Eosinophils mediate inflammation in the skin through the activity of eosinophil peroxidase

By

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CERTIFICATE OF APPROVAL

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List of Abbreviations

CGRP: calcitonin gene related peptide

CSF2: colony stimulating factor 2

CSF3: colony stimulating factor 3

DRG: dorsal root ganglia

ECP: eosinophil cationic protein

EDN: eosinophil derived neurotoxin

EPX: eosinophil peroxidase

GRP: gastrin releasing protein

GRPR: gastrin releasing protein receptor

ICAM-1: intercellular adhesion molecule 1

ISAAC: international study of asthma and allergy in children

LPA: lysophosphatidic acid

MBP: major basic protein

MRGPR: mas related G-protein coupled receptor

NGF: nerve growth factor

PAR-2: protease activated receptor 2

SP: substance P

sPLA2: secreted phospholipase A2

TMA: trimellitic anhydride

TNF: tumor necrosis factor

TRPA1: transient receptor potential cation channel ankyrin 1

TSLP: thymic stromal lymphopoietin

VCAM-1: vascular cell adhesion molecule 1

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Abstract

Atopic dermatitis is a chronic relapsing inflammatory disease of the skin characterized by chronic itch. Eosinophils, and eosinophils products, are present in the skin in patients with atopic dermatitis, though what role they play in mediating itch and inflammation in the skin is unknown. Potential mechanisms by which eosinophils cause itch and inflammation in the skin were studied in this dissertation.

The cytokine thymic stromal lymphopoietin (TSLP) causes itch by activating sensory nerves. The effect of eosinophil coculture on expression of the receptor for TSLP was tested. Eosinophils were found to increase gene expression for the receptor for TSLP in sensory nerves in cell culture in a mechanism that required cell contact (chapter 3).

Next, the role of eosinophil peroxidase on increasing TSLP in an animal model of chronic contact dermatitis was tested. Blocking peroxidase activity of eosinophil peroxidase reduced numbers of eosinophils in the skin and reduced the proinflammatory cytokine TSLP in the animal model of chronic contact dermatitis (chapter 4). It was also found that eosinophil peroxidase directly increases gene expression for TSLP, CSF2, CSF3, TNF and IL1α in keratinocytes, requiring its peroxidase activity to do this. This was further found to require signaling through LPA to increase TSLP expression, and IL-1 to increase expression of CSF2, CSF3, TNF and IL1α (chapter 5). Blocking IL-1 in the animal model of chronic contact dermatitis caused a significant reduction in

eosinophil recruitment and inflammation, similar to what was observed when blocking the peroxidase activity of EPX. However, blocking LPA in the animal model of chronic contact dermatitis did not reduce TSLP levels, and caused an increase in general markers of inflammation (chapter 6).

These data indicate that eosinophils can influence itch and inflammation in atopic dermatitis through interactions with both sensory nerves and keratinocytes. Eosinophils directly interact with sensory neurons, increasing the receptor for TSLP. At the same time, they increase keratinocyte derived mediators that cause inflammation and further recruitment of eosinophils, as well as TSLP, which can directly activate sensory nerves to cause itch. These data implicate eosinophils as important mediators of itch and inflammation in the skin, and highlight eosinophils as potential targets of therapy in patients with atopic dermatitis.

Chapter 1: Introduction

The focus of this dissertation is the characterization of the mechanisms that lead to itch and inflammation in the disease atopic dermatitis. Chronic itch can be debilitating in patients with atopic dermatitis, and while we have recently made great strides in developing novel therapies to treat these patients[1,2], the mechanisms causing chronic inflammation and itch are still unclear and are in need of further study. A better understanding of the mechanisms that lead to itch will greatly enhance our ability to develop effective new therapies for these patients.

1.1 Atopic Dermatitis

Atopic dermatitis has gone by many names throughout history, including neurodermatitis, Von Hebra Dermatitis, and Besnier's prurigo [3]. These names often encompassed several different diseases, including atopic dermatitis. Even now atopic dermatitis will be referred to by several different names, including atopic eczema or eczema (technically, eczema is a broad term for several different types of dermatitis, though atopic dermatitis is the most common of these [4]). This can cause confusion throughout the literature, as these terms are often used interchangeably. In this document the term atopic dermatitis will be used.

1.1.1 Clinical features

Atopic dermatitis is a chronic relapsing inflammatory disease of the skin characterized by intense itch. The disease has a typical distribution, which changes depending on the age of the patient, with flexor involvement common in adults and involvement of the face and extensors in infants [5]. Constant inflammation in chronic lesions leads to lichenification, or thickening, of the skin [6]. Atopic dermatitis most commonly begins in the first 2 years of life, with about 50% of patients showing symptoms before the age of two [7]. Many will grow out of the disease, with about 50% of patients not showing symptoms after the age of two [7,8]. However, in about 30% of patients, the disease will last a lifetime [7,8].

Patients with atopic dermatitis suffer from reduced quality of life [9,10]. Poor sleep due to chronic itch and scratching and concerns about appearance due to skin rashes contribute to poor quality of life. While we can't cure atopic dermatitis, treatments that reduce symptoms also effectively improve quality of life [11,12].

Atopic dermatitis is also commonly associated with other atopic diseases such as asthma and hay fever[8,13]. Typically, patients will first present with atopic dermatitis early in life, then will progress to asthma in childhood, and finally will develop seasonal allergies later in life. This phenomenon has been termed the atopic march. While there are many hypotheses about what causes the atopic march, we still do not have a proven answer, and no current treatments prevent this progression.

While the cause of atopic dermatitis is still unknown, there are clearly both genetic[14-16] and environmental factors [17]. The association of atopic dermatitis and atopy (the predisposition to develop allergic type 1 inflammatory reactions) was one of the first noted associations[18-20]. While this was initially thought be a driving factor in atopic dermatitis, there is still debate about the nature of this association, with conflicting evidence about the benefit of allergen avoidance in patients[21-24].

Insights from the study of genetic polymorphisms that are associated with atopic dermatitis have been enlightening about the potential causes of this disease. The most strongly associated gene mutations with atopic dermatitis are filaggrin mutations [14,15], which are present in roughly 20% of patients with atopic dermatitis [25]. These mutations lead to disruption of the skin barrier, which will be discussed in greater detail in the following sections.

1.1.1.1 Diagnostic criteria

There is no single laboratory test that can diagnose atopic dermatitis, and no single marker that separates this disease from other skin diseases. Diagnosis is made clinically using morphology and distribution of lesions, medical history, other clinical findings, and by excluding other causes of dermatitis, such as contact dermatitis[26]. More recently there have been validated diagnostic criteria[5,27] in use for clinical trials.

The term atopic dermatitis was first used by Sulzberger and Wise in 1933[28] to describe a disease that began as infantile eczema that occurred

typically on the face and flexor areas, in patients with a family history of atopic disorders. Even after the coining of the term atopic dermatitis it took another 40 years before Hanifin and Lobitz published the first consensus diagnostic criteria[6].

Hanifin and Rajka refined these criteria in 1980 [5]. Under these criteria patients must have 3 of 4 basic features including pruritis, typical morphology and distributions, chronic or chronically relapsing dermatitis, and personal or family history of atopy. Patients must also have 3 or more of 23 minor features, including xerosis (dry skin), elevated IgE, and early age of onset. The full list of these criteria are in Table 1.

Several attempts have been made to refine these criteria to make them less complex. The Hanifin and Rajka criteria were further refined by the UK working party[27]. These criteria state that a patient must have a pruritic skin condition and three of the following symptoms; history of flexural involvement, history of dry skin, onset of age under two years, personal history of asthma or allergic rhinitis, or visible flexural involvement as seen by a clinician. These criteria were validated in Britain and along with the Hanifan and Rajka criteria are considered the gold standard.

1.1.1.2 Epidemiology

While the UK working party and Hanifin and Rajka criteria for diagnosis of atopic dermatitis are effective clinically, they are difficult to use with children, since children do not have a long personal medical history. This limitation made it difficult for epidemiologic studies on the prevalence of atopic dermatitis,

especially in developing countries. Due to the fact that many patients with atopic dermatitis are young children there was an effort to generate diagnostic criteria that are effective in children to be used for epidemiologic studies[29]. These diagnostic criteria were developed and used in the International Study of Asthma and Allergy in Children (ISAAC) to determine worldwide prevalence of allergic diseases including atopic dermatitis.

In 1998 ISAAC published its first findings on the prevalence of atopic dermatitis symptoms, and found that rates of atopic dermatitis ranged from 2% in China and Indonesia to 20% in the United Kingdom, Finland, Sweden[30]. In 2007 ISAAC published results showing a leveling off of atopic dermatitis rates in many countries, though there was an increase in incidence in countries that had previously had low incidence[31]. A study of Oregon school children found an incidence rate of 17.8% [32].

Patients with atopic dermatitis often will develop other allergic disease, including asthma, allergic rhinitis, and food allergies[33-36]. This often follows a typical course, with atopic dermatitis onset in young children, followed by the development of asthma and allergic rhinitis during adolescence. Some individuals may have an improvement of their disease as they age[8]. This is often called the atopic march, with atopic disease marching from the skin to the airways.

1.1.1.3 Genetics of atopic dermatitis

There are clear effects of genetics on the risk of developing atopic dermatitis. Twin studies have shown a strong link between genetics and risk of developing atopic dermatitis, with correspondence rates of 0.86 for monozygotic twins and 0.50 for dizygotic twins[37,38]. Familial history of atopic dermatitis is also a strong risk factor for developing atopic dermatitis, so much so that family history is one of the diagnostic criteria[5,39].

While there is a clear genetic component to atopic dermatitis, it has been difficult to identify genes that are important for increasing risk for atopic dermatitis. This is due to heterogeneity of risk causing genes in atopic dermatitis, as well as clear interactions with environment that also play a role. The gene most linked to atopic dermatitis, which has been validated in many studies, is filaggrin[14-16,25,40]. Some studies identified other genes such as IL-4, thymic stromal lymphopoietin (TSLP), and IL-13, but none are as well associated with atopic dermatitis as filaggrin [41-44]. These other factors are associated with Th2 inflammatory profiles, which has some interesting implications that will be discussed in more detail in the inflammation in atopic dermatitis section.

1.1.1.4 Costs of atopic dermatitis

Atopic dermatitis produces a dramatic economic burden. Part of this is due to the high prevalence of atopic dermatitis, but also the difficulty in controlling this disease in some patients. Patients with atopic dermatitis spend from

$3,000-$10,000 a year on direct medical costs, much of this cost is carried by patients with severe atopic dermatitis [45,46].

These patients also have an increased risk of other atopic diseases, such as asthma and allergic rhinitis, which also places a large cost burden on these patients[45]. This is further compounded due to costs associated with missed work, either as sick days due to dermatitis, doctor's visits, or doctor's visits for children[47].

New therapies that have recently been approved, specifically dupilumab (a monoclonal antibody that inhibits the IL4Rα receptors, blocking signaling of IL-4 and IL-13), are biologics that are very effective in treating patients with atopic dermatitis. While there have not been studies on the costs of biologics in atopic dermatitis, for other diseases such as psoriasis, costs of biologics can exceed $20,000 a year[48]. The net effect these therapies have on cost to patients with atopic dermatitis is yet to be seen.

1.1.1.5 Treatment of atopic dermatitis

Treatment of atopic dermatitis involves several different stages. For patients with active disease, the goal is to achieve inactive disease[4]. This can be achieved with several different treatments. Typically, topical corticosteroids are used, and these can be very effective in some patients[4,49]. Other options include calcineurin inhibitors, such as tacrolimus, as well as oral steroids for very severe unresponsive disease[4,49]. While topical steroid treatment can be effective,

unpleasant side effects of these drugs reduce their effectiveness in treating atopic dermatitis[50,51].

Once control of the disease is achieved, treatment then usually focuses on maintaining remission. Moderate use of topical steroids is also sometimes used to achieve this, though care must be taken to ensure that steroids are used at a low enough dose to limit side effects[4,49]. Maintaining hydration of the skin also aids in maintaining remission of atopic dermatitis. This involves use of moisturizers after bathing, and avoidance of irritating substances, such as some types of soaps[4,49].

New therapies for patients with very severe uncontrolled disease have recently had successful clinical trials for patients with atopic dermatitis. The most successful of these is dupilumab [1,11]. Nemolizumab also had a recent successful clinical trial in reducing itch in atopic dermatitis. This drug binds to the IL-31 receptor and inhibits its signaling[2]. IL-31 is thought to activate sensory nerves and cause itch, so this drug does not reduce inflammation in patients, but does reduce itch[52]. These new therapies show that progress is being made in developing new therapies for atopic dermatitis.

It may also be possible to prevent the development of atopic dermatitis. Two recent studies found that daily use of emollients in infants at high risk for developing atopic dermatitis significantly reduced their rates of development of the disease[53,54]. Importantly, methods of educating parents, especially those of children at high risk of developing atopic dermatitis, could greatly benefit those

patients and prevent many from developing atopic dermatitis. These studies showing prevention of atopic dermatitis are exciting, though it is still yet to be seen if these children never develop atopic dermatitis as they age.

Even with the advances we have made in treating patients with atopic dermatitis many patients with atopic dermatitis still struggle with chronic itch. Gaining a greater understanding of the mechanisms that cause itch in these patients will allow the development of more effective therapies to treat these patients.

1.1.2 Histology and anatomy of normal skin[55]

Skin can be separated into three general layers. The outermost layer is the epidermis, which provides both protection from external pathogens and irritants and reduces water loss through the skin. The dermis is the next layer, which helps to provide strength to the skin and contains the blood vessels and many nerves. Finally, the subcutaneous fat forms the final layer, called the hypodermis.

1.1.2.1 Epidermis

The epidermis is the outermost layer of skin. Keratinocytes represent 80% of the cells in the epidermis, and form the barrier that prevents access of pathogens and irritants to the body and prevents water loss. The basal cells of the epidermis are the stem cell population that provides the continuous renewal of keratinocytes in the skin. As keratinocytes migrate from the basal layer of the epidermis they will continue to differentiate until they reach the outer layer of the

epidermis, where they form the cornified surface of the skin. This layer is continuously sloughed off during normal skin turnover. Many other cell types are also present in the epidermis, though in much lower numbers then keratinocytes. The epidermis is divided into 4 layers[1], which each contain keratinocytes at different steps of differentiation and which perform distinct functions critical for maintaining the barrier function of the skin.

The stratum basale forms the first layer of the epidermis. This layer sits just on top of the basement membrane between the epidermis and the dermis. Keratinocytes in the stratum basale are responsible for attaching the epidermis to the basement membrane. Keratinocytes are attached to the basement membrane through integrins[56]. $\alpha 6\beta 4$ integrins are critical for the formation of hemidesmosomes that aid in adhering the epidermis to the basement membrane[57-59]. These integrins are connected to the intermediate filaments in keratinocytes, forming strong bonds between the basement membrane and the cells[58,60]. The keratinocytes in the stratum basale also generate some components of the basement membrane and organize the components into an organized structure[61]. Keratinocytes in this layer also express fibronectin and laminin that make up the basement membrane[62-64].

The keratinocytes in the stratum basale are the only keratinocytes proliferating under normal conditions. There are both slow-dividing stem cells and fast dividing transit amplifying cells that arise from the slow dividing stem

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[1] The epidermis contains 5 layers in the palms of the hands and the soles of the feet.

cells. It is likely that the connection with the basement membrane is responsible for this, as cells will halt proliferation as they separate from the basement membrane[59,65].

The stratum spinosum is the next layer and is named after the spine-like appearance of the cells in this layer, caused by the abundant desmosomes holding these cells together through intracellular keratin bundles. As keratinocytes migrate from the stratum basale and differentiate, they express cadherins, such as E-cadherin and P-cadherin, to form desmosomes[66]. Due to the large number of desmosomes in this layer it provides much of the shear strength of the epidermis. These cells also begin to form lamellar granules which are found in the next layer of the epidermis.

The stratum granulosum is characterized by keratinocytes with lamellar granules that deliver critical components to form the stratum corneum, the final layer of skin. Keratinocytes in this layer also form tight junctions, which are critical in forming the protective barrier and allowing the passage of only small molecules and ions through the skin[66]. The lamellar granules contain profillagrin, loricrin and keratin filaments that are components of the stratum corneum. Keratinocytes in this layer will also undergo the final stage in their differentiation into corneocytes by undergoing programmed destruction of the nucleus.

The final layer of the epidermis is the stratum corneum. This layer contains stacked layers of flattened anuclear corneocytes, surrounded by

crosslinked proteins such as loricrin and involucrin. This layer provides the barrier to water loss and access of outside pathogens and irritants to the body.

Filaggrin performs critical functions in establishing the barrier of the epidermis. Filaggrin is expressed as profilaggrin in the stratum granulosum and packaged into the lamellar granules in the keratinocytes. Profilaggrin is a large precursor protein consisting of many filaggrin repeats flanked by N and C terminal sequences[67,68]. Filaggrin monomers are cleaved by proteases such as profilaggrin endopeptidase 1 from profilaggrin[69]. Filaggrin monomers then bundle keratins, which allows crosslinking of the keratin bundles, and eventually compaction of the keratinocytes, critical in formation of the stratum corneum[70]. Finally, filaggrin will be further processed into free amino acids, which form an important component of the natural moisturizing factor in the skin[70]. Loss of filaggrin function will lead to a disruption in skin barrier and a decrease in skin hydration due to the loss of the natural moisturizing factor in the skin.

There are many cell types other then keratinocytes found in the epidermis. These cells include Merkle cells, Langerhans cells, melanocytes, and free sensory nerve endings. Merkel cells are mechanosensive cells, typically located in areas of the body that are highly sensitive to touch, such as the finger tips. These cells are associated with sensory nerve endings and will activate sensory nerves in response to touch. Langerhans cells are specialized dendritic cells that reside in the epidermis, which present antigen to T cells. Melanocytes are the pigment generating cells that reside in the epidermis.

1.1.2.2 Dermis

The dermis is the largest layer of the skin and provides the strength and flexibility of the skin. The strength and elasticity of the skin is provided by the large amount of collagen bundles and elastin fibers that form the bulk of the extracellular components of the dermis. The fibrous extracellular matrix also contains proteoglycans, glycosoaminoglycans, and glycoproteins. These components bind and retain water and bind growth factors, which have influences on tissue growth and repair. The dermis contains many cell types, including fibroblasts, inflammatory cells (both resident cells such as macrophages, mast cells, and transit circulating cells such as T cells), as well as blood vessels, lymphatics and nerve bundles that supply the skin. Epidermal appendages, such as hair follicles and sweat glands are also partially located in the dermis and will reach up into the epidermis from here.

The dermis can be segregated into two layers: the papillary dermis and the reticular dermis. These two regions can be readily distinguished, with the papillary dermis containing small collagen fibers and the reticular dermis containing large collagen bundles surrounded by elastin fibers that get progressively larger as they approach the hypodermis, where they transition to adipose connective tissue. Separating the papillary dermis and the reticular dermis is the subpapillary plexus, a horizontal plane of blood vessels.

1.1.3 Morphology of atopic dermatitis

Depending of the stage of disease, there can be different changes observed in the skin in patients with atopic dermatitis. Importantly, there is not normal skin on patients with atopic dermatitis. Skin that is not currently visually affected by the disease is termed non-lesional skin. Non-lesional skin may not have grossly observable changes, inflammation or itch, but it does have some changes compared to normal healthy skin.

Non-lesional skin contains minimal perivascular T-cell infiltration, indicating that there may be low levels of inflammation even in normal appearing skin[71,72]. There is also mild thickening of the epidermis, known as hyperkeratosis.

In lesional atopic dermatitis, there are much more dramatic changes to the skin. Hyperkeratosis is more severe in lesional skin, and spongiosis is also observed in the epidermis in lesional skin[71]. There is a much larger infiltration of perivascular inflammatory cells consisting of a large number of T cells, as well as some eosinophils[71]. Isolation and examination of the T cells from skin in atopic dermatitis has found that many of these cells are CD4+ T cells[73,74]. Activation of these T cells with IL-2 causes release IL-4 and TNF[74], indicating that many of these T cells are Th2 cells. IL-4 is also expressed at higher levels in the skin in patients with atopic dermatitis[75].

1.1.4 Barrier dysfunction

Patients with atopic dermatitis have a baseline barrier dysfunction in skin that is present both in inflamed lesional skin and in normal appearing non-lesional skin. This barrier dysfunction is worse in lesional skin and will improve as the disease improves, though it never improves to normal[76]. However, it wasn't until more recently that this loss of barrier function of the skin was thought to be the critical driver of atopic dermatitis in patients.

The consequence of this disruption of barrier function of the skin allows infiltration of irritants, pathogens or allergens into deeper layers of the skin, potentially driving inflammation in atopic dermatitis[77]. However, the disruption of barrier function of skin even without infiltration of irritants is sufficient to increase cytokine expression in the skin[78]. These findings have led to the current belief that barrier dysfunction of the skin is a driving factor that causes atopic dermatitis.

The hypothesis that atopic dermatitis is caused by an underlying barrier dysfunction of the skin has gained support from recent findings. The association of filaggrin mutations with atopic dermatitis is one such finding[14-16,41]. Filaggrin is an important protein for forming the stratum corneum in the skin and maintaining skin hydration. Mouse models with filaggrin knocked out have dry skin, increased penetrations of external factors, and increased inflammation[77,79,80]. These findings support the hypothesis that barrier dysfunction is critical for

causing atopic dermatitis, since filaggrin mutations are well associated with atopic dermatitis and it play such an important role in forming the barrier.

Further support for a role in barrier dysfunction in the development of atopic dermatitis was demonstrated in two recent studies that used daily emollient to protect skin in children at high risk to develop atopic dermatitis. These studies found a 50% reduction in atopic dermatitis rates in patients that used emollients daily compared to normal patients[53,54].

These findings support the hypothesis that barrier dysfunction is the root cause of atopic dermatitis, and that the immune dysfunction observed in these patients is not the basis for these barrier defects observed in the skin. However, this does not mean that the activated immune system has no effect on the barrier function of the skin. Cytokines, that are elevated in the skin and serum in patients with atopic dermatitis, have been found to reduce expression of important barrier factors, including filaggrin[81]. This supports data showing that barrier function is worse in lesional skin and improves during treatment[76].

1.1.5 Inflammation in atopic dermatitis

Inflammation is a critical component of atopic dermatitis and is likely the connection between barrier dysfunction and itch in atopic dermatitis. Inflammation in atopic dermatitis is generally described as a Th2 inflammation, including elevated levels of immunoglobulin-E (IgE), TSLP, IL-4, IL-5 and IL-13[82]. IgE levels are elevated in the serum in about 80% of patients with atopic

dermatitis[20,83,84]. The role of these factors on mediating inflammation and itch in atopic dermatitis is still being studied.

1.1.5.1 Role of keratinocytes

Keratinocytes form more than just a physical barrier preventing entrance of pathogens and loss of water, they are active participants in mediating inflammation and protecting against pathogens. Keratinocytes possess receptors such as toll like receptors (TLR), which allow them to respond to invading pathogens[85,86]. Keratinocytes also produce factors that are important for killing invading pathogens to control colonization of the skin, including antimicrobial peptides[87], as well as cytokines and chemokines that recruit and activate the immune system. Keratinocytes will express and release antimicrobial peptides in response to bacterial infection[87,88]. Levels of the antimicrobial peptides produced by keratinocytes are significantly reduced in patients with atopic dermatitis[89]. This reduction in antimicrobial peptide expression in the skin may explain the association of staphylococcus aureus colonization in lesional skin in patients with atopic dermatitis[80,87,90]. Importantly, expression of the antimicrobial peptides is reduced by proinflammatory cytokines like IL-4 and IL-13, which are increased in the skin in patients with atopic dermatitis[91].

Keratinocytes also produce proinflammatory cytokines to initiate inflammation, including TSLP and IL-1α. The role of these cytokines will be discussed below. Keratinocytes will produce these cytokines in response to

many different stimuli, including barrier damage and pathogens[92-95]. Expression of these proinflammatory cytokines are a mechanism by which keratinocytes can initiate inflammatory reactions in the skin.

1.1.5.2 TSLP

TSLP levels are elevated in both the skin and the plasma in patients with atopic dermatitis[82,96,97]. TSLP plays critical roles in mediating inflammation, and expression of TSLP in the skin in mice causes inflammation and itch that is characteristic of atopic dermatitis[98-100]. TSLP stimulates expansion of CD4+ T cells and plays critical roles in mediating differentiation of T cells into Th2 cells[96,101]. TSLP is required for sensitization to allergens in animal models of atopic dermatitis and asthma[102,103]. In a clinical trial in patients with asthma, blocking TSLP has been shown to reduce response to allergen, as well as reduce number of exacerbations[104,105].

TSLP also has significant effects on other cells present in atopic dermatitis, including eosinophils. TSLP can stimulate eosinophilopoiesis in cell culture and regulate eosinophil degranulation, survival, and recruitment[106-109]. TSLP also activates sensory nerves to cause itch[110], which may play a role in mediating itch in atopic dermatitis.

Given its critical role in initiating allergic inflammatory reactions, many have suggested that TSLP is critical in mediating the atopic march in patients with atopic dermatitis. In animal models dermal TSLP increases airway

inflammation[99,111,112]. Blocking TSLP in atopic dermatitis has some promise in preventing the atopic march.

1.1.5.3 IL-1α

IL-1α is one of the first genes to be upregulated in the skin after barrier damage[78,113,114] and is elevated in the serum of patients with atopic dermatitis[82]. IL-1α has important effects on recruitment of T-cells into the skin[115], which may partially be caused by its ability to increase expression of adhesion molecules in the skin to allow recruitment of inflammatory cells[116]. IL-1 treatment of reconstructed human skin in cell culture causes upregulation of classic atopic dermatitis cytokines like TSLP and reduces expression of barrier proteins like filaggrin[117]. What role IL-1α has in mediating inflammation in atopic dermatitis is unknown, but there is more and more evidence that this proinflammatory cytokine may play an important role[117].

1.1.5.4 IL-4 and IL-13

IL-4 and IL-13 signal through related receptors and have many redundant effects on inflammation in the skin. IL-4 binds to IL4Rα and can form a signaling competent receptor by dimerizing with either the common γ chain or IL-13Rα1. IL-13 signals through the IL-13Rα1 and IL4Rα. Both cytokines are elevated in serum and skin from patients with atopic dermatitis[75,82,118] and are known to cause many of the changes that are observed in the skin in these patients. Expression of either IL-4 or IL-13 in the skin of mice leads to itch and inflammation with characteristics similar to what is observed in atopic

dermatitis[119,120], including recruitment of inflammatory cells and a reduction of important barrier proteins in keratinocytes[121]. Most importantly, blocking the receptor that is critical for both IL-4 and IL-13 signaling, IL4Rα, reduces itch in patients with atopic dermatitis[1].

1.2 Sensory innervation of the skin

The majority of nerves innervating the skin are sensory nerves, whose cell bodies reside in dorsal root ganglion (DRG). DRG neurons are pseudounipolar neurons, with one process innervating target tissue and the other synapsing on interneurons in the spinal cord. Every level of the skin is innervated, including all layers of the epidermis[122]. The skin is innervated by a variety of nerve subtypes, which are responsible for sensing a broad range of stimuli, including heat, cold, touch, pain and itch. There is a small amount of sympathetic innervation in the skin, which controls sweat glands and some other structures in the skin, though the majority of nerves in the skin are sensory nerves.

All nerves can be labeled with antibodies for PGP 9.5, a ubiquitin C-terminal hydrolase expressed by all neurons[123]. Many of the studies looking at innervation of the skin have stained for PGP 9.5 to label and visualize nerves. Importantly, this marker is not selective for sensory nerves, but will label all nerves. To account for this many studies will focus on nerves in the epidermis, where it is assumed only sensory nerves innervate.

