EOSINOPHILS INCREASE SENSORY NEURON BRANCHING

By

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LIST OF ABBREVIATIONS

AD: atopic dermatitis

CNS: central nervous system

DRG: dorsal root ganglion(a)

ECP: eosinophil cationic protein

ELISA: enzyme-linked immunoabsorbence assay

EDN: eosinophil-derived neurotoxin

EPO: eosinophil peroxidase

GM-CSF: granulocyte-macrophage colony stimulating factor

H&E: hematoxylin and eosin

HLA: human leukocyte antigen

IL-: interleukin-

K5, K14: keratin 5, keratin 14

L: lesional atopic dermatitis skin

MHC: major histocompatibility complex

MBP: eosinophil major basic protein

NGF: nerve growth factor

NL: non-lesional atopic dermatitis skin

PGP 9.5: protein gene product 9.5, a marker for all subtypes of neurons

RT-PCR: reverse-transcriptase polymerase chain reaction

SP: substance P

TGFβ-1: transforming growth factor beta-1

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ABSTRACT

Cutaneous nerves are increased in atopic dermatitis, and itch is a prominent symptom. The functional interactions between eosinophils and nerves were studied in human and mouse skin and in culture. Human atopic dermatitis skin has eosinophil granule proteins present in the same region as increased nerves. Epicutaneous sensitization of wild-type mice, but not mice deficient in eosinophils, increased cutaneous nerves predominantly in the papillary dermis. Transgenic mice in which interleukin-5 (IL-5) expression is driven by a keratin-14 (K14) promoter had eosinophil proteins in the epidermis, and the number of nerves was also significantly increased in the epidermis. Mice lacking filaggrin, which are a model for atopic dermatitis, also had increased nerves in skin, as did keratin-5-TGFβ-1 transgenic mice, which have high IgE levels and eosinophils in the dermis. In co-cultures, eosinophils dramatically increased branching of sensory neurons isolated from the dorsal root ganglia (DRG) of mice. This effect did not occur in DRG neurons co-cultured with mast cells or with dead eosinophils. Physical contact of the eosinophils with the neurons was not required, and the effect was not blocked by an antibody to nerve growth factor. DRG neurons recruit and bind eosinophils *in vitro* and synthesize eotaxin-1 and ICAM-1 and VCAM-1. These data indicate a new therapeutic target in atopic dermatitis and other eosinophilic skin conditions with neuronal symptoms such as itch.

CHAPTER ONE: INTRODUCTION

I. ATOPIC DERMATITIS

A. Clinical Presentation, Diagnosis, Epidemiology, Genetics and Costs

1. Introduction and Clinical Presentation

Atopic dermatitis is characterized by pruritus, or itch, which greatly affects the quality of life of patients (1). Often, the itch sensation begins before any lesions appear, and marks on the skin can be limited to excoriations, or scratches made by the patient. The disease has a characteristic distribution, depending on the age of the patient: face and extensor surfaces of arms and legs in infants, and flexural surfaces (knees and elbows) in adults. Lesions are characterized as acute or chronic, depending on their clinical appearance and duration. Acute lesions are papulovesicular, red, weeping lesions. Chronic lesions become lichenified, thickened skin with accentuation of the natural lines, after repeated trauma from rubbing and scratching the skin (2).

The first use of the term "atopic dermatitis" was in 1933 by Wise and Sulzberger. They defined it "as a fairly distinct and clear-cut entity… best called atopic dermatitis, generally known as …diffuse pruritus with lichenification." They defined nine cardinal qualities, including atopic family history and emphasized that these cases should be "sharply isolated and distinguished from other forms of lichenification" (3). Previous reports had not differentiated this disease from other eczemas, which were defined histologically prior to 1912 (reference unknown, used in Wise and Sulzberger). Unfortunately, the terms "atopic dermatitis" and "atopic

eczema" are used interchangeably throughout the literature, although the primary term used by dermatologists, and throughout this document, is atopic dermatitis.

Atopic dermatitis is characterized as "exaggerated responses to a variety of environmental stimuli (irritants, allergens and microbes)" (4), and it is possible that these exaggerated responses are due to underlying defects in skin barrier, altered sensation of itch, or hyperactive immune function. It is hypothesized that patients become sensitized to allergens in early infancy, possibly due to a skin barrier defect. This leads to itching, followed by scratching, which induces trauma to the skin, allows greater entry of allergens, and results in the typical lesions and lichenification of atopic dermatitis (5).

However, understanding the development and exacerbation of atopic dermatitis is complicated by the heterogeneity of the disease. The natural history and progression vary among individuals, based on the age of development and disease severity. Allergen sensitization is heterogeneous, and other forms of atopic disease, such as allergic rhinitis, asthma and food allergy, do not occur in all patients. Sensitization to allergens, defined as high levels of total serum immunoglobulin (Ig)-E and allergen-specific IgE, was once thought to precede development of atopic dermatitis, but it is now clear that defects in the skin barrier can allow subsequent sensitization rather than the other way around.

There is a genetic predisposition to atopic dermatitis, but the disease is also influenced by the environment. Trauma to the skin, emotional stress, bacterial infections, irritation, temperature changes, sweat, or light touch can all lead to exacerbations, which prompt scratching, which produces chronic lichenified skin

(6). The disease is also characterized by increased water loss through the skin due to a dysfunctional epidermal barrier, which results in dry skin, or xerosis (7), discussed in detail below.

Atopic dermatitis is often a mild disease of childhood that remits during adulthood. However, patients that develop severe atopic dermatitis early in childhood have a more prolonged course of disease that can persist for their entire lifetime (8). Of 1123 children enrolled in the German Multicenter Atopy Study, 21.5% developed atopic dermatitis in the first two years of life. Upon entry to the study, the children were divided into groups based on their frequency of scratching and severity of disease, and then followed until they were 7 years old. At the age of 7 years, 18.7% of the children with atopic dermatitis had had symptoms every year and were categorized as having persistent atopic dermatitis, while 38% had intermittent bouts of atopic dermatitis up to age 7. Patients with persistent disease later in life were primarily those with more severe disease that developed before the age of 2 years. Of those with persistent atopic dermatitis (18.7% of all patients with atopic dermatitis), 72% reported frequent scratching with early disease, thereby indicating severity and early age of onset as strong risk factors for persistent disease (Figure 1.1).

In addition to severity of early disease, early sensitization, especially to food allergens, is crucial for determining the prognosis of atopic dermatitis. It is not clear whether atopic sensitization or atopic dermatitis occurs first. However, sensitization clearly predicts duration of disease (8). A systematic review of 11 earlier studies looking at the natural history of atopic dermatitis concluded that the predictive

factors for prognosis are severity of disease, age of onset, and association with respiratory allergy or food hypersensitivity (9).

2. Diagnostic Criteria

Unlike other common dermatological diseases, atopic dermatitis cannot be diagnosed simply by histology or clinical presentation; accurate medical and family histories are crucial. Definitions of atopic dermatitis have been complicated by the range of clinical manifestations as well as the uncertain linkage with atopy, or allergy. Consensus diagnostic criteria established in 1977 helped standardize the definition (10), and subsequent modifications in 1980 by Hanifin and Rajka simplified the diagnosis of this disease to the criteria found in most publications (11):

1. History of itching
2. Lichenification
3. Chronically relapsing course of disease
4. Atopic history, either personal or familial

More recently, in 1994, the U.K. Working Party validated and refined the original Hanifin and Rajka diagnostic criteria to evidence of a pruritic skin condition plus 3 or more of the following:

1. history of flexural (skin crease) involvement
2. history of dry skin
3. onset under the age of 2 years
4. personal history of asthma or allergic rhinitis
5. visible flexural dermatitis as seen by a clinician (12).

This clinical gold standard for diagnosis was implemented in Britain after quantifying the level of agreement among 14 physicians on patients with typical atopic dermatitis. The physicians agreed strongly when a rash was present but disagreed on the contribution of some minor signs, such as dry skin, infraorbital creases, or periorbital darkness, that often accompany atopic dermatitis. Therefore, the Working Party excluded signs on which there was poor agreement and determined the five most useful diagnostic criteria for atopic dermatitis (13). These were tested in an independent sample and compared to the gold standard of the Hanifin and Rajka criteria and were found to have similar sensitivity (87.9% versus 93.1%), but increased specificity (92.8% versus 77.6%). Diagnosis in children required modifications in the diagnostic criteria to replace personal history of atopy with family history of atopy and removal of early onset disease, to result in 85% sensitivity and 96% specificity (12, 14). A consensus report in 2006 agreed that the Hanifin and Rajka criteria or U.K. Working Party criteria should be used for diagnosis across Europe and North America (14).

3. Epidemiology

A worldwide study of 715,033 children found that atopic eczema, defined as an "itchy rash in skin folds" affects 5-20% of children in the age ranges surveyed (6-7 years old and 13-14 years old) (15). Developed countries have the highest prevalence, and urban centers have higher rates of disease than rural areas. A review of 25,000 children in five European countries indicated a prevalence of atopic eczema up to 24% (16). Physician-diagnosed atopic dermatitis was present in 17% of 4783 Norwegian two year olds (17), between 15-24% of Japanese

schoolchildren (18), and in 20% of British children, aged 3-11 years (19). In 45% of British children with atopic dermatitis, the disease developed during the first 6 months of life. Between 6.8 and 17.2% of Oregon schoolchildren have atopic dermatitis, depending on the stringency of diagnostic criteria (20). 60% percent of all people with atopic dermatitis develop the disease by their first birthday, while 85% develop it within the first five years of life[8]. A larger and more recent survey, by self-administered questionnaires, in the United States found that 6% of 116,202 individuals reported itching/scratching in conjunction with one of the following:

1. red/inflamed rash
2. excessive dryness/scaling
3. skin fold location
4. early onset
5. symptoms lasting for 14 days or more, or
6. a physician diagnosis of asthma, allergic rhinitis or hay fever (21).

This percentage was extrapolated to 17.8 million people in the United States meeting the criteria for atopic dermatitis. A retrospective analysis of private insurer and Medicaid claims for atopic dermatitis found a prevalence of disease of around 2.5% of all eligible beneficiaries (22).

4. Genetics of Atopic Dermatitis

Family history and genetics are clearly important in the pathogenesis of atopic dermatitis. Schultz-Larsen and Holm examined positive responses from patients to a mailed atopic dermatitis questionnaire, followed by clinical examination. They found that monozygotic twins had a pair wise concordance of

0.77 for atopic dermatitis, compared to 0.15 for same sex dizygotic twins (23), indicating a strong genetic component. An increasing incidence of atopic dermatitis, between twin groups born from 1960-64 and those born between 1970-74, from 0.03 to 0.10 was also noted. However, while having a parent with atopic dermatitis is a strong predictor for atopic dermatitis in a child, allergic asthma or rhinitis in a patient's parent is not (24).

Genome-wide linkage and association studies have proven difficult, because the effect of a single gene/polymorphism in a complex disease such as atopic dermatitis is anticipated to be relatively modest. "It can be assumed that variants in multiple genes will cooperate in an additive or synergistic manner to affect disease risk, a phenomenon referred to as epistasis. A major difficulty in testing for epistasis is power..." (25). Because of the heterogeneity of the disease and its complexity, few clear genetic associations have been made in atopic dermatitis. One that has been confirmed in more than 20 studies is filament-aggregating protein, or filaggrin, which is discussed below. Other molecules that have been identified in 3 or more studies are interleukin (IL)-4, IL-4RA, SPINK5, IL-13, and RANTES (which stands for regulated upon activation, normal T cell expressed and secreted). IL-4 is a cytokine that is produced by T cells, eosinophils and basophils, and IL-13 is a cytokine that uses the same receptor for signaling (26, 27). RANTES is a chemokine that signals through the CCR3 chemokine receptor, which is present on T cells and eosinophils (28, 29). SPINK5 is a serine protease inhibitor that has been implicated in the etiology of Netherton's syndrome (30, 31). None of the genome-wide association studies identified any candidate genes that approached significance. A model of

genes and their functions which have been identified in atopic dermatitis genetic screens is shown in Figure 1.2.

5. Costs of Atopic Dermatitis

Because of its prevalence and interference with quality of life, atopic dermatitis incurs high costs to the medical system and to individuals. By examining hospital reimbursement for fees related to atopic dermatitis, Lapidus et al. estimated that the national costs of atopic dermatitis were at least $364 million in 1990 (32). The authors believed that this was a conservative estimate, since it excluded fall and winter, when atopic dermatitis exacerbations are at their highest level. In 2002, using a retrospective analysis of state Medicaid and private insurer claims, the costs to these third-party payers were estimated at $20 million for private insurers and $111 million for Medicaid. The price per patient was $580 or $1250, respectively, and included inpatient care, emergency department fees, outpatient office visits, and therapeutics (22). This did not include patient co-payments or cost of charges that were not reimbursed.

Atopic dermatitis is also costly to families. Depending on the severity of the disease, costs in an Australian study ranged from A$480-2545 (US$307-$1628) per year per family in 1997 (33). In a 2006 study, both direct (costs of health services and products) and indirect (costs of time, work lost, etc) costs were determined for atopic dermatitis in the United States. Direct costs for medical management of atopic dermatitis were $1.01 billion, while indirect costs (largely the lost workdays of caregivers taking children to seek medical attention) were $248.5 million. The authors also found that atopic dermatitis is the #4 dermatologic condition in the

United States in terms of willingness to pay for symptom relief, after all hair and nail disorders, acne, and seborrheic keratosis (benign warts)(34).

B. Anatomy and Histology of Healthy Control and Atopic Dermatitis Skin

1. Introduction and Comparison of Healthy Skin to Atopic Dermatitis Skin

Human skin can be divided descriptively into three compartments: the epidermis, the dermis and the hypodermis. Each layer contributes to the skin's barrier function and its ability to eliminate microorganisms and maintain homeostasis. However, when cells are either deficient in protective capabilities or hyperreactive to non-noxious stimuli, the skin loses its protective function and can severely compromise health.

Patients with atopic dermatitis have no regions of normal skin. Uninvolved skin, called *non-lesional skin* through the rest of this document, appears normal clinically, but has significant alterations compared to control skin in a healthy person without atopic dermatitis (Figure 1.3). Eighty-eight percent of atopic dermatitis patients show some form of dry skin, either as a clinical disorder called *ichthyosis vulgaris*, or as milder overall dry skin (35). In the dermis of patients with atopic dermatitis, there is an infiltration of immune cells, including lymphocytes, monocytes, mast cells and rare eosinophils (36), including a perivascular mononuclear infiltrate in non-lesional skin (37). Therefore, the changes in atopic dermatitis skin begin before lesions appear and include changes in both the epidermis and dermis.

2. Anatomy and Histology of Healthy Skin (36)

Skin is composed of cells from all three embryonic cell layers. Ectodermal cells form the epidermis, hair follicles and glands, including sebaceous, apocrine and eccrine glands. The mesoderm gives rise to fibroblasts, adipocytes, blood vessels and lymphatics, and the neural crest forms nerves and melanocytes.

a. Healthy Epidermis

The epidermis is the outer protective layer of the skin and is separated from the dermis by the basement membrane. The epidermis is composed mainly of keratinocytes, which produce keratins, important structural proteins in the epidermis. Keratinocytes are ectoderm-derived cells that are organized into distinct sublayers according to their state of differentiation. The cells begin in the deepest, least differentiated layer and move to the most superficial, fully differentiated layer in humans in two weeks.

The basal layer of the epidermis (*stratum basale*) contains slowly dividing, self-renewing epidermal stem cells, as well as rapidly dividing transiently amplifying keratinocytes and post-mitotic cells. As these cells differentiate, they move upward into the spinous layer (*stratum spinosum*), which is the cell layer responsible for resistance to mechanical stress and shear forces. This layer is strengthened by intercellular adhesion through desmosomes, linked to keratin intermediate filaments in the cytoplasm of each cell. In addition, lamellar granules, which contain lipid precursors, are synthesized in this layer. The next outer layer is the granular layer (*stratum granulosum*). The dense granules seen in this layer contain keratohyalin granules, composed of profilaggrin, keratin, and loricin, which

are processed and then aggregate to form macrofilaments. The granular layer is also the site of keratinocyte apoptosis. Cells, having reached the end of their differentiation, are genetically programmed to self-destruct, and leave behind only a matrix of keratin and filaggrin molecules. The final, outermost layer of the skin is the *stratum corneum*, or cornified envelope. This layer is composed of stacked, anucleate cell remnants and a lipid extracellular matrix, and is the main barrier to water loss and entry of molecules from the outer environment, as well as the major protection against ultraviolet radiation damage to the cells below. The timing of entry to sloughing off of a dead keratinocyte through the layers of the *stratum corneum* is approximately two weeks, which gives a keratinocyte approximately one month to leave the basal layer, move through the epidermis, die and be removed.

Other cells also reside in the epidermis in small numbers. Melanocytes occupy a portion of the basal layer and synthesize pigment molecules. Langerhans cells are a specialized subset of dendritic cells that process and present antigen within epithelia and are located throughout the epidermis. Merkel cells are mechanoreceptors that signal to nerves upon stimulation and are present in tactile-sensitive regions of the skin. Finally, free unmyelinated sensory nerve endings innervate all layers of the epidermis.

b. Healthy Dermis

The dermis is the thickest layer and is composed of many different cell types and ultrastructural components, especially collagen and elastic fibers. These fibers give the skin strength and flexibility. The non-fibrous components of the dermal extracellular matrix include proteoglycans, glycoproteins and glycosaminoglycans,

which bind water and growth factors and influence cellular growth and repair. In addition, smooth muscle, such as erector pili muscles, and epidermal-derived appendages, such as hair follicles and sweat glands, transverse the dermis.

