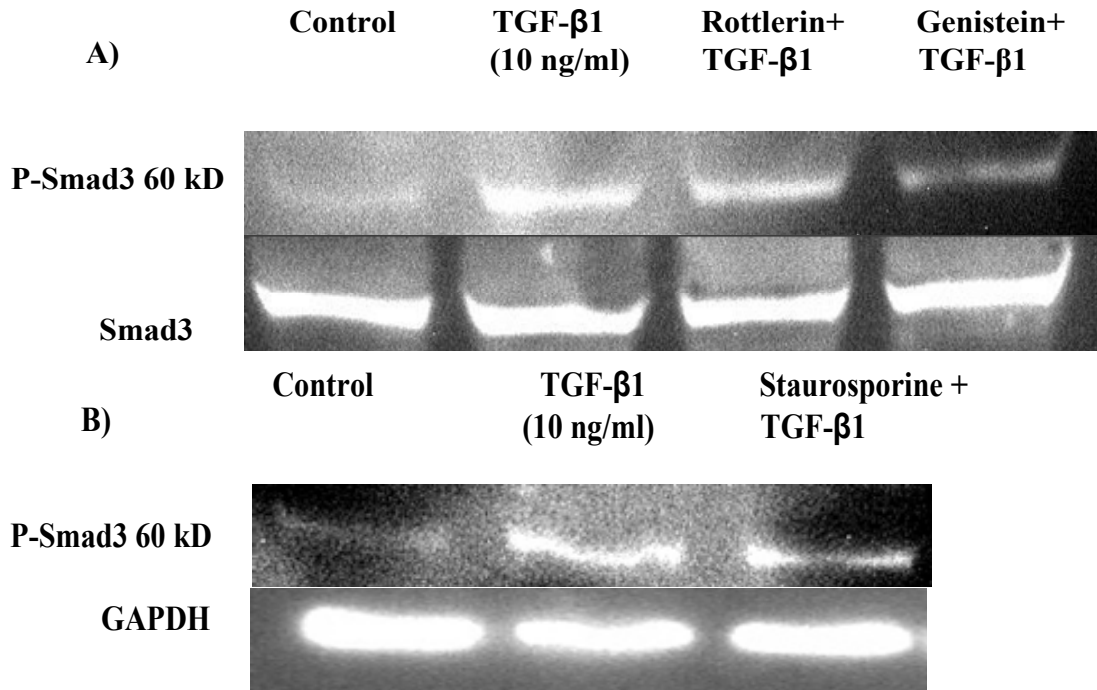


Figure 13. Phosphorylation of Smad3. Western blot showing protein expression phosphorylated Smad3 in human eosinophils after stimulation with **A)** TGF- β 1 (10 ng/ml) for 30 minutes, rottlerin (10 μ M) for 30 minutes prior to TGF- β 1, or genistein (10 μ M) for 30 minutes prior to TGF- β 1; N=2 or **B)** stimulation with TGF- β 1 (10 ng/ml) for 30 minutes or staurosporine (1 μ M) for 30 minutes prior to TGF- β 1; N=2.

3.8 Expression of CLC-3 in Asthmatic Eosinophils

Western blot analysis of CLC-3 protein expression in eosinophils isolated from the peripheral blood of allergic asthmatics when compared to nonasthmatic subjects demonstrated increased CLC-3 expression in allergic asthmatic eosinophils than in nonasthmatic eosinophils (Figure 14). CLC-2, CLC-4, CLC-5 showed no protein expression in either allergic asthmatic or normal eosinophils.