1.2.1 Development of the sensory nervous system

Development of the DRG sensory nerve system requires not just differentiation of cells into sensory nerves, but also proper connection of these nerves to target tissues, such as skin, as well as connection to proper areas of the spinal cord. DRG sensory nerves are derived from neural crest cells that migrate from the neural tube. These cells are responsible for differentiating into multiple different structures, including the DRG sensory system and sympathetic neurons. Within the DRG, neural crest cells will differentiate into both the sensory nerves and the supportive glia that are present in the adult. Commitment to differentiation into sensory nerves is controlled by a series of factors that program the neural crest cells, some of which come from exposure to factors expressed by the neural tube itself[124,125].

As these sensory nerves develop, they quickly send projections to the dorsal lateral margin of the spinal cord. This innervation of the spinal cord is controlled by both extrinsic factors and intrinsic factors in the sensory nerve. Extrinsic factors include repulsive forces that prevent these axons from innervating the wrong area, such as the semaphorins[126]. Intrinsic factors include the transcription factor RUNX1, and loss of RUNX1 leads to improper innervation of the spinal cord by sensory nerves[127].

Innervation of the skin is separated into dermatomes, where each dermatome is an area of skin that is supplied by one spinal nerve[128]. Dermatomes are established early and axons from DRGs will innervate their

specific dermatomes during development[129]. The development of these dermatomes is coordinated such that nerves do not just innervate nearby skin, but will innervate distinct dermatomes[129].

1.2.2 Cutaneous sensory nerves

The epidermis is innervated by un-myelinated free nerve endings[122]. The amount of innervation of the epidermis varies dramatically depending on the area of the body[130]. In general, the farther from the trunk that area of the body is, the lower the amount of innervation of the epidermis there is[130]. There are obvious exceptions to this, the fingertips have much more innervation of the dermis in than in the palm of the hand[131]. Importantly, innervation of the skin is similar across many different species, including human, mouse, and rat[132].

The epidermis is innervated by many different classes of sensory nerves responsible for mediating sensations of touch, pain, temperature and itch. While sensory nerves are critical for relaying detection of these sensations to the brain and initiate responses, other structures in the skin also initiate these responses. One examples is Merkel cells, which are innervated by sensory nerves in the skin and are critical for sensations of touch[133]. Another particularly interesting examples is noxious heat sensations. TRPV1 is a cation channel responsive to noxious heat[134], and mice that lack TRPV1 have a significantly reduced response to noxious heat[135]. Interestingly, simply expressing TRPV1 only in keratinocytes in mice that lack TRPV1 reconstitutes their ability to respond to noxious heat[136].

In addition to providing afferent activity to the brain, sensory nerves also have local responses and effects on inflammation. Neuropeptides such as substance P (SP) and calcitonin gene related peptide (CGRP) can both trigger inflammation. Sensory nerves also express adhesion molecules and chemokines that recruit inflammatory cells.

1.2.3 Classification of sensory neurons

Sensory nerves can be classified in several ways. Methods of classification are often based on the following factors, morphology, conduction velocity, functions, and expression of receptors and neuropeptides.

1.2.3.1 Classification based on morphology and conduction velocity

Initially, sensory nerves were split into different fiber types based on size. Sensory nerves with large cell bodies were labeled A fibers, and sensory nerves with small cell bodies were labeled C fibers (Duce and Keen 1977; Yoshida and Matsuda 1979; Harper and Lawson 1985). An early observation was made that cell body size correlated well with conductance velocity. Sensory nerves with small cell bodies, or C fibers, have slow conductance velocities of roughly 1 m/s. Conversely, sensory nerves that have large cell bodies, or A fibers, typically have faster conductance velocities [137,138]. A fibers can be separated into multiple subgroups, again depending on conductance velocity. Aδ fibers have an intermediate conductance velocity, roughly 2-10 m/s. Finally, Aβ fibers have a fast conductance velocity of 15-30 m/s[138]. Differentiating between Aβ and Aδ fibers based on cell body size is not possible. Importantly, cell body size does

not perfectly differentiate between A and C fibers, as there is a distribution of cell body size of each fiber type.

1.2.3.2 Classification based on function

Sensory nerves will also be classified based upon the type of stimulus the respond to. General classes of sensory nerves include mechanosensors, nociceptors, and thermosensors. Some sensory nerves will respond to multiple stimuli. Mechanosensors are sensory nerves that respond to mechanical stimuli such as touch and are generally Aβ fibers[139]. Nociceptors respond to stimuli that cause damage, and both Aδ and C fibers are nociceptors[139,140].

Sensory nerves classified based on function does not always fit an exact profile based on morphology. An example are nociceptive neurons, which can be both slow conducting C fibers, and medium speed conducing Aδ fibers[139]. Mechanosensors can also be both fiber types, with pleasure of touch sensations mediated by slow conducting C fibers[141].

1.2.3.3 Classification based on receptor and neuropeptide expression

Finally, sensory nerves can also be classified based on what receptors and neuropeptides they express. Some sensory nerves express neuropeptides such as SP and CGRP, while others do not. Sensory neurons that express neuropeptides are peptidergic sensory nerves. These sensory nerves are generally C fibers, though some Aδ fibers are peptidergic.

Sensory nerves will also be classified based on what cell receptors they express. There are too many receptors to name all different receptors that can classify sensory nerves, though more detailed discussions of some of these nerves responsible for mediating itch are mentioned below.

1.2.4 Itch sensations

Itch and pain are both sensed by C fibers in the skin[142]., and there are clear connections between pain and itch, with painful stimuli inhibiting itch. The biologic purpose of these two sensations is to initiate protective behaviors to noxious stimulus, but in very different ways. Pain stimulates a withdrawal of the area from the stimuli causing pain. On the other hand, itch causes you to actively remove the irritant from the body by scratching or rubbing the area.

There has been some controversy about the mechanism by which sensory nerves transmit sensations of itch. Many theories have been put forth to explain the neurobiology of itch. An early theory postulated that itch is just a low threshold form of pain and is not coded by a distinct population of itch specific neurons but just pain sensory nerves[143]. This was supported by the finding that all itch sensitive nerves express TRPV1 a receptor that is activated by the capsaicin and causes pain. However, recent work has found that loss of neurons that cause itch does not reduce pain making this theory unlikely to be true.

Another theory was established to explain the neurobiology of itch and is called the labeled line theory. In this theory, neurons are specifically responsible for mediating sensations of itch, or sensations of pain, but not both. This gained

traction with the finding that inhibiting itch specific neurons does not reduce pain sensations in mice[144]. There has been an explosion in the identification of what appear to be itch specific nerves.

Studies in mice have found the best evidence for selective neurons that sense itch. One examples are neurons that express the mas-related g-protein coupled receptor member A3 (mrgpra3). Sensory nerves that express mrgprA3 innervate the epidermis. Activation of these neurons causes itch in mice, while loss of these neurons does not influence pain behavior induced by inflammation. Loss of these neurons also reduces itch in response to many stimuli including histamine and chloroquine[145].

Many factors that activate sensory nerves to cause itch have recently been identified. While some of these factors include exogenous factors, such as proteases, many are endogenous signaling molecules. These include cytokines, such as TSLP[110] and IL-31[146], as well as other signaling molecules like lysophosphatidic acid[147]. Typically, these pathways are discovered by performing intradermal injections of the mediator into the cheek of a mouse, and the resultant behavior of the mouse is observed[148]. Typical behaviors for itch in mice are scratching the area with their hind limbs, while pain causes wiping responses with the forepaw[148]. Using these methods, TSLP, lysophosphatidic acid (LPA), and others have been identified as inducers of itch in mice[110,147].

DRG sensory nerves are glutamatergic. Glutamate is required for mediating pain sensations in the skin, however, loss of glutamate signaling from

these neurons actually sensitizes mice to itch[149]. This shows that itch neurons do not require glutamate to induce itch and likely rely on neuropeptides to mediate sensations of itch. One neuropeptide that has been implicated is gastrin-releasing peptide (GRP). Sensory nerves express GRP, and synapse on GRP receptor (GRPR) expressing neurons in the spinal cord[150]. Loss of GRPR expressing neurons leads to a significant reduction in itch[151].

1.2.4.1 TSLP and LPA

The finding that TSLP triggered itch caused some excitement[152], since TSLP levels are elevated in the skin in patients with atopic dermatitis, and this could potentially explain chronic itch in these patients. Work identifying the mechanisms by which TSLP caused itch found that the both subunits of the TSLP receptors (IL7Ra and the TSLP specific subunit TSLPR) are expressed on sensory nerves in both mice and humans. It was further found that TSLP activated sensory nerves, measured by calcium influx through the cation channel TRPA1. TRPA1 knockout mice also had significantly less itch in response to intradermal injection of TSLP[110].

LPA also causes itch after intradermal injection in mice. In this case, LPA likely acts through the receptor LPAR5, which is expressed by sensory nerves. It was found that LPA also causes calcium influx in sensory nerves in cell culture. However unlike TSLP, LPA requires both TRPV1 and TRPA1 to cause itch in mice[147].

1.2.4.2 Substance P

Substance P (SP) is a neuropeptide expressed by sensory nerves, as well as other cells such as eosinophils and mast cells, and has been implicated in mediating itch[153-155]. SP will activate sensory nerve through one or more of the mas-related G protein coupled receptors (mrgpr) to cause itch[156]. Blocking SP has been shown to reduce itch in patients with atopic dermatitis that have intractable itch[157].

1.2.4.3 Histamine

Histamine is the prototypical itch mediator. Histamine is released by mast cells in response to allergen, leading to itch. Itch causing sensory nerves express the H1 and H4 histamine receptors, and loss of both receptors significantly reduces itch in response to histamine[158,159]. However, blocking histamine is not effective in reducing itch in atopic dermatitis, which will be discussed in more detail later.

1.2.5 Itch in atopic dermatitis

Patients with atopic dermatitis suffer from chronic itch. While scratching does provide some immediate relief for these patients, scratching causes damage to the skin and increased inflammation. Inflammation then increases itch in these patients, causing a terrible cycle called the itch-scratch cycle. While we have developed a better understanding of mechanisms that lead to inflammation in patients with atopic dermatitis, it is still not clear what mechanisms tie inflammation and itch.

Patients with atopic dermatitis have altered responses to stimuli that cause itch. Patients with atopic dermatitis will respond to stimuli that normally cause pain, mechanical or heat induced, with sensations of itch[160]. Patients with atopic dermatitis are also much more sensitive to stimuli that cause itch and will itch when sweating or changing clothing[161]. Protease activated receptor 2 (PAR-2) agonists will induce itch in healthy patients but will induce worse itch in patients with atopic dermatitis[162]. While there is a clear increase in itch response in patients with atopic dermatitis, it has not been proved what mechanisms cause this. There are several possibilities that could potentially explain this. Local changes in sensory nerves, which will be discussed in more detail in the following section, may be responsible, if expression of itch receptors are increased. There could also be a change in processing of itch in the spinal cord, causing the mis-sensation of itch in response to pain stimuli. Finally, these changes in response may be mediated by other cells, such as inflammatory cells and keratinocytes, that influence sensory nerves activity. Understanding why patients with atopic dermatitis are more sensitive to itch responses would provide potentially effective new therapies for patients.

Histamine is a classic initiator of itch, though response to histamine is not increased in atopic dermatitis[162]. Treatment with antihistamines is also not effective in reducing itch in patients with atopic dermatitis[163]. However, antihistamines are still used some to treat patients, with the thought that they may help patients to sleep when itch is disrupting their sleep.

1.2.6 Changes of sensory nerves in atopic dermatitis

There have been many observations of changes of sensory nerves in the skin in patients with atopic dermatitis. While there are hypotheses about what consequences these changes have, it has yet to be proven that changes in sensory nerves drive symptoms in these patients. This is partially due to the fact that it is incredibly difficult to directly test the consequence of these changes. Many of these changes were identified by immunohistochemical staining of the skin for either general markers of nerves or subsets of nerves in the skin.

There are clear increases in nerve innervation in lesional skin in atopic dermatitis, compared to non-lesional skin from the same patients[164-166]. Changes in nerve innervation in the skin in these patients has been observed in the dermis and some in the epidermis, meaning that these changes likely are caused by sensory nerves. There has also been observed changes in neuropeptide expression, such as CGRP[167]. Again, while the consequences of these changes are not directly addressed, the observation of increased innervation of the skin may explain the increased sensitivity to itch.

Using electron microscopy, it was found that many of the nerves that are present in the skin in patients with atopic dermatitis are unmyelinated[113]. This is a characteristic of C fibers, which are responsible for mediating sensations of itch. It is also possible that myelin is degraded by chronic inflammation in these patients. However, if these nerves are C fibers, this data would show that the increase in nerves in the skin is driven by increases in C fiber sensory nerves,

which would fit with the hypothesis that increases in sensory nerves explains why patients have exaggerated responses to itch stimuli.

Neurotrophins, which are important growth factors for nerves, are also elevated in the serum in patients with atopic dermatitis[168]. The levels of nerve growth factor (NGF), one of the neurotrophins, in serum also correlates with severity of disease in patients with atopic dermatitis[168]. Neurotrophins, including NGF, have effects on peripheral innervation of tissue, and NGF has also been well shown to increase substance P levels in sensory nerves[169]. Importantly, NGF also has dramatic effects on inflammatory cells as well.

1.3 Eosinophils

Eosinophils are inflammatory cells that make up a small proportion (~2-4%) of inflammatory cells in peripheral blood in healthy individuals and were first described by Paul Ehrlich in the late 1800's. Paul Ehrlich was the first to extensively to study the ability of dyes to differentially label cells in the blood and used this to identify different leukocytes. In 1879, he first described granular cells that labeled with acidic coal tar dyes, especially eosin, and named them eosinophils. Paul Ehrlich was also one of the first to describe the association between elevated eosinophils in peripheral blood in several diseases, including asthma and helminth infections[170].

The interest in eosinophils has waxed and waned dramatically over the last couple of decades. Initial failures of mepolizumab, a drug targeting the eosinophil growth factor IL-5, in treating asthma in a clinical trial led to a

significant pushback in the study of eosinophils[171], as these trials convinced some that eosinophils were not important in disease[172]. Given serious concerns about study design, the trials were repeated, but only patients who were shown to have elevated peripheral eosinophils blood counts were admitted to the trial. Here, mepolizumab showed great success in reducing asthma exacerbations, and the study of eosinophils in these diseases has been renewed in the last decade[173].

1.3.1 Development

Homeostatic differentiation of eosinophils is regulated by coordinated expression of master transcription factors, including PU.1, C/EBP, and GATA-1[174-176]. Eosinophils differentiate from committed eosinophil progenitors (EoP), which split from other hematopoietic lineages at different times depending on the species. In humans EoPs are a subset of common myeloid progenitors that can be defined as expressing the receptor for IL-5[177]. In mice, EoPs separate at a later stage of development from the granulocyte macrophage progenitor (GMP) [178]. EoPs express high levels of the transcription factors mentioned above and are committed to differentiating into eosinophils. Targeting these transcription factors will all lead to a loss in eosinophils in mice, though there is often a loss of other inflammatory cell types as well. C/EBP knockout mice lack eosinophils but also lack neutrophils, showing that these transcription factors play important roles in development of multiple cell types and that the pattern of expression is also critical in cells deciding what path of differentiation to take[179].

Growth factors also have significant influences on the differentiation of eosinophils in the bone marrow. IL-5 specifically and dramatically increases the differentiation of eosinophils[180]. Importantly, loss of IL-5 due to genetic ablation does not reduce baseline eosinophil numbers, but blocks increases in eosinophil numbers above this[181]. Loss of IL-5 will block the increase in eosinophils that is associated with helminth infections in mice[181] and reduces peripheral eosinophils numbers in patients with asthma[173,182]. Other growth factors, such as GM-CSF also increase eosinophil differentiation, though it does not selectively effect eosinophils like IL-5 [183].

These findings on the pathways leading to eosinophil differentiation have led to the development of biologics which target these pathways in patients in an attempt to reduce eosinophil numbers. The most successful of these has been mepolizumab, which targets the IL-5 receptor, in patients with asthma. Mepolizumab significantly reduces exacerbation rates in patients with eosinophilic asthma, as defined by an elevated peripheral eosinophil levels[184]. Whether blocking IL-5 will be effective in treating patients with atopic dermatitis has not been effectively answered.

1.3.2 Granules

The granules in eosinophils can be separated into two distinct populations, primary and secondary granules[185]. Primary granules are positive for the Charcot–Leyden crystals[186]. The role of these granules is not well understood in disease or under normal conditions in humans, and mice do not have Charcot-

Leyden crystals. Eosinophil secondary granules consist of large amounts of strongly cationic proteins and are the component that labels with eosin giving eosinophils their pink appearance after staining[187]. These granules contain a complex collection of cationic eosinophil granule proteins, along with preformed cytokines and chemokines that can be rapidly released at sites of inflammation[187-190]. Importantly, while granules have many different granule proteins, cytokines, and chemokines, eosinophils can selectively release different components of the granules once activated (reviewed in [191]). These secondary granules are the main focus on studies of eosinophil activity and will be the only granules discussed in the rest of this dissertation.

When observed using electron microscopy, the eosinophil secondary granule contains two distinct regions[192]. In the center is a dense crystalline core, containing major basic protein (MBP) [187]. Outside of this is the outer matrix, containing the other granule proteins eosinophil peroxidase (EPX), eosinophil cationic protein (ECP) and eosinophil derived neurotoxin (EDN) [187]. These granule proteins, along with the Charcot-Leyden crystals, make up the vast majority of the protein content of mature eosinophils. Within the eosinophil granule there are also preformed cytokines that the eosinophil can rapidly release when activated[188-190].

Eosinophil granule formation starts early during eosinophil development in eosinophil progenitors. The eosinophil granule proteins are translated into the endoplasmic reticulum and transported to the Golgi. There, the granule proteins bud off in small vesicles that will fuse to form the larger granules. The precursor

of MBP will then mature as it forms the crystalline core at the center of the granule, and once this process is completed, the granule will contain the electron dense crystalline core that is the hallmark of a mature granule[193].

Of the granule proteins, EPX is the most selective for eosinophils, since these are the only cells known to express this protein. The other granule proteins can be found in other cells types, such as neutrophils and basophils[187,194].

These cationic proteins are all toxic at high levels[195-197]. The cationic granule proteins will kill parasites[198], as well as epithelial cells including keratinocytes[199]. However, this view that the eosinophil granule proteins are mainly toxic cationic products does not account for the other effects these proteins can have.

MBP has been shown to bind and inhibit M2 muscarinic receptor function, causing airway hyperreactivity in the lungs[200-202]. ECP and EDN both contain RNAse activity, which is thought to potentially play a role in host defense against viruses[203-205]. The role of EPX will be discussed in more detail in the next section. As these granule proteins are studied, there is clearly complex roles in the way that they interact with host cells to drive inflammation and symptoms in different diseases including asthma and potentially atopic dermatitis.

Release of eosinophils granules can take many different forms, but granule release is defined as release of some or all of the contents of eosinophil granules or release of whole granules from eosinophils. Release of total granule

content is achieved through exocytosis of the granules themselves. This type of degranulation is typically observed in cell culture and not typically observed in human tissue. There may be some evidence that this wholesale release of granule contents occurs in the context of parasite infections and in inflammatory bowel disease, but this is not typical of eosinophils[206].

Piecemeal degranulation is much more commonly observed in eosinophils, with ~65-80% undergoing this form of degranulation in humans[207,208]. Piecemeal degranulation involved the selective release of different components of the granules, without wholesale release of all contents. Here, selective granule proteins can be released, depending on the different stimuli that activates the eosinophil[209]. Using electron microscopy on eosinophils undergoing piecemeal degranulation, many small vesicles are observed in the cytoplasm, with the granules either disappearing or appearing degraded[210].

The mechanism by which eosinophils release selective preformed cytokines from granules may require the receptors for the cytokines. The best described mechanism of this is with the cytokine IL-4. Work using electron microscopy showed that the receptor for IL-4 will bind IL-4 in the granules and then transport the cytokine to the cell membrane for release[211]. Whether release of all components of eosinophil granule proteins occurs in a similar way is not known. Importantly, eosinophils can modulate their activity in response to different stimuli using piecemeal degranulation to have different effects under different conditions.

Finally, eosinophils can release whole granules through cell cytolysis, where their cell membrane ruptures and free granules are released. This type of eosinophil degranulation explains the observation of free eosinophil granules in tissue from patients with asthma and atopic dermatitis[212,213]. Free eosinophil granules maintain the capability of responding to environmental stimuli, and release its contents into tissue[214].

Eosinophil granules are critical for the function of eosinophils in disease, and understanding the mechanisms by which eosinophil granule content is released could have profound influences on our understanding of eosinophil functions in these situations.

1.3.3 Eosinophil peroxidase

Eosinophil peroxidase was the first eosinophil granule protein discovered[215]. EPX was discovered as released peroxidase content from granules during development of methods to purify the granules [215]. EPX is a heme peroxidase that is closely related to myeloperoxidase. Importantly, EPX is the most selective marker of eosinophils, and the gene for EPX has only been found to be expressed by eosinophils and eosinophil progenitors.

Like other eosinophil granule proteins, EPX is highly cationic, and through this cationic nature, it is toxic to many cell types. However, EPX has roles outside of its toxicity through this cationic nature. Early work showed that like MBP, EPX can bind and inhibit M2 muscarinic receptors[200], though it was much less potent then MBP in this aspect.

Like all peroxidases, EPX will use hydrogen peroxide to oxidize substrates. However, EPX is unusual in the substrates that it prefers to oxidize. Under physiologic conditions, EPX will oxidase both bromide and thiocyanate as preferred substrates, different from the related myeloperoxidase which will mainly oxidize chloride[216]. The activity of EPX can be measured in tissue by formation of bromotyrosines, which are a selective product of EPX activity in inflammation[217].

The influence of EPX on other cell types has also been studied as well. EPX has been shown to increase expression of GM-CSF in airway epithelial cells[218]. To increase GM-CSF, it was found that EPX did not require the presence of its substrates to increase gene expression, though it was able to have a more potent effect in the presence of its substrates. EPX will also increase expression of remodeling factors such as TGF-beta in airway epithelial cells[219]. In these experiments, EPX was capable of increasing cytokine expression in airway epithelial cells through its cationic nature and did not require peroxidase activity.

The role of EPX in diseases associated with eosinophils has not been determined at this point. EPX is certainly active in the lungs in patients with asthma, as we can observe brominated proteins in the lungs in these patients[220]. However, EPX knockout mice do not have any reduced inflammation, tissue remodeling or airway hyperreactivity in an animal model of asthma, calling into question the importance of EPX in this disease[221].

More recently, EPX was shown to be critical for development of diarrhea in a mouse model of inflammatory bowel disease. Importantly this was mediated predominantly by the peroxidase activity of EPX and could be inhibited using the EPX peroxidase activity inhibitor resorcinol. Interestingly, in these experiments they found that acute inhibition of EPX reduced the activity of eosinophils in tissue, suggesting that EPX is an important signal of activated eosinophils to activate other eosinophils in tissue[222].

Recent unpublished work by our collaborator (James Lee, unpublished communication) has found that EPX is critical for itch and inflammation in a mouse model of chronic contact dermatitis in mice. Like the above model, we found that EPX requires its peroxidase activity to drive itch in this model. This fits with what is known about atopic dermatitis, where there is extensive release of EPX in the skin in patients[164]. When this project was started it was unknown how EPX activity could drive both itch and inflammation in this model of chronic contact dermatitis.

1.3.4 Eosinophil recruitment

Recruitment of eosinophils to tissue is a critical component of eosinophils in mediating effects during inflammation. There are many components of eosinophil recruitment into tissue. There are chemotactic factors that will attract eosinophils to specific tissues, as well as adhesion molecules that allow binding of eosinophils to endothelium and extravasation of the eosinophils out of the

circulation. Each of these steps has different players that are critical for the overall recruitment of eosinophils into inflamed tissue.

Eotaxins are a family of 3 chemokines that selectively recruit eosinophils, except for eotaxin-2 that will also recruit basophils. Eotaxin was the first chemokine of this family discovered. Eotaxin-1, or CCL11, was first discovered as a factor in guinea pig lungs that attracted eosinophils to the skin when intradermally injected[223]. Eotaxin-1 is expressed at baseline in the gastrointestinal tract, not including the esophagus[224]. This helps explain why eosinophil levels are high in the gastrointestinal tract under normal conditions. During inflammation, eotaxin-1 levels can be induced in epithelial cells by proinflammatory cytokines IL-1 alpha, IL-1 beta, TNF, and the Th2 cytokines IL-4 and IL-13[225].

Other members of the eotaxin family are eotaxin-2 and eotaxin-3. These two factors share little sequence similarities to eotaxin-1, though both bind the same receptor, CCR3, and seem to be mostly selective for the recruitment of eosinophils[226]. These chemokines are also induced by Th2 cytokines like eotaxin-1, though there are distinct temporal differences in how these cytokines are expressed, which may have distinct effects on recruitment of eosinophils[226,227].

The receptor for the eotaxins on eosinophils is CCR3. CCR3 is expressed at high levels on eosinophils from healthy donors. While CCR3 is the receptor

for eotaxins, it is also a receptor for other chemokines, including RANTES, another important chemokine that recruits eosinophils[228].

Eotaxin appears to be critically important for the accumulation of eosinophils specifically around nerves, at least in the lungs[229]. Blocking CCR3 with an antagonist significantly reduced airway hyperreactivity in a sensitization and challenge animal model of asthma. Importantly, eosinophil numbers were not reduced in the lung in total, but their localization around nerves was blocked by the CCR3 antagonist[229].

While chemokines play an important role in signaling to recruit eosinophils to tissue, adhesion molecules are required for eosinophils to adhere to endothelium and migrate into tissue. Adhesion in the endothelium is mediated by a group of selectins and integrins that mediate rolling of eosinophils along the endothelium, tight binding, then extravasation into the tissue. Different steps of these pathways involve different adhesion molecules on eosinophils and endothelial cells. Eosinophils express L-selectin under baseline conditions, which binds to E and P selectin on endothelial cells to allow rolling of eosinophils on the endothelium[230].

Integrins are also critical components of allowing eosinophils to migrate into and in tissue. VLA-4 and LFA-1 are also constitutively expressed on eosinophils and allow binding to VCAM-1 and ICAM-1 respectively[231,232]. Adhesion of LFA-1 to ICAM-1 is critical for tight binding of eosinophils to endothelium and allowing extravasation into the tissue. VLA-4 is also critical for

recruitment of eosinophils into tissue, as blocking VLA-4 with an antibody blocks recruitment of eosinophils to the lung in an animal model of asthma and reduces airway hyperreactivity[233]. VLA-4 also has a role in binding extracellular matrix proteins, specifically fibronectin[234].

Binding of eosinophils to these adhesion molecules also has effects on eosinophil function. Binding of eosinophils leads to increased eosinophil survival, as well as an increase other eosinophil functions such as degranulation, and chemokine and cytokine production[235-237]. The purpose of this is thought to reduce eosinophils activity in the periphery and only allow activation at sites of inflammation.

1.3.5 Eosinophil nerve interactions

During times of chronic inflammation, such as in lesional skin in atopic dermatitis, eosinophils will typically associated with nerves in tissue. This is not limited to the skin, but also occurs in the lungs in patients with asthma, nasal nerves in allergic rhinitis, and nerves in the gut in inflammatory bowel disease[164,238-240].

Nerves can actively recruit eosinophils into close localization to nerves. Both parasympathetic and sensory nerves express the chemokine eotaxin[164,229], which was discussed in more detail above. Expression of eotaxin is increased in nerves in the airways in animal models of asthma and is expressed by sensory nerves in cell culture.