The dermis has two sections: the upper papillary dermis, near the epidermal boundary, and the deeper reticular dermis, near the hypodermal compartment. Each section has distinct organization of collagen apparent on histological examination with hematoxylin and eosin (H&E) staining. The papillary dermis has small units of individual collagen fibers, while the deeper reticular dermis has larger bundles surrounded by elastic fibers. The sub-papillary plexus of blood vessels is a dividing marker between the two regions of the dermis.

The papillary dermis is named for the intersection of dermal papillae with epidermal rete pegs and is the area immediately adjacent to the epidermis. Cells that normally occupy the papillary dermis include macrophages, mast cells, and fibroblasts, which synthesize collagen. Sensory nerves often end in the papillary dermis as well. In times of infection or inflammation, neutrophils, eosinophils and T cells enter the skin via the sub-papillary plexus of blood vessels and migrate throughout the papillary and reticular dermis, and can also infiltrate the epidermis. Small lymphatics are located in the papillary dermis, which drain into larger lymph vessels near the sub-papillary blood vessels and transverse through the reticular dermis. The reticular dermis occupies the majority of space in the dermis. Large collagen fibers, organized into bundles and surrounded by elastic fibers, are characteristic of this layer.

Deep in the reticular layer of the dermis, the fibrous connective tissue transitions into adipose connective tissue, which forms the hypodermis. The cutaneous vascular network begins from vessels that enter the hypodermis and course through the reticular dermis. The nerve fibers supplying the skin begin as large networks below the hypodermis and branch into a more superficial plexus in the sub-papillary space between the papillary and reticular dermis. Free nerve endings supply the papillary dermis and epidermis. There is very little autonomic innervation of the skin, relative to the overwhelming number of sensory fibers (discussed further below).

The hypodermis is the deepest compartment of the skin and provides insulation and a source of stored energy in the adipocytes that compose it. Lobules of adipocytes are divided by walls of connective tissue that contain nerves, blood vessels and lymphatics, which sub-divide into smaller branches through the dermis and epidermis.

3. Anatomy and Histology of Atopic Dermatitis Skin

a. *Atopic Dermatitis Epidermis*

Histologically, atopic dermatitis is defined as one of several eczematous diseases, after the Greek *eczeo*, "to boil over or effervesce." This is due to intercellular edema in the epidermis, called *spongiosis* (2). Atopic dermatitis exhibits variable *acanthosis*, or thickening of the stratum spinosum of the epidermis. The stratum corneum keratinized layer, is often lost in acute weeping eczema, while *hyperkeratosis*, or thickening of the stratum corneum, occurs in chronic cases (2).

Epidermal area is increased 3- to 4-fold in patients with atopic dermatitis, due to hyperplasia. In non-lesional skin, the increase in area is solely due to epidermal hyperplasia, while in lesional skin, hyperplasia and increased folding of the dermal-epidermal junction occur, creating rete pegs into the papillary dermis (Figure 1.3) (37). Non-lesional skin is clinically normal, but has mild eczematous features histologically, including hyperkeratosis, parakeratosis, acanthosis, and focal spongiosis in the epidermis (37).

i. Barrier Defects in Atopic Dermatitis Epidermis

As mentioned above, a large percentage of atopic dermatitis patients have dry skin. These patients exhibit greater transepidermal water loss when their disease is active. With clinical improvement, a gradual reduction in transepidermal water loss is observed, and appearance of skin returns to normal at the same time as transepidermal water loss is decreased to normal levels (7).

As early as 1976, a hypothesis on the etiology of atopic dermatitis was suggested in which "a defective barrier layer, which is brought about by scratching or inadequate keratinization secondary to increased alpha adrenergic stimulation and depressed beta adrenergic stimulation, allows excess antigens from environment, epidermis or bacteria to penetrate the epidermis" (38).

Recently, mutations in epidermal barrier proteins have been found in atopic dermatitis skin, pointing to a potential mechanism for the observations of xerosis and increased water loss in atopic dermatitis skin. The first barrier protein mutations associated with atopic dermatitis were found in filaggrin, which helps form the epidermal barrier, aggregates the keratin cytoskeleton, and facilitates

terminal differentiation of keratinocytes (reviewed in (39)). Specific filaggrin mutations predispose people toward atopic dermatitis (40, 41), and two loss-of-function mutations in filaggrin cause ichthyosis vulgaris, or severe dry skin, in humans (42). Atopic dermatitis has visibly less filaggrin in the stratum corneum when compared to healthy control skin (Figure 1.4).

Studies using knockout mice lacking filaggrin (flaky tail mice) in the stratum corneum indicate that the skin barrier is more permissive to entry of protein antigens, and that the threshold for inflammation is lowered after application of irritants (43, 44). These mice exhibit dry, scaly skin and hyperkeratosis (45-47).

Other genes important for the formation of the stratum corneum can also be affected in atopic dermatitis. Using mRNA expression analysis, an overall reduction in expression of genes associated with terminal differentiation was observed. The largest changes were seen in loricrin, which is the main component of the cornified envelope and corneodesmosin, which attaches the cornified layer to the granular layer (48, 49).

b. Atopic Dermatitis Dermis

An early study using anti-T cell serum on lesional skin from atopic dermatitis patients determined that the majority of the cells in the dermal infiltrate were T cells (50). These skin-infiltrating lymphocytes were isolated from skin biopsies, characterized by flow cytometry and determined to be 97% CD3+ cells, with 83% CD4+ T cells, compared to 16% CD8+ T cells. These cells proliferated best, measured by tritiated thymidine incorporation, when exposed to either IL-2 alone or a combination of IL-2 and IL-4 *in vitro* (51-54)

Additional studies characterizing T cells in atopic dermatitis skin also have found that the majority were CD4+ cells and that the main cytokines they released after isolation from biopsies were IL-4, IL-5 and IL-13, marking them as type 2 helper (Th2) cells (53, 55, 56). In addition, mRNA isolated from atopic dermatitis skin has high levels of IL-4 in acute lesions and IL-5 and IFN-γ in chronic lesions (57). Cytokine receptor mRNA paralleled this finding, with IL-4Rα increased in acute lesions, and IL-5Rα increased in chronic lesions (58). Non-lesional skin had more IL-4 and IL-4Rα message than normal skin, while IL-5 and its receptor and IFN-γ did not differ between non-lesional and normal skin (57, 58). Lesional atopic dermatitis skin lacks regulatory T cells, with no *foxp3* mRNA found in skin T cells (56), and NK cells are also absent (55).

c. Intrinsic versus Extrinsic Atopic Dermatitis

Two distinct phenotypes of atopic dermatitis were formally recognized in 1994 (59). Elevated IgE levels and sensitization with skin disease was termed allergic, or extrinsic-type, atopic dermatitis, while skin disease in the absence of high serum IgE or high allergen-specific IgE was called non-allergic, intrinsic-type atopic dermatitis. Differentiation between the two forms of disease is made by determining atopy, defined as IgE antibody reactivity to environmental or food allergens, or skin prick test positivity (60). Importantly, Hanifin and Rajka's criteria modified the previous requirement of high serum IgE in diagnosing atopic dermatitis to a personal or family history of atopy, and "exclusion of specific IgE

measurements … did not change the sensitivity and specificity" of those criteria (60).

Clinical presentation of skin disease does not differ between atopic and non-atopic patients (61). In addition, in one study, intrinsic and extrinsic atopic dermatitis had similar cellular infiltrates into the skin, and similar ratios between blood and skin T cells, indicating no differences in recruitment of cells to the skin (62). However, a separate group found that there were histologic differences, including fewer infiltrating eosinophils and eosinophil granule proteins into the skin of non-atopic patients (63). Peripheral circulating lymphocytes in extrinsic atopic dermatitis produced high levels of both IL-4 and IL-5, while lymphocytes from intrinsic patients made high levels of IL-5 and no IL-4 (59).

Many studies, especially early ones, do not differentiate intrinsic from extrinsic atopic dermatitis when looking at blood and skin findings. Concerning the blood, both intrinsic and extrinsic atopic dermatitis patients have eosinophilia, although the numbers of eosinophils are higher in patients with extrinsic atopic dermatitis (62). Unless noted otherwise, the studies mentioned in this introduction did not distinguish between samples from intrinsic or extrinsic atopic dermatitis patients. However, in our own studies, we only accepted human subjects with a personal and family history of atopy, in addition to their atopic dermatitis. In this way, without serum IgE measurements, we tried to limit our study to only extrinsic atopic dermatitis.

In an attempt to standardize definitions, "atopic dermatitis" was redefined as "atopic eczema," which is dominated by IgE antibody-associated reaction, based on

IgE sensitization (64). However, “atopic dermatitis” is still used most commonly, and can pertain to either form of the disease.

d. Summary

The histology of atopic dermatitis skin differs according to the stage of disease, whether non-lesional, acute lesional or chronic lesional. Non-lesional skin showed traces of hyperkeratosis and epidermal hyperplasia, intercellular edema, slight dermal cellular infiltrate with primarily lymphocytes. In acute lesional sites, there is intercellular edema, or spongiosis, of the epidermis, with an infiltration into the dermis of lymphocytes and macrophages, with rare eosinophils and neutrophils. In chronic lesional sites, histology shows epidermal hyperplasia with minimal spongiosis and a dermal inflammatory cell infiltrate of lymphocytes and monocyte-macrophages. Lichenified plaques demonstrate hyperkeratosis and hyperplasia of the epidermis with focal areas of intercellular edema, while the dermis has moderate cellular infiltrate with monocyte-macrophagess and lymphocytes (65).

In addition, in lesional skin, cutaneous nerves at all levels of the dermis exhibit alterations, including apparent demyelination and fibrosis, and occasional vacuolated areas within nerve fibers (65). In non-lesional skin, occasional fibrosis and focal demyelination of cutaneous sensory nerves were also observed.

4. Treatment of Atopic Dermatitis

The pruritus found in atopic dermatitis is resistant to anti-histamines and leukotriene antagonists. A study of children who took sedating or non-sedating antihistamines determined that the sedation effect inhibited scratching, rather than antihistamines decreasing the itch (66). However, a later study with a particular

sedating antihistamine, chlorpheniramine, found that it was no more effective than placebo for childhood atopic dermatitis with itching at night (67).

The current standard treatment for atopic dermatitis is reactive, using topical corticosteroids combined with calcineurin inhibitors, such as tacrolimus, to treat flares (68) (5). Maintaining skin hydration and an intact barrier with moisturizers after bathing is also important in preventing exacerbations. Compliance with treatment is a problem, especially among children (69) . Experimental treatments such as type 4 phosphodiesterase inhibitors, serotonin agonists, antibiotics and immunosuppressants are currently under study (70-74).

II. EOSINOPHILS

A. Identification of eosinophils and general characteristics

Eosinophils were first described in 1878, in a doctoral dissertation by Paul Ehrlich at the University of Leipzig, on the staining of tissues with dyes. The cells were named by Ehrlich for their retention of the acidic pink dye, eosin [http://nobelprize.org/nobel_prizes/medicine/laureates/1908/ehrlich-bio.html]. Eosinophils are immune cells, measuring approximately 8um in diameter. Mature human eosinophils have bilobed nuclei, while nuclei in rodent eosinophils can take a variety of forms, but are most often ring- or donut-shaped (75). Eosinophils are classified as granulocytes, due to cytoplasmic granules apparent upon differential staining. They develop from bone marrow precursors and act as terminal effector cells, antigen-presenting cells, phagocytes, and mediators of cell signaling. Eosinophils are increased in helminthic infections, in allergic disorders and in the

hypereosinophilic syndrome, a heterogeneous collection of idiopathic disorders defined solely by the presence of elevated numbers of eosinophils in the blood.

B. Eosinophil granules

The pink staining by eosin that gives the eosinophil its name is due to granule proteins present in the cytoplasm of mature cells. Eosinophils have two populations of granule proteins (76): primary granules, which make up about 5% of the total granule population, and secondary granules, which comprise the remaining 95% (77). Primary granules contain Charcot-Leyden crystal protein, which is also present in basophils, and is made of the enzyme lysolecithin acylhydrolase, or lysophospholipase (78, 79). Secondary, or specific, granules form unique structures which are elliptical, contained by a membrane, and filled with an "amorphous matrix," with an embedded dense core of protein (80). Granules are formed as vacuoles that pinch off the Golgi apparatus, which aggregate to form larger immature granules and then condense. In secondary granules, crystals appear shortly afterward (77).

Secondary granules have four major protein components: Major Basic Protein (MBP), which comprises most of the crystalline core, and a matrix surrounding the core containing Eosinophil Peroxidase (EPO), Eosinophil Cationic Protein (ECP), and Eosinophil-derived Neurotoxin (EDN) (75, 81). The granule proteins are exceptionally basic, which is why they bind acidic stains like eosin.

The first of the granule proteins to be identified, EPO was found in rat eosinophils during development of a protocol to isolate granules (81). Peroxidase was released from intact cells and granules placed in acidic solutions with pH

between 5.5 and 2. Subsequently, rat and horse eosinophil granules were isolated and found to have high specific activity of peroxidase, compared to the other enzymes present (82).

MBP was identified in 1973 by freezing and thawing purified guinea pig eosinophil granules. It constitutes about 55% of the protein in the secondary granules in guinea pig eosinophils, or around 30% in human eosinophils (83, 84). As its name indicates, it is extremely basic due to its arginine-rich composition, and it has a strong tendency to form insoluble aggregates that are linked with disulfide bonds (83). A different form of MBP, named MBP-2, was identified in 2000 and is specifically expressed in eosinophils, while MBP-1 is expressed in eosinophils and basophils (85, 86).

ECP was first isolated from eosinophil granules from individuals with leukemia and subsequently was identified in humans with asthma (87). Isolated ECP injected into the brains of guinea pigs induces inflammation, degradation of neurons, and progressive neurologic disease, called the Gordon phenomenon (88-90). It is cytotoxic and destructive to helminths, and it can function as a ribonuclease (91). Moreover, it is a more specific marker for eosinophils and their precursors than EDN (below) or MBP-1 (91).

EDN was first purified in 1981 using chromatography of circulating eosinophils from patients with hypereosinophilic syndromes. It was named based on its trait of inducing neurologic pathology in animals after intrathecal injection, through degradation of myelinated neurons in the central nervous system (88, 92). It was identified for its neurotoxic effects before ECP, but has substantially weaker

effects. EDN is a member of the ribonuclease family, as is ECP, has ribonuclease activity, and is expressed in many different types of cells, including neutrophils (93).

Eosinophil granules are released from the cell in a variety of regulated ways (reviewed in (94)).Whole cell cytolysis occurs when the eosinophil ruptures its plasma membrane and releases all of its cytoplasmic contents, including granules, as can be seen on the surface of invading helminthic parasites (95). This happens more rarely with eosinophils than with basophils or mast cells (96).

Alternatively, piecemeal degranulation (97) occurs after eosinophil stimulation and involves a change in the larger structure of secondary granules, as well as their polarization to one end of the cell. A vesicular system is generated to bud off granules and sequester them into subcompartments and tubules for specific release (97). Granules released intact to the extracellular milieu are able to maintain regulated release of proteins, including cationic proteins and cytokines, based on the expression of activating receptors on their membrane surfaces, which must be triggered by specific binding of their ligands (98). This is independent of RNA or protein synthesis and requires none of the cellular organelles that the granule is missing.

Once eosinophils have degranulated, they are difficult to identify. This leads to confusion as to the presence of eosinophils in some diseases, including atopic dermatitis (see below), and their role in pathogenesis. Stains for extracellular eosinophil proteins may mark the presence of eosinophils and circumvent this problem.

Eosinophil granules are cytotoxic when applied at high concentrations to a variety of cells in culture (99-101). However, co-culture of intact eosinophils with other cell types does not result in degranulation or higher nerve cell mortality (102), indicating that eosinophils are able to regulate release of their granules based on signaling from the extracellular milieu, in a similar manner to other immune cells. In fact, one paper found that distinct forms of degranulation occurred in different eosinophilic diseases, with cytolysis occurring more often in allergen-induced disease, and piecemeal degranulation more frequent in nasal polyps and inflammatory bowel disease (103).

C. Development of eosinophils and role of growth factors

Eosinophils develop from hematopoietic stem cells in the bone marrow and fetal liver (104). In mice and humans, eosinophil lineage-committed progenitors have been identified in the bone marrow (105, 106). In mice, eosinophil progenitors derive from the common myeloid progenitor, downstream of the granulocyte/macrophage progenitor population (107). In healthy mice and during helminth infection, there are no eosinophil progenitors found in the spleen or intestine, indicating that the main location of eosinophil production is the bone marrow (106). In humans, the eosinophil progenitor is derived directly from hematopoietic stem cells and not a subtype of any other previously described progenitor population (105).

The differentiation of eosinophils from progenitor cells has mostly been investigated *in vitro*, with a trial and error method of testing various growth factors. Without inclusion of growth factors into cultures, eosinophils die after 7 days, in

contrast with neutrophils, which can be maintained and survive in culture for several weeks (104). The earliest successful culturing of eosinophils involved adding antigen-stimulated supernatants from mouse splenocytes, implicating a T cell-derived, secreted factor as the component with "eosinophil differentiation activity," or "EDA" (104). Isolation, purification and sequencing of this factor resulted in the identification of interleukin-5 (IL-5) (108-110). Until the identification of IL-5, it was known that adding IL-3 and granulocyte macrophage colony stimulating factor (GM-CSF) to bone marrow cultures increased the number of eosinophil colonies, but also stimulated growth of basophil colonies and other lineages (111). IL-5 shared some traits with GM-CSF and IL-3 but was the only growth factor up till that point that had specific effects on the growth of eosinophils. Due to the rapidity of identification of new immunologic growth factors at the time, it had many names within a few years, including T cell-replacing factor, eosinophil differentiation factor, interleukin-4, and finally IL-5 (110, 112).

i. *Interleukin-5*

IL-5 was first isolated from parasite antigen-reactive T cell clones from mice infected with *Mesocestoides corti*. It was a selective inducer of eosinophils, but not other blood cell types, from bone marrow (108, 109). Human IL-5 shares about 70% primary sequence homology with murine IL-5, and signals through a heterodimeric receptor that is composed of a unique IL-5α chain and the common beta chain shared by GM-CSF and IL-3 (113-116).