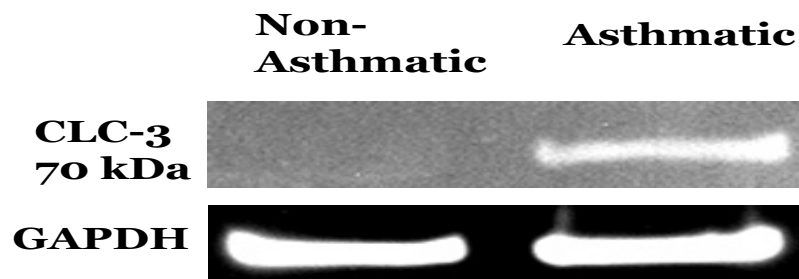


Figure 14. Effect of asthma on CLC-3 protein expression in eosinophils. CLC-3 protein expression in human blood eosinophils from healthy and allergic asthmatic donors. N=4

Chapter 4

Discussion

The action of eosinophils is important in some of the key features of airway remodeling that are seen in the asthmatic lung. TGF- β activity is also a potent inducer of many aspects of airway remodeling. The activity of TGF- β and eosinophils have shown a close correlation in regards to airway remodeling and other aspects of asthma such as inflammation. Eosinophils release TGF- β , which can stimulate such responses as increased collagen synthesis (119). Eosinophil release of TGF- β also stimulates increased fibrosis of the airway tissue by converting fibroblasts to a more fibrogenic phenotype (124). TGF- β 1 stimulation of eosinophils leads to increased expression of IL-13R α 1, helping to increase affinity for IL-13, which is a potent activator of eosinophils (113). TGF- β stimulation of eosinophils can also induce migration of the cells (10). Therefore, TGF- β 1 may be directly linked to eosinophils through its stimulation of eosinophils, which activates eosinophils and produces some functional effects, such as chemotactic migration.

If TGF- β 1 can stimulate eosinophils, there is a question as to what functions might be induced in the eosinophils upon stimulation. Chloride channel activity can be induced by TGF- β 1, which has been shown in, for example, fibroblasts (9). RT-PCR and western blot results from the experiments showed that CLC-3 was upregulated in eosinophils after stimulation with TGF- β 1 (Figure 6). TGF- β 1 stimulation increased activity of the chloride current in eosinophils, as well (Figure 7A). Chloride channels also affect cell migration. Cells such as monocytes and neutrophils have their cell migration regulated by chloride channel activity (7, 8). TGF- β 1 stimulation has also been shown to induce migration of macrophages (173). Therefore, the role that the TGF- β 1-induced increase of chloride channel activity had on chemotaxis of eosinophils was examined. TGF- β 1 stimulation increased chemotaxis of the

eosinophils (Figure 8A). The chemotaxis induced by TGF- β 1 was also blocked by the chloride channel inhibitors DIDS and NPPB (Figure 8B).

When eosinophils migrate by chemotaxis from the blood vessels to the lungs, they may require a change in shape to migrate out of the vasculature and into the lung tissue. TGF- β 1 stimulation induces shape change (Figure 9A) in eosinophils. Moreover, the chloride channel inhibitors DIDS and NPPB block the TGF- β 1-induced shape change (Figure 9B), thus demonstrating other facets of TGF- β 1-induced cell migration that chloride channel activity is necessary for in eosinophils.

These results demonstrate a role for CLC-3 in inducing migration of eosinophils in response to TGF- β 1. These experiments do not address the question of which isoform of CLC-3 could be the most prominent in the induction of cellular migration. Two isoforms which have been examined in human cells such as Calu-3 and HEK 293 lines are CLC3A and CLC3B for their ability to interact with the CFTR protein, of which only CLC-3B does. These two isoforms are located on the membranes of intracellular organelles, with CLC3A being located on the endosomes and CLC3B being found on Golgi bodies (174). Other experiments involving human epithelial cell lines show that CLC-3B, but not CLC3A, can be expressed on the plasma membrane, as well as in cytoplasmic organelles. The CLC-3B protein found on the cell membrane is generally seen in membrane ruffles, which are cellular protrusions made of newly polymerized actin filaments used for motility (175). Since membrane ruffles are formed during cytoskeletal reorganization, and movement of cytoskeletal proteins is necessary for the cell to change shape in order to migrate, CLC-3B becomes a candidate for the CLC-3 isoform whose activity induces eosinophil migration.