Neuropeptide release from nerves may also play an important role in recruiting eosinophils to tissue, as eosinophils migrate along neuropeptide concentration gradients[241]. Neuropeptides also stimulate expression of adhesion molecules on endothelium that allows the migration of inflammatory cells into tissue[242,243]. Stimulating nerves in vivo also leads to an increase in adhesion of eosinophils to endothelium [244].

Interactions of eosinophils with nerves has effects on eosinophil activity. Nerves express adhesion molecules that allow the binding of eosinophils to nerves. Both parasympathetic and sensory nerves will express VCAM-1 and ICAM-1, which bind to CD11b and VLA-4 expressed on eosinophils[164,237]. In cell culture the binding of eosinophils to nerves leads to degranulation of eosinophils, in a mechanism that involves more than just adhesion, but also a signal coming from the nerves[237]. This is shown by the fact that fixed nerves will still allow eosinophil adhesion but will not lead to degranulation. However, eosinophil binding also leads to release of other factors from eosinophils, including LTB4, which is triggered simply by adhesion of eosinophils to nerves[245].

Eosinophils also have effects on nerve function. Eosinophils increase sensory nerve growth and branching both in the skin in mice and in cell culture[164]. The mechanism by which this is accomplished does not appear to involve nerve growth factor, though what pathway is involved still needs to be described.

Eosinophil granule proteins influence nerve functions as well. Major basic protein (MBP) binds to and inhibits M2 muscarinic receptors on parasympathetic nerves[200]. M2 receptors are critical for feedback inhibition of parasympathetic nerve release of acetylcholine, and inhibition of M2 receptors leads to excess release of acetylcholine and increased bronchoconstriction[246]. The effect of MBP on M2 receptors can be blocked using the polyanion heparin[202]. Eosinophil granule proteins also reduce thresholds of activation for sensory nerves. Specifically, MBP and EPX treated sensory nerves are more sensitive to capsaicin, ATP and electrical stimulation[247,248].

1.4 Eosinophils in atopic dermatitis

The first mention of eosinophils being present in the skin of patients with atopic dermatitis was made in 1975 [249]. The relevance of eosinophils has been questioned since, as intact eosinophils are not always seen in skin biopsies from patients. However, it has been noted that most biopsies from patients with atopic dermatitis have either eosinophil infiltration or presence of free eosinophil granule proteins, implying that eosinophils have been recruited and degranulated in the skin[250,251]. This is particularly true of patients who develop atopic dermatitis before adulthood[249]. Eosinophils can even be observed undergoing cytolytic degranulation in the skin of patients with atopic dermatitis using electron microscopy[212]. These studies highlight the difficulty in measuring eosinophils in tissues.

Eosinophils also correlate well with severity of atopic dermatitis in patients. Peripheral blood eosinophils and levels of their granule proteins in serum correlate with severity of disease [252-254]. Tissue eosinophils are also usually present in areas that have increased spongiosis and epidermal hyperplasia [250]. These factors will also change during treatment as symptoms improve. The levels of the eosinophil cationic protein also decreases in response to treatment[255].

While there are clear associations of eosinophils and disease severity in atopic dermatitis, there is still a dearth of research into understanding mechanisms by which eosinophils participate in this disease. Evidence of eosinophil degranulation in the skin makes it likely that eosinophils are active in atopic dermatitis, but what specific roles they have in mediating symptoms are still unknown.

There has been one attempt to target eosinophils in a clinical trial in atopic dermatitis. In this trial, patients were treated with mepolizumab, to block IL-5 and reduce eosinophils, and followed for 2 weeks[256]. Mepolizumab reduced peripheral blood eosinophils in these patients but did not have an effect on itch. However, there are concerns that the timeline for this trial did not allow for effective reduction of eosinophils in the skin, and subsequently did not effectively test if eosinophils are important for itch in atopic dermatitis. The half-life of eosinophils in circulation in humans is roughly 12 hours[257]. However, in tissue the half-life of eosinophils is much longer, up to 8 days in the mouse lung during

allergen challenge[258]. Given these observations, a two week long trial may not have allowed for turnover of the eosinophils in the skin in these patients.

1.4.1 Eosinophil recruitment in atopic dermatitis

The increase in peripheral blood eosinophils in atopic dermatitis suggests that there is an increase in production of eosinophils in the bone marrow. Important factors for triggering development of eosinophils in bone marrow include IL-5 and GM-CSF[180,183], both of which are known to be elevated in plasma in patients with atopic dermatitis[82]. Interestingly, fitting with the idea that successful treatment of atopic dermatitis reduces eosinophils, IL-5 levels will reduce during treatment[259]. TSLP is also an important regulator of eosinophil development. TSLP levels are also elevated in the skin and plasma in patients with atopic dermatitis[97,260]. Inhibition of TSLP in humans with asthma leads to a dramatic decrease in peripheral blood eosinophils[105], though whether this would have the same effect in atopic dermatitis is still unknown.

Peripheral blood eosinophils will also delay apoptosis if from an atopic patient[261]. This increase in cell survival may also explain the increase in both peripheral blood eosinophils and levels of eosinophils in the skin. While the exact mechanisms that lead to increased eosinophil survival have not been proven in vivo, in vitro studies have shown that TSLP, IL-5 and GM-CSF have all increase eosinophil survival[108,262]. Since these cytokines are all elevated in serum in atopic dermatitis, they are likely candidates to drive this effect[82].

There are several pathways that are potentially involved in recruitment of eosinophils into the skin in atopic dermatitis. The first step in leukocyte recruitment requires expression of adhesion molecules on endothelial cells to allow migration from the blood into the skin. Expression of adhesion molecules on endothelial cells can be triggered by common cytokines like TNF, IL-1, IL-4 and IL-13[263-267]. While we do not know exactly what adhesion molecules are required for eosinophil recruitment in atopic dermatitis, it has been shown that eosinophils will adhere to endothelial cells expressing VCAM-1[268], and that IL-4 and IL-13 will both stimulate VCAM-1 expression on endothelial cells[264,267].

Both eotaxin and RANTES are important chemotactic factors for eosinophils[223,228]. Expression of these factors have both been found to be elevated in atopic dermatitis, both in the skin and in plasma[259,269]. Importantly, the levels of eotaxin and RANTES will also decrease during successful treatment of lesions in atopic dermatitis patients[259]. Expression of eotaxin and RANTES is enhanced by common Th2 cytokines IL-4 and IL-13[270,271]. Eosinophils will also express both of these cytokines, potentially setting up a feed forward loop where eosinophil recruitment can increase expression of factors that will then increase further eosinophil recruitment[272,273].

1.4.2 Eosinophil functions in skin

Our group has made some progress in understanding what potential roles eosinophils have in mediating some of the symptoms in atopic dermatitis. We have found that eosinophils are preferentially located around sensory nerves in

the skin in patients with atopic dermatitis[164]. As mentioned earlier, it is typical to observe an increase in sensory innervation in the skin in atopic dermatitis[164,274], which may be important in causing the increased sensitivity to stimuli that cause itch. We found that eosinophils are associated with areas of increased innervation in the skin, and that eosinophils can directly stimulate sensory nerve growth and branching in cell culture[164].

We have also found eosinophils play important roles in mediating inflammation and itch in an animal model of chronic contact dermatitis [275]. While this model does not perfectly recapitulate human atopic dermatitis, it does share many similarities, and allows us to identify potential mechanisms by which eosinophils mediate itch that can be tested in human atopic dermatitis. Using this chronic contact dermatitis model, we have been able to further show that eosinophils mediate itch through eosinophil peroxidase. We stained lesional skin biopsies from patients with atopic dermatitis for eosinophil peroxidase, and found that there is extensive release of this granule protein from eosinophils[164]. This finding supports the idea that EPX is important in atopic dermatitis.

All of this research into the role of eosinophils in atopic dermatitis suggests of eosinophils are important mediators of symptoms in this disease. However, much of this work is only an association of eosinophils with disease severity.

1.5 Hypothesis and research question

We know that eosinophils are present in the skin in patients with atopic dermatitis and that they correlate with severity of itch in this disease. We also know that eosinophils, and specifically EPX, is important for itch in a mouse model of chronic contact dermatitis. Previous work by our group has also found that eosinophils can interact with sensory nerves in the skin and increase growth and branching of sensory nerves. It has also been shown that the proinflammatory cytokine TSLP can activate sensory nerves to cause itch.

Based on these observations I hypothesized that eosinophils would mediate itch and inflammation through interactions with both keratinocytes and sensory nerves. Specifically, I hypothesized that eosinophils would interact with keratinocytes to increase TSLP expression, producing a ligand to activate sensory nerves to cause itch. I also hypothesized that eosinophils interact with sensory nerves to increase TSLP receptor expression, to hypothetically increase their response to TSLP and increase itch.

This hypothesis was tested by answering the following questions:

1. Do eosinophils increase TSLP receptor expression in sensory nerves?
2. Is EPX required for increase in TSLP levels in the skin in an animal model of chronic contact dermatitis?
3. Are eosinophils sufficient to increase TSLP expression in keratinocytes?

Hanifin and Rajka diagnostic criteria for atopic dermatitis from {Hanifin:1980vw}:

Must have 3 of the following:

- Pruritis
- Typical morphology and distribution
  - Flexural lichenification or linearity in adults
  - Facial and extensor involvement in infants and children
- Chronic or chronically-relapsing dermatitis
- Personal or family history of atopy (asthma, allergic rhinitis, atopic dermatitis)

Plus 3 or more minor features:

- Xerosis
- Ichthyosis/palmar hyperlinearity/keratosis pilaris
- Immediate (type I) skin test reactivity
- Early age of onset
- Tendency towards cutaneous infections/impaired cell-mediated immunity
- Tendency toward non-specific hand or foot dermatitis
- Nipple eczema
- Cheilitis
- Recurrent conjunctivitis
- Dennie-Morgan infraorbital fold
- Keratoconus
- Anterior subcapsular cataracts
- Orbital darkening
- Facial pallor/facial erythema
- Pityriasis alba
- Anterior neck folds
- Itch when sweating
- Intolerance to wool and lipid solvents
- Perifollicular accentuation
- Food intolerance
- Course influence by environmental/emotional factors
- White dermographism/delayed blanch

Table 1 – List of diagnostic criteria established by Hanifin and Rajka

Chapter 2: Methods

2.1 In Vivo Methods

2.1.1 Selection of animal model

The hypothesis of this thesis tests the role of eosinophils on mediating itch and inflammation in atopic dermatitis. To test the role of eosinophils on mediating itch an inflammation an animal model of atopic dermatitis was required, as itch and inflammation require a whole animal to occur. Many animals will develop atopic dermatitis like lesions, such as dog[276], and mouse[277]. There has been extensive effort to develop effective mouse models of atopic dermatitis, as mice provide a wide-ranging array of tools to determine mechanisms that promote this disease. We and a collaborator have developed many tools that are unique to mice to explore the biology of eosinophils, including eosinophil deficient mice[278], selective knockout of eosinophils granule proteins in mice[221,279], as well as others that were not specifically used to generate data in this project, but have proven crucial for enhancing our understanding of eosinophil biology. Because of this we have chosen to use a mouse model to determine the role of eosinophils in mediating itch and inflammation in the skin.

Unfortunately, there are currently no models of atopic dermatitis that effectively recapitulate all aspects of human disease, so we use models that mimic specific characteristics of atopic dermatitis. We are interested in studying mechanisms by which eosinophils cause itch and inflammation, and as such

have used a chronic contact dermatitis model that is dependent on sensitization and challenge to the chemical irritant trimelitic anhydride (TMA) [280]. This model has been shown to cause robust inflammation and itch in mice, and has an inflammatory profile that is well representative of the skin in atopic dermatitis. One particular advantage to this model is the activation state of eosinophils in the skin. We have shown that eosinophils degranulate extensively in the skin in this model, which is rare for sensitization and challenge models in mice[275]. Importantly, when looking at eosinophils in the skin in patients with atopic dermatitis there is often extensive degranulation of eosinophils as well[212,281]. We have also shown in this model that eosinophils are important mediators of itch and inflammation[275], making this model particularly effective for identifying mechanisms eosinophils use to drive these symptoms.

2.1.2 Animals

All experiments involving animals were approved by the Institutional Animal Care and Use Committee (IACUC). Animals were housed in 12:12 hour light:dark cycle at 20^{o} C. C57Bl/6 mice were purchased from Jackson labs, and bred on site for all experiments.

NJ1638 these mice were a generous gift from James and Nancy Lee laboratory, Scottsdale, AZ[282]. NJ1638 mice express IL-5 under the control of the CD3δ gene, leading to constitutive expression of IL-5 in peripheral T lymphocytes. This causes a massive increase in numbers of eosinophils in

circulation, allowing isolation of a large enough number of eosinophils from peripheral blood for experiments.

2.1.3 TMA induced chronic contact dermatitis mouse model

A chronic contact dermatitis model was used as described in the literature[275,280], with some modifications. This model of chronic contact dermatitis was achieved by sensitizing C57Bl/6 mice to trimellitic anhydride (TMA), a sensitizer in humans[283-285]. Subsequently, mice are challenged by exposing the right ear to TMA chronically.

In detail, C57Bl/6 mice aged 10-15 weeks were sensitized with a solution of 5% TMA in acetone/olive oil (4:1). 50 μL of this solution was applied to a shaved portion of the back on days 0 and 5. Mice were challenged by application of 10 μL 2% TMA in acetone/olive oil (4:1) to the back of the right ear on days 6 – 14. Contralateral control ears were treated with vehicle solution during every challenge. Mice were anesthetized with isoflourane prior to every sensitization and challenge.

Mice were weighed prior to every challenge to ensure that the treatments were well tolerated. After the mice were anesthetized with isoflourane, ear thickness was measured using a digital caliper (Mitutuyo) before each challenge, so that ear thickness was measured each day. On day 15 mice were euthanized by i.p. injection of pentobarbitol (300mg/kg), and tissue was collected. Ears and draining lymph nodes were collected, weighed and either flash frozen in liquid nitrogen or fixed in zamboni's fixative for 24 hours. Trigeminal ganglia were

collected and flash frozen in liquid nitrogen or fixed in zamboni's fixative for 24 hours. Blood was collected by cardiac puncture, a small amount was used to make a blood smear, and then the blood was spun to collect plasma, which was flash frozen in liquid nitrogen and stored at -80^{o}C.

Mice were treated with the following drugs, as noted in the individual experiments: Resorcinol, 1.25 mg/kg i.p., Kineret (anakinra), 30 mg/kg i.p., BrP-LPA 30 mg/kg, i.p.

2.1.4 Tissue Eosinophil Counts

After ears were fixed, they were embedded in paraffin, sectioned and stained with hematoxylin and eosin. Eosinophil counts were performed by a blinded observer. Eosinophils were identified by both nuclear shape and the presence of pink granules in the cells. The number of eosinophils which were present in three high powered fields (63x) per ear. High powered fields were selected at random, and eosinophils in both the dermis and the epidermis were counted, though eosinophils were only rarely observed in the epidermis. The number of eosinophils was averaged within each ear, and this was treated as one sample.

2.1.4.1 Validation of animal model and identification of draining lymph node

To test the animal model in our lab a small control cohort of animals was put through the protocol. This was also used to determine what lymph node was the draining lymph node from the ear that would respond to this treatment.

Potential lymph nodes were identified based on the literature[286], and the Superficial parotid, mandibular, accessory mandibular lymph nodes were harvested. To determine if lymph nodes responded to treatment weight of the lymph nodes was measured and compared to contralateral control lymph node.

I found a significant increase in ear thickness in this model, as had been previously shown in the literature (**Figure 2-1** B) [280]. I also found a trend towards an increase in EPX activity in extracted protein, and a significant increase in TSLP levels in extracted protein (**Figure 2-1**B-C). I also found that the superficial parotid lymph node was the only lymph node that significantly increased in weight compared to contralateral control (**Figure 2-2**). Due to this finding, for all experiments the superficial parotid lymph node was the only harvested lymph node.

2.2 In Vitro Methods

2.2.1 Cell culture medium

KGM-2

Keratinocyte growth medium-2 (KGM-2) was purchased as base media with a bullet kit from Lonza. Bullets include bovine pituitary extract, hEGF, insulin, hydrocortisone, epinephrine, transferrin, and GA-1000. All bullets were thawed and added to the base medium, except GA-1000, which was never added, to generate basic KGM-2. For experiments, KGM-2 without hydrocortisone or GA-1000, though with all other supplements, was used.

C2 Medium

C2 medium is made from 1:1 F12 media:DMEM media, with 0.05% bovine serum albumin, 1 x ITS (cellgro), and 1x L-Glut (sigma).

2.2.2 Primary mouse keratinocyte cell culture

Keratinocytes were isolated from mouse tail skin using modifications of previous protocols[287]. C57BL/6 mice were euthanized by i.p. injection of pentobarbital (30mg/mL) followed by cervical dislocation. After euthanasia tails were removed and skin was pulled from the tail using sterile forceps. The skin was placed dermis side down on thin layer of 0.25% trypsin (Gibco) in a 100mm petri dish overnight at 4^{o}C. The next day the skin was placed epidermis side down on a culture plate, and the epidermis was separated from the dermis by pulling the dermis with sterile forceps from the epidermis. The epidermis was minced and triturated in Minimum Essential Media (MEM, Gibco) with 5% Fetal Bovine Serum (FBS, Hyclone), penicillin (100 units/mL), streptomycin (100 μg/mL) and amphotericin B (0.25 μg/mL, PSF, Thermo Fisher Scientific). The solution containing the Keratinocytes was then passed through a 100 um cell strainer (Corning) to remove stratum corneum sheets. The cells were then centrifuged at 300 x g for 10 minutes, and the pelleted cells were re-suspended in MEM media with supplements (5% FBS, PSF). Keratinocytes were then cultured on plates coated with fibronectin (from human plasma, Sigma) and bovine type I collagen (Sigma), in MEM media with supplements at a density of 2 x 10^5 cells/cm^2, in a cell culture incubator at 5% CO_2 and 37^{o}C. After four hours media was replaced

with Keratinocyte Growth Medium 2 (KGM-2) with supplements (bovine pituitary extract, hEGF, insulin, hydrocortisone, epinephrine, and transferrin). Keratinocytes were cultured until they reached 100% confluence, and then cells were washed in PBS and media was replaced with KGM-2 with all supplements added except hydrocortisone. After 24 hours cells were used for experiments.

2.2.3 Primary mouse dorsal root ganglion cell culture

Dorsal root ganglia (DRG) were isolated and grown in culture using modifications to previous protocols[288]. C57BL/6 mice were euthanized by i.p. injection pentobarbital (30mg/mL). The mice were then perfused with cold Hanks Buffered Saline Solution (HBSS, Gibco), and the spine was extracted and placed into HBSS on ice. All lumbar, cervical and thoracic DRGs were harvested and placed into ice cold HBSS. Ganglia were then spun at 300 x g for 10 min, and were incubated in 600 U/mL type IV Collagenase (Worthington) in HBSS with calcium and magnesium for 5 min at room temp. Digestion was stopped by adding 5 times volume of ice cold HBSS. The DRGs were then spun at 300 x g, resuspended in HBSS with 20 U/mL Papain (Sigma), 0.4 mg/mL L-Cystein (Sigma), 0.5 mM EDTA (Abrisco) and incubated for 20 minutes at 37°C. After incubation cells were centrifuged at 410 x g for 5 minutes, and resuspended in HBSS containing 600 U.mL type IV collagenase and 2.5 U/mL Dispase II (Roche/Sigma) for 20 min at 37°C. The ganglia are again spun at 410 x g for 5 min, then are resuspended in HBSS. The ganglia are then triturated using a p1000 to dissociate the ganglia, and spun at 1100 x g for 3 min. The ganglia are resuspended in C2 Medium containing penicillin (100 units/mL), streptomycin

(100 μg/mL). The cells were then plated on chamber slides or 24 well plates that had been coated with Matrigel. Cells were placed in a cell culture incubator at 5% CO_2 and 37°C. Cells were then ready for use in experiments the following day.

2.2.4 Mouse eosinophil isolation from peripheral blood

NJ1638 mice, age 15 to 20 weeks, were anesthetized with pentobarbital (300mg/kg, i.p.). Blood was collected through a cardiac puncture, and placed in to a tube with 0.05% BSA, 5mM EDTA in PBS at a 1:1 volume. The peripheral blood is then layered over room temperature histopaque 1119 (Sigma) and spun at 600x g for 30 minutes. The buffy coat is then collected and placed into a new conical tube. If necessary, red blood cells are lysed by adding 36 mL of ice cold water, mixed briefly, and then 4 mL of 10 PBS is added. The cells are spun at 300x g for 10 minutes, and are resuspended in PBS with 0.05% BSA and 5mM EDTA. Cells were counted and incubated with anti CD45.2 and anti CD90.2 antibodies conjugated to MACS beads (Miltenyl) on ice for 30 minutes. Cells are then washed in PBS, spun and resuspended in PBS with 0.05% BSA and 5mM EDTA. Cells are then passed through Miltenyl LS columns in Miltenyi Vario to separate labeled cells. The flow through from the column contained the eosinophils. Viability of isolated eosinophils was determine using trypan blue staining. Purity of eosinophils was determined using Hemacolor assay on cytospin slides. Eosinophils were then resuspended in C2 media and used for experiments.

2.2.5 Reagents

Purified human eosinophil peroxidase was provided by Dr. Gerald Gleich, Department of Dermatology and Medicine, University of Utah. Keratinocytes were treated with the following: EPX (30nM), Horseradish Peroxidase (Life Technologies, 1 uM), Hydrogen Peroxide (Fisher Chemical, 100 uM), Sodium Bromide (Sigma, 100uM), Resorcinol (Sigma, 30 uM), Kineret (Anakinra, 30ug/mL). Sensory nerve cultures were treated with the following: TSLP (Peprotech, 50ng/mL), IL-33 (Peprotech, 100 ng/mL), IL-4 (Peprotech, 30 ng/mL), or IL-6 (10 ng/mL).

2.2.6 Cell viability assay

Keratinocytes in a 96 well plate were treated with 500 ug/mL Thiazoyly Blue Tetrazolium Bromide (MTT, Sigma) in KGM-2 medium without hydrocortisone for 3 hours at 37^{o}C in tissue culture incubator. Keratinocytes were protected from light throughout the assay. MTT was then removed, and the media was replaced with 5% triton X-100 (Sigma) in PBS for 2 hours at 37^{o}C. Absorbance was measured at 562 nm on a VersaMax plate reader.

2.3 RNA and Protein Isolation

2.3.1 mRNA isolation and quantitative real-time reverse transcriptase PCR

RNA was isolated from keratinocytes cultures using a RNeasy Kit (Qiagen) following manufacturer's instructions. cDNA was prepared using 100 ng of total RNA by reverse transcription using Superscript III (Invitrogen). Gene

expression for all genes were measured using the primers listed in table 1. qRT-PCR was performed using SYBR Green assay in Applied Biosystems 5500 Fast Thermocycler. Changes in gene expression were normalized to 18s, and relative quantities of RNA were determined using the delta-delta CT method[289].

2.3.1.1 Qiagen RT2 Profiler Array

Cytokine gene expression was measured in keratinocytes using the common cytokine RT2 profiler array (Qiagen, see table for specific list of genes). For these experiments, RNA was isolated as described above using the RNeasy kit. cDNA was prepared using 600 ng of total RNA by reverse transcription using Superscript III (Invitrogen). Master mix was made using SYBR Green assay, and 5 ng total cDNA was added to each well in the profiler array, which had primers already present in each well. qPCR was performed in an applied biosystems 5500 Fast Thermocycler. Changes in gene expression were normalized to a β-actin, and analyzed using a python program, using the Delta-Delta CT method.

2.3.2 Protein isolation and quantification

Frozen ears were thawed and homogenized (Tissue Terror, Biospec Products) in protein extraction buffer (10 mM Tris-HCL, pH 7.5; 0.5 mM EDTA; 0.5 mM EGTA; 1% Triton X-100; 0.5 mM PMSF; and Protease Inhibitor Cocktail [Thermo]). Samples were then spun at 500 x g for 10 minutes to remove debreis, and supernatante was collected and stored at -80^{o}C until use. Protein concentration was measured using a BCA protein assay (Thermo). Briefly, protein samples were mixed with the BCA working reagent for 30 minutes at

37°C, then absorbance was measured at a wavelength of 562 on VersaMax plate reader.

TSLP levels were measured using a TSLP ELISA (R and D, DY555). For cell culture experiments, media was collected and spun at 300 x g for 10 minutes, then used with the TSLP ELISA using manufacturer's instructions.

2.3.3 Peroxidase activity assay

EPX peroxidase activity was measured using an o-phenylenediamine assay. 30 nM EPX was placed into solution with 50 mM Tris-HCL, 0.1% Triton X-100, 8.8 mM Hydrogen Peroxide, 10 mM o-phenylenediamine, and 6mM NaBr. This was incubated for 5 min at 37°C, and the reaction was stopped by adding H_2SO_4 to the reaction. Absorbance was measured at 490 nm on a VersaMax plate reader.

2.4 Immunocytochemistry of sensory nerve cultures

Sensory nerve cultures were fixed for 15 minutes with zamboni's fixative, then washed with PBS. For staining, cells were blocked and permeabilized with 10% normal goat serum, 0.1% triton X-100 in tris buffered saline (blocking solution) for 1 hour at room temperature. Cells were then labelled with primary antibody, rabbit anti PGP 9.5, antibody at a 1:500 dilution in blocking solution. Samples were then washed with TBS, and cells were labelled in secondary antibody, goat anti-rabbit 555, or goat anti-rabbit 488 in blocking solution. Finally, the slides were washed, and coverslipped with Vecta Shield mounting medium with DAPI, to label nuclei.

2.4.1 DRG imaging and quantification

All nerves in slides were imaged at 20x magnification. Only single sensory nerves that were not touching cell bodies or neurites with other sensory nerves were analyzed. Using Imaris software computer models of the neurites were created, and total number of branchpoints and total neurite length was measured for each neuron, and averaged across each single culture.