In initial experiments, IL-5 treatment of mouse and humans bone marrow and cord blood from humans generated more eosinophil colonies than with other colony stimulating factors such as GM-CSF or IL-3 (108, 117, 118). Pre-treatment of cultures with IL-3 increased the yield of eosinophils when exogenous IL-5 was added. Murine IL-5 was able to stimulate the selective growth of eosinophils from human bone marrow, and IL-5 in any type of bone marrow culture had no effect on other cell lineages (117, 119).

Although IL-5 was identified as a murine eosinophil *differentiation* factor, it was important to understand which eosinophil precursors the cytokine acted on, as well as its specific functions. Because bone marrow can contain a variety of cells in different stages of differentiation, it was unclear whether IL-5 was responsible for the initial differentiation of the eosinophil lineage from hematopoietic stem cells or acted on later stages of cells instead. While human cord blood and bone marrow cultured with IL-5 selectively differentiated into eosinophils, the role of non-hematopoietic cells in providing additional growth factors was not yet determined (118).

Several *in vitro* studies were critical in discriminating the functions of IL-5 on eosinophils. Eosinophil progenitors express the IL-5Rα subunit, but earlier progenitors and those from other lineages do not (106). Two experiments used ectopic expression of the IL-5Rα subunit to try to force differentiation of progenitors toward an eosinophilic lineage. Expression of IL-5Rα in a multipotent progenitor cell line did not increase eosinophil development *in vitro*, (120), and

transduction of IL-5Rα into mouse granulocyte/macrophage progenitors did not turn the progenitors into eosinophil progenitors or eosinophils (106). Therefore, both sets of authors concluded that IL-5 signaling is a *result* of commitment to the eosinophil lineage, rather than a requirement. This conclusion was supported by the finding that splenocytes treated with 5-fluorouracil, which enriches for primitive hematopoietic progenitors, did not form eosinophil colonies after treatment with IL-5, unless additional growth factors were added (119). Subsequently, clear evidence in single-cell cultures indicated that IL-5 signaling supports proliferation and termainal maturation of eosinophils, but not their differentiation (106).

Finally, the creation of several strains of transgenic mice that express high levels of IL-5 in various tissues have confirmed that this causes massive eosinophilia, but not through regulation of the early myeloid or granulocyte progenitors. Rather, extramedullary hematopoiesis caused increases in later stage eosinophil-committed cell types, especially fully mature cells (121).

ii. Generation of eosinophils from bone marrow progenitors

Since the early days of differentiating bone marrow cells into various lineages, more specific protocols for producing eosinophils have emerged. Isolated CD34+ progenitor cells from normal human blood were differentiated into eosinophils, using stem cell factor, IL-3, IL-6, GM-CSF, and IL-5, and the resulting cells produced EPO, EDN and Charcot-Leyden crystals, but only few were producers of MBP or ECP (122). More recently, fully functional eosinophils were differentiated from mouse bone marrow and human embryonic stem cells, using cytokine stimulation over time. For the mouse cells, stem cell factor and Flt3-ligand were

added to cultures for the first four days, and IL-5 for at least 6 days afterward (123). The human cells were first differentiated to hematopoietic precursors, then expanded with GM-CSF in a co-culture with feeder cells, and finally cultured in the presence of IL-3 and IL-5 for 12 to 14 days (124). The authors also performed the same protocol on induced pluripotent stem cells and found similar success.

The development of eosinophils is not only controlled by the external milieu of environmental cytokines, but also by its transcriptional program. Through elegant forced expression studies and analysis of knockout animals, the major eosinophil lineage transcription factors have been identified. They include GATA-1, CCAAT enhancer-binding protein (C/EBP)-α and C/EBP-ε, as well as PU.1 (125-128).

GATA-1 was named for its propensity to bind promoter sequences that contain GATA palindromes, and it is a required transcription factor for the development of eosinophils. Forced expression of GATA-1 is sufficient to induce progenitors to commit to the eosinophil lineage (125). Conversely, deletion of GATA-1 results in mice that completely lack eosinophils (129).

C/EBP-α knockout mice were found to lack the ability to signal downstream of G-CSF, rendering the mice devoid of neutrophils and eosinophils, while having large numbers of progenitor cells in the blood. Bone marrow transplantation experiments demonstrated that mice transplanted with C/EBP-α knockout marrow did not reconstitute with granulocytes, while wild-type mice recovered normal numbers of all cell populations (126).

Different isoforms of C/EBP-ε serve as activators or repressors of transcription, and transduction of progenitors with the 32/20 isoforms programmed them to differentiate into eosinophils, even without exposure to IL-5. This occurred even when other cytokines were added to the cultures to try to force differentiation down another lineage (130). C/EBP-ε and PU.1 appear to be most important for the later stages of differentiation, especially expression of secondary granules (128). C/EBP-ε mice were not able to generate neutrophil or eosinophil secondary granules, and transfection of cells with C/EBP-ε only restored the formation of granules present in neutrophils. However, co-transfection of PU.1 fully restored the activity of the MBP promoter. In addition, PU.1 mutant myeloid cell lines from PU.1 knockout mice also did not synthesize eosinophil granule proteins until PU.1 expression was restored.

D. Mature Eosinophils: Effects of IL-5 and Role of Eotaxins

Mature eosinophils occupy about 3% of healthy human bone marrow (131). There are very few eosinophils in the blood of healthy humans or mice, and the range encompassing normal is 0-8% of white blood cells (132). The half-life of eosinophils in the blood of healthy individuals is estimated to be about 18 hours, although that can extend in eosinophilic states, depending on exposure to growth and survival factors. They are estimated to remain in tissues for up to two weeks (133). Relevant to this report, IL-5 and eotaxins have effects on mature eosinophils that alter their activation and survival.

i. IL-5 and mature eosinophils

IL-5 has effects on mature eosinophils, besides facilitating terminal differentiation and maturation. It can prolong the survival of eosinophils isolated from human blood or mouse peritoneal cavities in culture (119, 134, 135). It also activates eosinophils, indicated by ruffling of the eosinophil membrane, polarization of granules, increased superoxide production, and better killing and phagocytosis of antibody-coated organisms (108, 109, 135). Further, IL-5 acts as both a chemokinetic and chemotactic factor for eosinophils, inducing their migration using a positive gradient of cytokine (134). IL-5 inhibits apoptosis in mature eosinophils isolated from healthy humans (136). Finally, recruitment of eosinophils from the bone marrow into the blood is a key function of IL-5. The presence of eosinophils in the blood allows them to respond to other chemotactic factors to enter tissue, including eotaxins (below) (137).

ii. *Eotaxins and their receptor, CCR3*

The eotaxin chemokine receptor, CCR3, is a potent regulator of eosinophil chemotaxis and is therefore important for eosinophil infiltration into tissues. To identify CCR3, human genomic libraries were screened with cDNA of homologous chemokine receptors, or total human eosinophil RNA was reverse-transcribed and amplified with oligonucleotides specific for other chemokine receptors (138, 139). Human CCR3 has high sequence homology to CCR1 and CCR2 (63% and 51%, respectively) (138, 139) and mRNA for the receptor has been detected in eosinophils and at very low levels in neutrophils and monocytes, but not lymphocytes, monocytes, or neutrophils (138, 140).

CCR3 has several ligands, including members of the eotaxin family and RANTES, each of which triggers release of intracellular calcium stores, although eotaxin-1 acts through CCR3 at lower concentrations than RANTES (29, 138, 139, 141). When injected into the skin of a rhesus macaque, eotaxin specifically recruited human eosinophils, and not neutrophils or monocytes, while RANTES was more promiscuous and also recruited monocytes around vessels (29). In addition, eotaxin-1 had no effect on intracellular calcium stores in human neutrophils or monocytes (141).

Eotaxin-1 (CCL11) was the first member of the eotaxin family discovered, and was originally isolated from HPLC-fractionated bronchoalveolar lavage fluid of sensitized guinea pigs (142, 143). When the fraction containing eotaxin-1 was injected into the skin of new guinea pigs, eosinophils were found at high numbers, identifying it as a new molecule responsible for eosinophil trafficking (143). Human eotaxin-1 is 62% homologous to mouse and guinea pig eotaxin-1 and is expressed at high levels in the heart, small intestine and colon, as well as in many tissues in nasal polyps from allergic subjects, especially epithelium (29, 141). Eotaxin-2 (CCL24) and eotaxin-3 (CCL26) were identified by homology to eotaxin-1, using the published human genome. Both contain the first two cysteine-cysteine (CC) residues that characterize CC chemokines, and both recruit eosinophils through chemotactic gradients (144-147).

Eotaxins regulate polymerization of the actin cytoskeleton to induce chemotaxis in cells that express CCR3 (148, 149). However, they also activate eosinophils in other ways, including stimulating release of intracellular calcium

stores, production of reactive oxygen species, and upregulation of surface activation markers, such as CD69 (148-150). In addition, CCR3 has a role in eosinophil differentiation from progenitor cells, as it is upregulated on the surface of CD34+ hematopoietic precursors upon stimulation with Th2 cytokines (and repressed with IL-12 and IFN-γ) (151). Addition of eotaxin with IL-5 to undifferentiated cultures increases eosinophil lineage production, and progenitors can also be activated, indicated by intracellular calcium release, by exposure to eotaxin-1 (151). Finally, stimulation through CCR3 also increases surface expression of CD11b integrin (149).

iii. ICAM-1, VCAM-1 and eosinophils

Cell adhesion molecules (CAMs) are cell surface molecules that promote binding of cells to each other or the extracellular matrix (152). Intercellular adhesion molecule (ICAM)-1, or CD54, was discovered using a monoclonal antibody that prevented white blood cell clumping, and it is expressed ubiquitously on endothelial, epithelial and fibroblast cells, as well as on tissue macrophages and other leukocytes (153, 154). Vascular cell adhesion molecule (VCAM)-1 is expressed by endothelial cells, and can be induced on their surfaces by treatment with inflammatory cytokines, such as IL-1 or TNF-α (153).

Migration into tissues from the blood by blood cells is a multistep process. Selectins must be expressed by endothelial cells, followed by an initial weak binding of leukocytes, which correlates with their activation and slower movement. Expression of integrins by the blood cells leads to interactions with cell-adhesion

molecules, such as VCAM-1, that are expressed on the vessel wall. These interactions lead to firm adhesion, and the blood cell flattens, creates a pseudopod and migrates through the endothelial cell lining of the vessel (152, 155, 156).

Human eosinophils can express cognate receptor integrins for cell adhesion molecules, which are lymphocyte function associated antigen (LFA)-1 for ICAM-1 and very late antigen (VLA)-4 for VCAM-1 (157-160). VLA-4 is expressed without exogenous stimulation (159). ICAM-1 and VCAM-1 are required for eotaxin-mediated migration of eosinophils, as there are no eosinophils present in the peritonea of cell adhesion molecule knockout mice injected with eotaxin (161). In addition, cells that express ICAM-1 and VCAM-1 can activate eosinophils and cause degranulation (162, 163). Eosinophils can bind to a neuron cell line via ICAM-1 and VCAM-1-mediated adhesion (164).

Eosinophils present in allergic airways and nasal polyps of human atopic patients are "hyperadhesive," compared to eosinophils isolated from the blood (165). Interestingly, treatment of blood eosinophils with IL-5 induced increased adhesion on *in vitro* assays. Therefore, ICAM-1 and VCAM-1 are important mediators of eosinophil trafficking and migration.

E. Functions of Eosinophils

Development of eosinophils, their maturation and correct trafficking, as detailed above, are crucial for eosinophil-mediated immune functions. Eosinophils can act as antigen-presenting cells, with HLA-DR (MHC II in mice) appearing on their surfaces during differentiation and after cytokine or growth factor stimulation (166-168). If stimulated and then incubated with peptide, eosinophils induce T cell

proliferation *in vitro* (167). If Staphylococcal enterotoxin B (SEB) is added to eosinophil and T cell cultures, T cell production of both Th1 and Th2 cytokines is increased, including IFNγ, IL-5 and IL-13 (102).

In addition to co-stimulatory functions to adaptive immune cells, eosinophils have many direct immune functions on their own. The first immune function identified for eosinophils was phagocytosis by Mesnil in 1895. Horse eosinophils easily phagocytosed zymosan, or yeast cell wall components, and red blood cells in the presence of immune serum (82). Human eosinophils were shown to be able to phagocytose polystyrene spheres, Gram-positive and Gram-negative bacteria, antibody-coated red blood cells, and *Candida albicans*, both alive and dead (169). Eosinophils were not as efficient as neutrophils at phagocytosis, but it was clearly one of their specified immune functions. In fact, after attachment and degranulation of eosinophils onto helminths, new eosinophils perform phagocytosis of the membrane particles left behind (95).

Rats, mice and humans with parasitic infections have higher circulating levels of eosinophils, and more bone marrow production of eosinophils than healthy controls (109, 170). Eosinophils can mediate antibody-dependent damage to helminths, such as *Schistosoma mansoni, Trichinella spiralis,* and *Nippostrongyloides brasiliensis* (95, 171, 172). Specifically, eosinophils surround parasites and attach to them, crowding out other cells, such as neutrophils (Figure 1.5). A condensed excerpt from Densen et al, 1978 is included below to describe the interaction of eosinophils with helminthic parasites:

"*Within minutes, eosinophils and neutrophils were attracted to and made contact with the schistosomules* (S. mansoni)*. Despite close contact of some neutrophils with schistosomules, eosinophils were observed to "push" their way in and establish contact by displacing neutrophils already at the surface. After eosinophil had established contact, degranulation was observed in the presence of immune sera (but not normal sera) within the first hour of observation. Granules appeared to pop or explode when viewed at normal speed. Degranulation occurred only at the interface of the eosinophil and schistosomula. No neutrophil degranulation was observed...*

In experiments in which the schistosomula were allowed to move freely, increasing numbers of granulocytes became adherent over several hours in the presence of either immune or normal serum. Despite active and vigorous movement of the schistosomules, the granulocytes remained firmly attached. Wright-stained preparations showed that both eosinophils and neutrophils were attached to the schistosomula (171).

Detailed studies using phase-contrast and electron microscopy determined that the eosinophils not only attached to the parasites, but polarized their granule proteins to the apposed, or basal, membrane and released the granules from aggregated vacuoles directly onto the surface of the parasites (Figure 1.6). The eosinophil subsequently degenerated, and the surface of the parasite disintegrated, with the tegument shedding in large sheets, resulting over time in the eventual loss of shape of the helminth (95, 173). This process was found to be dependent on peroxidase (174) and mediated also by Major Basic Protein, albeit with lower effectiveness than the total granule composition (175).

Eosinophils also constitutively express many types of cytokines with extremely regulated secretion (176). Human eosinophils contain intracellular stores of preformed cytokines, with IL-13 the most abundant, followed by IFNγ, TNFα, IL-12p70, IL-10, IL-6 and IL-4 (176). These cytokines are all located in the secondary granules, and release of cytokines is strictly dependent on the type (Th1, Th2, inflammatory or regulatory) of signals that the cells are exposed to *in vitro.* Interestingly, cells stimulated with IFNγ or TNFα primarily released IL-4. In addition, exposure to Th1 or Th2 cytokines or TNFα resulted in release of IFNγ, whereas IL-12p70 was only released in response to IL-10. Eosinophils had no change in their release of IL-4, IL-6 or IL-13 when cells were pretreated with IL-10, indicating a predisposition to release these cytokines, even in anti-inflammatory environments. Finally, when cells were simultaneously stimulated with two cytokines and assayed for their secretion, the cumulative effect was as if each trigger had occurred separately, indicating that eosinophils might respond to several cytokines, possibly with conflicting messages, simultaneously (176).

F. Eosinophilia

Eosinophilia, or high levels of eosinophils in the blood, is caused by several distinct pathologies. It occurs during helminth-induced eosinophilia and immunity (177) and results in higher numbers of circulating eosinophils to battle parasites in all regions of the body. In addition, bone marrow production of IL-5 is modulated by inflammatory stimuli (178).

Allergen sensitization and challenge rapidly increases the number of eosinophils in blood and bone marrow, as well as concentrations of serum IL-5 (179). Expression of the IL-5Rα chain is also upregulated on the surface of asthmatic human bone marrow CD34+ cells after allergen challenge, indicating an increased responsiveness to the cytokine among bone marrow precursor cells (180). Atopic patients, including those with atopic dermatitis, generally have higher numbers of blood eosinophils (see below). Patients with the hypereosinophilic syndrome also have high levels of circulating eosinophils, although there is no known cause except for the myeloid proliferative version (reviewed in (181)).

Finally, eosinophilia is the hallmark characteristic of transgenic mice that over express IL-5 (121, 182-184). Mice overexpressing IL-5 on a T cell-specific CD3δ promoter have enormously enlarged spleens and livers, due to eosinophil production, and the bone marrow composition changes from 3% eosinophils in wild-type mice to 70% in transgenic mice. These mice develop skin lesions, alopecia and rectal prolapse in an age-dependent manner, although these phenotypes are not 100% penetrant (121). The authors specifically note that it is not possible to determine whether the formation of skin lesions is a direct response to IL-5 or an indirect response instead to pruritus induced at affected sites. However, it is clear in this model, and in the keratin-14-IL-5 transgenic mice also used in the present study, that recruitment of eosinophils to the skin can cause dermatitis as a primary outcome of IL-5 expression.