Other experiments demonstrate a short isoform of CLC-3 in the human NIH/3T3 cells. Its activation is shown to be volume sensitive via its interaction with actin filaments on the cytoskeleton. It is also inhibited by phosphorylation of PKC (176). Studies in other mammals also provide evidence for more than one isoform of CLC-3. In rat hepatocytes, both a short mRNA form and a long mRNA form of CLC-3 are present. The CLC-3 protein in these cells is found both intracellularly and on the cell membrane (177). Thus, additional studies may be required to determine which CLC-3 isoform is involved in eosinophil migration in response to TGF- β 1, as well as where the CLC-3 channel is located within the cell.

If chloride channel activity is required for increased migration of eosinophils, how does change in chloride ion concentration, due to increased chloride channel activity, activate promigratory molecules and induce migration of the cell? It is possible that an increase in chloride ion concentration activates the Rho molecular pathway, which reorganizes the cytoskeleton. Chloride channel activation induces changes in cell volume, which causes activation of molecules such as Src. Src activates Vav, which leads to activation of Rho GTPases such as RhoA or Rac1 to reorganize the cytoskeleton (171). Chloride channel activation may also lead to activation of myosin light chain proteins by PI3 kinase or PLC, which produces tension on the cytoskeleton (172). If PLC activity produces changes in the cytoskeleton which could induce migration of the cell, it is also possible that PLC activates PKC molecules to produce tension on the cytoskeleton independently of chloride channel transcription and/or activation, but this has yet to be investigated.

The aforementioned PKC is one downstream molecule involved in many cell processes and signaling pathways that can be activated by TGF- β . TGF- β 1 stimulation of various cells can activate PKC- δ , leading to gene transcription, cell migration, or post-transcriptional mRNA

stabilization (12, 178, 179). PKC activity can regulate expression and activity of chloride channels. General PKC activation increases expression of volume-sensitive outwardly rectifying chloride channels (13). Specifically, PKC- β 1 increases the expression of CFTR chloride channel (14). Both PKC- α and PKC- ζ are also involved in regulating chloride channels (15, 16).

Since TGF- β 1 leads to upregulation of PKC, the potential involvement of PKC isozymes in the increased chloride activity induced by TGF- β 1 was investigated. The protein expression of PKC- β 1 and PKC- δ was significantly increased in eosinophils upon stimulation the cells with TGF- β 1 (Figure 10). One finding of note was that PKC- β 1 and PKC- δ showed an increase in protein expression in response to TGF- β 1 after only 30 minutes (Figure 10B). It is possible that this rapid increase in total protein expression is due to the release of sequestered mRNA transcript of these PKC isozymes, thus allowing for nearly immediate translation. However, this speculation has yet to be tested. Once this was shown, the role of PKC in functional aspects of eosinophils (chloride current and chemotaxis) was assessed. The PKC- δ inhibitor rottlerin blocked the TGF- β 1 induced increase in the chloride current of the cells (Figure 11). Thus, PKC- δ is involved in the increased chloride channel activity seen in eosinophils after stimulation with TGF- β 1. Increased chloride channel activity has been shown to play a role in the activation of eosinophils by, for example, increasing the release of superoxide ions from eosinophils (6). Therefore, this pathway of increased chloride channel activity caused by TGF- β 1 stimulation via PKC signaling may play a significant role in activating eosinophils.

Next, the involvement of PKC in the cell chemotaxis that is induced by TGF- β 1 and ClC-3 activity was assessed. The TGF- β 1-induced chemotaxis was blocked by the PKC- δ

inhibitor rottlerin (Figure 12). Thus, eosinophil migration can be induced by TGF- β 1 via a PKC- δ -dependent pathway which involves increasing chloride channel expression and activity. The chemotaxis was not completely abolished, however, suggesting that PKC- δ may not be the only molecules involved in the TGF- β 1-induced chemotactic pathway in eosinophils.