2.5 Statistical analysis

All data is expressed as mean +/- SEM. For most experiments one way ANOVA with multiple comparisons and a Sidak correction was used to determine statistical significance. Comparisons of nerve length and branching in cell culture was made using a paired T test, as each culture had a treated and untreated pair. RT-qPCR experiments were compared using one way ANOVA with sidak correction for multiple comparisons, except when there are only two groups present, then a T test was used. Time course experiments used a two way ANOVA with repeated measures to determine statistical significance between groups. Experiments comparing differences in ear thickness over time were compared using a two way ANOVA with repeated measures to determine statistical significance between groups. A p value of < 0.05 was considered statistically significant. For dose response curve data r values were calculated from

Gene	5’ Primer	3’ Primer
18S	GTAACCCGTTGAACCCCATT	CCATCCAATCGGTAGTAGCG
Substance P	ACTGTTTGCAGAGGAAATCG	CAAAGAACTGCTGAGGCTTG
TRPV1	TTTTGGGAAGGGTGACTCAG	CCTGCGATCATAGAGCCTTG
TSLPR	AGTCGTCTGCCATGACCT	GCTGCCTAGCCTTAAACAC
TSLP	CTTGTCTCCTGAAAATCGAG	ATTCTGGAGATTGCATGAAG
TNF	CTGAACTTCGGGGTGATCGG	GGCTTGTCACTCGAATTTTGAGA
IL-1α	CGAAGACTACAGTTCTGCCATT	GACGTTTCAGAGGTTCTCAGAG
CSF2	TCGTCTCTAACGAGTTCTCCTT	CGTAGACCCTGCTCGAATATCT
CSF3	ATGGCTCAACTTTCTGCCCAG	CTGACAGTGACCAGGGGAAC
sPLA2G1B	GTGTGGCAGTTCCGCAATATG	CCTGTCTAAGTCGTCCACTGG
sPLA2G2A	TGGCTCAATACAGGACCAAGG	GTGGCATCCATAGAAGGCATAG
sPLA2G2C	GCTGCCAACCCATCTTGAATG	CACAGACTGTTTGTCACACTCA
sPLA2G2D	TGCTGGCCGGTATAACTGC	CTGTGGCATCTTTGGGTTGC
sPLA2G2E	CCAGTGGACGAGACGGATTG	AGCAGCTCTCTTGTCACACTC
sPLA2G2F	GCCTCTCCCTCTAAAACCTCC	AGCACCAGTCTACCTCATCCA
sPLA2G3	AGAGACCACAGGGCCATTAAG	GCTGTAGAATGACATGGTGCT
sPLA2G5	CCAGGGGGCTTGCTAGAAC	AGCACCAATCAGTGCCATCC
sPLA2G7	CTTTTCACTGGCAAGACACATCT	CGACGGGGTACGATCCATTTC
sPLA2G10	GTGCAGGTGTGACGAGGAG	CACTTGGGAGAGTCCTTCTCA
sPLA2G12A	AGATAGACACGTACCTCAACGC	GCTGCACTTGTACTGGCAGA
LPAR1	AGCCATGAACGAACAACAGTG	CATGATGAACACGCAAACAGTG
LPAR2	ATGGTAGCTGTCTACACACGA	AACCGTCTTGACTAGGCTGAG
LPAR3	CAAGCGCATGGACTTTTTCTAC	GAAATCCGCAGCAGCTAAGTT
LPAR4	AGTGCCTCCCTGTTTGTCTTC	GCCAGTGGCGATTAAAGTTGTAA
LPAR5	ACCTGGACATGATGTTTGCCA	GAGACCAGTCGCCAATACCA
LPAR6	ACGGGTGCATGTTCAGCAT	TGCCAGGTTAATCATGTACGTTG

Table 2 – Primer sequences for RT-PCR

UniGene	GenBank	Symbol	Description
Mm.3969	NM_009605	Adipoq	Adiponectin C1Q and collagen domain containing
Mm.235137	NM_007926	Aimp1	Aminoacyl tRNA synthetase complex-interacting multifunctional protein 1
Mm.27757	NM_009755	Bmp1	Bone morphogenetic protein 1
Mm.103205	NM_007553	Bmp2	Bone morphogenetic protein 2
Mm.209571	NM_173404	Bmp3	Bone morphogenetic protein 3
Mm.6813	NM_007554	Bmp4	Bone morphogenetic protein 4
Mm.428950	NM_007555	Bmp5	Bone morphogenetic protein 5
Mm.385759	NM_007556	Bmp6	Bone morphogenetic protein 6
Mm.595	NM_007557	Bmp7	Bone morphogenetic protein 7
Mm.4861	NM_011616	Cd40lg	CD40 ligand
Mm.42228	NM_011617	Cd70	CD70 antigen
Mm.290924	NM_170786	Cntf	Ciliary neurotrophic factor
Mm.795	NM_007778	Csf1	Colony stimulating factor 1 (macrophage)
Mm.4922	NM_009969	Csf2	Colony stimulating factor 2 (granulocyte-macrophage)
Mm.1238	NM_009971	Csf3	Colony stimulating factor 3 (granulocyte)
Mm.389954	NM_007795	Ctf1	Cardiotrophin 1
Mm.3355	NM_010177	Fasl	Fas ligand (TNF superfamily member 6)
Mm.317323	NM_008002	Fgf10	Fibroblast growth factor 10
Mm.31325	NM_011819	Gdf15	Growth differentiation factor 15
Mm.422844	NM_019506	Gdf2	Growth differentiation factor 2
Mm.4744	NM_008109	Gdf5	Growth differentiation factor 5
Mm.9714	NM_008110	Gdf9	Growth differentiation factor 9
Mm.14091	NM_010503	Ifna2	Interferon alpha 2
Mm.377088	NM_010504	Ifna4	Interferon alpha 4
Mm.1245	NM_010510	Ifnb1	Interferon beta 1
Mm.240327	NM_008337	Ifng	Interferon gamma
Mm.874	NM_010548	Il10	Interleukin 10
Mm.35814	NM_008350	Il11	Interleukin 11
Mm.239707	NM_008352	Il12b	Interleukin 12B
Mm.1284	NM_008355	Il13	Interleukin 13
Mm.4392	NM_008357	Il15	Interleukin 15
Mm.10137	NM_010551	Il16	Interleukin 16
Mm.5419	NM_010552	Il17a	Interleukin 17A
Mm.59313	NM_019508	Il17b	Interleukin 17B

Mm.222808	NM_145834	Il17c	Interleukin 17C
Mm.222807	NM_145856	Il17f	Interleukin 17F
Mm.1410	NM_008360	Il18	Interleukin 18
Mm.131480	NM_001009940	Il19	Interleukin 19
Mm.15534	NM_010554	Il1a	Interleukin 1 alpha
Mm.222830	NM_008361	Il1b	Interleukin 1 beta
Mm.882	NM_031167	Il1rn	Interleukin 1 receptor antagonist
Mm.14190	NM_008366	Il2	Interleukin 2
Mm.103794	NM_021380	Il20	Interleukin 20
Mm.157689	NM_021782	Il21	Interleukin 21
Mm.125482	NM_031252	Il23a	Interleukin 23 alpha subunit p19
Mm.196691	NM_053095	Il24	Interleukin 24
Mm.90154	NM_080729	Il25	Interleukin 25
Mm.222632	NM_145636	Il27	Interleukin 27
Mm.983	NM_010556	Il3	Interleukin 3
Mm.276360	NM_021283	Il4	Interleukin 4
Mm.4461	NM_010558	Il5	Interleukin 5
Mm.1019	NM_031168	Il6	Interleukin 6
Mm.3825	NM_008371	Il7	Interleukin 7
Mm.3006	NM_008373	Il9	Interleukin 9
Mm.1100	NM_010564	Inha	Inhibin alpha
Mm.8042	NM_008380	Inhba	Inhibin beta-A
Mm.378911	NM_010094	Lefty1	Left right determination factor 1
Mm.4964	NM_008501	Lif	Leukemia inhibitory factor
Mm.87787	NM_010735	Lta	Lymphotoxin A
Mm.1715	NM_008518	Ltb	Lymphotoxin B
Mm.2326	NM_010798	Mif	Macrophage migration inhibitory factor
Mm.3514	NM_010834	Mstn	Myostatin
Mm.202727	NM_021524	Nampt	Nicotinamide phosphoribosyltransferase
Mm.131422	NM_001013365	Osm	Oncostatin M
Mm.22802	NM_170727	Scgb3a1	Secretoglobin
Mm.288474	NM_009263	Spp1	Secreted phosphoprotein 1
Mm.248380	NM_011577	Tgfb1	Transforming growth factor beta 1
Mm.18213	NM_009367	Tgfb2	Transforming growth factor beta 2
Mm.3943	NM_009379	Thpo	Thrombopoietin
Mm.1293	NM_013693	Tnf	Tumor necrosis factor

Mm.15383	NM_008764	Tnfrsf11b	Tumor necrosis factor receptor superfamily member 11b (osteoprotegerin)
Mm.1062	NM_009425	Tnfsf10	Tumor necrosis factor (ligand) superfamily member 10
Mm.249221	NM_011613	Tnfsf11	Tumor necrosis factor (ligand) superfamily member 11
Mm.344820	NM_011614	Tnfsf12	Tumor necrosis factor (ligand) superfamily member 12
Mm.8983	NM_023517	Tnfsf13	Tumor necrosis factor (ligand) superfamily member 13
Mm.28835	NM_033622	Tnfsf13b	Tumor necrosis factor (ligand) superfamily member 13b
Mm.483369	NM_019418	Tnfsf14	Tumor necrosis factor (ligand) superfamily member 14
Mm.208152	NM_177371	Tnfsf15	Tumor necrosis factor (ligand) superfamily member 15
Mm.276823	NM_183391	Tnfsf18	Tumor necrosis factor (ligand) superfamily member 18
Mm.4994	NM_009452	Tnfsf4	Tumor necrosis factor (ligand) superfamily member 4
Mm.4664	NM_009403	Tnfsf8	Tumor necrosis factor (ligand) superfamily member 8
Mm.41171	NM_009404	Tnfsf9	Tumor necrosis factor (ligand) superfamily member 9
Mm.268863	NM_001005506	Txlna	Taxilin alpha
Mm.282184	NM_009505	Vegfa	Vascular endothelial growth factor A
Mm.328431	NM_007393	Actb	Actin beta
Mm.163	NM_009735	B2m	Beta-2 microglobulin
Mm.343110	NM_008084	Gapdh	Glyceraldehyde-3-phosphate dehydrogenase
Mm.3317	NM_010368	Gusb	Glucuronidase beta
Mm.2180	NM_008302	Hsp90ab1	Heat shock protein 90 alpha (cytosolic) class B member 1

Table 3 – List of cytokines from the Qiagen RT2 Array

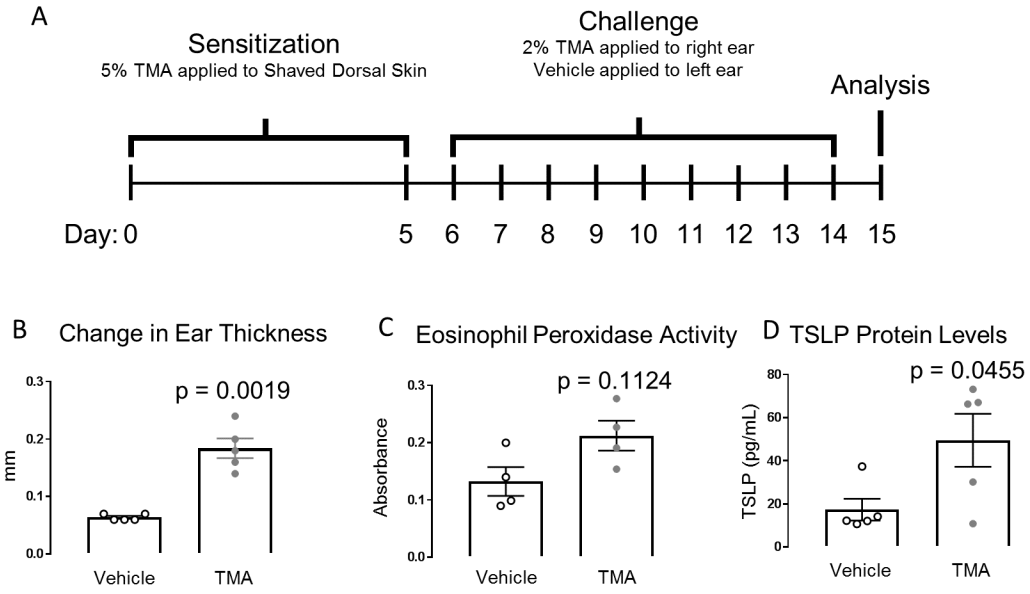

Figure 2-1 Chronic exposure to TMA increases inflammation, including TSLP.

A) Mice were sensitized by application of a 5% TMA solution (4:1 acetone:olive oil) on a shaved portion of dorsal back. Mice were then challenged on the right ear with a 2% TMA solution (4:1 acetone:olive oil). Contralateral ears were treated with vehicle for controls. B) Ear thickness was measured on the day 6 before the first challenge, and on day 15, data is represented as the change in ear thickness between the two samples. C) EPX activity was measured using the EPX activity assay, 20 ug of total protein was loaded into each reaction. D) TSLP protein levels in extracted protein were measured using TSLP ELISA.

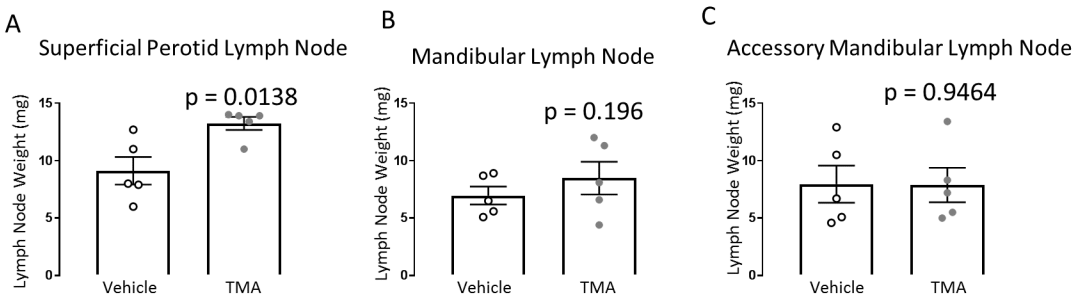

Figure 2-2 Chronic TMA exposure increases weight of the superficial parotid lymph node.

A) Superficial Parotid, B) Mandibular, C) Accessory Mandibular lymph nodes were harvested and weighed on day 15 after challenge

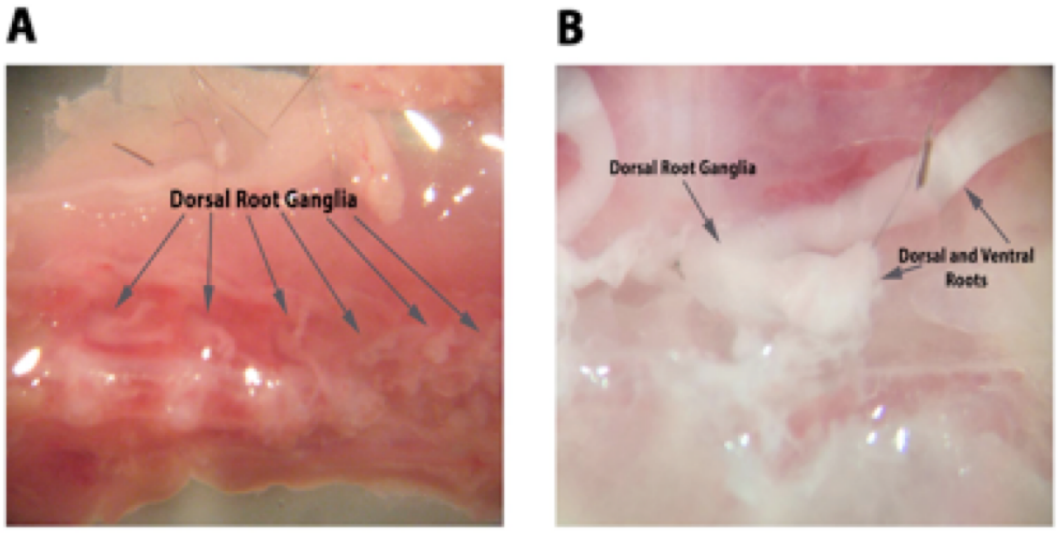

Figure 2-3 Location of mouse DRG

A) The DRG's can be seen in the spinal canal after removal of the spinal cord.

B) High magnification view of a DRG, with the dorsal and ventral roots labeled.

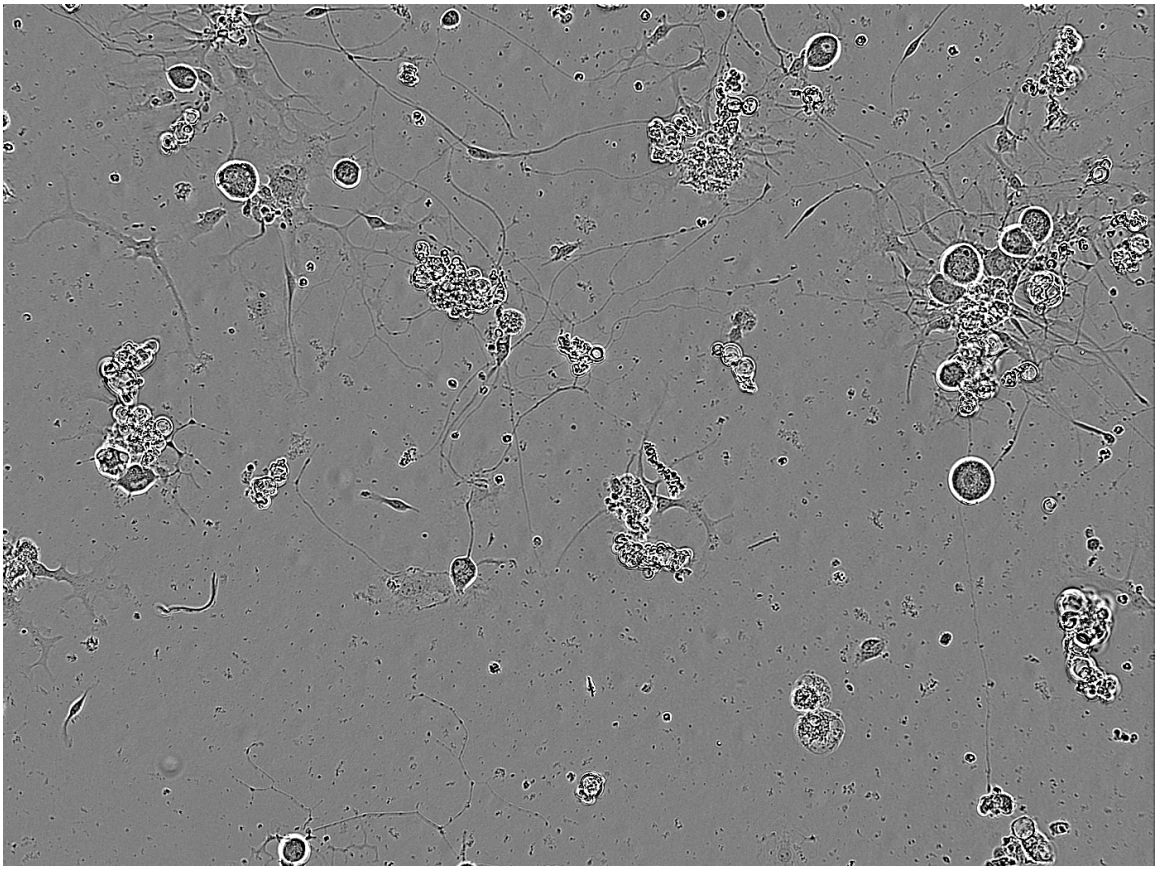

Figure 2-4 Unstained mouse DRG in cell culture for 24 hours.

Phase contrast image at 10x of untreated mouse sensory nerves in cell culture after 24 hours.

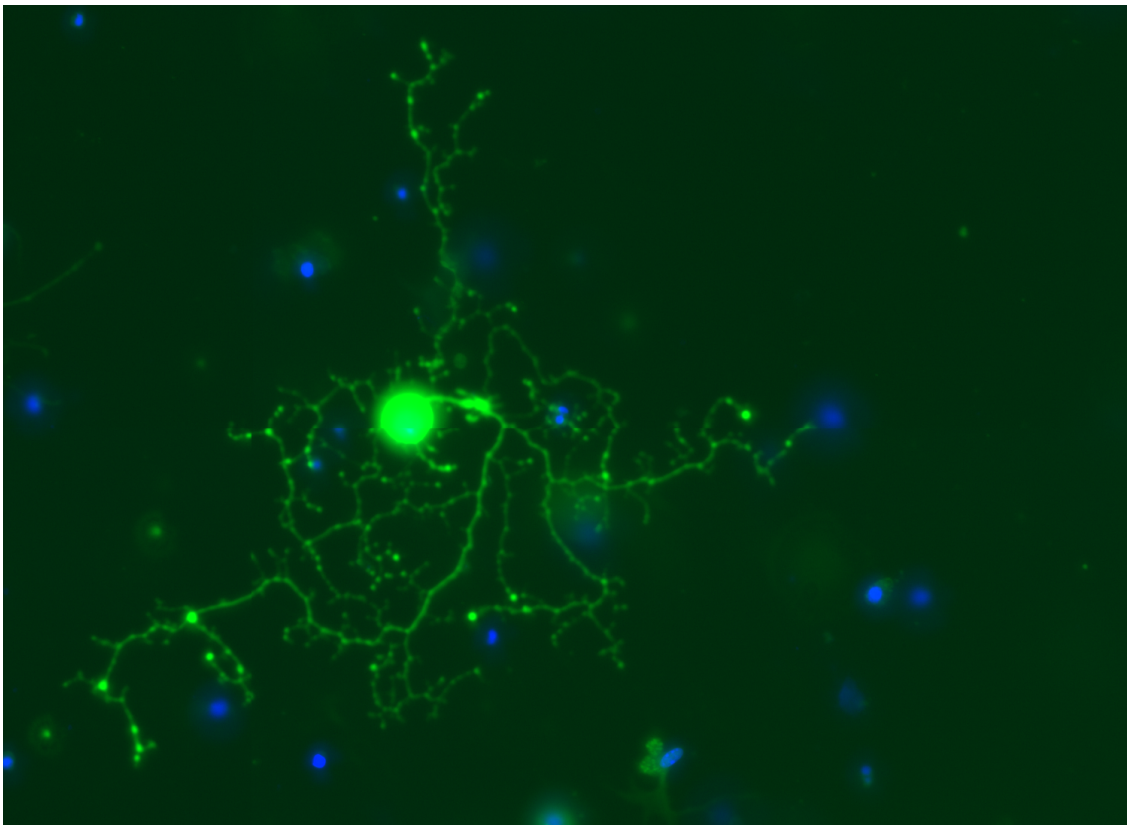

Figure 2-5 – Mouse DRG in cell culture stained with PGP 9.5 in green and DAPI in blue.

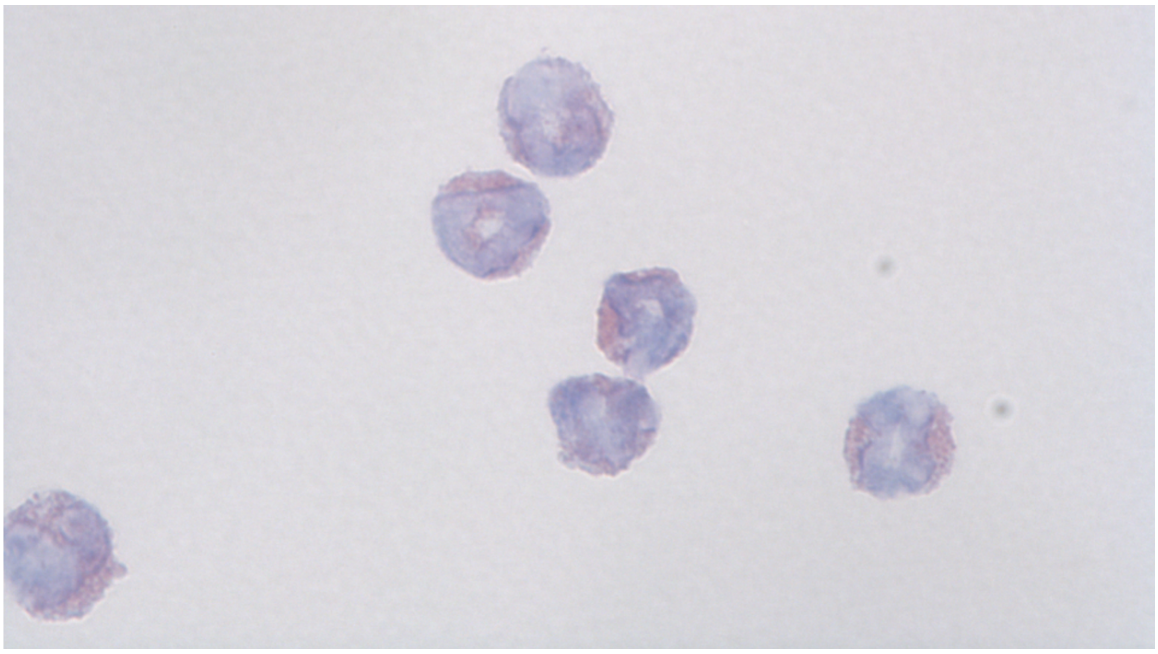

Figure 2-6 Hemacolor stained purified mouse eosinophils

40x bright field image of mouse eosinophil isolated from peripheral blood as described above, stained with hemacolor. Nuclei stained in blue, granules stained pink.

Chapter 3: Eosinophils increase TSLP receptor expression in sensory nerves

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3.1 Introduction

Patients with atopic dermatitis have dramatically changed responses to stimuli on the skin, leading to itch in response to stimuli that are normally received as touch (Ikoma et al. 2004; Andersen et al. 2017). This is termed alloknesis. The mechanism that accounts for this has not been definitively proven, though changes in sensory nerves in the skin may be responsible, since these have been well described in atopic dermatitis. We, and others, have shown that there is an increase in the length and branching of sensory nerves in inflamed skin in patients with atopic dermatitis[164,166,290,291]. Along with increased nerves, levels of neuropeptides, such as CGRP and substance P, are also increased in sensory nerves in the skin[167]. Importantly, many of these changes observed take place specifically in lesional, itchy skin, compared to non-lesional skin in the same patients [164].

We have shown that eosinophils likely play a critical role in mediating these changes in sensory nerves. Eosinophils are present in lesional skin of patients with atopic dermatitis [212,281] and specifically associate with nerves in the skin[164]. Eosinophils often localize around nerves in tissue in the setting of chronic inflammation, including the airways[238], nasal tract[240] and gastrointestinal tract[239,292]. We have also shown that in animal models eosinophils are sufficient

to increase sensory innervation in the skin and that eosinophils increases sensory nerve length and branching in cell culture[164]. These findings implicate eosinophils in driving changes in sensory nerves in the skin in atopic dermatitis.

Thymic stromal lymphopoietin (TSLP) causes itch by directly activating sensory nerves[110]. This may be relevant to chronic itch in patients with atopic dermatitis, as TSLP levels are increased in lesional skin in patients with atopic dermatitis[96,97,293]. Wilson et al. found that sensory nerves, both in mouse and in human, express both receptor subunits for TSLP[110].

Given these observations, I hypothesized that eosinophils sensitize sensory nerves to stimuli that cause itch by increasing expression of receptors and neuropeptides responsible for mediating itch. This includes TRPV1 (a critical receptor for histamine induced itch), substance P (a neuropeptide that causes itch), and TSLPR (which causes itch when activated on sensory nerves).

3.2 Results

Eosinophils increase sensory nerve length in cell culture.

Previous work from our group found that eosinophils increase sensory nerve branching and length in cell culture[164]. I first performed experiments to identify conditions where eosinophils influence sensory nerve function.

Sensory nerves were isolated from mouse dorsal root ganglia, dispersed into single cells and grown in cell culture. Eosinophils were isolated from peripheral blood from NJ1638 mice, and added to the sensory nerve cell

cultures. After 24 hours the sensory nerve cultures were fixed, and stained for the pan neuronal marker PGP 9.5 and nerve length and branching was measured.

Coculture with eosinophils significantly increased sensory neurite length, and caused a trend towards an increase in number of neurite branch points per cell (Figure 3-1 A-D). Each symbol represents the measurement of neurite length and number of branch points from many neurons in a distinct culture. The number of measurements from each experiment is shown in Figure 3-1 E-J.

Eosinophils specifically increase TSLP receptor expression in sensory nerves in cell culture.

To determine if eosinophils had an effect on the expression of factors relevant to itch (specifically substance P [SP], TRPV1, and TSLPR), eosinophils were cocultured with sensory nerves and gene expression for these factors was measured. Sensory nerves and eosinophils were isolated and prepared as described in the above section. Eosinophils were cocultured with sensory nerves at concentrations of $3 \times 10^4 - 3 \times 10^5$ eosinophils/mL. After 24 hours eosinophils were washed out of the cultures, and RNA was isolated from the sensory nerves. After 24 hours of coculture, eosinophils dose dependently increased gene expression for TSLPR in sensory nerves, but not TRPV1 or SP (Figure 3-2 A-C). Importantly, levels of the eosinophil specific genes eosinophil peroxidase and major basic protein were not detected in the samples, showing that eosinophils RNA was not contaminating the samples

Eosinophils require cell contact to increase TSLPR expression in sensory nerves in cell culture.