G. *Eosinophils in atopic dermatitis*

Eosinophils have been linked to atopic dermatitis for at least forty years, due to high numbers circulating in the blood of atopic dermatitis patients (185, 186). In addition, programmed cell death of eosinophils is significantly delayed in *in vitro* assays, compared to healthy eosinophils from non-atopic subjects, even above other atopic diseases (187). Serum concentrations of eosinophil granule proteins, including ECP and EDN, correlate with the severity of atopic dermatitis, as do the number of circulating eosinophils (188, 189). Serum levels of IL-5 are well above control levels, and recede during successful treatment (190). Peripheral blood eosinophils also have altered phenotypes, with more neurotrophin receptors and more functional activity in response to neurotrophins than eosinophils from healthy controls (191).

Intact eosinophils are not found in high numbers in atopic dermatitis biopsies, leading some to question whether these cells have a role in the pathogenesis of this disease. However, several studies have found evidence of eosinophil granules in lesional atopic dermatitis skin biopsies (192-195). One study, using electron microscopy, even specifically determined that the release of eosinophil granules was due to cytolysis and degeneration, rather than piecemeal degranulation (194) (Table 1). While the authors found no normal eosinophils in atopic dermatitis skin, many samples had eosinophils with abnormal granules, uropods, a significant loss in membrane, and regions of free eosinophil granules. This indicates that eosinophils are present in skin and have been activated to induce release of granules and cytokines from their intracellular stores, leaving no

recognizable eosinophils behind. However, not all atopic dermatitis patients have eosinophil granule proteins deposited in their lesions. This is likely due to the difference between intrinsic and extrinsic disease, the limits of detection of eosinophil granule proteins in the skin, and other unknown factors (196-198).

However, correlations between atopic dermatitis and eosinophils and their products in the skin and blood of patients do not identify the role of eosinophils in the disease. Atopic dermatitis is considered a hyperactivation of the immune system in response to innocuous stimuli (4), and it is possible that eosinophils are important mediators of this overreaction. The fact that they are activated and degranulated in tissues indicates an active participation in the process, although this does not indicate whether they are primary effector cells or recruited later to participate in tissue remodeling.

Eosinophils are not present in normal uninflamed human skin. Therefore, the presence of eosinophils in atopic dermatitis skin signifies recruitment to the skin. In atopic dermatitis, eotaxin-1 expression in the skin is increased, compared to healthy skin, with concomitant increases in CCR3+ cells (Figure 1.7). The main cells making eotaxin in lesional skin in this study were mononuclear cells, fibroblasts and eosinophils themselves (199). A subsequent study identified increases in eotaxin-1 in the epidermis of atopic dermatitis lesions, as well as in infiltrating inflammatory cells (200). Human keratinocytes can make eotaxins-1, -2 and -3, and this production is enhanced by IL-4 and IL-13, two Th2 cytokines that are elevated in atopic dermatitis and can be made by eosinophils (176, 201-204). Eosinophils produce IL-4 rapidly upon stimulation, indicating a potential feed forward

mechanism for further recruitment of eosinophils and other CCR3-expressing cells, such as mast cells and T cells (176). Endothelial cells can also produce eotaxins (205) and might increase eosinophil infiltration into the skin if upregulated in atopic dermatitis.

The known functions of eosinophils include antigen presentation, phagocytosis, and rapid release of cytokines, chemokines and granule proteins. Barrier defects in the skin allow deeper penetration of antigen, so it is possible that eosinophils act as phagocytes and antigen-presenting cells in atopic dermatitis by taking up foreign and endogenous particles and degrading or processing them.

Eosinophils can participate in tissue remodeling, mainly through their production of TGF-β (206, 207). Eosinophils can induce fibroblasts to increase production of α-smooth muscle actin and tenascin-C, which are important extracellular matrix proteins, and this activity is blocked with a TGF-β blocking antibody (206). Reduction of eosinophils in humans with asthma, using anti-IL-5 monoclonal antibodies, results in less airway remodeling and extracellular matrix deposition (208). IL-5 knockout mice, and mice treated with antibodies to IL-5, develop significantly less fibrosis and airway remodeling after antigen sensitization and challenge than wild-type or untreated mice (209).

Importantly, the most effective treatment for atopic dermatitis, topical tacrolimus, has effects on eosinophils and related molecules (190). In a time course biopsy study of patients using tacrolimus for 8 weeks, as disease was alleviated, serum levels of IL-5 were significantly decreased. In addition, expression of CCR3

and RANTES in the skin was also significantly lower, by immunohistochemistry, compared to the initial levels in each patient. Finally, intact eosinophils were decreased in tissue, although there was no significant decrease in blood eosinophils. These data indicate that eosinophils are important for the pathogenesis of atopic dermatitis, although their specific contributions are not yet fully understood.

III. SENSORY INNERVATION OF THE SKIN

A. Identification and general characteristics

Spinal sensory nerves that innervate the skin have cell bodies that reside in the dorsal root ganglia (DRG) or, for those that innervate the face, in the trigeminal ganglia. These ganglia lie in a chain on either side of the spinal cord, are 20-100um in diameter, and are derived from the neural crest, which migrates to the sites of final differentiation. Dorsal root ganglia nerves are pseudounipolar afferents, with one axon emerging from the cell body and dividing into one process that is directed centrally and a second process that is directed peripherally. The central process synapses on interneurons in the spinal cord that are located in lamina organized roughly according to their functions. The peripheral process extends directly to the region of skin to be innervated and arborizes across the dermis and epidermis. One peripheral process that innervates one region of skin can have many terminal branchpoints, and these terminals transduce sensory stimuli into electrical action potentials that travel to the central nervous system (CNS). Skin innervation begins during embryogenesis and is ongoing in adult skin. Regeneration of injured nerves occurs in skin, with characteristic expression of molecules that signify plasticity.

B. Development of DRG sensory neurons

Dorsal root ganglion neurons develop from the migrating neural crest, which separates from the neural tube during embryogenesis. The formation of spinal DRG is dependent on signals from the somites, which are masses of mesoderm alongside the neural tube, and the neural tube, as total removal or blockade of signals from either results in a complete absence of DRGs (210, 211). Tracking the progenitors by using tritiated thymidine indicates that the DRG cells, Schwann cells and the primary sympathetic chain complex all arise from this population of migrating crest cells (212). Differentiation occurs based on the distance each progenitor population migrates from the neural tube, and the local environmental signals (212).

Each DRG derives from neural crest cells from two successive levels of somites, the posterior of the one above and the anterior of the one below. (213). In a matter of hours after neural crest migration begins, aggregations of cells at distinct segmental locations began to appear and divide, forming each intact ganglion of cell bodies in a segmental manner between somites. These are located outside the spinal cord (Figures 1.8, 1.9).

C. Development of skin innervation during embryogenesis

The first nerve fibers arrive in the skin between the second and third trimester in humans as bundles of coarse fibers in deep layers of developing skin, then single fibers extend toward the epidermis, hair follicles, sweat glands and blood vessels (reviewed in (214)). Sensory neurons arrive first, then autonomic and motor neurons, and these can be differentiated by their individual cell markers. Autonomic nerves have many functions, including vasoconstriction, regulation of

sweat glands, and stimulation of arrector pili muscles to form “goose bumps,” based on emotional and thermoregulatory activators (reviewed in (215)).

The segmental development of the somites and spinal nerves leads to segmental innervation by sensory neurons. In humans, there are a total of 30 spinal nerves, 8 cervical, 12 thoracic, 5 lumbar and 5 sacral. Mice have 31, not including the tail. The region of skin specifically innervated by a single spinal nerve, which is a group of axons with cell bodies all located in a single DRG, is called a dermatome. The creation of human dermatome maps has been based mostly on observations of denervation after surgical removal of ganglia, outbreaks of shingles (which occur along one or two spinal sensory nerves), or other clinical cases of intact patients. Considering the paucity of actual physiologic data, the maps have been extremely variable. A recent attempt to formulate a dermatome map based on existing strong evidence is shown in Figure 1.10 (216).

Studies of skin innervation during development have determined that peripheral axons from skin sensory neurons in each DRG grow directly to their target skin, make a single invasion, and then arborize and add fine peripheral branches (217). The central process of each sensory neuron arrives at the substantia gelatinosa (sensory region) in the spinal cord before the peripheral process reaches the skin, at embryonic days 14-15 in humans and embryonic day 16 in the rat (218). Outgrowth of axons to the skin is not random. When neurons are relocalized to different parts of the trunk (for example, cervical to thoracic), correct innervation of the distal target limb by the peripheral neurite occurs, as if the DRG

had always been located in the thorax, and the central process of the DRG also innervates the correct level of spinal cord (219).

D. Skin innervation in the mature animal

Pre-natal sensory innervation of the skin is completed in the first week of life in rats, and the last weeks of gestation in humans (218). In a study of common laboratory animals, it was found that the pattern of innervation in the skin was similar in all animals studied, including mouse, rat, rabbit, guinea pig and hamster (220). A nerve plexus located under the skeletal muscle in the deep reticular dermis sends branches externally to supply the hair follicles, dermis and epidermis (220). Nerves can be detected using immunocytochemistry targeting a ubiquitin carboxy-terminal hydrolase, protein gene product 9.5 (PGP 9.5), which is specifically located in the cytoplasm of all neurons (221).

The epidermis is innervated by free unmyelinated nerve endings. One description of reconstructed thick-section biopsies by confocal microscopy with PGP 9.5 began, "The superficial layers of skin contain an interlacing horizontal network of nerve bundles that give off a series of diagonal branches that are clearly demonstrated...," and this is illustrated in Figure 1.11, taken from this paper (222). All layers of the epidermis are innervated, generally by small diameter fibers.

Interestingly, the extent of epidermal innervation in humans varies according to a rostral-caudal gradient, with decreasing nerve density in more distal regions of the leg (223). This also occurs in mice, based on my own observations of different nerve densities between dorsal cervical and lumbar skin. Density can be measured

as number of nerve terminals per high-powered field, or can be expressed as a ratio to the epidermal basement membrane zone length (223).

The dermis is innervated by a variety of sensory neuron subtypes that detect heat and cold, mechanical stimulation, pain and itch. In addition to afferent activities, neurons innervating the skin can also act as efferents, releasing molecules that cause vasodilation, inflammation and recruitment of immune cells.

E. T*ypes of Sensory Neurons*

Subtypes of sensory neurons can be identified in a variety of ways: through their morphology, functions or expression of receptors, transcription factors and signaling molecules. These different specifications delineate overlapping subpopulations of neurons, and no single set of descriptors is sufficient to fully describe a single neuron.

i. Classification by morphology

The earliest delineation of sensory neurons was by cell morphology in the DRG. This technique separated cells into large and small cell bodies (224). While this separation is not completely accurate, because each population has a normal distribution of cell body sizes and the two distributions overlap, it gives a rough approximation of the two main sensory neuron populations known today by morphology. These are the neurons with A fibers (generally larger cell bodies) and those with C fibers (generally smaller cell bodies).

Neurons with C fibers make up approximately 60% of DRG neurons. These neurons have unmyelinated small diameter nerve fibers (~1um or less) and relatively slow conduction velocities of around 1 m/s (224, 225). Neurons with A

fibers can be subdivided into those with Aβ fibers, which are myelinated, have thick diameters and conduct action potentials around 100 m/s, and those that have intermediate Aδ fibers, which are thinly myelinated and have intermediate conduction velocities.

ii. Classification by function

Nerve terminals in the skin are triggered to fire by a variety of different stimuli, and this serves as a separate method of classification. Neurons are classified as mechanoreceptors, thermoreceptors, chemoreceptors, or nociceptors, based on the type of stimulus that induces action potentials within them. Mechanoreceptors transduce contact that deforms the skin, thermoreceptors detect heat and cold, chemoreceptors fire when exposed to products of injury and inflammation, and nociceptors fire only when the intensity of a stimulus approaches levels at which damage to the tissue could occur. Because of this, many nociceptors, or pain sensors, are polymodal and can transduce several different types of stimuli (reviewed in (225)). Nociceptors include neurons with C and Aδ fibers.

iii. Classification by expression of transcription factors, surface molecules and neurotransmitters.

More recently, with modern molecular identification techniques, subtypes of neurons have been classified by their expression of certain functional molecules, transcription factors and neurotransmitters. Expression of different transcription factors determines sensory sublineages. This occurs during development, long before innervation of the peripheral or central targets occurs (reviewed in (226)).

Because this classification is ongoing, and not immediately pertinent to the body of work presented here, it will not be discussed in detail. Some mention of different nerve types will be detailed below, especially relating to future directions.

F. Itch sensation

Until recently, it was not clear that there was a dedicated neural pathway for itch (a "labeled line") that was separate from pain sensation (controversy reviewed in (227)). However, it is now known that itch sensation is transduced from the skin via primary afferents with cell bodies in the DRG, which then synapse on neurons in the outer lamina of the dorsal horn (228-233). Itch is transmitted by C fibers, and there are distinct populations for histamine-induced itch and histamine-independent itch. Histamine-induced itch activates nociceptors that are not responsive to mechanical or heat stimuli, and these fibers have no role in transducing pain (233). Activation of these neurons can be visualized by the rapid production of Fos protein, which occurs in lamina II of the dorsal horn after intradermal injection of histamine (230).

A different population of afferent C fibers is stimulated by itch induced by cowhage spicules. These histamine-independent neurons have a different time response to itch, with a faster dissipation of the itch sensation than injection with histamine (234). In addition, these fibers are mechano- and heat-sensitive, otherwise known as polymodal nociceptors (235, 236). The itch-inducing factor in cowhage is a cysteine protease called mucunain that triggers proteinase-activated receptor-2 (PAR-2, also known as protease-activated receptor-2). Histamine and

cowhage never activate the same neuron, even though they are located near each other in the spinothalamic tract of the spinal cord (231).

Besides histamine receptors and protease-activated receptors, a third receptor for itch is Mas-related GPR, or Mrgpr. Expression of this family of G-protein couple receptors only occurs in a subset of small-diameter neurons in the DRG and trigeminal ganglia (237). One activator of these receptors is chloroquine, which is known for producing itch as a major side effect of malaria prophylaxis (228). Targeted deletion of the Mrgpr family locus in mice reduced chloroquine-induced scratching, while retaining normal responses to a variety of painful stimuli.

The three itch receptors discussed above were identified on primary afferent neurons that supply the skin. In addition, recent discoveries have occurred related to the second-order neurons in the spinal cord, which are important for receiving the primary itch signals and passing them on. Both genetic mutation and targeted destruction of gastrin-releasing peptide receptor (GRPR)-expressing neurons in the dorsal horn completely prevented scratching by mice after exposure to a variety of pruritogens (232, 238). However, the mice did not lose motor capabilities or their ability to sense pain. Interestingly, both histamine-dependent and –independent itch was inhibited by deletion of GRPR+ neurons, indicating a single common pathway for transducing different kinds of itch sensations from the periphery. These recent reports have given the first direct evidence for a labeled-line neural circuit specifically for itch and have given patients suffering from chronic itch hope for respite.

G. Itch and atopic dermatitis

Unlike pain, which provokes a motor reflex of withdrawal, itch sensation provokes reflex and conscious mechanical movement *toward* the stimulus, even in sleep. Speculation about the role of pain (after scratching) in inhibiting the itch sensation has been confirmed by recent data in primates. After intradermal injection of histamine, spinothalamic tract neurons are activated for many minutes. However, scratching in the cutaneous field that had been treated inhibited responses to histamine in the spinothalamic tract. This only provided relief if the skin was scratched *after* histamine was applied. There was no change in responsiveness to painful stimuli with scratching (229).

Atopic dermatitis patients suffer from chronic itch, and scratching yields immediate relief. Unfortunately, scratching also damages the epidermal barrier, allows penetration of surface microbes into deeper layers, and aggravates the disease. People with atopic dermatitis have abnormal sensation in their skin and feel itch instead of pain when given mechanical, chemical, electrical and heat stimulation (239, 240). A similar phenomenon occurs in healthy subjects after pre-treatment with histamine. However, histamine is not responsible for the itch in atopic dermatitis, as evidenced by the lack of efficacy of non-sedating anti-histamines against scratching (241). Currently, it is not known what causes the increased sensation of itch in atopic dermatitis skin. One possible mechanism is central sensitization to itch, or modification of the threshold for second-order itch neurons. Other hypotheses include increased expression of PAR-2 receptors in the

skin, increased itch-inducing interleukins, activation of keratinocytes by infiltrating antigens, or an increase in the number of nerve terminals that transduce itch (242).

H. Quantification of nerves in skin

The quantification of nerves in skin by immunohistochemical staining has been used in the diagnosis of peripheral neuropathy, and published guidelines were created by the European Federation of Neurological Societies (243). These guidelines specifically focused on intraepidermal nerve fibers (IENF), but the methods for determining density, usually by anti-PGP 9.5 immunostaining, are applicable for full biopsy thickness, including dermal nerves (244, 245). PGP 9.5 also labels some neuroendocrine cells, which are found in the lungs and elsewhere (246).

Interestingly, the relationship between density of IENF and neuropathic pain is not completely clear. In some diseases, such as HIV or diabetic neuropathies, a lower density of nerves correlated with more pain. In other situations, including recovery after steroid treatment, having more nerves correlated with more pain (reviewed in (244)). It is not known what causes the chronic pain of peripheral neuropathy, but in general, denervation of cutaneous structures such as sweat glands, hair follicles and epidermis is a characteristic hallmark of severe sensory neuron deficit.