Because PKC is an intracellular kinase that is involved in this signaling pathway in eosinophils upon TGF- β 1 stimulation, there is a question as to what, if any, transcription factors may be involved downstream of both TGF- β 1 and PKC. Upon TGF- β stimulation of human blood eosinophils, the expression of TGF- β 1 and TGF- β 2 are increased via a pathway involving Smad2 and Smad3 (112). TGF- β 1 is known to lead to an upregulation of Smad3, which in turn can affect some airway remodeling-related processes such as induction of apoptosis in fibroblasts to decrease negative effects of remodeling (83). Smad molecules such as Smad3 can also be regulated by PKC- δ upon TGF- β 1 stimulation, leading to outcomes such as cell spreading and gene expression (11, 12). The results showed that TGF- β 1 induced phosphorylation of Smad3, and that the PKC- δ/ϵ inhibitor rottlerin and the general PKC inhibitor staurosporine partially blocked this phosphorylation (Figure 13). Interestingly, the tyrosine kinase inhibitor genistein inhibited TGF- β 1-induced Smad3 phosphorylation the most (Figure 13A), suggesting that some other kinase could be involved in this signaling pathway.

Activator protein (AP)-1 is another transcription factor that can be downstream of TGF- β , as it can, among other things, lead to increased IL-2 expression in T cells upon TGF- β 1 stimulation (19). In addition, NF- κ B is a candidate for a transcription factor that could be involved in the transduction pathway described here. NF- κ B inhibition decreases eosinophil infiltration into the lungs during asthmatic airway inflammation (180). NF- κ B can also induce

TGF- β 1 secretion from ASM cells (181), suggesting that it may play a role in airway remodeling because of its connection with ASM cells and TGF- β .

It is also possible that PKC molecules may not be involved upstream of the transcription factors that induce transcription of CLC-3, but may only phosphorylate the already translated CLC-3 protein, since PKC is known to have a phosphorylation site on the CLC-3 protein (182). This would activate the channel to lead to eosinophil shape change and migration. It is also unclear as to whether TGF- β 1 stimulation leads directly to activation of the PKC isozymes, or whether it goes through transcription factors such as the Smads to increase transcription and translation of the PKC molecules (Figure 15). The involvement of other kinases, such as MAPK or ERK, could also be studied.

Most of these experiments, which examine the effect of TGF- β 1 on eosinophil migration and the associated intracellular mechanisms, make use of nonasthmatic eosinophils. However, since this research is meant to assess mechanism involved in the pathogenesis of asthma, preliminary experiments were begun on differences in some of these intracellular mechanisms between normal and asthmatic eosinophils. Protein expression of CLC-3 was found to be higher in asthmatic eosinophils when compared to normal eosinophils (Figure 14). Although much more work on potential differences in other aspects of the pathway examined in these experiments is warranted, these initial results demonstrate that mechanism involving chloride channels within eosinophils may contribute to the pathogenesis of asthma.