To determine if eosinophils secrete a soluble factor that increases TSLP receptor expression in sensory nerves two experiments were performed. In the first experiment, conditioned media was generated by culturing eosinophils sensory nerve media for 24 hours at a concentration of 3 x 10^5 cells / mL. Eosinophils were then spun at 300 x g for 10 minutes and the supernatant was used as conditioned media. Sensory nerve cultures were treated for 24 hours with the conditioned media, and then gene expression for TSLPR, TRPV1, and SP was measured. In these experiments, eosinophil conditioned media did not increase TSLPR, TRPV1, or SP in sensory nerves (Figure 3-3 A-C).

In the second experiment to further identify if eosinophil cell contact is required, transwell experiments were used to allow soluble communication between nerves and eosinophils. In these experiments, sensory nerve cultures were established, and eosinophils were isolated from mouse peripheral blood as described above. Transwell inserts were placed into the well with established sensory nerve cultures, and eosinophils were placed in the upper chamber of the transwell so the concentration of eosinophils was 3 x 10^5 cells/mL for the total volume of media. After 24 hours RNA was isolated from the sensory nerves and gene expression was measured. Eosinophils did not increase on TSLPR gene expression, nor TRPV1 or SP gene expression, in sensory nerves across transwells (Figure 3-3 D-F). These data indicate that cell contact is required for eosinophils to increase TSLPR expression in sensory nerves.

The cytokines TSLP, IL-33, IL-4 and IL-6 do not mimic the effect of eosinophils on TSLPR gene expression.

To determine if common Th2 cytokines (TSLP, IL-33, IL-4 and IL-6) could mimic the effect of eosinophil coculture on TSLPR expression in sensory nerves, sensory nerve cultures were established as described above and were treated for 24 hours with the noted concentration of the cytokines TSLP, IL-33, IL-4 and IL-6. RNA was isolated and TSLPR gene expression was measured. These cytokines did not affect TSLPR gene expression in sensory nerves (Figure 3-4 A-D).

3.3 Discussion

Here I show that eosinophils increase neurite length in sensory nerves in cell culture, which is in agreement with previous work from our lab[164]. I also found that eosinophils specifically increase TSLPR expression in sensory nerves, in a mechanism that requires cell contact. Finally, typical Th2 cytokines were not able to mimic the effect of eosinophils on TSLPR expression in sensory nerves.

The finding that eosinophils require cell contact to increase TSLPR expression on nerves fits with data from animal models of allergic disease. Eosinophils associate with nerves in tissue, in the skin in patients with atopic dermatitis[164], or in the lungs in patients with asthma[238]. Nerves express adhesion molecules for eosinophils, including ICAM-1 and VCAM-1[164,237,294]. This association with nerves in tissue is critical for airway hyperreactivity in animal models of asthma[229]. The finding that cell contact was required for eosinophils to

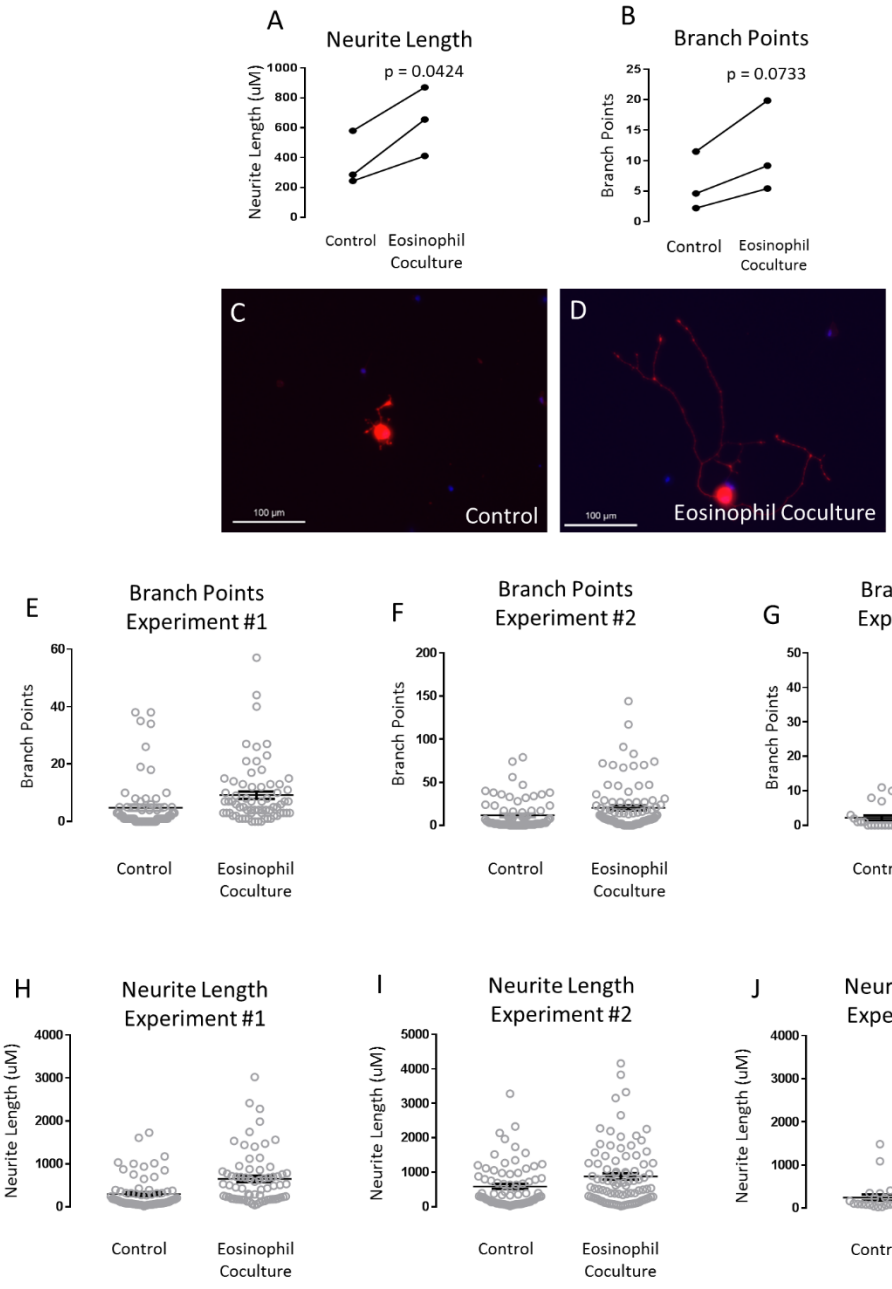
increase TSLPR expression in sensory nerves fits with these observations in vivo. Whether cell contact between eosinophils and nerves is required for itch in the animal model of chronic contact dermatitis has not been tested.

Cell contact of eosinophils with nerves has been shown to have distinct effects on eosinophil function. Sensory nerves, and parasympathetic nerves express adhesion molecules to allow direct contact between eosinophils and nerves[164,237,294] both in vivo and in cell culture. This adhesion has been shown to activate eosinophils to release inflammatory mediators such as LTB4 [237,245,295]. Contact between eosinophils and nerves has also been shown to stimulate eosinophil degranulation, in a mechanisms that requires signaling from the nerves[237,245,295]. Activation of eosinophils after adhesion to nerves could mean that a soluble mediator released by eosinophils is responsible for increasing TSLPR expression in sensory nerves, but that adhesion between eosinophils and nerves is required for eosinophils to secrete this mediator.

Two possible explanations could explain the increased TSLPR gene expression. The TSLPR is expressed in about 5% of sensory nerves in the DRG[110]. One mechanism could be increasing expression of TSLPR in nerves not previously expressing it. During times of inflammation, sensory nerves have been shown to express neuropeptides in nerves that normally do not express these neuropeptides[296]. The other possibility is an increase in the level of expression in sensory nerves that already express TSLPR. TSLPR expression has been shown to be increased by proinflammatory cytokines such as TNF[297].

Regardless of the mechanisms, we hypothesize an increase in TSLPR would increase the sensitivity of these nerves to TSLP, and result in increased itch.

Future experiments will need to be performed to test these different possibilities, both in cell culture as well as in the skin in atopic dermatitis. Calcium imaging would be capable of determining the proportion of sensory nerves respond to TSLP, and how this changes after eosinophil coculture[110]. Staining for TSLPR in lesional skin biopsies from patients with atopic dermatitis could be used to determine if TSLPR levels change in the skin in patients with atopic dermatitis, using methods we have established in the lab[275,298]. These methods would determine what functional consequences changes in TSLPR expression has in sensory nerves, and whether these changes are directly relevant in patients with atopic dermatitis.


A
Neurite Length
p = 0.0424
Neurite Length (uM)
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0
Control
Eosinophil Coculture
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p = 0.0733
Branch Points
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15
10
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Eosinophil Coculture

Figure 3-1. Eosinophil increase sensory nerve neurite length and branching after coculture for 24 hours.

Mouse DRG's were isolated, dispersed into single cells and grown in cell culture for 24 hours. Eosinophils were then added to the sensory nerve cell cultures at 30,000 eosinophils/mL and cocultured for 24 hours. After fixation and staining for PGP 9.5 to label all nerves, cultures were imaged and neurites were modeled using Imaris software. Neurite length and branchpoints were determined using the model from Imaris. A) Average neurite length from three experiments, with lines connecting paired cultures. B) Average number of neurite branchpoints from three experiments, with lines connecting paired cultures. C-D) Representative images from control and eosinophil coculture. Sensory nerves are labeled in red for PGP 9.5. DAPI is labeled in blue to label cell nuclei. E-G) Quantification of branch points in three independent cultures. Each symbol represents total number of branchpoints for a single sensory nerve. H-J) Quantification of neurite length in three independent culture. Each symbol represents total neurite lengths for a single sensory nerve.

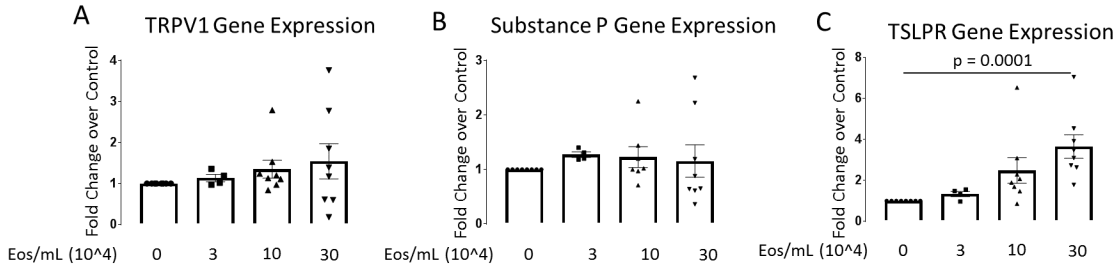

Figure 3-2 Eosinophils dose-dependently increase TSLPR expression in sensory nerves.

Mouse DRG's were grown in cell culture as described above. After 24 hours, 3 x 10^4 - 30 x 10^4 eosinophils/mL were added to the cultures for 24 hours. Eosinophils were then washed away, and RNA was isolated from the sensory nerves in culture. Gene expression was measured using real-time RT-PCR. A) TRPV1 gene expression in sensory nerves. B) Substance P gene expression in sensory nerves. C) TSLPR gene expression in sensory nerves.

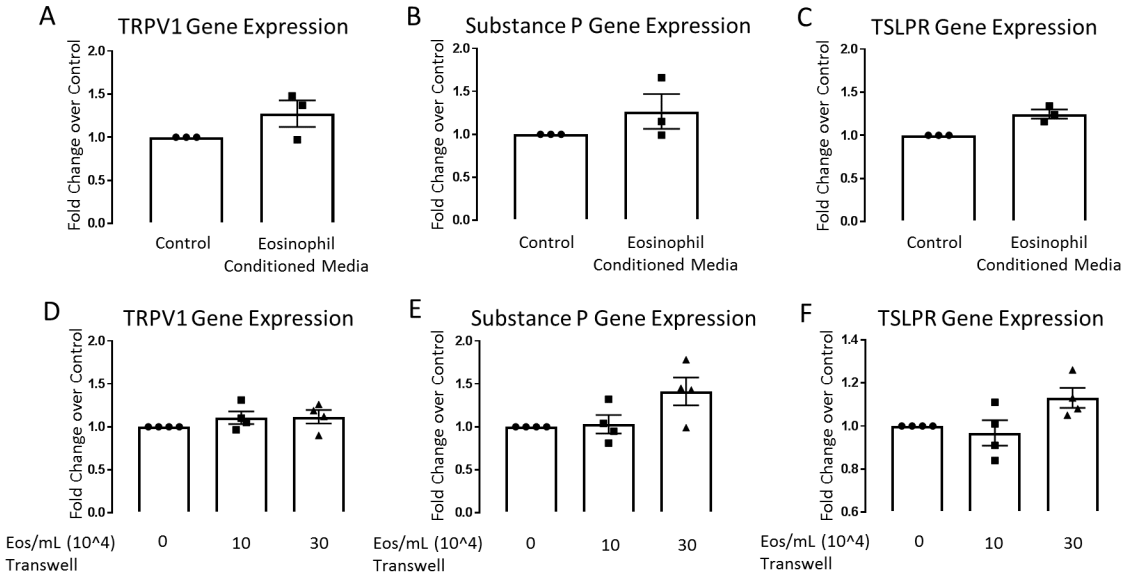

Figure 3-3 Eosinophils require cell contact to increase TSLPR expression in sensory nerves.

Sensory nerve cultures were established as described above. A-C) Eosinophil conditioned media was created by culturing eosinophils for 24 hours in sensory nerve media at a concentration of 3 x 10^5 cells/mL for 24 hours. Eosinophils were removed from the media, DRG cultures were treated with the conditioned media for 24 hours. TRPV1, SP, and TSLPR gene expression was then measured in the sensory nerves by real time RT-PCR. D-F) Sensory nerves were cultured in plates, and transwell inserts (0.4μm) were placed in the cell culture wells. Eosinophils were added to the upper chamber of the transwells at 10 or 30 x 10^4 eosinophils/mL. After 24 hours TRPV1, SP and TSLPR gene expression was measured in the sensory nerves by real time RT-PCR.

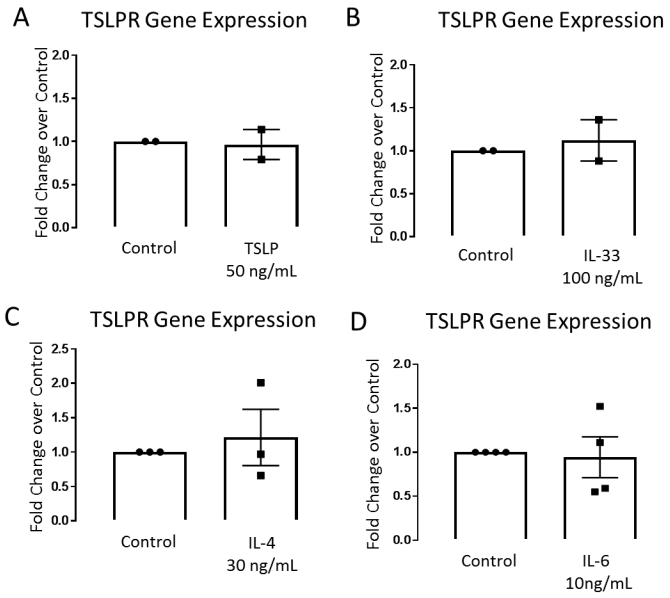

Figure 3-4 TSLP, IL-33, IL-4 and IL-6 did not affect TSLPR expression in sensory nerves.

Mouse DRG's were grown in cell culture and treated with A) 50 ng/mL TSLP, B) 100 ng/mL IL-33, C) 30 ng/mL IL-4, or D) 10 ng/mL IL-6 for 24 hours. TSLPR gene expression was measured 24 hours later by real time RT-PCR.

Chapter 4: The eosinophil peroxidase inhibitor resorcinol reduces TSLP levels and inflammation in an animal model of chronic contact dermatitis

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4.1 Introduction

Eosinophil peroxidase (EPX) is an important mediator of itch in a mouse model of chronic contact dermatitis. In this setting EPX requires its peroxidase activity to increase itch, as the peroxidase inhibitor resorcinol blocks itch in the mouse model. Importantly, extensive release of EPX is observed in the skin in this mouse model[275], as well as in the skin in patients with atopic dermatitis[164]. However, while EPX is important for itch in this model the mechanism by which it causes itch were unknown, and its effects on inflammation in the skin were also unknown.

The cytokine TSLP has many described functions that place it in a unique position to be a mediator of both itch and inflammation in the skin. The receptor for TSLP is expressed on sensory nerves, and intradermal injection of TSLP causes itch by directly activating these sensory nerves[110]. Given the fact that TSLP levels are elevated in patients with atopic dermatitis[96,97,293], it is possible that TSLP plays a critical role in mediating chronic itch in these patients. Along with TSLPs described role on mediating itch, TSLP also has important roles on inflammation, including many effects directly on eosinophils. These include stimulating eosinophil recruitment to tissue[107,108], which may play a role in

eosinophil recruitment in atopic dermatitis. TSLP also increases survival of eosinophils[108], which may explain the increased survival ex vivo of eosinophils isolated from peripheral blood from atopic dermatitis patients compared to normal[261]. Finally, TSLP causes eosinophil degranulation[106], which may explain why there is such extensive degranulation in the skin in patients with atopic dermatitis[251].

This is just a snapshot of all the described roles TSLP has on inflammation, with a focus on specific effects on eosinophils. TSLP also has dramatic roles in shaping the inflammatory response that is generated in response to allergen. TSLP is critical for sensitization in animal models of allergy[299], and may be critical for the development of allergies in patients with atopic dermatitis[98,300]. Understanding the mechanisms that lead to increased levels of TSLP in the skin in patients with atopic dermatitis would provide potential targets for therapy that would aid patients.

The mechanisms leading to chronically elevated levels of TSLP in atopic dermatitis are not known. TSLP is predominantly derived from epithelial cells, including keratinocytes in the skin. Common inducers of TSLP include TLR3 agonists, such as viruses[301,302], the synthetic TLR3 agonist Poly I:C[303], and proteases[304]. However, the role of these receptors on increasing TSLP levels in the skin in patients with atopic dermatitis has not been tested.

Based on the observation that EPX activity was required for itch in the mouse model of chronic contact dermatitis I hypothesized that EPX would

mediate itch through increasing TSLP levels in the skin. Given the roles of TSLP on mediating inflammation in the skin, I also hypothesized that EPX is required for inflammation in the skin as well, including a reduction in eosinophils numbers in the skin.

4.2 Results

Resorcinol dose dependently inhibits the activity of human EPX

To test if resorcinol inhibits human eosinophil peroxidase, purified human eosinophils peroxidase activity was measured in the presence of increasing concentrations (3 nM – 1 x 10^5 nM) of resorcinol and compared to that without resorcinol. Specifically, human EPX activity was measured using a eosinophils peroxidase activity assay as described in chapter 2. Similar to the effect of resorcinol on mouse EPX, resorcinol inhibited the activity of human EPX in a dose dependent manner (Figure 4-1).

Resorcinol significantly reduces ear inflammation induced by chronic exposure to TMA

The role of eosinophil peroxidase activity in mediating both general markers of inflammation and TSLP expression was tested in an animal model of chronic contact dermatitis. C57Bl/6 mice were sensitized and chronically challenged with TMA on the ear (Figure 4-2 A). As described previously, treatment of WT mice with TMA markedly increased ear thickness compared to the vehicle treated contralateral control ear, demonstrating inflammation is induced by chronic TMA exposure (Figure 4-2 B). Lymph node weight was also

measured to determine general levels of inflammation, and was increased after chronic TMA treatment (Figure 4-2 C). Representative images of H&E stained ear tissue sections suggest an increase in epidermal thickening in the skin after chronic TMA treatment (Figure 4-3). To inhibit the peroxidase activity of EPX, mice were treated with resorcinol during challenge. Resorcinol did not have any effect on ear thickness, or lymph node weight in untreated ears, but resorcinol significantly inhibited the increase in ear thickness caused by chronic treatment with TMA (Figure 4-2 B) compared to control TMA treated ears. However, Resorcinol did not reduce lymph node weight after treatment with TMA (Figure 4-2 C). Representative images of H&E stained ear tissue sections suggest resorcinol reduces the increase in epidermal thickening caused by chronic TMA treatment (Figure 4-3).

Resorcinol significantly reduces eosinophil levels in skin after chronic TMA exposure.

Due to the fact that there was reduced inflammation in mice treated with resorcinol, I measured the recruitment of eosinophils in the skin after treatment with resorcinol. Under normal conditions very few, if any, eosinophils are observed in the skin. After chronic treatment with TMA there was a significant increase in eosinophils numbers in the skin (Figure 4-4). Resorcinol significantly reduced the numbers of eosinophils that were present in the skin after treatment with chronic TMA compared to control ears treated with TMA only (Figure 4-4).

Resorcinol significantly reduced TSLP levels in skin after chronic treatment with TMA.

To test if resorcinol reduced levels of TSLP after exposure to chronic TMA, TSLP protein levels were measured in extracted protein from mouse ears. Chronic treatment with TMA caused an increase in TSLP protein compared to contralateral control ears treated with vehicle only (Figure 4-5). Resorcinol significantly reduced TSLP levels in ears after chronic TMA treatment compared to control ears treated with TMA only (Figure 4-5). TSLP levels were normalized in three different ways: total pg TSLP per ear (pg TSLP/Ear), pg TSLP per mg extracted protein (pg TSLP/mg Protein), and pg TSLP per mg tissue weight (pg TSLP/mg Tissue). TSLP levels were also compared as fold increase in treated ear over contralateral control ear. The effect of resorcinol on TSLP levels was seen no matter what method was used to normalize the data.

To ensure that these methods of normalization were not influenced by resorcinol, ear weight and protein extraction concentrations were measured. Ear weight was significantly increased by chronic TMA treatment, which was reduced by resorcinol (Figure 4-6). Protein extraction concentrations were also significantly increased in TMA treated ears over control, but resorcinol does not have any effect on these levels (Figure 4-7). Finally, resorcinol did not influence mouse body weight, and was well tolerated by the mice (Figure 4-8).

4.3 Discussion

Here I show that the EPX inhibitor resorcinol significantly reduced TMA induced inflammation in a model of chronic contact dermatitis, including a decrease in ear thickness, reduced eosinophil numbers in the skin, and lower levels of the proinflammatory cytokine TSLP. However, another general marker of inflammation, draining lymph node weight, was not reduced by resorcinol. The effect of resorcinol appeared to only have an effect on local inflammation, specifically in the ear, without effecting lymph node weight.

This finding agrees with what is known about inflammation in the skin in patients with atopic dermatitis. Eosinophils are present in the skin in these patients, and there is extensive degranulation and release of eosinophil granule proteins, such as EPX in the skin[164,251]. TSLP levels are also increased in the skin in these patients[96,97,293]. Our findings that blocking eosinophil peroxidase reduces TSLP provides evidence to support a connection between EPX and TSLP in the skin in patients with atopic dermatitis.

Here, inhibition of the peroxidase activity of EPX caused a significant decrease in the number of eosinophils in the skin. This could be explained through several mechanisms, including a reduction in eosinophil survival in skin, reduction in recruitment of eosinophils to the skin, and a reduction of eosinophilopoiesis of new eosinophils in the bone marrow. Based on these data, it is likely that eosinophils activate a feedforward loop that triggers an increase in eosinophils in skin, and that this is mediated by the peroxidase activity of EPX.

Resorcinol has been found to inhibit the activity of other heme containing peroxidases, though in this model of chronic contact dermatitis resorcinol has a similar effect to the loss of specifically eosinophil peroxidase, by genetic ablation (unpublished observations, James Lee). EPX activity can also clearly be detected in the lungs in patients with asthma, a disease associated with elevated eosinophils, and is likely active in the skin in patients atopic dermatitis as well.

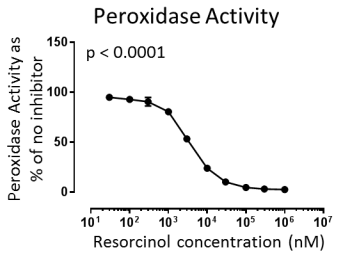

Figure 4-1 Resorcinol inhibits activity of EPX.

EPX activity in the presence of resorcinol, compared to EPX activity alone.

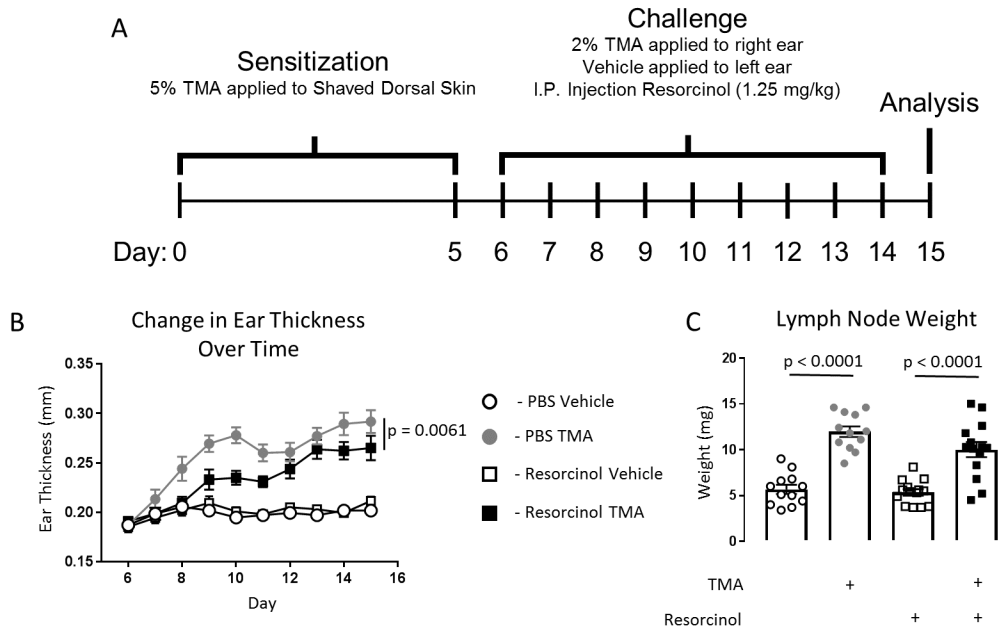

Figure 4-2 Resorcinol significantly reduces ear inflammation induced by TMA.

A) Mice were sensitized by application of a 5% TMA solution (4:1 acetone:olive oil) on a shaved portion of dorsal back. Mice were then challenged on the right ear with a 2% TMA solution (4:1 acetone:olive oil). Contralateral ears were treated with vehicle for controls. Mice were treated with daily injections of resorcinol (1.25mg/kg), or PBS as control during challenge. B) Ear thickness was measured daily using a Mitutoyo Digital Caliper. Ear thickness was measured just prior to treating the ears with TMA or vehicle. C) Draining lymph nodes were harvested and weighed on day 15.