In contrast to peripheral neuropathy, there are other diseases that are linked to increases in nerve density in the skin, including prurigo nodularis, notalgia paresthetica and, most importantly for this report, atopic dermatitis. Prurigo nodularis is a disease solely characterized by itch, in which patients scratch their

skin surfaces until lesions form. There is a massive expansion of nerves in the dermis of lesional skin biopsies from these patients compared to healthy control skin (247). Later examinations found that there were increased numbers of PGP 9.5-immunoreactive fibers throughout the dermis and epidermis (247). Interestingly, a subsequent study of lesional prurigo nodularis skin found large depositions of eosinophil granule proteins (248).

Notalgia paresthetica is a form of sensory neuropathy which is often characterized by itch, burning pain or tenderness between the scapulae on the back. When examined for PGP 9.5 immunoreactivity, there were more positive fibers, particularly in the epidermis and the upper papillary dermis near the basement membrane, in affected skin than in unaffected areas from the same patients, or compared to healthy control skin (249). Because these two diseases, as well as atopic dermatitis, are mainly characterized by itch and have more nerve terminals in the skin, it is possible that nerve density and itch are not only correlated, but possibly have a causal interaction.

I. Nerves in atopic dermatitis

1. Increase in number of nerves in atopic dermatitis

Atopic dermatitis also has an increase in the number of nerves in lesional skin compared to healthy control skin, as well as compared to uninvolved, non-lesional skin (250-254). In a study of 64 patients with atopic dermatitis, defined by the Hanifin and Rajka criteria, Sugiura et al found increases in PGP 9.5-immunoreactive nerves in atopic lesional skin compared to healthy control skin and non-lesional skin from the same atopic dermatitis patients (Figure 1.12). There was a significant

increase in nerve density in the papillary and upper dermis, and near the dermal-epidermal junction in all but the earliest lesional skin (Figure 1.13). These authors reported hypertrophy of nerve bundles in the subepidermal area. Another study with 16 atopic dermatitis patients found increases in PGP 9.5 staining in the papillary dermis and dermal-epidermal junction of atopic dermatitis lesional skin (252).

Using electron microscopy, it has become clear that the nerves in lesional atopic dermatitis skin also have altered ultrastructural appearance. The axons of nerve fibers are often bulging, are swollen and vacuolized, and/or are undergoing demyelination and fibrosis (65, 251). The distribution density and diameter of nerve fibers is also significantly higher in lichenified atopic skin compared to normal controls, upon electron microscopic evaluation (252).

2. Types of nerves increased in atopic dermatitis

The subtypes of neurons that are increased in atopic dermatitis have been characterized by their location, morphology, and expression of characteristic molecules in several studies. Because the major regions of difference in PGP 9.5 staining were in the papillary and upper dermis, and occasionally the epidermis, several groups argued that sensory neurons were specifically increased, because the main nerves populating those regions are sensory nerves (251, 253).

Nearly all of the nerves in atopic dermatitis papillary dermis and epidermis are unmyelinated (252). This morphology is most characteristic of C fibers, but it is possible that A fiber myelin sheaths have been degraded by the disease process.

Several other studies investigated particular subtypes of nerves, using specific markers and found varying results, depending on the marker (252, 253, 255). In general, markers for sensory neurons, such as neurofilament-200kD, were increased in atopic dermatitis skin. Results with Substance P (SP), a C-fiber product released upon activation, were equivocal due to differing results in different studies (252, 253, 256). One C fiber marker that was consistently increased was calcitonin gene-related peptide (250, 252, 253). Autonomic nerve fiber markers did not change in the papillary or upper dermis (252). Therefore, the small amount of existing evidence currently implicates unmyelinated sensory fibers that express molecules characteristic of C fibers as the source of the increased nerve growth in atopic dermatitis.

Finally, growth factors for nerves, known as neurotrophins, are increased in atopic dermatitis patients. Elevated levels of nerve growth factor (NGF) have been found in the skin lesions and plasma of patients with atopic dermatitis (257, 258). Importantly, plasma levels of NGF and SP correlated with disease activity assessed using two scales: the objective Severity Scoring of AD (SCORAD), and the Eczema Area and Severity Index (EASI) (258). More severe clinical disease correlated with higher levels of NGF and SP.

IV. EOSINOPHILS AND NERVES

Eosinophils were initially described as neurotoxic, based on experiments injecting isolated granules into the brains of laboratory animals. However,

interactions between eosinophils and nerves *in vitro* and *in vivo* appear to be more subtle, and can contribute to pathogenesis of allergic diseases, such as asthma.

A. Cytotoxicity of isolated eosinophil granule proteins

Eosinophil granules are cytotoxic when applied at high concentrations to a variety of cells in culture (99-101) and when injected intracerebrally into live animals (90, 259). In addition, isolated granule proteins have been instilled intratracheally into animals, which induced tachypnea and decreases in tidal volume. These effects were inhibited by treatment with capsaicin, which triggers sensory nerves to release neurotransmitters (260).

However, the release of granule proteins, preformed cytokines and other regulatory molecules from eosinophils is tightly controlled and can occur through regulated vesicle secretion, called piecemeal degranulation, or whole cell degradation, called cytolysis (94). After release from the cell, eosinophil granules continue to be regulated by receptor-ligand interactions and can activate signal transduction to secrete cytokines or any of the granule proteins (98).

In all of the experiments which found eosinophil cytotoxicity, the granule proteins were isolated by sonication, low pH, freezing and thawing, or otherwise complete disruption of the cell, which resembles neither cytolysis nor piecemeal degranulation. In addition, the concentrations of granules used were supraphysiological, from enormous numbers of eosinophils, which would increase the probability of seeing an effect. In the papers describing eosinophil effects on sensory neurons in the lung, there were no dose-response curves demonstrated, so it was difficult to determine how the appropriate dose of protein was determined.

Therefore, the experiments describing neurotoxicity of eosinophil granules after sonication and collection of total granule protein are unlikely to be modeling true cell-cell interactions.

B. Intact eosinophils have beneficial effects on nerves in vitro.

In contrast to the reports of cytotoxicity, co-culture experiments with nerves indicate a pro-survival function for eosinophils. Eosinophils isolated from various compartments constitutively synthesize specific neurotrophins, including nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), and neurotrophin-3 (NT-3), and they can be triggered to release these factors upon stimulation (191, 261). In co-cultures with a cholinergic nerve cell line, eosinophils adhere to the nerves, and activate release of acetylcholine, upregulation of muscarinic receptors on the surface, and surface adhesion molecule expression (262, 263). Furthermore, when nerves were cultured in serum-deprived medium or exposed to cytokines to trigger apoptosis, adding live eosinophils prevented programmed cell death (264). Later characterization showed that MBP-1, but not EDN, dose-dependently inhibited apoptosis of neurons, through phosphorylation of ERK 1/2 and expression of the anti-apoptotic gene Bfl-1.

C. Interactions between eosinophils and nerves in vivo

Eosinophils and nerves also interact *in vivo*, and this interaction is responsive to changes in the health and inflammatory state of the animal or human. The gastrointestinal tract is a rich source of eosinophils under normal conditions, and eosinophil-nerve interactions are increased in response to parasite infection in the gut-associated lymphoid tissue (GALT) (265, 266). In rats infected with *Fasciola*

hepatica, the numbers of eosinophils and mast cells significantly increase in the colon, probably due to increased expression levels of IL-5 and eotaxin-1. Eosinophils contact nerves significantly more often in infected colon, compared to the predicted amount of random association, based on the numbers of cells present. Importantly, in gut parasitic infections, when eosinophils are recruited into the GALT and contact nerves, the number of nerve fibers per millimeter is significantly increased compared to control. In addition, in certain regions, the overall volume occupied by these nerves is also significantly increased. The nerves in the lamina propria of colons from infected rats express neuronal growth and plasticity markers, GAP-43 and NCAM, signifying their state of activation and growth (265).

Eosinophils also interact with nerves in the airways of patients with asthma, in animal models of asthma, and in culture. Eosinophils are found adjacent to nerves in airway biopsies of humans who died from asthma attacks, a histological finding that is recapitulated in antigen-challenged guinea pigs and rats (267). Airway nerves in asthmatic human tissue express eotaxin-1, which could be a mechanism to recruit the eosinophils that surround them (268). The association of eosinophils with airway nerves is important in the pathophysiological changes that lead to airway hyperreactivity (267, 269), as the airways with the most eosinophils around nerves had the most extreme muscarinic receptor dysfunction in antigen-challenged guinea pigs.

D. Sensory neurons and eosinophils

However, with all of these descriptive and physiological data, it is not known which subtype(s) of airway nerves attract eosinophils, since nerve bundles include

both sensory and autonomic fibers, nor which nerves in the GALT are in contact with eosinophils. Primary cultures of parasympathetic neurons from guinea pig and human airways express eotaxin-1, as well as ICAM-1 and VCAM-1, and these participate in binding of eosinophils to neurons in co-cultures (164, 268).

In clinical case reports describing interactions of eosinophils with nerves in human patients, sensory neuropathy is the most common symptom. In patients with hypereosinophilic syndrome, symptoms can include multifocal neuropathy, diffuse and chronic itching, rash, and peripheral and central nervous system dysfunctions (270-273). These data indicate that eosinophils have effects on sensory neurons *in vivo*, and that normal interactions between these two cell types that might be helpful for disease prevention or immunity could be disrupted in disease states.

Evidence for this hypothesis comes from a single paper (274). A patient with idiopathic hypereosinophilic syndrome (HES) who was exhibiting substantial peripheral neuropathy was examined and electromyogram (stimulation of the muscle with an electrode to detect nerve activity) studies indicated both denervation and reinnervation occurring in the anterior leg. Eosinophils were isolated from the HES patient and three healthy subjects to culture with chick sensory neurons. After 3-4 days of culture, there was a major decrease in survival of neurons cultured with HES eosinophils, compared to healthy eosinophils. This was demonstrated very clearly with different titrations of eosinophils to nerve cells. A sural nerve biopsy did not show infiltration with intact eosinophils, but the authors suggested that eosinophil products could have been present without detection.

V. HYPOTHESIS AND RESEARCH QUESTIONS

From this background, we know that eosinophil granules are present in the skin in atopic dermatitis lesions, and that nerve density is also increased in these lesions. We know that eosinophils have dramatic effects on the function of nerves in the airways in allergic airway disease. Therefore, we wanted to determine whether eosinophils could exert direct effects on sensory neurons in ways that would be relevant to atopic dermatitis, by increasing the density of their nerve terminals, altering their sensitivity to firing upon stimulation, or changing the release of neurotransmitters or other mediators.

To determine this linkage, we hypothesized that the eosinophil granules in the skin of atopic dermatitis would be located in the papillary dermis, where neurons were increased in number, and that eosinophils would directly affect sensory neurons in culture and in mouse models. More specifically, we hypothesized that neuron morphology, function or neurotransmitter release would be altered by eosinophils.

Therefore, we asked the following research questions in pursuit of this hypothesis:

1. Are nerves and eosinophils or their granules located in proximity in human atopic dermatitis skin?
2. Are eosinophils required for increased nerve growth in mouse models of atopic dermatitis or models of recruitment of eosinophils to the skin?
3. Do eosinophils have a direct effect on neuron morphology, function, or neurotransmitter release *in vitro*?

VI. TABLES.

Table 1.1. Selected epidemiologic studies of atopic dermatitis prevalence.

Location of study	Age of subjects	Prevalence of AD	Reference, Year
United Kingdom	3-11 years	20%	Kay, 1994
Worldwide	6-7, 13-14 years	5 - 20%	Williams, 1999
Oregon, United States	5-9 years	6.8 - 17.2%	Laughter, 2000
Japan	7-12 years	15 - 24%	Yura, 2001
United States	All ages	2.4 – 2.6%	Ellis, 2002
United States	All ages	6%	Hanifin, 2007

Table 1.2. Histologic characterization of different stages of atopic dermatitis by epidermal and dermal findings. Adapted from (65).

Type of lesion	Epidermal findings	Dermal findings
Acute lesional	spongiosis	lymphocytes, macrophages, rare eosinophils and neutrophils
Chronic lesional	hyperplasia, minimal spongiosis	lymphocytes, monocyte-macrophages, increased mast cells
Lichenified plaque	hyperkeratosis, hyperplasia, focal spongiosis	moderate infiltrate of lymphocytes and monocyte-macrophages
Non-lesional	hyperkeratosis, hyperplasia, spongiosis	slight infiltrate of lymphocytes

Table 1.3. Electron microscopic observation of eosinophils in atopic dermatitis skin. From (194).

TABLE I. Eosinophils in atopic dermatitis

Patient No.	Age (yr)	Gender	Serum IgE (U/ml) (Normal, < 367)	Serum MBP (ng/ml) (normal, 87-287) (mean = 185, SD = 46)	Body surface involvement (%)	Tissue MBP extracellular* (normal, 0)	Electron microscopy					
							Normal eosinophils	Eosinophils with abnormal granules	Eosinophils with abnormal granules and uropods	Loss of cytoplasmic and/or nuclear membrane (>75%)	Regions of free eosinophil granules	Total identifiable eosinophils†
1	39	M	23,738	1,264	70	+3	0	10	10	30	10	50
2‡	11	F	76	292	50	0	0	0	0	0	0	0
3	52	M	8,250	778	80	+2	0	2	6	4	2	12
4	30	F	1,917	2,430	20	+3	0	0	0	0	4	0
5‡	10	F	459	398	75	+2	0	1	1	1	0	3
6‡	31	F	27,002	334	45	+2	0	2	6	2	3	10
7‡	9	M	1,257	389	30	+1	0	0	0	0	0	0
8‡	50	M	9,557	300	45	+1	0	1	0	1	0	2
9	41	M	10,281	385	70	+1	0	3	1	2	0	6
10‡	39	M	8,400	337	60	+1	0	0	0	0	0	0
Total							0	19	24	40	19	83

SD, Standard deviation

*Extracellular protein deposition based on intensity and extent of fluorescence in tissue.

†Sum of normal eosinophils, intact eosinophils with granule abnormalities, intact medusa cells, and cells with loss of cytoplasmic and/or nuclear membranes; note that regions of free granules are not included in the total.

‡Patients 2, 5, 6, 7, 8, and 10 in this study were patients 6, 16, 12, 4, 7, and 11 in the study by Ott et al.[9]

VII. FIGURES

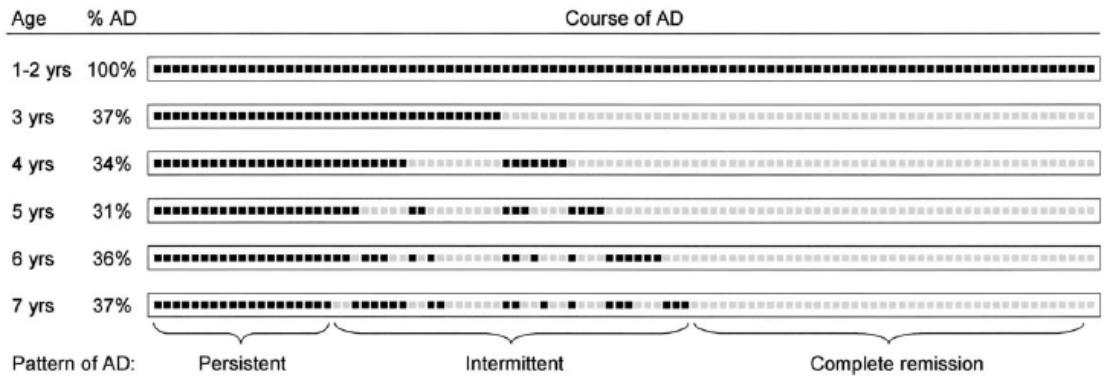


Figure 1.1. Natural course of atopic dermatitis up to 7 years of age in children with early manifestation of disease (<2 years). Each symbol represents 1% of children with early atopic dermatitis, and natural course of disease can be traced vertically. Of those with persistent disease, all developed it in the first two years of life and most began with severe disease. Used with permission, RightsLink license #2371580086428, (8).