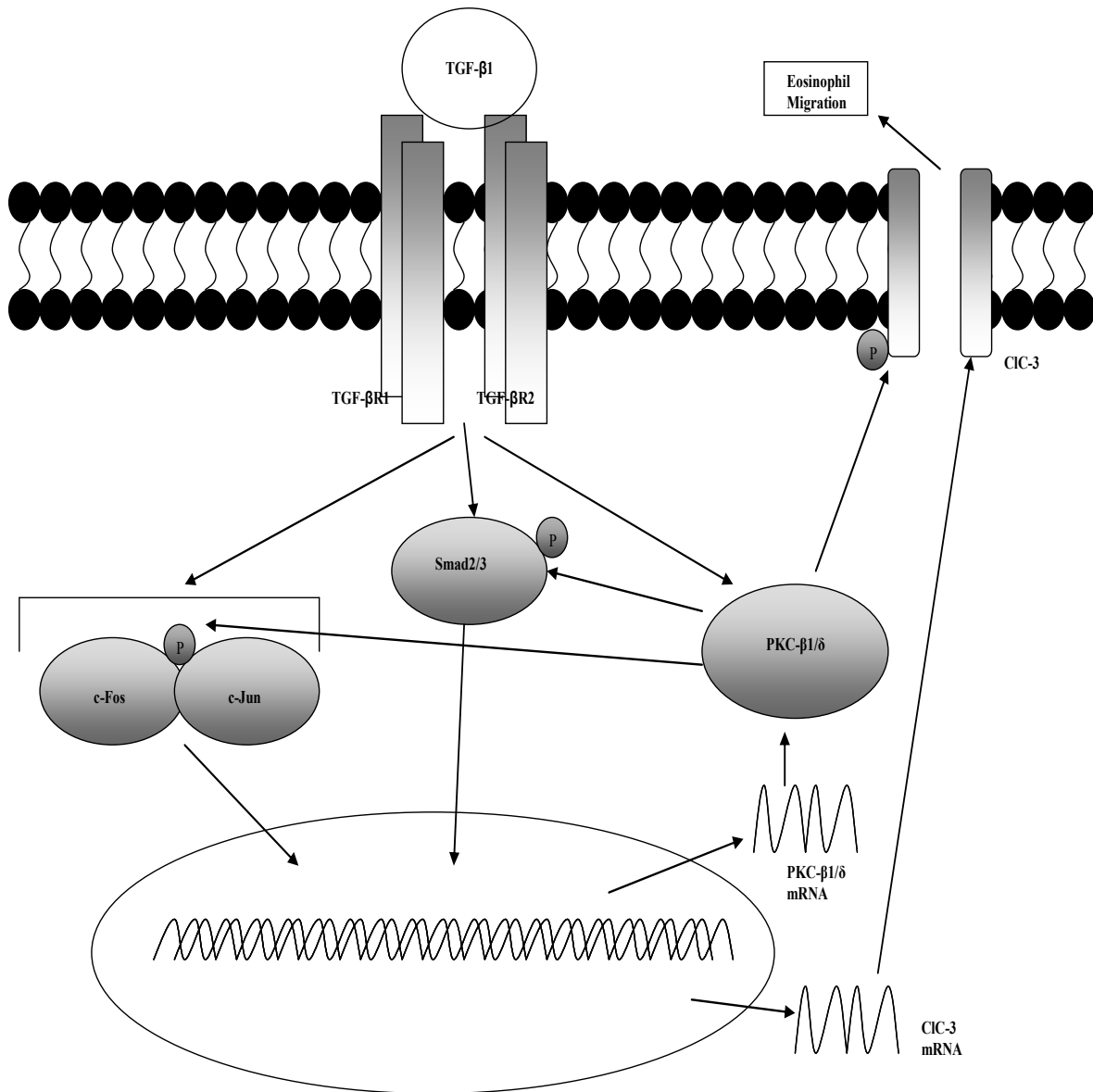


Figure 15. Proposed signaling pathway in eosinophils after TGF-β1 stimulation inducing chloride channel activity and migration. TGF-β1 stimulation of eosinophils activates PKC-β1/δ, either directly or via transcription factors such as Smad2/3 or AP-1. PKC-β1/δ may also phosphorylate these transcription factors to induce transcription of CLC-3, and the PKC molecules may phosphorylate the already translated CLC-3 protein. The increased activity of chloride channels leads to the induction of eosinophil migration.

Taken together, these results show how TGF- β 1 induces eosinophil chloride channel activity and eosinophil migration. Due to the fact that eosinophils exert many deleterious effects on the lungs during asthma, such as airway remodeling, the possibility exists that the pathway discussed here may lead to eosinophil induction of asthmatic features. Since TGF- β 1 activity influences the cell migration of eosinophils via this pathway, this pathway may also be a cause of eosinophils migrating to the lung tissue during an asthmatic response and releasing certain mediators or cytokines that may lead to airway remodeling. If mechanisms involved in the eosinophil functions from these studies could be more clearly elucidated and a strong relationship between this pathway and airway remodeling found, it would provide new targets in the attempt to help alleviate some of the features seen in the lungs during asthma that are caused by airway remodeling.

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