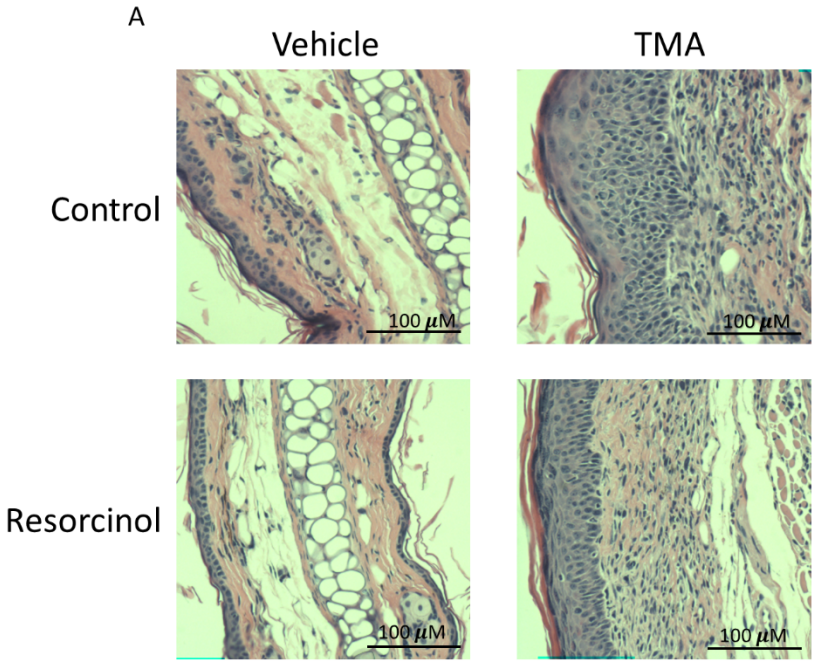

Figure 4-3 Representative images of H&E stained ear sections.

Ear sections from mice were harvested and fixed in Zambonis fixative. Samples were then paraffin embedded, sectioned, and stained with H&E.

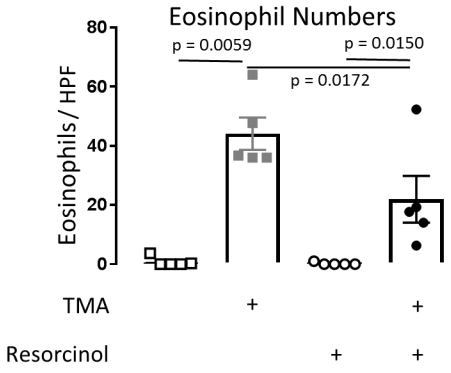

Figure 4-4. Resorcinol reduces eosinophil recruitment to skin after chronic exposure to TMA

Eosinophils were counted in 3 randomly selected high powered fields in each ear, and averaged.

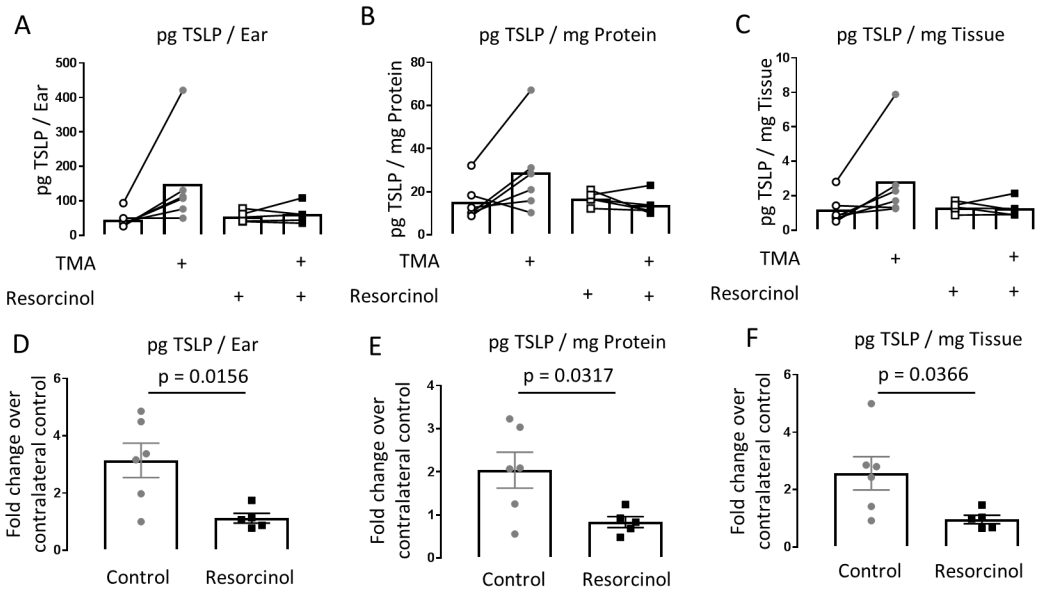

Figure 4-5 Resorcinol significantly reduces the increase in TSLP caused by TMA.

TSLP was measured from total protein extracted from mouse ears using an ELISA. A) Total TSLP extracted from mouse ear. Connected lines indicate paired samples from one mouse. B) TSLP levels normalized to as pg TSLP per mg protein. Connected lines indicate paired samples from one mouse. C) TSLP levels normalized to weight of tissue protein was extracted from. Connected lines indicate paired samples from one mouse. D-F) TSLP levels were graphed as fold increase over contralateral control ear from the same mouse.

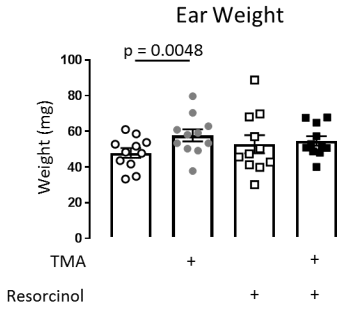

Figure 4-6 Resorcinol blocks the increase in ear weight caused by TMA.

Ears were harvested and weighed on day 15.

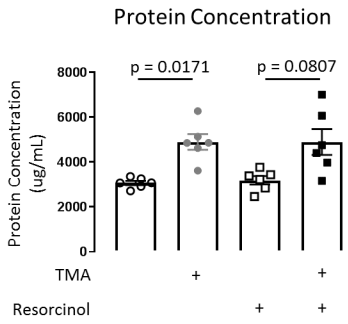

Figure 4-7 Resorcinol does not affect the increase in protein extraction levels caused by TMA.

Total protein was extracted from mouse ears by homogenizing in protein extraction buffer, and measured using a BCA assay.

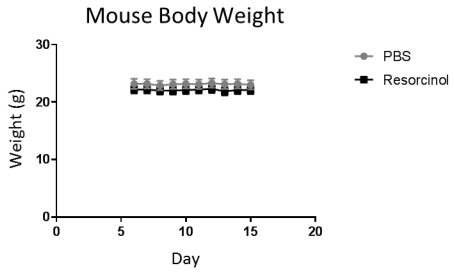

Figure 4-8 Resorcinol does not reduce mouse body weight.

Mice were weighed daily during challenge.

Chapter 5: Eosinophil peroxidase increases cytokine expression in keratinocytes through LPA and IL-1

5.1 Introduction

The mechanism by which EPX increases TSLP and inflammation in the skin is unknown. The consequence of EPX release in tissue can be toxic, EPX causes death in bacteria and parasites, death in epithelial cells, and lesions in skin[195,197,305,306]. In many of these instances, EPX is a more potent toxin in the presence of its substrates, though the strong cationic nature of EPX can cause some toxicity alone. However, EPX also has other effects on cytokine expression in epithelial cells, other than just causing cell death, including increasing GM-CSF release from airway epithelial cells[218].

EPX uses hydrogen peroxide and halides as substrates to produce hypohalous acid[216]. Bromide is normally present in plasma at concentrations of 10-100 uM[307], and at normal plasma concentrations of bromide and chloride EPX will almost exclusively use bromide as the halide substrate[216]. Importantly, bromide is not present in cell culture media, and must be added in cell culture experiments using EPX.

TSLP is predominantly an epithelial cell derived cytokine, and is generally found to be expressed in keratinocytes in the skin in patients with atopic dermatitis[97]. Due to the fact that EPX activity is required for the increase in TSLP

in the skin, I hypothesized that EPX would directly stimulate TSLP expression in keratinocytes.

5.2 Results

Horseradish peroxidase, a substitute for EPX, increases TSLP expression in keratinocytes.

Initial experiments to determine conditions permissive for EPX to increase TSLP expression used horseradish peroxidase. Horseradish peroxidase was used as a surrogate of peroxidase activity similar to EPX peroxidase activity. To test the effect of horseradish peroxidase on TSLP gene expression primary keratinocyte were treated with HRP (1μM) and H_2O_2 (100 μM) and TSLP gene expression and protein release into media was measured. HRP increased gene expression and protein release of TSLP from keratinocytes in the presence of its substrate hydrogen peroxide (Figure 5-1 A-B). Hydrogen peroxide alone had no effect (Figure 5-1 A-B). To determine if this stimulus caused cell death, an MTT assay was used on keratinocytes treated with horseradish peroxidase and/or hydrogen peroxidase for 8 hours. Horseradish peroxidase and hydrogen peroxidase caused a significant reduction in keratinocyte cell viability, while either alone had no effect on cell viability (Figure 5-1 C).

Eosinophil peroxidase increases cytokine expression in keratinocytes.

Using the conditions identified in the horseradish peroxidase experiments the effect of EPX on cytokine expression in keratinocytes was measured. Keratinocytes were treated with purified EPX (30nM) along with its substrates

hydrogen peroxide (100 μM) and bromide (100 μM) (referred to as "substrates" from here) for 6 hours and cytokine gene expression was measured using the Qiagen RT2 common cytokine profiler array. EPX plus substrates increased gene expression of TNF, CSF2, CSF3, and IL1α (Figure 5-2). To determine whether the substrates of EPX are required or sufficient to increase cytokine expression, keratinocytes were treated with EPX alone, substrates alone, or EPX with its substrates. EPX in the presence of its substrates increased gene expression for TSLP, CSF2, CSF3, TNF, and IL-1α (Figure 5-3 A-E), and increased TSLP protein levels in cell culture media (Figure 5-3 F) however neither the substrates alone nor EPX alone increased cytokine gene expression or TSLP protein release. Unlike what was observed for horseradish peroxidase, EPX plus its substrates did not have any effect on cell viability as measured by MTT assay (Figure 5-3 G).

EPX requires peroxidase activity to increase cytokine gene expression in keratinocytes.

In the chronic contact dermatitis model, peroxidase activity of EPX was required for itch and inflammation, and the increase in TSLP levels in the ears. The finding that EPX requires its substrates to increase cytokine expression in keratinocytes suggests that peroxidase activity is required. To further demonstrate this, the EPX peroxidase activity inhibitor resorcinol, and heat inactivated EPX were used. Heat inactivated EPX did not increase cytokine gene expression in keratinocytes (Figure 5-4 A). Resorcinol (30 μM) significantly

inhibited the EPX-induced increase in TSLP, CSF2, CSF3, TNF and IL1α in keratinocytes (Figure 5-4 B). These data show that EPX requires peroxidase activity to increase cytokine gene expression in keratinocytes.

EPX increases cytokine expression in keratinocytes through a soluble mediator.

One surprising finding when performing the time-course experiments was the significant delay in increase in cytokine gene expression after the cells were treated. Based on this observation, I hypothesized that EPX increased cytokine expression through a soluble mediator, since this delay would be required for the soluble mediator to accumulate to sufficient concentrations to drive expression.

To test this hypothesis I used conditioned media experiments. To generate the conditioned media, keratinocytes were treated with EPX plus substrates for 8 hours. After 8 hours, the media from the treated keratinocytes was removed and centrifuged at 300 x g for 10 minutes, and the supernatant was used as conditioned media. Naïve keratinocytes were then treated with conditioned media or EPX plus substrates for 4 hours and cytokine gene expression was measured. This time point was selected because previous experiments had shown that EPX did not increase cytokine expression at 4 hours. At 4 hours conditioned media significantly increased TSLP, CSF2, CSF3, TNF and IL1α gene expression, while EPX did not(Figure 5-5). This findings supports the hypothesis that EPX increases cytokine expression through a soluble mediator.

Lysophosphatidic Acid (LPA) increases TSLP expression, but does not affect the gene expression for other cytokines.

To determine what soluble factors are required for the EPX-induced increase in cytokine expression in keratinocytes, we tested the role of LPA as this mediator. LPA has previously been shown to increase TSLP expression in airway epithelial cells[308], though it is unknown what effect it has on keratinocytes. Initially I tested whether keratinocytes express the LPA receptors, and thus can potentially respond to LPA. Gene expression for the 6 LPA receptors in keratinocytes were measured by real time RT-PCR. All six receptors for LPA (LPAR1-6) were expressed at the level of gene expression in keratinocytes (Figure 5-6 A). Knowing that keratinocytes express the LPA receptors, I next tested if LPA could mimic the effects of EPX on cytokine gene expression. Treating keratinocytes with LPA dose dependently increased TSLP gene expression in keratinocytes (Figure 5-6 B). However this effect was selective for TSLP, as LPA did not affect expression of CSF2, CSF3, TNF or IL1α (Figure 5-6 C-F). These findings suggest EPX increases cytokine expression through several different mediators.

EPX increases LPA levels in cell culture

To further investigate the role of LPA in EPX-increased TSLP expression, I measured the level LPA in cell culture media from keratinocytes treated with EPX. LPA 16:0 and 18:0 were the only two species detectable in media from keratinocytes. Both species were increased in cell culture media of keratinocytes

treated with EPX and substrates compared to media from untreated keratinocytes (Figure 5-7).

LPA is required for EPX to increase TSLP expression in keratinocytes

To determine if LPA was required for the increase in TSLP expression caused by EPX keratinocytes were treated with the pan LPA receptor BrP-LPA[309]. First, to test if BrP-LPA would block the increase in TSLP expression caused by LPA I treated keratinocytes with 3 μM LPA, in the presence of 1-100 μM BrP-LPA. BrP-LPA significantly inhibited the increase in TSLP expression caused by LPA (Figure 5-8 A). Next, to test if LPA was required for the increase in TSLP expression caused by EPX, keratinocytes were treated with EPX and its substrates, with 100 μM BrP-LPA. BrP-LPA significantly reduced the increase in TSLP expression caused by EPX (Figure 5-6 B).

Extracellular LPA production is dependent on the activity of secreted phospholipase A2 enzymes (sPLA2) [310]. These enzymes cleave at the sn2 position of phospholipids, releasing lysophospholipids and free fatty acids. There are 11 sPLA2 proteins in mammals[311]. To further support the finding that BrP-LPA inhibits EPX-induced increase in TSLP expression, keratinocytes were treated with the pan sPLA2 inhibitor MJ33. MJ33 (3-30 μM) dose dependently inhibited the increase in TSLP expression in keratinocytes treated with EPX and its substrates (Figure 5-9 A). MJ33 did not affect keratinocyte cell viability using a MTT assay (Figure 5-9 B). Together, these data identify LPA as the soluble mediator that is responsible for the increase in TSLP expression in keratinocytes

treated with EPX. Interestingly, LPA is selective for increasing gene expression of TSLP, since other cytokines were not affected by LPA.

IL-1 signaling is required for EPX to increase cytokine expression, except for TSLP

While LPA signaling plays a key role in the increase in TSLP expression in keratinocytes in response to EPX, LPA did not stimulate expression of the other cytokines that were increased by EPX. The role of IL-1 was tested as a possible mediator of CSF2, CSF3, TNF and IL1α in keratinocytes treated with EPX, since IL-1 has been shown to increase expression of TNF and IL1α in keratinocytes[312]. Treating keratinocytes with IL-1α caused a significant large increase in gene expression for CSF2, CSF3, TNF and IL1α in keratinocytes, but caused a comparatively smaller increase in TSLP gene expression (Figure 5-10). Treating keratinocytes with IL-1β had a similar effect on cytokine gene expression, without increasing TSLP gene expression (Figure 5-11). The IL-1 receptor antagonist anakinra blocks signaling of both IL-1 α, and IL-1β. To test if IL-1 was required for the increase in cytokine expression caused by EPX in keratinocytes, keratinocytes were treated with EPX and anakinra. Treating keratinocytes with anakinra (30 μg/mL) significantly reduced gene expression of CSF2, CSF3, TNF, and IL-1α in keratinocytes treated with EPX (Figure 5-12 B-E). Anakinra did not reduce TSLP gene expression in keratinocytes treated with EPX (Figure 5-12 A). These data show that IL-1 plays an important role in increasing expression of CSF2, CSF3, TNF and IL1α in keratinocytes, but not the expression of TSLP.

5.3 Discussion

Here I show that EPX increases the expression of cytokines in keratinocytes. EPX requires peroxidase activity to increase cytokine expression in keratinocytes, and its effects can be blocked using the peroxidase inhibitor resorcinol. The mechanisms by which EPX increases expression of these cytokines in keratinocytes breaks into two distinct pathways. For TSLP, EPX drives production of LPA which then increases TSLP expression in keratinocytes, which is a novel finding. For the other cytokines CSF2, CSF3, TNF and IL-1α, EPX increases gene expression through IL-1 signaling, since this effect can be blocked with the IL-1 receptor antagonist anakinra.

I also found that horseradish peroxidase increases expression of TSLP in keratinocytes, similar to what is observed with EPX. It is unknown if horseradish peroxidase also increases expression of the other cytokines that are increased in by EPX, or if LPA is also required for horseradish peroxidase to increase TSLP expression. However, this may indicate that the mechanism for peroxidases to increase cytokine expression in keratinocytes is not limited to eosinophil peroxidase alone. This may have implications on regulation of cytokine expression under settings of inflammation that do not involve eosinophils. Myeloperoxidase is expressed by other inflammatory cells, including neutrophils and macrophages, and may also increase expression of these cytokines when active.

Although LPA has previously been shown to increase TSLP expression in airway epithelial cells[308], this is the first evidence that LPA increases TSLP expression in keratinocytes. Whether LPA levels are increased in the skin of patients with atopic dermatitis is not known, however it is known that the level of autotaxin, an enzyme that plays a role in LPA production, is elevated in the serum of patients with atopic dermatitis[313]. The level of autotaxin also correlates with disease severity in these patients[313]. This finding linking LPA and TSLP in keratinocytes has two interesting implications. Along with TSLP, LPA has also been shown to directly activate sensory nerves and stimulate itch[147]. This hints at a redundancy in pathways that can trigger itch in caused by EPX. This finding also provides more evidence that LPA is linked to atopic dermatitis and opens potential new targets for therapy.

Work from our group has found that eosinophils are an important mediator of itch in the chronic contact dermatitis model[275], and unpublished work from our group (James Lee, unpublished communication) has identified EPX as a critical mediator of itch in this model as well. Based on the observation that both TSLP and LPA can activate sensory nerves to cause itch[110,147], we propose that this is potentially the pathway by which eosinophils cause itch in this model. Determining whether these pathways are involved in itch in atopic dermatitis requires more study, though there is some evidence that this may be the case. TSLP levels are elevated in the skin in patients with atopic dermatitis. Staining for EPX in the skin of patients with atopic dermatitis also reveals extensive release of EPX from eosinophils[164]. The link between EPX activity and TSLP in

these experiments suggests that eosinophils increase TSLP expression in the skin in patients with atopic dermatitis, potentially by stimulating production of LPA. This pathway may also be critical for chronic itch in patients with atopic dermatitis.

IL-1 is a well characterized mediator of inflammation, though what role it may have in atopic dermatitis is still being identified. IL-1 levels are known to be elevated in the plasma of patients with atopic dermatitis[82]. Here we show that IL-1 is critical for EPX to increase gene expression of several proinflammatory cytokines (CSF2, CSF3, TNF and IL-1α) in keratinocytes. All of these cytokines have been found to be elevated in the plasma of patients with atopic dermatitis[82]. Each cytokine has known influences on inflammation that may be important as a whole in atopic dermatitis. GM-CSF is a growth factor for inflammatory cells, including eosinophils[183]. GM-CSF also has important roles in priming and activating eosinophils[314]. TNF has many effects on eosinohphils. TNF was recently shown by our group to stimulate eosinophilopoiesis after exposure to ozone[315]. TNF also increases expression of eotaxin in epithelial cells, a protein that is elevated in the skin in patients with atopic dermatitis and is a potent chemokine for eosinophils[225]. When given as an intradermal injection TNF also will also cause eosinophil recruitment to skin by increasing expression of adhesion molecules on endothelial cells[316]. IL-1 also directly stimulates expression of adhesion molecules on endothelial cells that eosinophils use to migrate into tissue[317]. Together these cytokines may have a potent effect on inflammation in atopic dermatitis, an effect that could be regulated by IL-1.

IL-1 signaling can be mediated by IL-1α and IL-1β. Determining whether IL-1α or IL-1β mediated the increase in cytokine expression caused by EPX was not directly tested here, though there is some evidence that IL-1α is the more likely candidate. IL-1α is constitutively expressed in keratinocytes, where it is stored and released during insults[318]. In these experiments, IL-1β gene expression does not change after keratinocytes are treated with EPX and is not detectable at baseline before the cells are treated. Given these observations it is most likely that IL-1α is responsible for the increase in cytokine expression.

While we propose that LPA and TSLP have important effects on itch, it is impossible to ignore the potential roles TSLP may also have on inflammation. TSLP has been shown to stimulate eosinophil degranulation in vitro [106], to have a role in increasing eosinophilopoiesis [109], and to increases eosinophil survival [108]. Taking TSLP together with the other proinflammatory cytokines increased by EPX, we hypothesize that EPX activity initiates a feedforward loop that recruits and activates more eosinophils, leading to chronic inflammation.

The mechanism by which EPX stimulates production of LPA and IL-1 has not been determined. However since EPX peroxidase activity is required to increase cytokine expression in keratinocytes, it is likely that EPX requires peroxidase activity to increase production of LPA and IL-1 as well. EPX uses hydrogen peroxide to oxidize halides to produce hypohalous acid[216], which is a potent oxidizing agent that is toxic to both parasites and human cells[195,305,306]. The toxicity of EPX and its products are generally thought to be the main

mechanism by which EPX influences other cell types. The findings in these experiments suggest that EPX can activate signaling pathways without triggering cell death, as these treatments did not have any effect on cell viability. While the products of EPX are certainly toxic at high concentrations, at low concentrations these products may be signaling molecules that stimulate LPA and IL-1 production in keratinocytes.

We propose that EPX stimulates two distinct pathways in the skin, one through LPA the other through IL-1 to mediate itch and inflammation. In the first pathway, LPA and TSLP both act on sensory nerves to drive itch in the skin, providing a link between EPX to itch. At the same time, EPX increases IL-1 activity, which then increases the expression of other cytokines that increase and propagate eosinophilic inflammation. These findings highlighting potential targets for therapy in patients with atopic dermatitis.

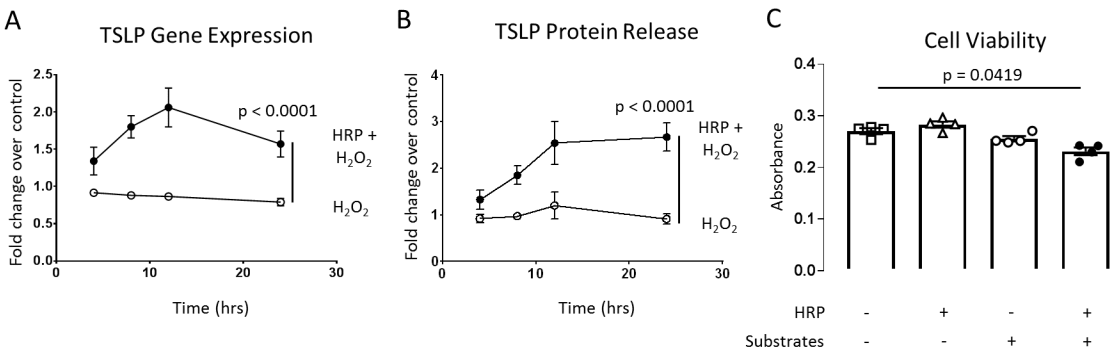

Figure 5-1 Horseradish peroxidase increases TSLP expression in keratinocytes.

Mouse keratinocytes were isolated from mouse tail skin, dispersed into single cells, plated and grown to confluency. Keratinocytes were cultured in hydrocortisone free media for 24 hours, and then treated with horseradish peroxidase (HRP, 1 uM) , and/or H_2O_2 (100 uM) for 4, 8, 12, and 24 hours. A) TSLP gene expression was measured from keratinocytes using real-time RT PCR. B) TSLP protein release into the media was measured in cell culture media using an ELISA. C) Keratinocyte viability was measured at 12 hours using a MTT assay.

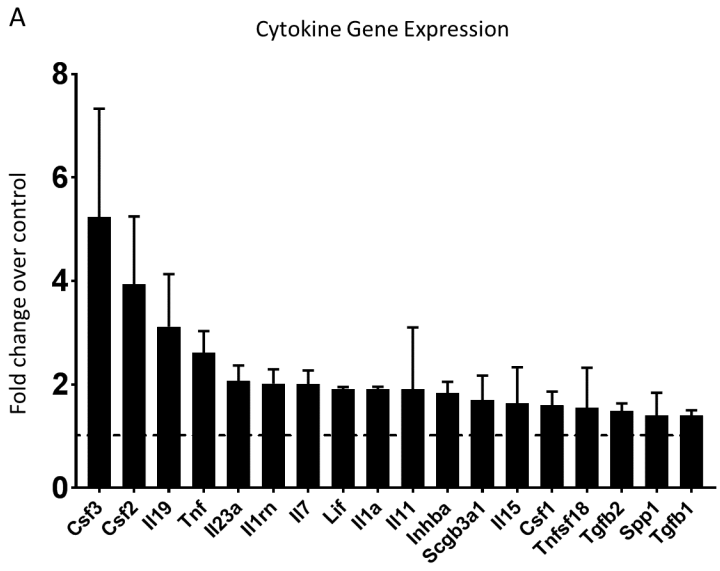

Figure 5-2 Eosinophil peroxidase increases cytokine expression in keratinocytes.

Keratinocytes were grown in cell culture as described above. Keratinocytes were treated for 6 hours with EPX plus its substrates, RNA was isolated, and gene expression was measured using the common cytokine RT2 profiler array from Qiagen.

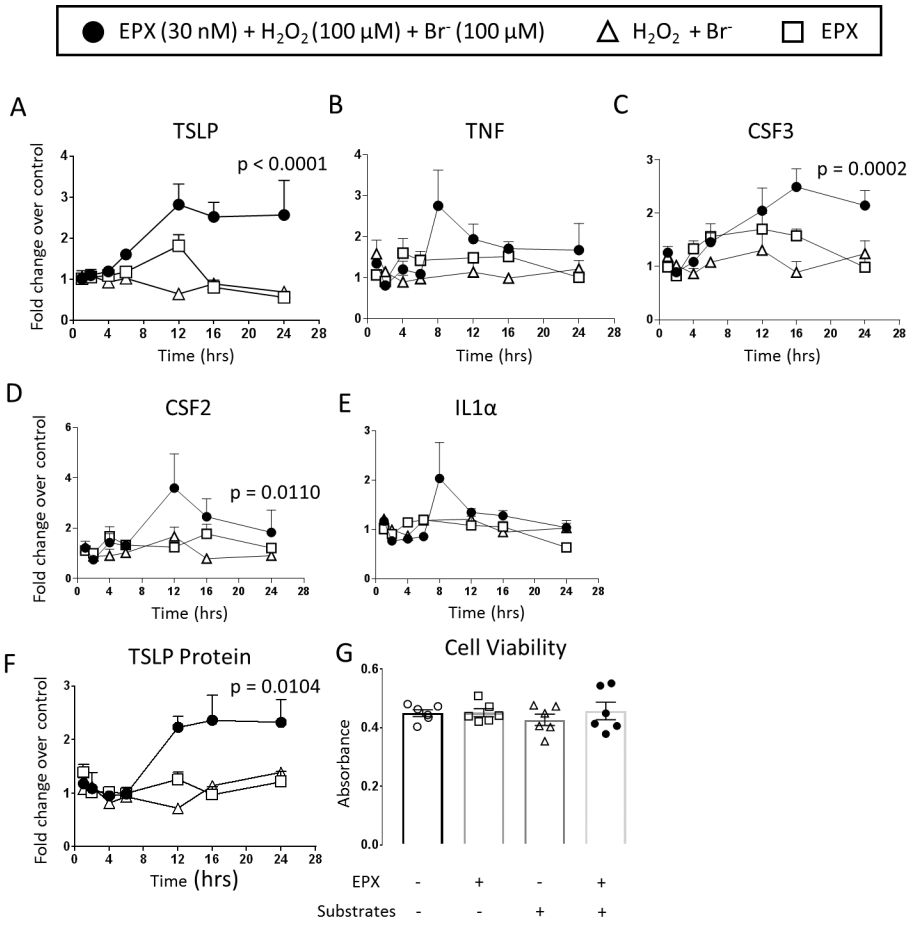

Figure 5-3 Eosinophil peroxidase increases expression of TSLP, CSF3, and CSF2, and trends towards increasing TNF and IL1α.