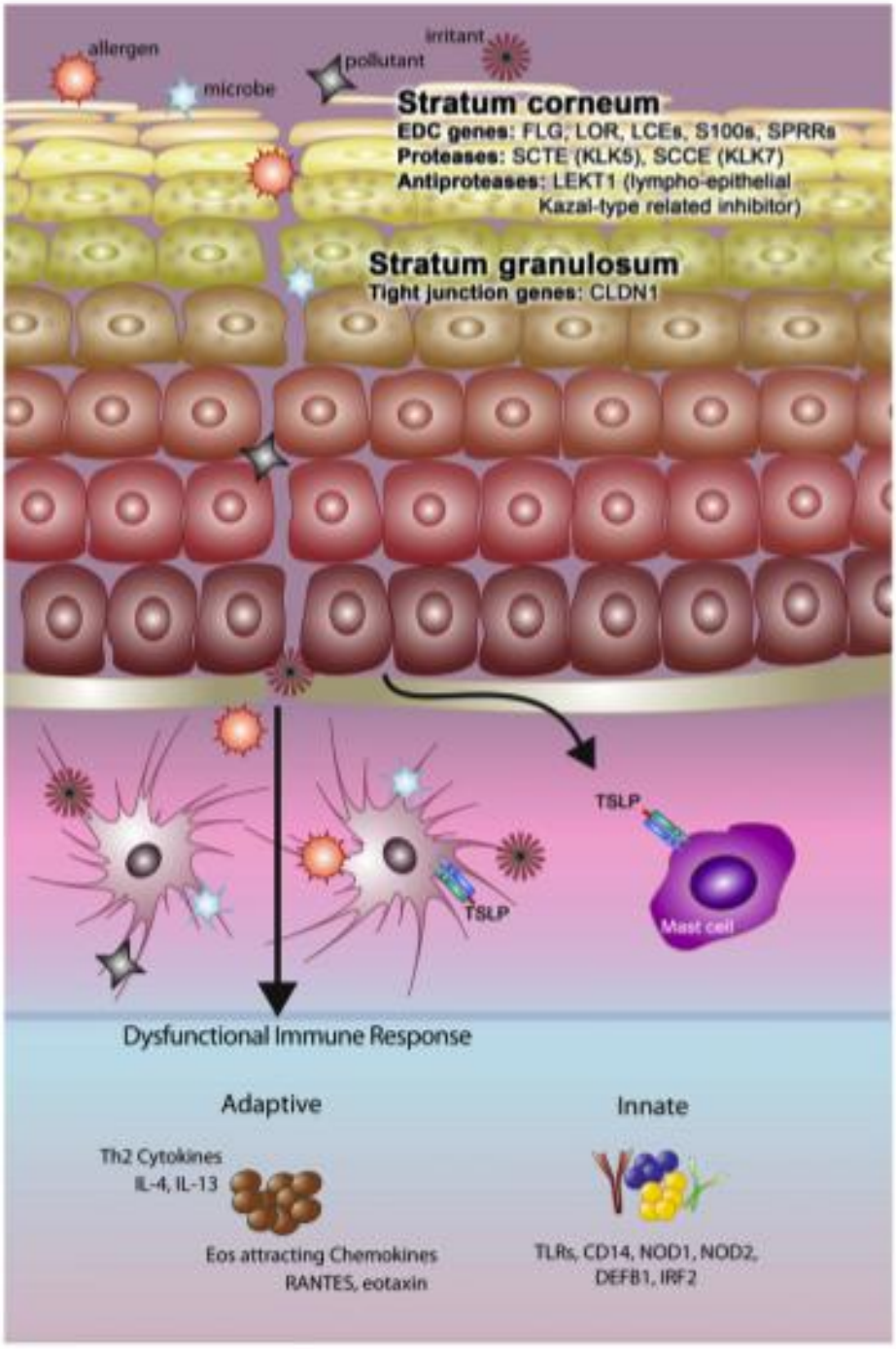


Figure 1.2. Model of genetic associations in atopic dermatitis. Each gene listed has been identified in genetic testing in atopic dermatitis. Note the "eosinophil-attracting chemokines:" RANTES and eotaxin. Used with permission from (25).

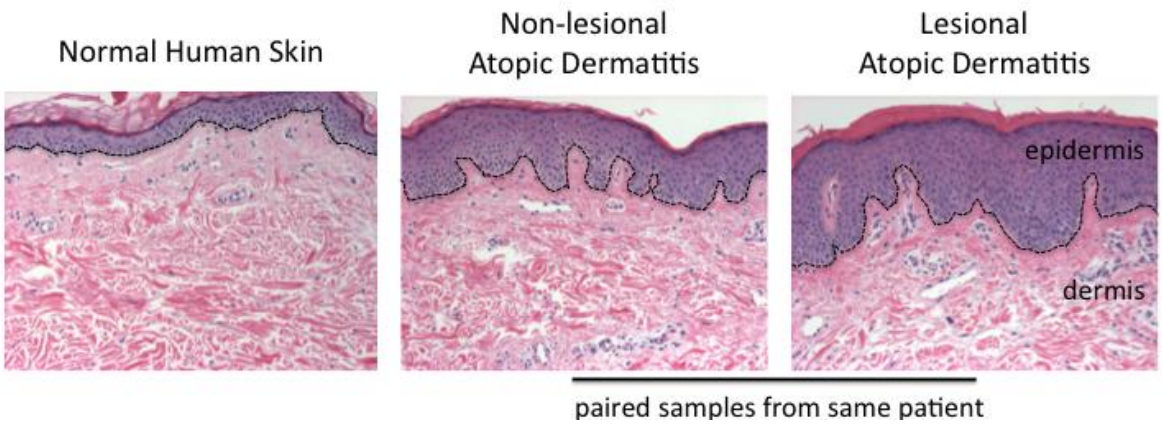


Figure 1.3. H&E stained sections of skin. Normal human skin (left) has a thin epidermis with little dermal infiltrate. Histologic changes are apparent in clinical normal-appearing non-lesional atopic dermatitis (middle), with the beginnings of rete pegs from the epidermis and infiltration of cells into the dermis. Lesional atopic dermatitis from the same individual (right) has much thicker epidermis, with focal areas of spongiosis and many more immune cells invading the dermis.

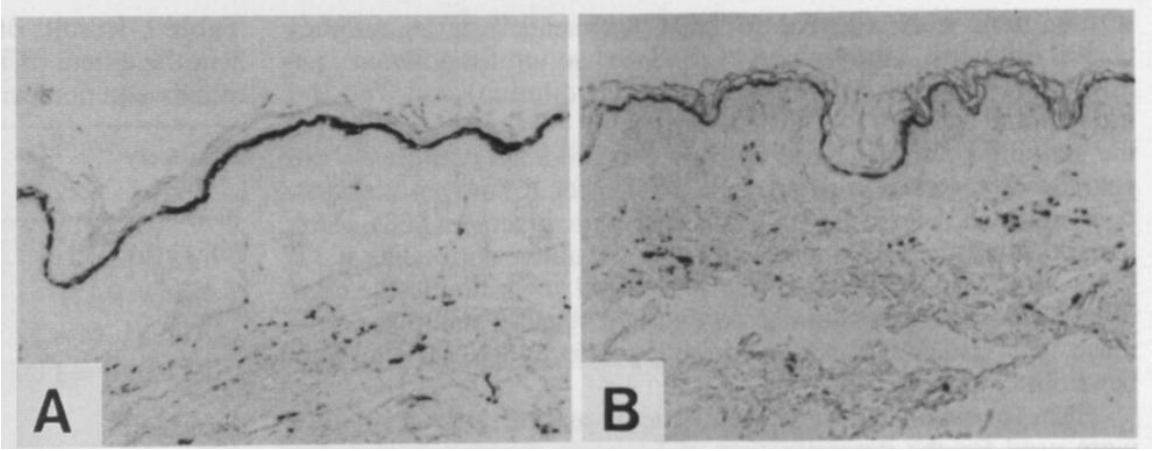


Figure 1.4. Expression of filaggrin is decreased in atopic dermatitis lesions. Paraffin-embedded skin biopsy sections were stained using anti-filaggrin antibody (dark black). The stratum corneum of healthy skin (A) has plentiful filaggrin staining, while atopic dermatitis skin (B) does not. From (275), with permission.

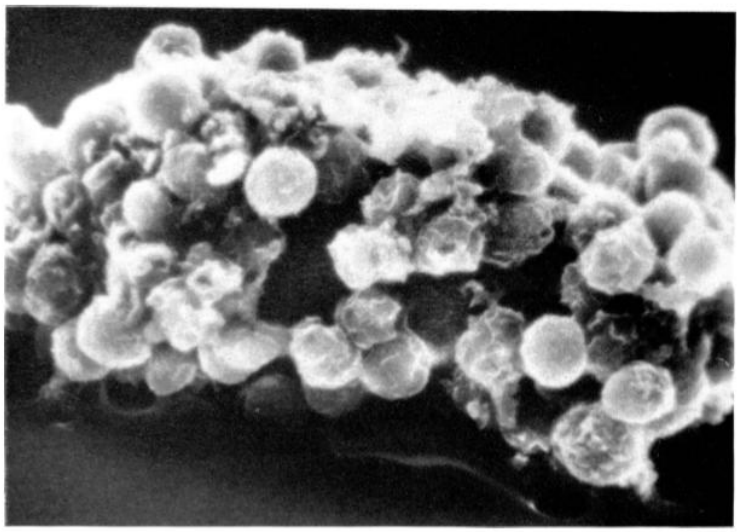


FIG. 2. Scanning electron micrograph (SEM) showing eosinophils adhering to the surface of schistosomulum of *S. mansoni* after 1·5 hr in culture. (Magnification × 157C.,

Figure 1.5. Eosinophils attached to surface of a schistosomulum. From (95).

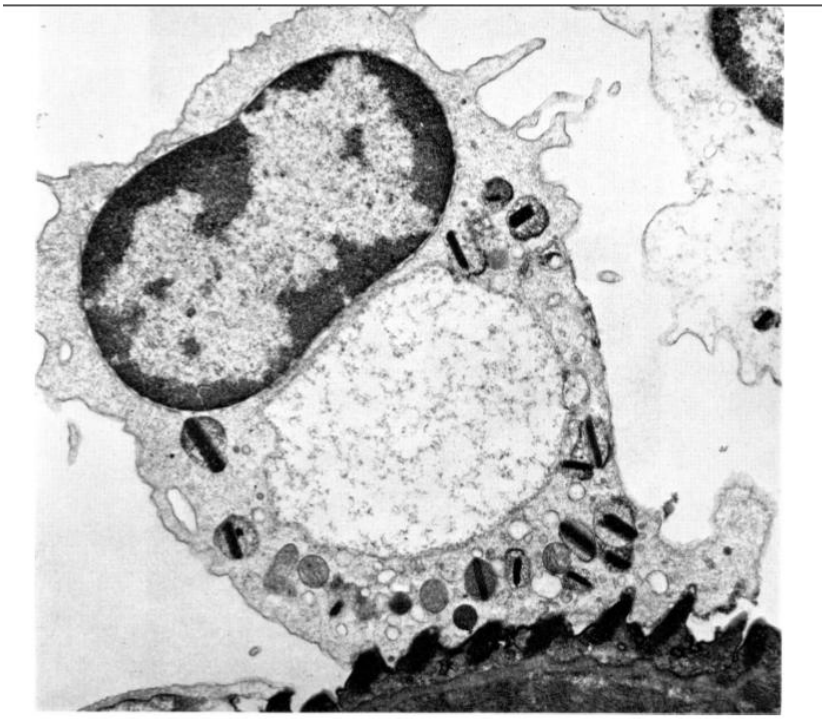


FIG. 9. TEM of an eosinophil with a large cytoplasmic vacuole. The cell is adhering to the surface of a schistosomulum. (Magnification ×16,250.)

Figure 1.6. Eosinophils release granule proteins onto helminths. Eosinophil granules polarized toward helminth surface, then fusing with internal vacuole in preparation for release. From (95).

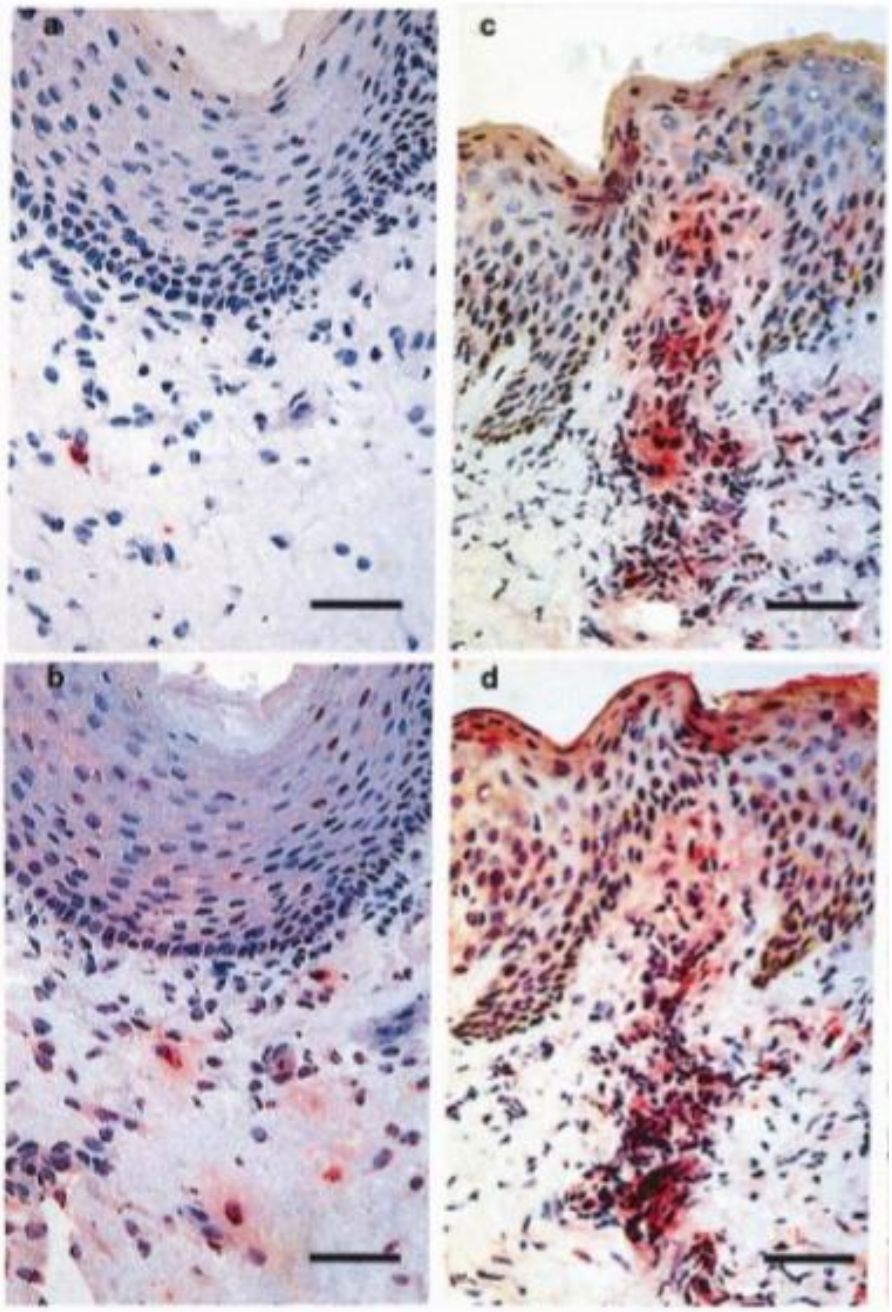


Figure 1.7 Eotaxin expression is increased in atopic dermatitis skin. Normal healthy skin does not express much eotaxin-1 (a) or CCR3 (b), while atopic dermatitis lesional skin has higher expression of both (c, eotaxin-1; d, CCR3). From (199), with permission.

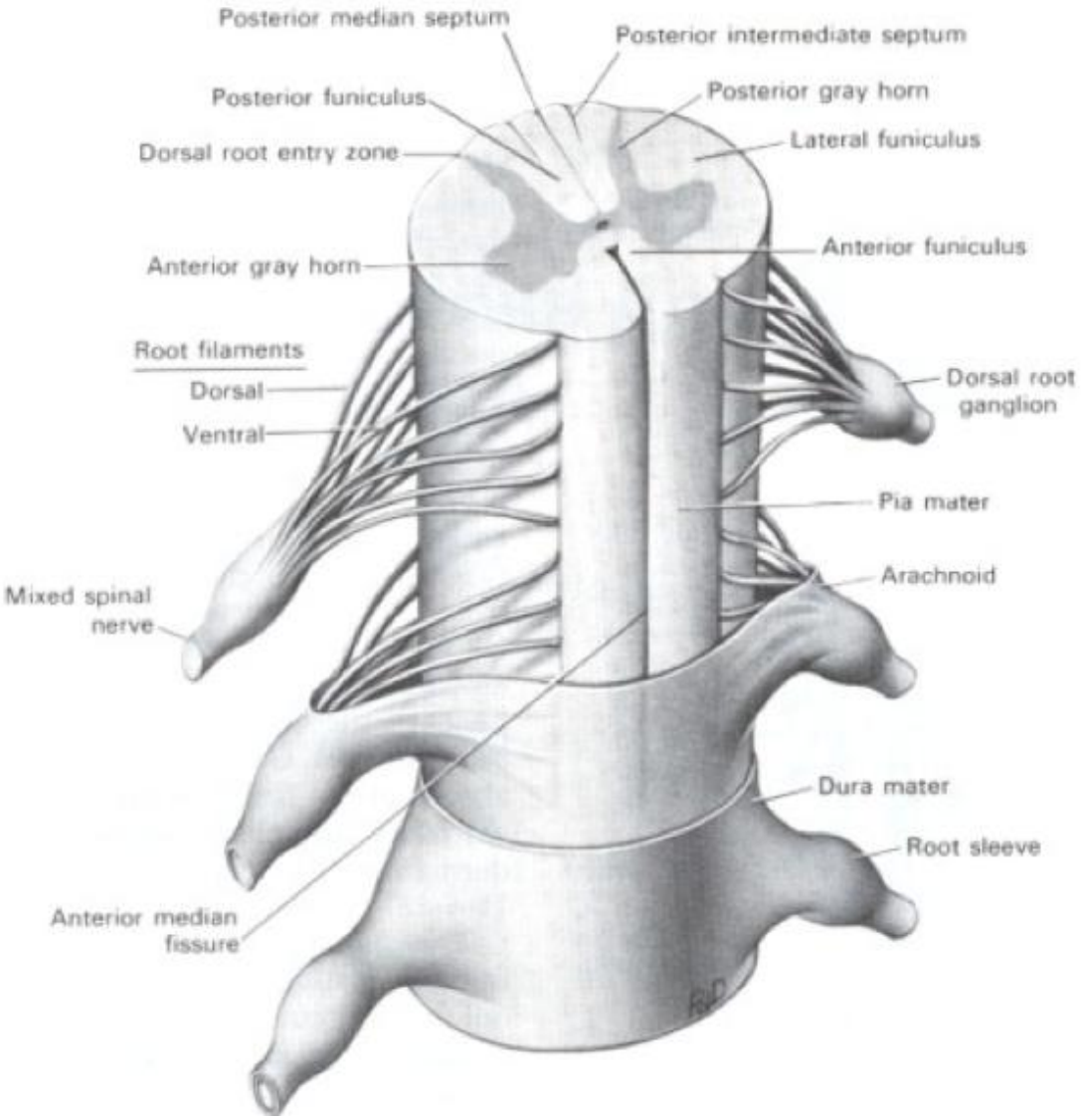


Figure 1.8 Representation of the spinal cord. This illustrates the location of the dorsal root ganglia, outside the spinal cord, in the intraforaminal space. Unknown artist's rendition.

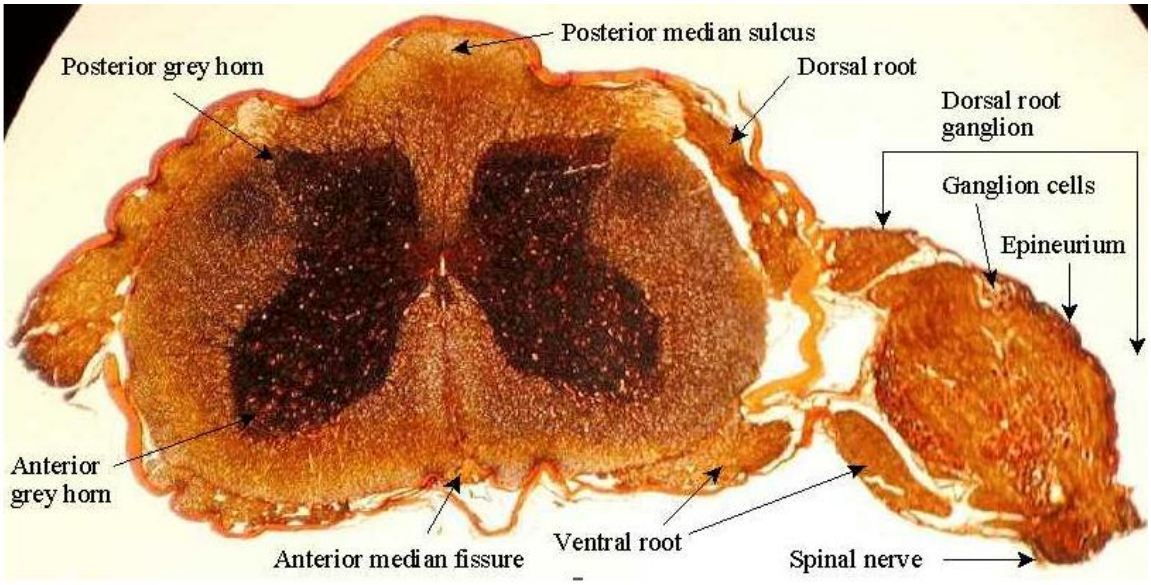
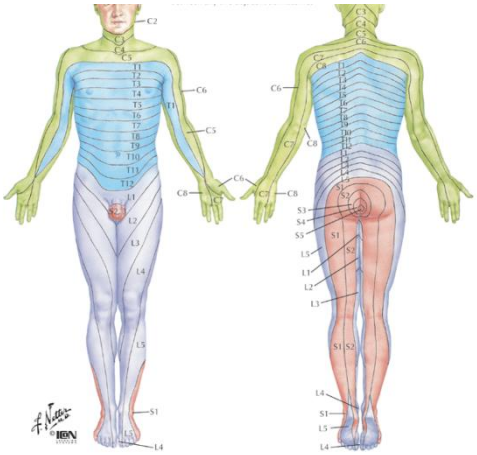


Figure 1.9. ***Ex vivo*** **image of intact spinal cord with attached dorsal root ganglion.** Photograph used with permission of David Fankhauser, PhD, University of Cincinnati Clermont College.

(A)



(B)

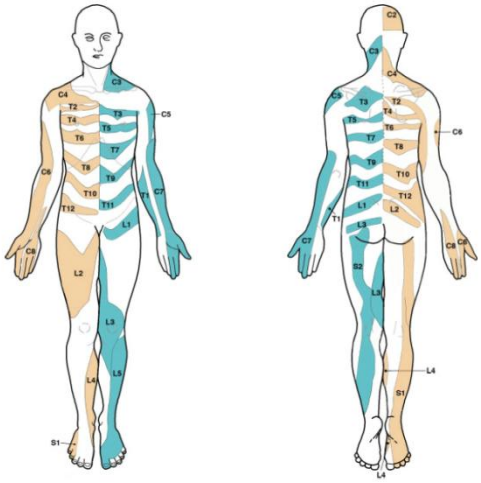


Figure 1.10. Dermatomes of the human body. (A) Traditional dermatome map of the body with spinal nerves. Each region of skin is supplied by a single afferent spinal nerve, color-coded with the skin. C = cervical ganglion, T = thoracic, L = lumbar, S = sacral. Drawing by Frank Netter.

(B) "Evidence-based" dermatome map, based on data from all previous studies. Blank areas are regions of considerable variability or overlap. Used with permission of Mark Stringer, University of Otago, New Zealand.

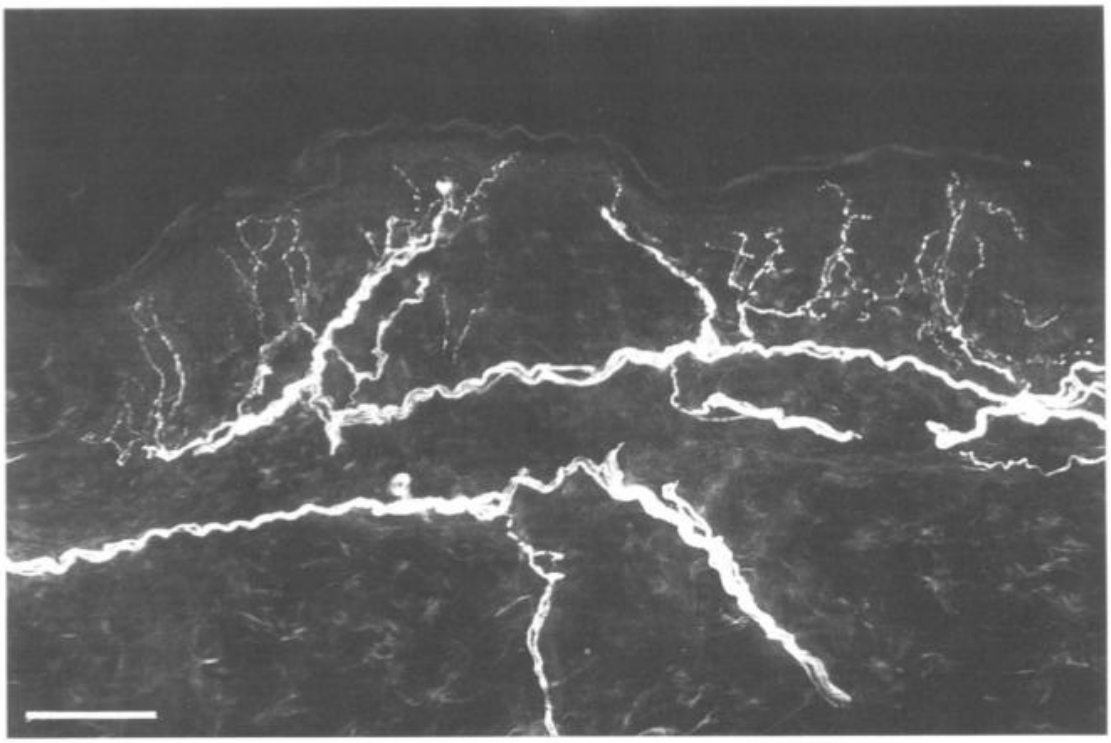
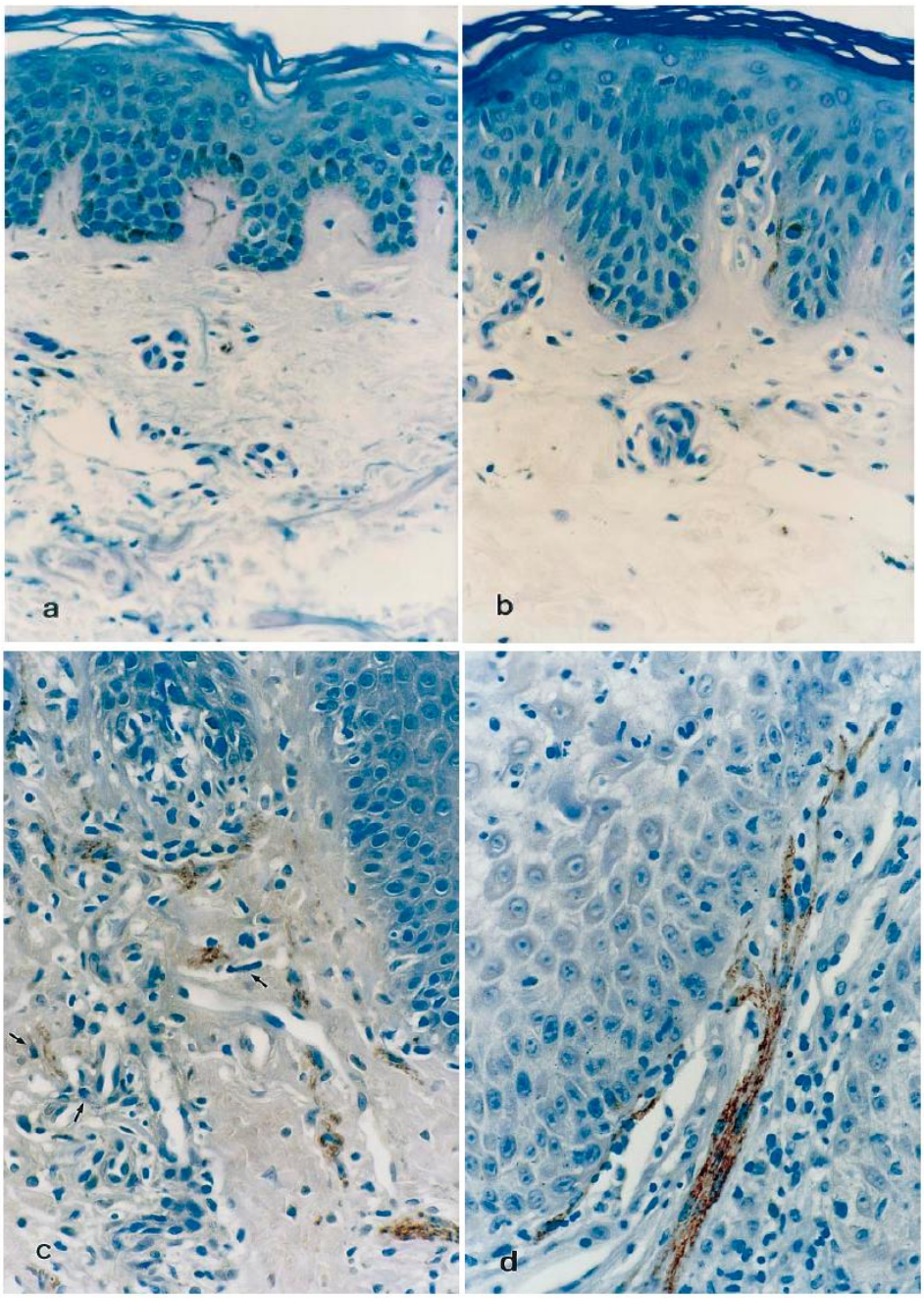


Fig. 1. Confocal micrograph of normal human skin (over biceps) cut vertical to the skin surface and immuno-stained for PGP 9.5 with Cy-3 fluorophore. The dense horizontal nerve network has multiple branches toward the epidermis. Bar = 50 μm.

Figure 1.11. Confocal micrograph of a thick section (50um) of paraffin-embedded normal human skin stained for PGP 9.5, using immunofluorescence. Note the thicker nerve bundles in the papillary dermis, which branch to send thin tributaries into the epidermis. From (222).

Figure 1.12. PGP-immunoreactive neurons stained in skin (next page). Paraffin sections of skin were stained for PGP 9.5 (brown). (A) Healthy control skin from a non-atopic individual; (B) non-lesional atopic dermatitis skin; (C) atopic dermatitis lichenified skin; (D) atopic dermatitis prurigo skin. Both (C) and (D) are classifications of chronic atopic dermatitis lesions. From (251), with permission.


a
b
c
d

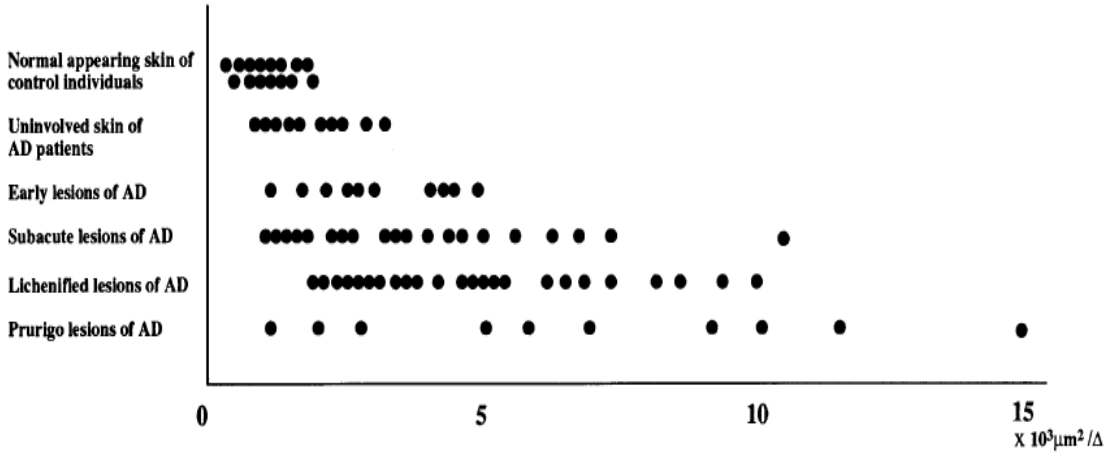


Figure 1.13. Quantification of PGP 9.5-immunoreactive nerve density in healthy and atopic dermatitis skin. Nerves were quantified in paraffin sections stained for PGP 9.5, in the papillary dermal area and upper dermis. Normal appearing skin of control individuals is on the top, and the severity of atopic dermatitis skin increases with lower positions on the Y axis. The categories of atopic dermatitis skin are, in increasing order of severity: uninvolved skin of AD patients, early lesions, subacute lesions, lichenified lesions, and prurigo lesions. $\Delta = 0.24$ mm^2. Subacute, lichenified and prurigo lesions had significantly more PGP 9.5-positive nerves than normal skin or uninvolved (non-lesional) atopic dermatitis skin. From (251), with permission.

CHAPTER TWO: METHODS

I. IN VIVO METHODS

A. Human Skin Biopsies

Human studies were approved by the Institutional Review Board of Oregon Health & Science University, and all subjects gave informed consent. Subjects were paid for their participation. Atopic dermatitis patients were interviewed for their personal and family history of atopy and atopic dermatitis and then examined by a dermatologist to determine extent of disease and chronic versus acute state. A self-reported history of atopy was required for inclusion as an atopic dermatitis patient, and all patients had a previous diagnosis of atopic dermatitis by dermatologists at OHSU. A sample subject intake form is included in the Appendix. Four millimeter punch biopsies of atopic human skin were removed from areas of normal-appearing (non-lesional) or active (lesional) disease respectively. In addition, biopsies of normal skin from healthy patients were taken from the inner arm or behind the knee. All biopsies were fixed in 10% neutral buffered formalin and embedded in paraffin.

B. Animals

Female wild-type C57BL/6 mice, 6-8 weeks of age, were purchased from Jackson Laboratories. Male or female CD3δ-IL-5 (NJ.1638) and PHIL mice were generous gifts from the James and Nancy Lee Laboratory, Mayo Clinic, Scottsdale, Arizona, and from the Jeffrey Gold Laboratory, OHSU.

The NJ.1638 IL-5 transgenic mouse was created using a construct that contained the full murine IL-5 sequence, with all the introns and 1.2kb of 3'-flanking sequences, but missing all of the upstream known regulatory elements (121). The construct is expressed in a manner regulated by the promoter and tissue-specific enhancer for CD3δ, ensuring that IL-5 is made by all subclasses of thymocytes and peripheral T cells (276). NJ.1638 mice have similar numbers of live births, weaned animals and similar sex ratios to wild-type mice (121). IL-5 transcripts were found at elevated levels in all tissues with high numbers of T cells, including thymus, bone marrow, spleen, peritoneal cavity, lung and blood, and high intracellular IL-5 was only found in CD3ε-positive cells. IL-5 protein levels in the serum of these mice persist around 400 pg/ml through the life of the animals, which is equivalent to serum IL-5 levels in wild-type mice that have a helminth infection.

The PHIL transgenic mouse was created using a construct that contained the diphtheria toxin A (DTA) chain open reading frame sequence. This was expressed under control of the eosinophil peroxidase (EPO) promoter, after finding that the upstream EPO sequences regulated a reporter protein's expression at high levels only in eosinophil lineage-committed cells. Therefore, in cells where the EPO promoter is active, i.e., eosinophils and their precursors, DTA is expressed, which results in the degradation of elongation factor-2, halting protein synthesis and death. PHIL mice did not have any eosinophils in peripheral blood and had wild-type numbers of B cells, T cells, mast cells and basophils. There were also no eosinophils in tissues that have resident populations in wild-type mice, including

bone marrow, uterus, small intestine and thymus. In addition, after allergen sensitization and aerosol challenge, the lungs of these mice had no eosinophils, unlike wild-type mice, who had significant infiltration of eosinophils (277).

K5.hTGF-β1 transgenic mice were a kind gift from Xiao-Jing Wang, University of Colorado (278). To ensure the proper genetic identification of mice, DNA was isolated from tails of mice at 3 weeks of age using a DNeasy Blood and Tissue Kit (Qiagen, Valencia, CA), according to the manufacturer's instructions. PuReTaq Ready-To-Go PCR beads (GE Healthcare, Piscataway, NJ) were used for DNA amplification, following the manufacturer's instructions. Primer sequences and product size were as follows:

K5.hTGF-β1 forward, 5' GCGTCTGCTGAGGCTCAAGTT-3' and reverse 5'-ACCTCGGCGGCCGGTAG-3', 271 bp. The annealing temperature for K5.hTGFb1 primers was 54°C.