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Figure 5-3 Eosinophil peroxidase increases expression of TSLP, CSF3, and CSF2, and trends towards increasing TNF and IL1α.

Mouse keratinocytes were grown in cell culture as previously described. Keratinocytes were then treated with eosinophil peroxidase (EPX, 30 nM), Br- (100 μM) and H_2O_2 (100 μM) for 1, 2, 4, 6, 8, 12, 16, and 24 hours. Cytokine gene expression was then measured in keratinocytes using real time RT-PCR, and TSLP levels were measured in cell culture media by ELISA. A) TSLP gene expression. B) TNF gene expression. C) CSF3 gene expression. D) CSF2 gene expression. E) IL1α gene expression. F) TSLP protein in cell culture media. G) Keratinocyte viability was measured at 8 hours using a MTT assay.

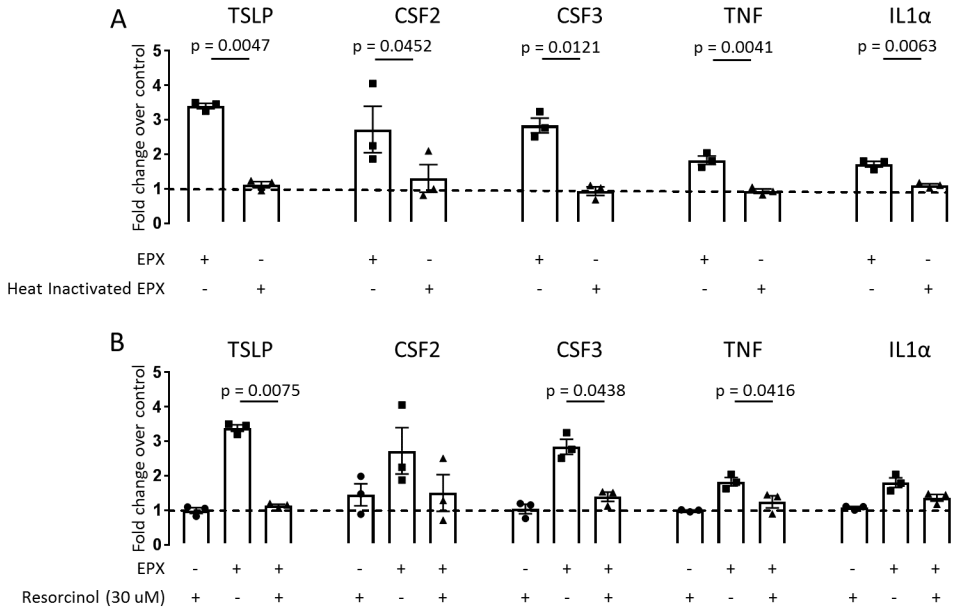

Figure 5-4 Eosinophil peroxidase requires peroxidase activity to increase cytokine expression in keratinocytes.

Mouse keratinocytes were grown in cell culture as previously described. A) Keratinocytes were treated with EPX or heat inactivated EPX with substrates Br- and H_2O_2 for 8 hours. EPX was heat inactivated by heating to 80^{o}C for 10 minutes, which abolished its peroxidase activity as determined by a peroxidase activity assay. B) Keratinocytes were treated with EPX plus substrates in the presence or absence of resorcinol (30μM) for 8 hours. Cytokine gene expression was measured by real time RT-PCR.

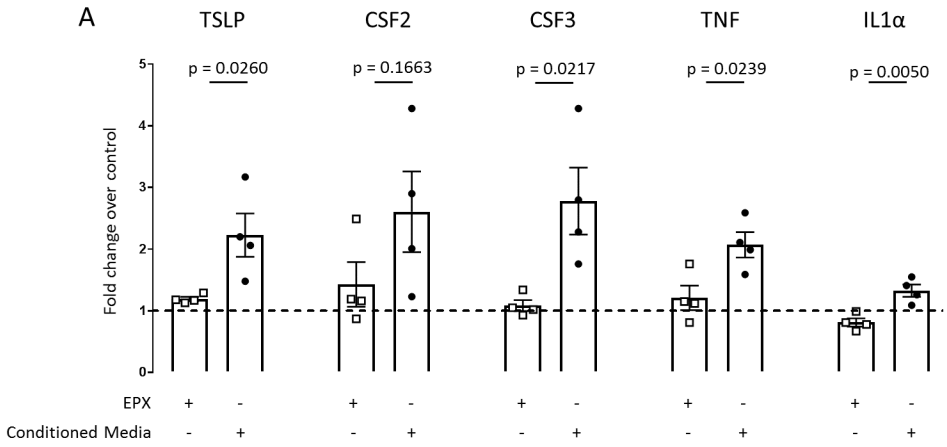

Figure 5-5 Eosinophil peroxidase increases cytokine expression through soluble factors.

Mouse keratinocytes were grown in cell culture as previously described. Conditioned media was harvested from keratinocytes treated with EPX plus substrates for 8 hours. Conditioned media was centrifuged at 300 x g for 10 minutes to remove cells and large debris. Naïve keratinocytes cultures were then treated with conditioned media or EPX plus its substrates. After 4 hours TSLP, CSF2, CSF3, TNF and IL1α gene expression was measured by real time RT-PCR.

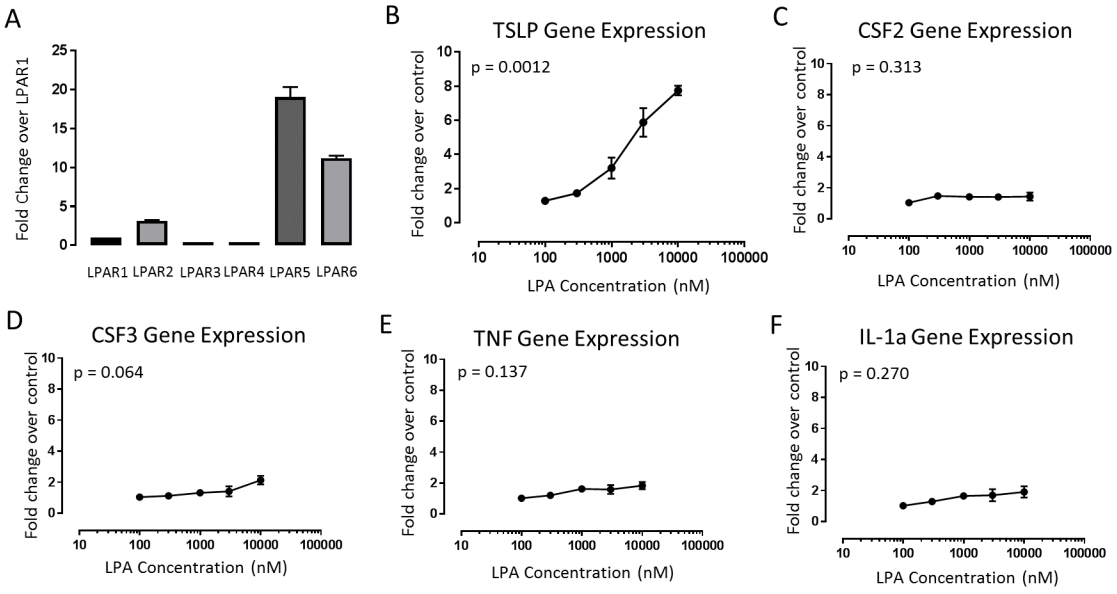

Figure 5-6 LPA increases TSLP expression in keratinocytes, but not CSF2, CSF3, TNF or IL1α.

A) Gene expression for the LPA receptors were measured using real time RT-PCR in primary culture keratinocytes. B-F) Mouse keratinocytes were grown in cell culture as described previously. Keratinocytes were treated with 0.1 – 10 μM LPA for 4 hours, and cytokine gene expression was measured using real time RT-PCR.

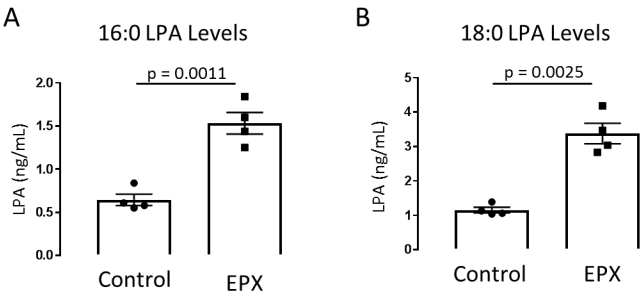

Figure 5-7 Eosinophil peroxidase increases LPA levels in cell culture media

Primary mouse keratinocyte cell cultures were grown as described above. Keratinocytes were treated with EPX plus its substrates for 8 hours, and LPA concentration was measured in cell culture media using LC/MS.

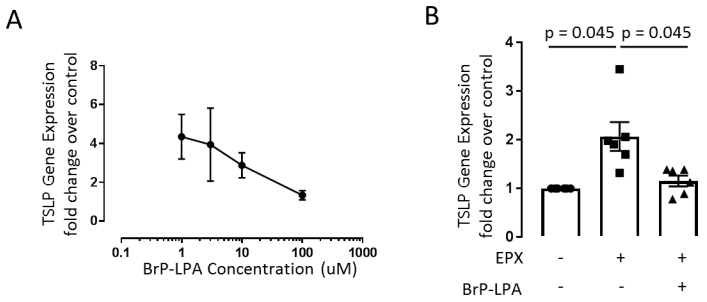

Figure 5-8 The LPA receptor antagonist BrP-LPA inhibits the increase in TSLP caused by EPX.

Mouse primary keratinocyte cultures were established as previously described. A) Keratinocytes were treated with 3 μM LPA and 1 – 100 μM BrP-LPA for 4 hours, then TSLP gene expression was measured using real time RT-PCR. B) Keratinocytes were treated with EPX plus its substrates, with and without BrP-LPA for 8 hours, then TSLP gene expression was measured using real time RT-PCR.

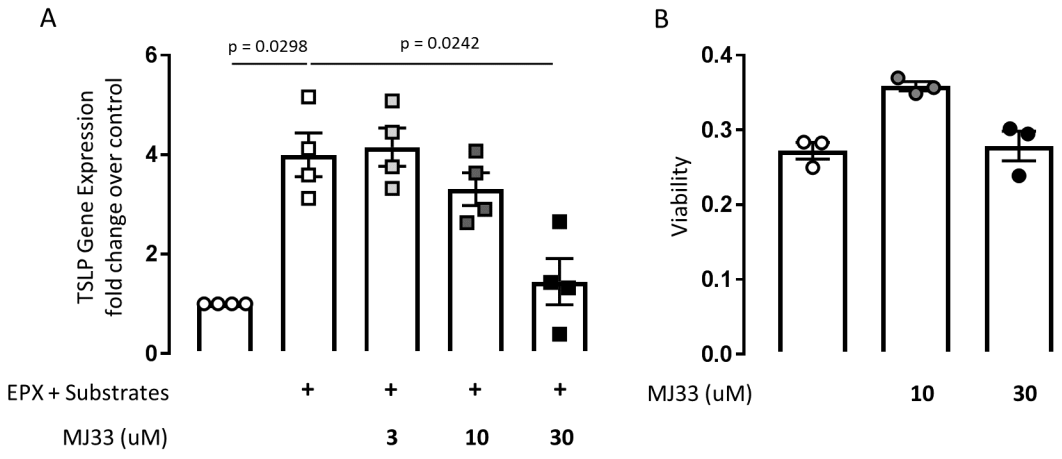

Figure 5-9 The sPLA2 inhibitor MJ33 inhibits the increase in TSLP gene expression caused by EPX in keratinocytes.

Mouse primary keratinocytes were grown in cell culture as described previously. A) Keratinocytes were treated with EPX plus substrates with or without 3-30 μM MJ33 for 8 hours, then TSLP gene expression was measured using real time RT-PCR. B) Keratinocyte were treated with MJ33 at 10 or 30 μM for 8 hours, and keratinocyte viability was measured using a MTT assay.

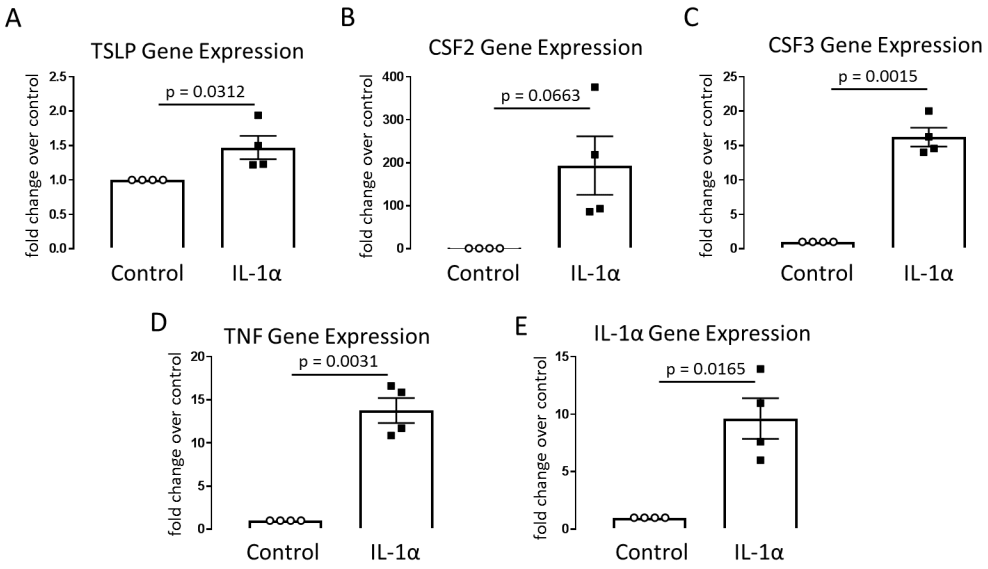

Figure 5-10 IL-1α increases cytokine gene expression in keratinocytes.

Primary mouse keratinocytes were grown in cell culture as described above. Keratinocytes were treated with 3 ng/mL Il-1α for 4 hours, and cytokine gene expression was measured using real time RT-PCR.

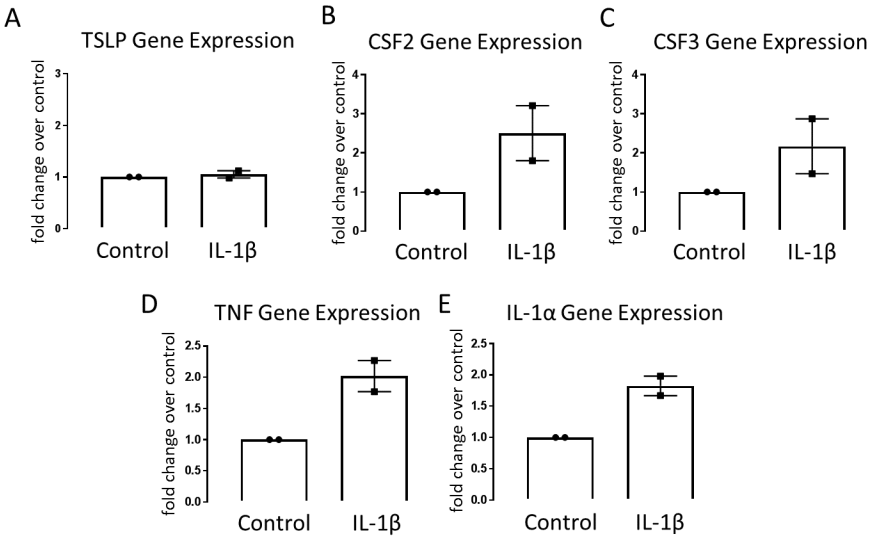

Figure 5-11 IL-1β increases cytokine gene expression in keratinocytes, but not TSLP expression.

Mouse primary keratinocyte cultures were established as previously described. Keratinocytes were treated with 10 ng/mL IL-1β for 4 hours, and cytokine gene expression was measured using real time RT-PCR.

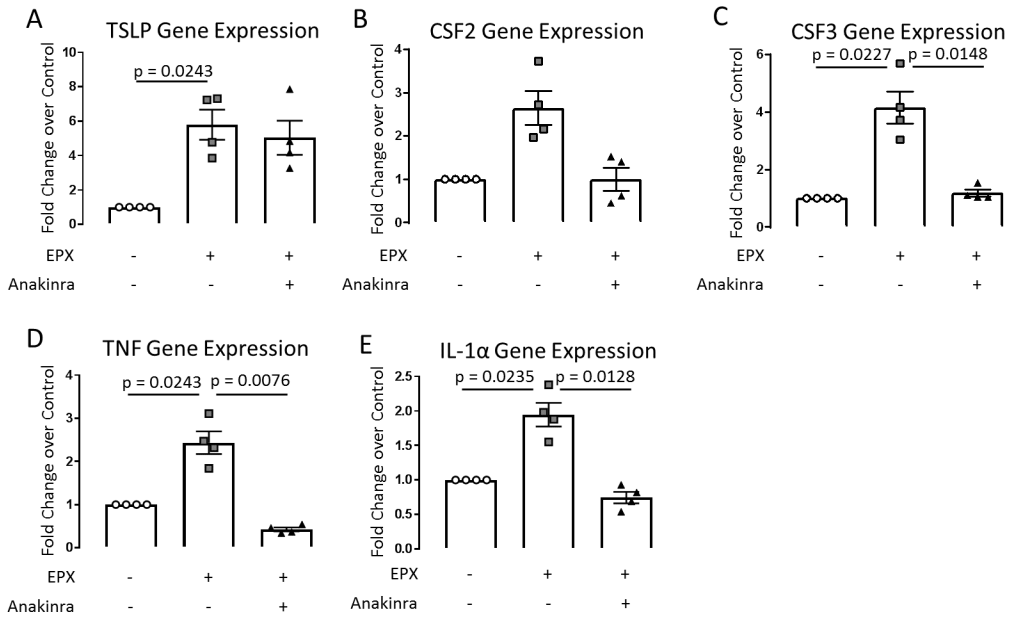

Figure 5-12 The IL-1 receptor antagonist anakinra blocks the increase in cytokine expression in keratinocytes caused by EPX, but does not affect TSLP expression.

Mouse primary keratinocyte cultures were established as described previously. Keratinocytes were treated with EPX plus its substrates, with or without anakinra (30 ug/mL) for 8 hours, then cytokine gene expression was measured using real time RT-PCR.

Chapter 6: Inhibition of IL-1 reduces inflammation in an animal model of chronic contact dermatitis

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6.1 Introduction

EPX recruits eosinophils, increases TSLP levels, and is an important mediator of itch in an animal model of chronic contact dermatitis. I have also shown that EPX activity increases TSLP, CSF2, CSF3, TNF and IL-1α gene expression in keratinocytes. To achieve this, EPX requires signaling of LPA to increase TSLP expression, and it requires IL-1 signaling to increase expression of CSF2, CSF3, TNF and IL1α. Here I tested whether IL-1 or LPA are critical for eosinophil recruitment and inflammation, and the increase in TSLP in the mouse model of chronic contact dermatitis.

IL-1α is elevated in serum in children in atopic dermatitis[319]. IL-1 is one of the early cytokines to increase in skin after barrier damage[78,113,114]. While the known roles of IL-1 on inflammation place it in a position to play a key role in atopic dermatitis, it still remains to be tested if it is critical for the development of inflammation. Given the known proinflammatory role of IL-1 in other settings, I hypothesized that IL-1 is a critical mediator of inflammation in the skin in the mouse model of chronic contact dermatitis, and that blocking IL-1 would reduce this inflammation.

LPA has only loosely been associated with atopic dermatitis. Levels of an enzyme that produces LPA, autotaxin, is elevated in serum of patients in atopic dermatitis, and this correlates with the severity of itch in the disease[313]. LPA levels are elevated in the lungs in patients with asthma[320,321], another disease with active eosinophils. However, what role LPA may have in increasing TSLP or inflammation in atopic dermatitis is unknown. Given the connection of LPA to TSLP and that both are capable of activating sensory nerves to drive itch[110,147], I hypothesize that blocking LPA may not have a dramatic effect on inflammation, but would reduce TSLP levels (and thus potentially reducing itch) in the skin in the chronic contact dermatitis model.

6.2 Results

Anakinra significantly reduces inflammation after chronic exposure to TMA.

The role of IL-1 on mediating inflammation in the model of chronic contact dermatitis was tested using anakinra to block IL-1 (Figure 6-1 A). Anakinra significantly reduced the increase in ear thickness caused by chronic treatment with TMA (Figure 6-1 B). Anakinra did not have an effect on the increase in lymph node weight after exposure to TMA (Figure 6-1 C). These data show that IL-1 has an important role in increasing inflammation in the ear after exposure to TMA.

Ears treated with vehicle or TMA were paraffin sectioned, H&E stained, and examined for changes in eosinophil numbers in the skin. The H&E stained

representative images suggest that anakinra reduces the increase in epidermal thickness caused by chronic treatment with TMA (Figure 6-2). Anakinra significantly reduced the number of eosinophils in the skin after chronic treatment with TMA (Figure 6-3).

To ensure that daily treatment with anakinra was well tolerated by the mice, body weight was measured daily. Anakinra did not have any effect on mouse body weight (Figure 6-4)

BrP-LPA significantly increases inflammation, but does not reduce TSLP levels in ears after chronic TMA treatment.

To test the role of LPA on the increase of TSLP, and its role in inflammation, mice were treated daily with the pan LPA receptor inhibitor BrP-LPA during challenge (Figure 6-5 A). Daily treatment with BrP-LPA significantly increased ear thickness in ears treated chronically with TMA (Figure 6-5 B) compared to TMA treatment alone. BrP-LPA also caused a significant increase in lymph node weight after chronic exposure to TMA (Figure 6-5 C). BrP-LPA had no effect on ear thickness (Figure 6-5 A) and lymph node weight (Figure 6-5 B) in vehicle treated ears.

To test if BrP-LPA reduced levels of TSLP after exposure to chronic TMA, TSLP protein levels were measured in extracted protein from mouse ears. TSLP levels were normalized in three different ways: total pg TSLP per ear (pg TSLP/Ear), pg TSLP per mg extracted protein (pg TSLP/mg Protein), and pg TSLP per mg tissue weight (pg TSLP/mg Tissue). TSLP levels were also

compared as fold increase in treated ear over contralateral control ear. Regardless of the method used to normalize the data, BrP-LPA did not reduce TSLP levels in ears after chronic TMA treatment compared to control TMA treated ears.

To ensure that these methods of normalization were not influenced by an effect of BrP-LPA effects on ears, ear weight and the amount of protein extracted were compared. Ear weight was significantly increased by chronic TMA treatment, and BrP-LPA did not significantly reduce ear weight (Figure 6-7). Protein extraction concentrations were significantly increased in TMA treated ears over control, but BrP-LPA does not have any effect on these levels (Figure 6-8). Finally, BrP-LPA did not influence mouse body weight and was well tolerated by the mice (Figure 6-9).

6.3 Discussion

These data test the role of the LPA and IL-1 signaling pathways that were identified using the keratinocyte cell culture experiments in an animal model of chronic contact dermatitis. Here, I found that anakinra caused a significant decrease in inflammation in the ear and reduction in eosinophil numbers in the skin. Conversely, I found that the LPA receptor antagonist BrP-LPA caused a modest but significant increase in inflammation in ears chronically treated with TMA, but did not reduce TSLP levels.

In this animal model of chronic contact dermatitis, blocking IL-1 signaling with anakinra showed that IL-1 plays an important role in mediating inflammation.

IL-1 is a well characterized mediator of inflammation, though what role it may have in atopic dermatitis is still unknown. IL-1 levels are known to be elevated in the plasma of patients with atopic dermatitis[82,319]. IL-1 is known to drive expression of factors that aid in eosinophil recruitment, including eotaxin in epithelial cells[225]. IL-1 can also increase expression of adhesion molecules on endothelial cells that allow recruitment of eosinophils into tissue[266,268]. IL-1 will also drive expression of proinflammatory cytokines as shown in chapter 5. These cytokines may also be important for IL-1 to drive inflammation and recruitment of eosinophils into the skin in this model of chronic contact dermatitis.

Like what was observed with resorcinol (EPX activity inhibitor, chapter 4), blocking IL-1 reduced local inflammation in the skin, but did not significantly reduce lymph node weight. Blocking IL-1 also caused a significant reduction in eosinophil numbers in skin chronically treated with TMA compared to TMA alone, like what was observed with resorcinol treatment. This supports the hypothesis that IL-1 is critical for a feedforward loop triggered by eosinophils to increase eosinophils numbers in skin.

It was surprising that BrP-LPA did not reduce TSLP levels in the skin and also increased general markers of inflammation, however, there are concerns about the efficacy of this drug in vivo. The half-life of BrP-LPA is very short, roughly 50 minutes[322], and thus it is possible that this drug is not effective at blocking LPA under conditions of chronic treatment that are present in this model. The modest increase in inflammation that occur after treatment with BrP-LPA may be due to chance, especially given the small sample size. Generating

controls to ensure that BrP-LPA is effectively blocking LPA signaling in the skin is difficult. Showing that BrP-LPA can block the effects of direct injection of LPA on TSLP would show that under acute situations BrP-LPA is effective. Due to the chronic inflammation generated in this model this control would not address the question of whether BrP-LPA can effectively block LPA over the long term.

Despite the concerns over the effectiveness of BrP-LPA to block LPA in vivo, the findings from these experiments may have some interesting implications. The role of LPA as an anti-inflammatory factor agrees with other findings in the literature. LPA reduces TNF expression in macrophages treated with endotoxin[323], and reduces free myeloperoxidase in lung after treatment with endotoxin[324]. Whether LPA reduces cytokine expression in the skin is unknown, though this may explain the increase in general markers of inflammation in the animals treated with BrP-LPA compared to controls.

LPA has also been found to increase barrier function of pulmonary epithelial cells in cell culture and to block the barrier disruption caused by endotoxin[325]. While the chronic contact dermatitis model used in this dissertation does not have underlying barrier dysfunction, chronic scratching does cause damage and barrier disruption in the skin. If LPA is critical for the recovery of barrier in the skin, blocking LPA would likely cause an increase in inflammation due to the inability of the skin to reform an effective barrier.

Since BrP-LPA did not block the increase in TSLP, this demonstrates that there are redundant pathways (not requireing LPA) to increase TSLP protein in

the skin. These pathways still require EPX activity, as blocking EPX activity does block the increase in TSLP expression in the skin. One potential factor that may be responsible for this is IL-13. IL-13 increases TSLP expression in epithelial cells[326]. Our collaborator has also found, in unpublished data, that blocking EPX reduces IL-13 levels in the mouse model of chronic contact dermatitis. These data possibly identify IL-13 as a redundant pathway responsible for increasing TSLP expression in this model.

Again, given the serious concerns about the efficacy of BrP-LPA in the in vivo model, these findings may not effectively test the role of LPA on mediating TSLP expression or inflammation. Identifying more specific pathways responsible for this effect in vitro, such as what specific LPA receptors or which specific sPLA2 enzymes, would aid in determining if this pathway for the increase in TSLP and itch in the animal model of chronic contact dermatitis. Using this knowledge to identify more selective inhibitors with better pharmacokinetic properties may provide a better method to determine the role of LPA on inflammation and TSLP levels in this model.

These findings highlight two distinct pathways active in the skin in this model, one pathway is proinflammatory and is mediated by IL-1, and the other pathway that aids in reducing inflammation and is mediated by LPA. If both of these pathways are regulated by EPX in the skin, it would imply that EPX both increases inflammation in the skin, as well as activating anti-inflammatory pathways. With this, it may be possible to block specifically the inflammatory

pathways in the skin in patients with atopic dermatitis, while sparing the pathways that are anti-inflammatory and may aid in skin barrier recovery.

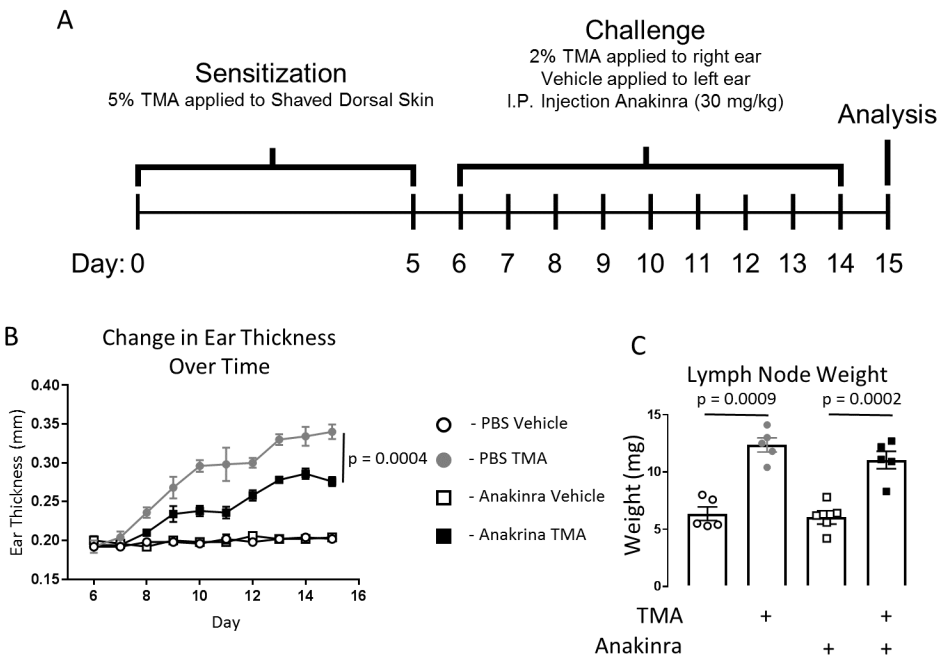

Figure 6-1. Anakinra reduces inflammation in ears treated chronically with TMA.

A) Mice were sensitized by application of a 5% TMA solution (4:1 acetone:olive oil) on a shaved portion of the back. Mice were then challenged on the right ear with a 2% TMA solution (4:1 acetone:olive oil). Contralateral ears were treated with vehicle only and used for controls. During the TMA challenge the mice were treated with daily injections of Anakinra (10 mg/kg i.p.), or PBS as control. B) Ear thickness was measured daily using a Mitutoyo Digital Caliper. Ear thickness was measured just prior to treating the ears with TMA or vehicle. C) Draining lymph nodes were weighed on day 15 after harvest.

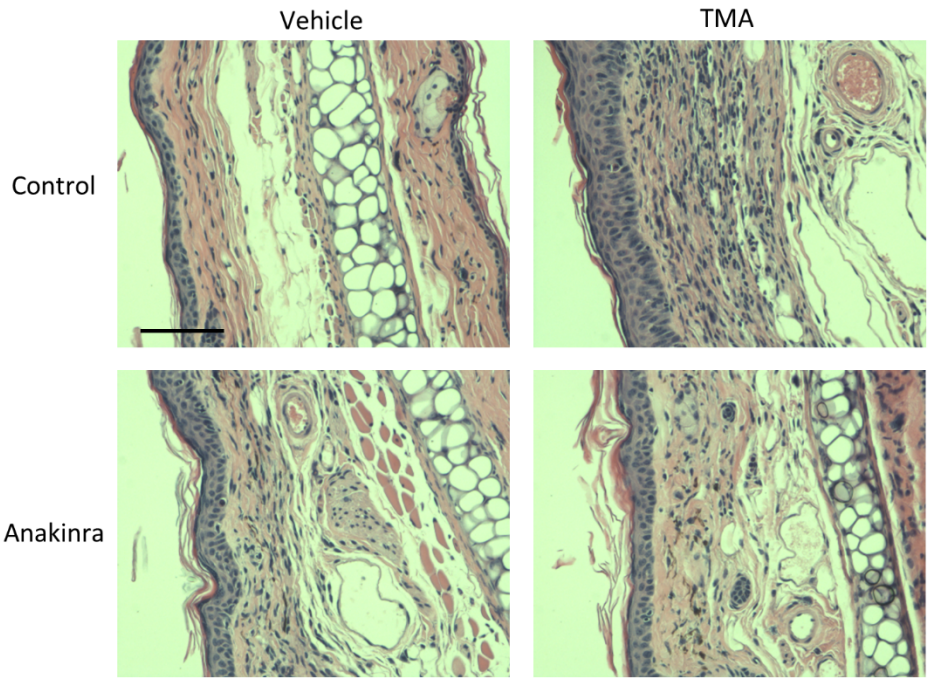

Figure 6-2. Representative images of H&E stained ear sections.

Ear sections from mice were harvested and fixed in Zambonis fixative. Samples were then paraffin embedded, sectioned, and stained with H&E.

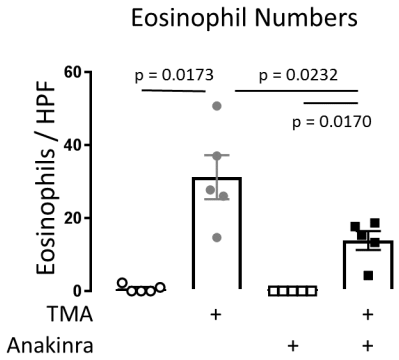

Figure 6-3 Anakinra significantly reduced the number of eosinophils in ears after chronic TMA exposure.

Eosinophils were counted in 3 randomly selected high powered fields in each ear and then averaged.

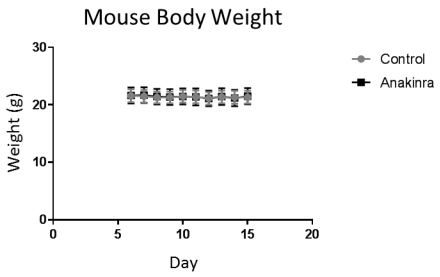

Figure 6-4 Anakinra does not reduce body weight of mice.

Body weight of mice was measured before every challenge.

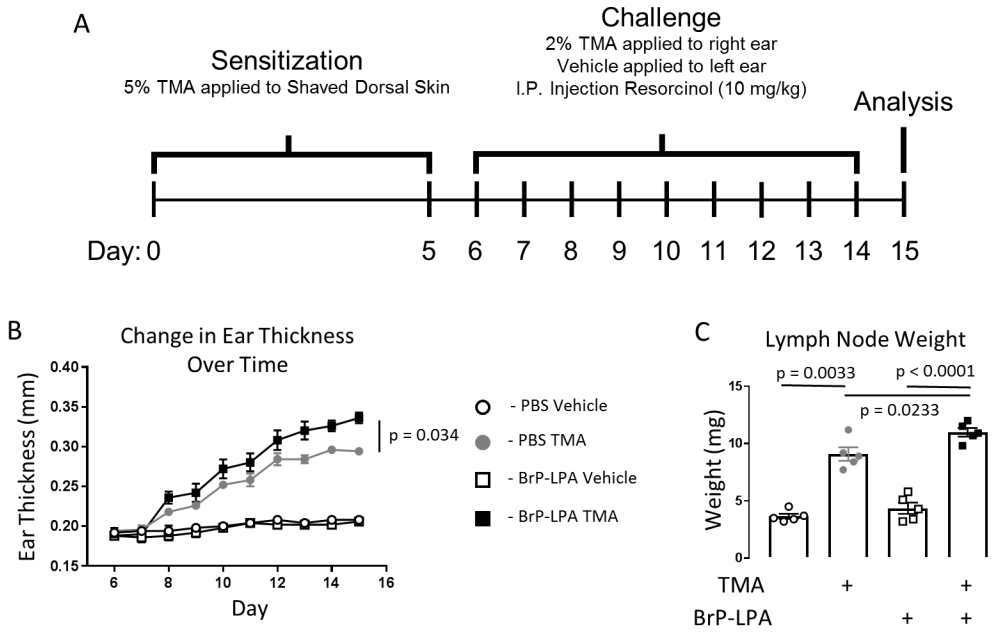

Figure 6-5 BrP-LPA slightly increases inflammation after chronic TMA exposure.

A) Mice were sensitized by application of a 5% TMA solution (4:1 acetone:olive oil) on a shaved portion of the back. Mice were then challenged on the right ear with a 2% TMA solution (4:1 acetone:olive oil). Contralateral ears were treated with vehicle for controls. Mice were treated with daily injections of BrP-LPA (30 mg/kg), or PBS as control. B) Ear thickness was measured daily using a Mitutoyo Digital Caliper. Ear thickness was measured just prior to treating the ears with TMA or vehicle. C) Draining lymph nodes were weighted on day 15 after harvest.

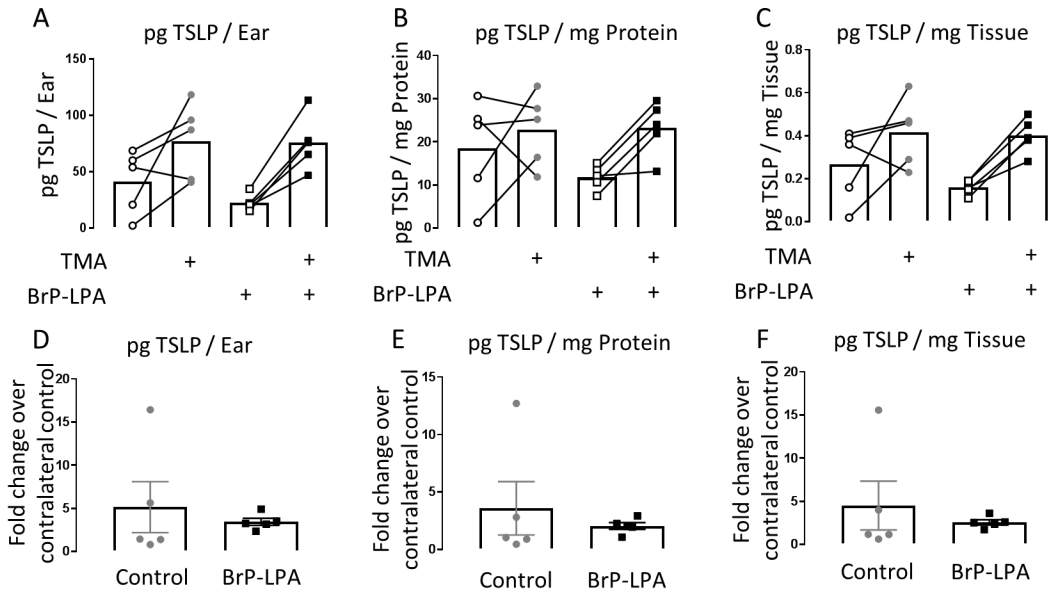

Figure 6-6 BrP-LPA does not reduce TSLP levels caused by chronic TMA exposure.

TSLP was measured from total protein extracted from mouse ears using an ELISA. A) Total TSLP extracted from mouse ear. Connected lines indicate paired samples from one mouse. B) TSLP levels normalized to as pg TSLP per mg protein. Connected lines indicate paired samples from one mouse. C) TSLP levels normalized to weight of tissue protein was extracted from. Connected lines indicate paired samples from one mouse. D-F) TSLP levels were graphed as fold increase over contralateral control ear from the same mouse.

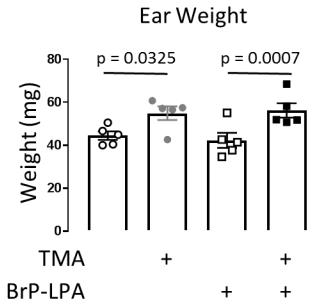

Figure 6-7 BrP-LPA does not reduce ear weight after chronic TMA exposure.

Ear weight was measured after harvest on day 15.

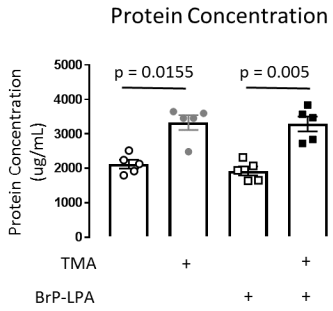

Figure 6-8 BrP-LPA does not reduce protein extraction from mouse ears.

Total protein was extracted from mouse ears by homogenizing in protein extraction buffer and measured using a BCA assay.

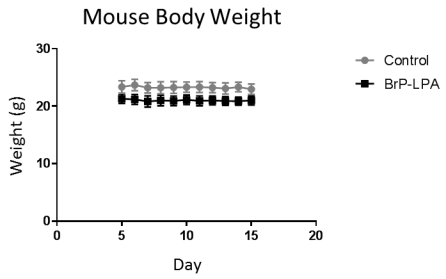

Figure 6-9 BrP-LPA does not reduce mouse body weight.

Mice were weighed before every challenge.

General discussion

Here, I provide novel findings showing potential mechanisms by which eosinophils mediate itch and inflammation in the skin. I found that eosinophils increase gene expression for the TSLPR in sensory nerves in cell culture, without affecting gene expression for other receptors like TRPV1 or gene expression for the neuropeptide SP. I further found that this mechanism requires cell-cell contact between the sensory nerves and eosinophils for this effect to occur (chapter 3). I also found that inhibiting the peroxidase activity of EPX caused a significant reduction in the increase in TSLP, ear thickness, and eosinophil numbers in skin caused by chronic exposure to TMA in a mouse model of chronic contact dermatitis (chapter 4).

Previous work from our group found that EPX is required for itch in the chronic contact dermatitis model. Together, these findings identify a potential mechanism by which eosinophils cause itch in the skin. I hypothesized that eosinophils mediate chronic itch by stimulating the TSLP/TSLPR pathway. Eosinophils can influence this pathway at multiple points. Eosinophils increase TSLP expression in the skin through the activity of EPX, and eosinophils increase TSLPR expression on sensory nerves to increase their response to TSLP and increase itch. This hypothesis is supported by the finding that EPX is required for the increase in TSLP and itch in the animal model of chronic contact dermatitis and that eosinophil increase expression of TSLPR in sensory nerves in

cell culture. However, these experiments have not proven that this pathway is critical for itch in the chronic contact dermatitis model. Future experiments will need to be performed to determine if TSLP is required for itch in the model, and if there are changes in itch responses to TSLP and an increase in TSLPR expression in the skin.

Evidence of eosinophil-nerve interactions in skin and changes in response to stimuli that activate sensory nerves to cause itch in patients with atopic dermatitis support this hypothesis. Eosinophils increase growth of sensory nerves in the skin and eosinophils localize around nerves in the skin in patients with atopic dermatitis[164]. Patients with atopic dermatitis also respond more strongly to agonists that induce itch, such as PAR-2/MRGPRC11 receptor agonists[162,327]. This would also not be the first evidence of increased receptor expression in sensory nerves in atopic dermatitis. Expression of receptors such as TRPA1 are also increased in sensory nerves in the skin in patients with atopic dermatitis[328]. While we have not yet tested if TSLPR is increased in skin in patients with atopic dermatitis, it is known that other receptors are, and that eosinophils mediate changes in sensory nerves, supporting this hypothesis.

Other findings from my research points towards potential mechanisms by which eosinophils increase inflammation in the skin. We had previously found that lack of eosinophils led to a reduction in general measures of inflammation in an animal model of chronic contact dermatitis[275]. We next found that the activity of EPX was important for itch in this model. Based on these observations I

tested the role of EPX in mediating inflammation in the skin in the chronic contact dermatitis model.

I found that EPX peroxidase activity was important for increasing inflammation, eosinophils recruitment, and TSLP in the animal model of chronic contact dermatitis (chapter 4). In vitro, I found that EPX increases expression of TSLP, CSF2, CSF3, TNF and IL1α in keratinocytes. I also found that EPX requires its peroxidase activity to increase expression of these cytokines in cultured keratinocytes in cell culture, matching what we found in vivo where the peroxidase activity was required for itch and inflammation. I next found that for EPX to increase expression of TSLP in keratinocytes in cell culture, LPA signaling was required, however LPA did not increase gene expression for CSF2, CSF3, TNF or IL1α. Instead, I found EPX requires IL-1 signaling to increase expression of CSF2, CSF3, TNF and IL1α in keratinocytes (chapter 5), but IL-1 signaling was not required for the increase in TSLP. In the chronic contact dermatitis model, I found that blocking IL-1 significantly reduced inflammation and recruitment of eosinophils into the skin after chronic treatment with TMA. Blocking LPA with the pan LPA receptor antagonist BrP-LPA in the animal model of chronic contact dermatitis did not reduce TSLP levels and, surprisingly, significantly increased ear thickness (chapter 6).

TSLP levels are elevated in the skin in patients with atopic dermatitis[96,97], though the mechanism leading to this is unknown in patients. Data in this thesis provides some evidence that increases in TSLP in the skin of patients with atopic dermatitis is mediated through eosinophils. Here I show that EPX activity in the

skin is important for the increase in TSLP in an animal model of chronic contact dermatitis, and I found that in vitro EPX can directly increase TSLP expression in keratinocytes. EPX required LPA signaling to increase TSLP in vitro, however, blocking LPA using the pan LPA receptor antagonist BrP-LPA did not reduce TSLP levels in the skin in the chronic contact dermatitis model. This likely implies that there are multiple mechanisms that are responsible for the increase in TSLP caused by EPX in the skin. As mentioned in chapter 6, IL-13 may play a role in increasing TSLP expression in the skin.

The identification of LPA in the cell culture experiments identified a potentially novel signaling pathway that is active in atopic dermatitis. This is the first evidence that LPA is an important factor in atopic dermatitis. Interestingly, blocking LPA in the animal model of chronic contact dermatitis did not reduce inflammation, but instead increased general measures of inflammation. This finding was surprising, since I had hypothesized that LPA was critical for mediating itch, through increasing TSLP, but would not be a critical factor in inflammation. LPA has been linked to some pro-inflammatory activities, including eosinophil recruitment[321,329]. Also, in the skin loss of a sPLA2G2F, an enzyme which produces LPA, protects from inflammation in a contact dermatitis model[330,331]. These findings seem at odds with the observation that blocking LPA increased inflammation in the animal model of chronic contact dermatitis. However, LPA has also been shown to have some protective roles as well, including enhancing barrier function of airway epithelial cells and playing an important role in wound healing[325,332]. These roles may explain why blocking

LPA increases inflammation, if barrier function in the skin is reduced when LPA signaling is blocked. Given concerns about the pharmacokinetics of BrP-LPA, such as its extremely short half-life, it may not be an effective drug for blocking LPA, and future experiments would need to be done to validate the findings of an anti-inflammatory role of LPA in this model.

Another implication of these findings is that eosinophils act to increase inflammation and to increase eosinophil recruitment in the skin through activity of EPX. Blocking EPX activity or IL-1 caused a similar significant reduction in TMA-induced increase in ear thickness, and eosinophil numbers in the skin. The finding that EPX requires IL-1 to increase expression of cytokines in keratinocytes would support the hypothesis that these pathways are linked in the animal model of chronic contact dermatitis.

Based upon this data I hypothesize that eosinophils mediate itch and inflammation in the skin through two distinct pathways. Eosinophils increase inflammation through the activity of EPX, which increases IL-1 leading to release of other proinflammatory cytokines and eventually recruitment and activation of eosinophils, causing a feed forward loop leading to increased inflammation. At the same time, eosinophils also influence itch by both increasing expression of TSLP in the skin and increasing TSLPR expression on sensory nerves, and causing itch. Eosinophils require EPX activity to increase TSLP levels in the skin, and require contact with the sensory nerves to increase TSLPR expression. This hypothesis would identify eosinophils as important mediators of itch and inflammation in the skin (Figure 7-1).

The findings from this dissertation may also be relevant in other tissues and in other diseases as well. The observation that horseradish peroxidase also increases TSLP expression in keratinocytes may imply that activation of this pathway is not limited to EPX activity, but is a general pathway activated by other peroxidases as well. Myeloperoxidase is expressed by several other inflammatory cells, including neutrophils and macrophages, and when active will produce hypochlorous acid, which is chemically similar to hypbromous acid produced by EPX. Myeloperoxidase levels are increased in psoriasis[333], and TSLP levels are increased in skin in psoriasis as well[334]. The data from this thesis supports a hypothesis where myeloperoxidase increases TSLP expression in the skin in psoriasis, though further tests would be required to prove this association.

I have also shown that horseradish peroxidase increases TSLP expression in airway epithelial cells. This data may indicate that EPX, which is active in the lungs of patients with asthma, may also increase TSLP levels in the lungs, and that this may be relevant in asthma. Blocking TSLP and reducing eosinophils in the lungs have both been shown to benefit patients with asthma[105,173,182], and this hypothesis may provide a link between eosinophil activity and increased TSLP levels in the lungs in these patients. Again, further tests would be required to show that the activity of EPX is the mechanism increasing TSLP levels in the lungs.

Future Directions

Many studies can still be performed to answer questions raised by the findings in these experiments. Experiments with eosinophil and sensory nerve coculture showed an increase in TSLPR expression in sensory nerves that required cell contact. However, future experiments could be performed to identify potential mechanisms driving this. Sensory nerves express ICAM-1 and VCAM-1 in cell culture[164] and experiments blocking these adhesion molecules using neutralizing antibodies could be used to determine if these are critical for eosinophils to increase TSLPR expression in sensory nerves. It is also possible that adhesion between eosinophils and nerves leads to activation of eosinophils and release of a soluble mediator from the eosinophils that is responsible for increasing TSLPR expression in sensory nerves. This could be tested by using conditioned media from sensory nerve/eosinophil cocultures.

Experiments showing changes in sensory nerve response after eosinophil coculture could also be pursued. TSLP activates sensory nerves and causes an increase in intracellular calcium[110]. The proportion of sensory nerves that respond to TSLP with calcium influx could be measured after eosinophil coculture, to determine if the proportion of nerves that respond to TSLP increase after coculture with eosinophils. Staining for TSLPR and all nerves using PGP 9.5 in skin could be used to determine if TSLPR levels are increased in the skin in patients with atopic dermatitis.

I have shown that blocking IL-1 reduces the increase in general measures of inflammation, as well as in the number of eosinophils. However, we have not demonstrated that there is reduced itch in animals that have been treated with IL-

1 blocking drugs. This experiment will be critical for supporting the hypothesis that IL-1 is an important mediator for itch in atopic dermatitis. Due to the reduction in eosinophils, I would also hypothesize that TSLP levels are reduced when blocking IL-1 in the chronic contact dermatitis model, and this could be tested as well.

The findings that blocking LPA caused an increase in inflammation in the animal model of chronic contact dermatitis, but did not reduce TSLP levels was surprising, and given concerns about the drug that was used, future experiments should be performed to validate these results. LPA has 6 different receptors, as well as 11 different sPLA2 enzymes that produce LPA that may be responsible for mediating these effects. Testing what specific receptor(s) and sPLA2 enzymes are critical for this in the primary keratinocyte cultures would provide insight into what specific pathways to block in the animal model of chronic contact dermatitis. I have tested several selective antagonists for LPA receptor 1 (LPAR1) and have found that these do not block the increase in TSLP caused by LPA in the cell cultures, meaning that LPAR1 is likely not involved in the increase in TSLP caused by LPA. Given the concentrations of MJ33 that were required to inhibit the increase in TSLP caused by EPX, I would hypothesize that sPLA2G2F is responsible for producing LPA, as the IC50 for inhibition of this enzyme by MJ33 is at a similar concentration[335]. Supporting this, sPLA2G2F gene expression was detected in primary mouse keratinocytes cultures. Unfortunately, there are not selective antagonists of sPLA2G2F, though other methods such as siRNA could be used in cell culture to determine if this enzyme is required.

Finally, the mechanism by which keratinocytes respond to EPX to release IL-1 and produce LPA was not identified in these studies. TRPA1 is a oxidant sensor that responds to oxidants such as sodium hypochlorite [336], which is chemically very similar to hypobromous acid, a product EPX peroxidase activity. TRPA1 is expressed in keratinocytes in the skin, and activation of TRPA1 has been shown to increase TSLP expression in keratinocytes[337]. Testing if TRPA1 is critical for the increase in cytokine expression in keratinocytes could determine if TRPA1 is critical for this and would support the hypothesis that TRPA1 is the receptor that responds to EPX activity in keratinocytes.

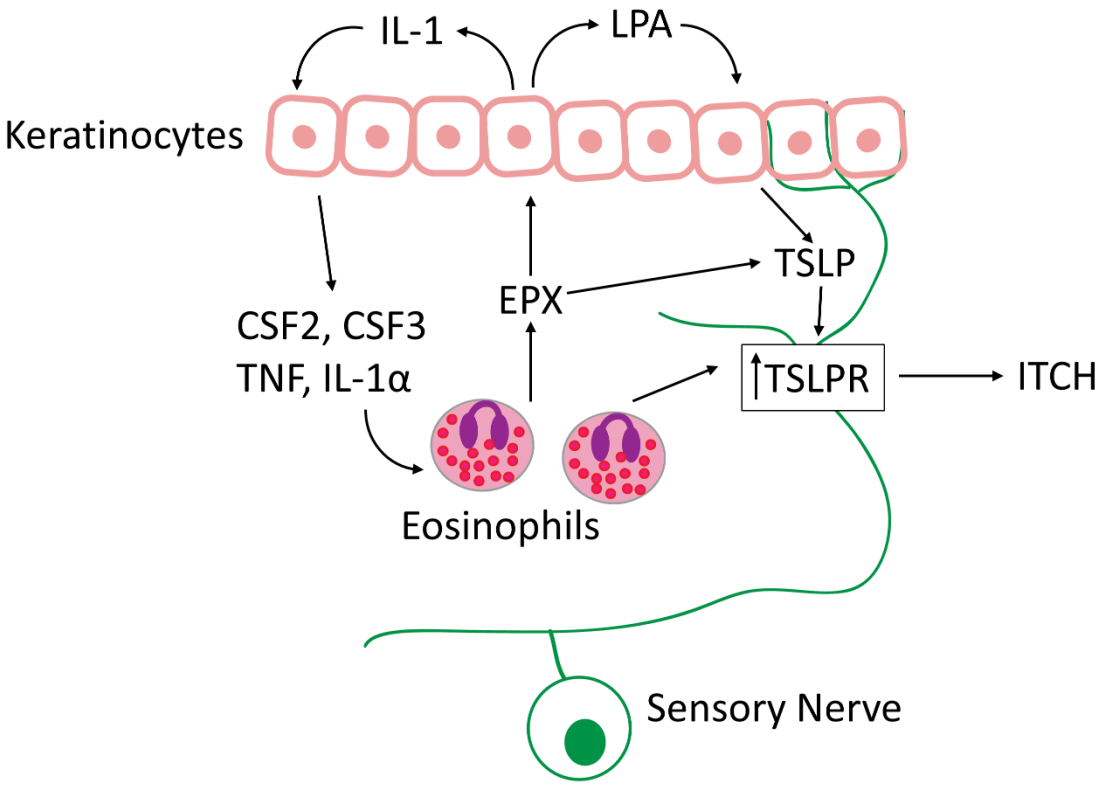

Figure 7-1 Model of eosinophil effects in skin on inflammation and itch.

Hypothesized model based on the data in this dissertation by which eosinophils initiate itch and inflammation in the skin.

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