All animals were housed in plastic cages with filtered cage tops in groups of five, except for epicutaneously sensitized mice, who were housed singly to prevent cooperative removal of patches. Personal protective equipment, including gloves, mask, gowns, shoe and hair covers, was worn at all times when interacting with the animals. All experiments were approved by the Institutional Animal Care and Use Committee of Oregon Health & Science University or the Portland Veterans Affairs Medical Center Institutional Animal Care and Use Committee.

C. Epicutaneous sensitization to ovalbumin

Wild-type or PHIL mice were sensitized to ovalbumin, according to a protocol established by Spergel et al (279), with modifications. Mice were anesthetized with i.p. ketamine (100 mg/kg) and xylazine (5 mg/kg). A small area between the shoulder blades was shaved, and the skin was gently abraded by tape stripping with Tegaderm six times. A gauze pad (1X1 square centimeters) saturated with 100ul of normal saline or 100ug/ul Grade V ovalbumin was applied to the shaved area and secured with Tegaderm occlusive dressing. Patches were replaced every other day for one week and then removed. The mice were rested for two weeks. The sensitization protocol was repeated, for a total of three weeks of sensitization and four weeks of rest. At the end of the experiment, mice were killed with an overdose of pentobarbital (300 mg/kg), and lesional and non-lesional skin were removed and fixed in 4% paraformaldehyde.

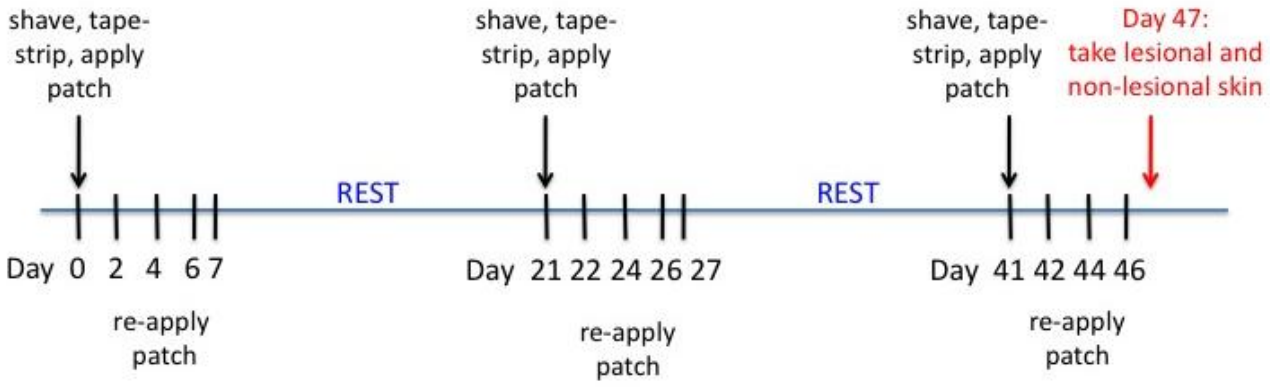


Figure 2.1. Timeline for epicutaneous sensitization of mouse skin.

II. IN VITRO METHODS

A. Isolation of Dorsal Root Ganglia (DRG)

Dorsal root ganglia were isolated according to modifications of previous protocols (280-283) Wild-type C57BL/6 mice were euthanized with an overdose of pentobarbital (300 mg/kg) and cervical dislocation. The spines were extracted, rinsed under running deionized water, and all cervical, thoracic and lumbar DRG were dissected and placed into Dulbecco's Modified Eagle Medium (DMEM) that contained 10% fetal bovine serum (FBS). Ganglia were centrifuged at 300Xg for 10 minutes at room temperature, and the pellet was resuspended in 0.05% collagenase in Hank's Buffered Salt Solution (HBSS) that contained calcium and magnesium and 100uU/mL penicillin and 100 μg/mL streptomycin. Ganglia were incubated in a shaking 37°C water bath for four hours, centrifuged at 300Xg for 10 minutes at room temperature, resuspended in 3 ml 1.25% Trypsin-EDTA, and incubated for 15 minutes at 37°C. Cells were centrifuged again and resuspended in DMEM, 10% FBS, penicillin-streptomycin, and pre-plated overnight on 100 mm Petri plates at 35.5°C with 5% CO2, to remove adherent cells, primarily fibroblasts. The next day, the supernatant was centrifuged at 300Xg for 10 minutes, and cells were resuspended in C2 nerve growth medium that contained penicillin-streptomycin at a range of $3X10^5$ cells/ml to $1X10^6$ cells/ml. C2 medium is defined in the Appendix, Medium Recipes section. Nerves were plated on chamber slides or 24 well Transwell plates that were previously coated with Matrigel and were incubated at 35.5°C with 5% CO2 for the duration of each experiment. In some cases, 2μM cytosine arabinoside

was added to each well to kill dividing cells, in order to enhance the purity of the nerve cellpopulation. After use in experiments, as described below, cells were fixed in 4% paraformaldehyde for 5 minutes, rinsed with HBSS, and stored in phosphate buffered saline (PBS) until analyzed.

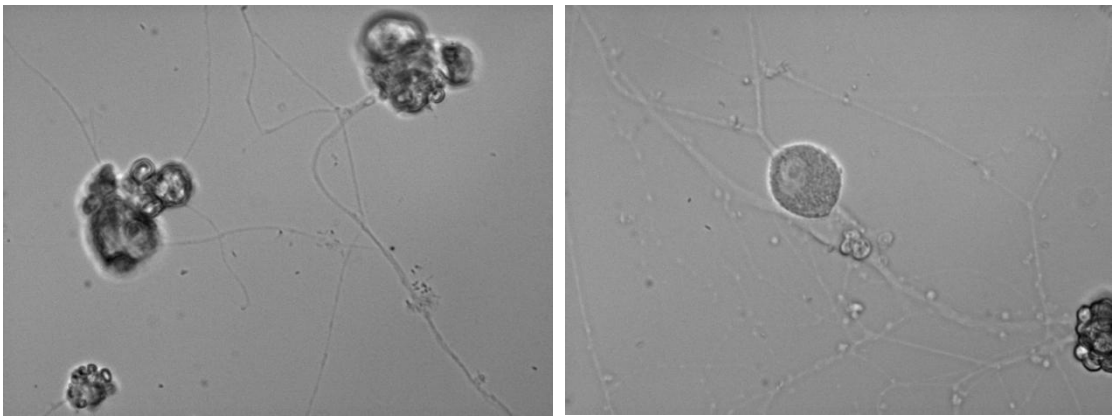


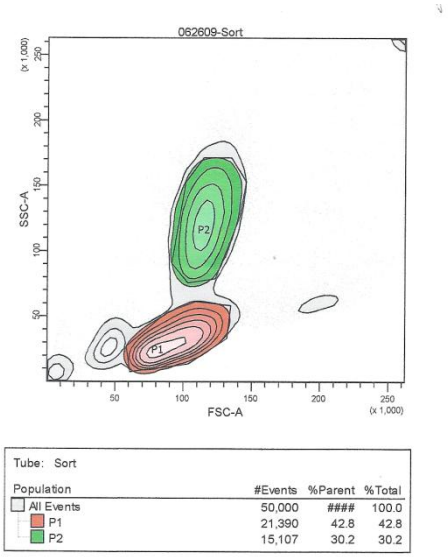
Figure 2.2. Unstained dorsal root ganglion neurons in culture, with no stimulation, after 24 hours.

B. Isolation of murine blood eosinophils

Male or female NJ.1638 (IL-5 transgenic) mice were killed with a lethal dose of pentobarbital (300mg/kg i.p.). The cervical area was cleaned with 70% ethanol, the skin was removed and muscle and fascia dissected to reveal the jugular vein. The jugular vein was severed, and blood was collected into a 1 mL syringe, then delivered to a 1 mL tube that contained 100 μL of 2% ethylenediaminetetraacetic acid (EDTA) to prevent clotting. The blood was subsequently diluted to 35 mL with 2% FBS in PBS, vortexed, and layered over 15 mL sterile Percoll (density 1.084 g/mL). The sample was centrifuged at 2000Xg for 45 minutes at 4°C, with no brake. The white layer at the interface, which contained the granulocytes, was collected, washed and centrifuged at 300Xg for 15 minutes at 4°C. The pellet was resuspended in 5 mL red blood cell (RBC) lysis buffer (NH4Cl, KHCO3, EDTA and RNase-free water), incubated at RT for 5 minutes, and then diluted with 2% FBS in PBS. Cells were centrifuged at 300Xg for 10 minutes at 4°C, washed once more with 2% FBS in PBS, and spun at 300Xg for 10 minutes. The pellet was resuspended in 1 mL 10% FBS in PBS for cell count, trypan blue exclusion assay for viability, and fluorescence-activated cell sorting (FACS) of eosinophils by size and granularity. After FACS sorting, cells were centrifuged at 300Xg for 10 minutes at 4°C, resuspended and counted, using trypan blue to determine viability. Percent purity was determined by Hemacolor assay on cytospin slides. Cells were resuspended in C2 nerve medium at the indicated concentrations and added to cultures of DRG neurons.

C. Fluorescence-activated cell sorting (FACS)

As previously described, whole blood was isolated from NJ.1638 transgenic mice and separated by density centrifugation, and RBCs were lysed. Cells were resuspended in 1ml 10% FBS in PBS and taken in ice to be sorted [Becton Dickinson FACSVantage SE]. Eosinophils are exceptionally autofluorescent when excited with blue or green light, and this is due to their intracellular granules (284, 285). This trait allows unstained cells to be sorted on the basis of size and granularity, corresponding to forward scatter (x axis) or side scatter (y axis) (286). Eosinophils have dense granular cores and high granularity, therefore, they scatter light at high levels and "are found above the neutrophil cluster in plots of forward versus side scatter" (287). The laser used to excite fluorescence was 488nm. A sample FACS plot for eosinophil isolation is shown below from one experiment of 17. After the Percoll and lysis steps, the mean percent of all non-RBC blood cells that were eosinophils was 39.4%, with a range from 22.5% to 74%. Percent purity of eosinophils after FACS sorting was determined by Hemacolor staining and differential cell counts under the 10X objective of a bright-field microscope. The average purity of eosinophils after sorting was 91%, with a range from 75% to 99%. Out of six experiments applying eosinophils to DRG neurons, five had eosinophil purities of 99%.



Tube: Sort

Population	#Events	%Parent	%Total
All Events	50,000	####	100.0
P1	21,390	42.8	42.8
P2	15,107	30.2	30.2

Figure 2.3. Sample flow cytometry plot of mouse blood, after red blood cell lysis, from a single transgenic NJ.1638 mouse. Eosinophils are green, and labeled as the P2 category, indicating 30.2% of the cells in this sample were eosinophils. The red population are likely neutrophils.

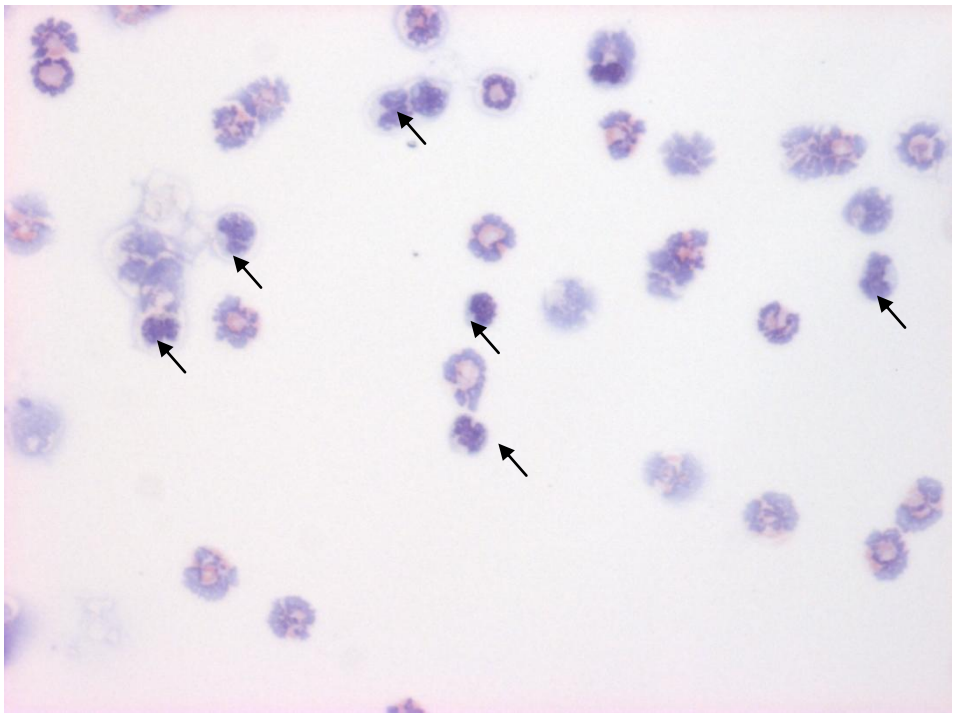


Figure 2.4. NJ.1638 blood cells stained with Hemacolor before FACS sorting. Arrows indicate cells that are *not* eosinophils. This blood contained 74% eosinophils before FACS sorting.

D. Isolation of murine peritoneal mast cells

Peritoneal mast cells were isolated according to the method of Jensen et al. (288). Mice were euthanized with a subcutaneous injection of pentobarbital (300 mg/kg), and cervical dislocation was performed. The ventral abdomen was cleaned with 70% ethanol, and abdominal skin was removed, leaving the peritoneal cavity intact. 10 mL of 1X calcium- and magnesium-free HBSS was injected into the peritoneal cavity, using a 10 mL syringe and 23 gauge needle. The abdominal area was shaken for 60 seconds with fluid inside, and the fluid was then recovered into the original 10 mL syringe. The lavage was repeated with a second 10mL syringe filled with HBSS. Peritoneal cell suspensions were pooled under sterile conditions and centrifuged at 400Xg for 5 minutes at room temperature. The pellet was resuspended in 5 mL lysis buffer ($NH4°Cl$, $KHCO3$, EDTA and RNase-free water) for three minutes to lyse red blood cells, then 5 mL 1X HBSS was added. Cells were centrifuged at 400Xg for 5 minutes, washed with HBSS and centrifuged again. The pellet was resuspended in 70% isotonic Percoll solution, and cells were layered over filter-sterilized peritoneal mast cell (PMC) medium and centrifuged at 580Xg for 15 minutes at room temperature. PMC medium and 70% Percoll are defined in the Appendix Medium Recipe section. The top layer of monocytes and macrophages and the middle Percoll layer were discarded, and the mast cell pellet was resuspended in PMC medium. Aliquots of cells were counted and stained by trypan blue to determine viability or toluidine blue to determine purity of mast cells, whose granules stain positively with toluidine blue. Finally, cells were adjusted to indicated concentrations, added to slides or wells and incubated at 35.5°C with 5% $CO2$.

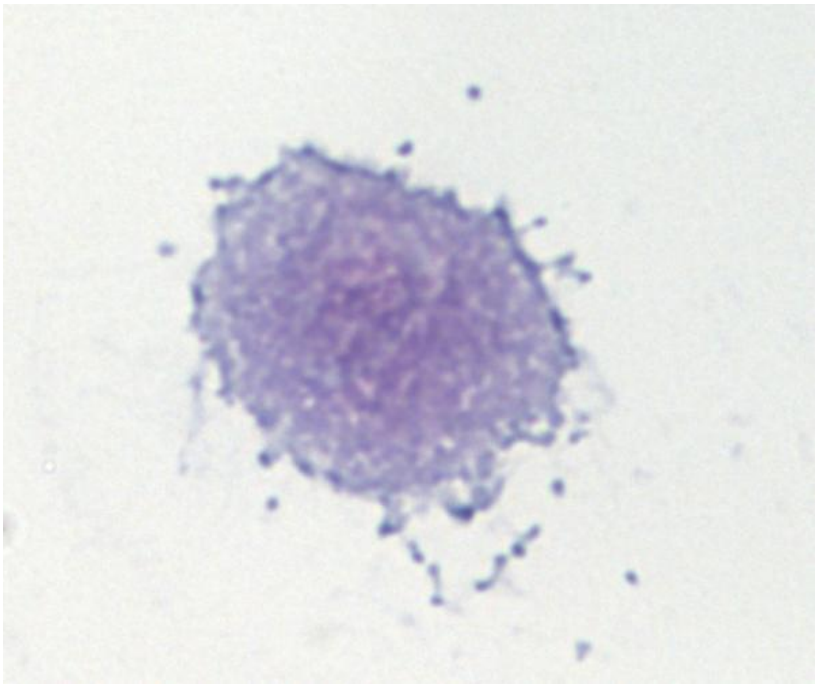


Figure 2.5. Mast cell, stained with toluidine blue, after isolation by peritoneal lavage of a single wild-type mouse.

III. IN SITU METHODS

A. Human Skin Immunocytochemistry

5um sections were cut onto slides, and these were rehydrated through xylenes and sequential ethanol dilutions. Antigen unmasking solution was used, according to the protocol, [Vector], and 3% hydrogen peroxide in methanol was applied for ten minutes, to quench endogenous peroxidase activity. Sections were blocked with 10% normal goat serum, and mouse anti-human protein gene product 9.5 (PGP9.5) [Serotec] was applied overnight at 4°C. Slides were rinsed in PBS and biotinylated goat anti-mouse IgG was applied for 30 minutes at room temperature. The Vectastain ABC kit and chromogenic substrates were used to visualize positive antibody staining. Slides were rinsed in tap water, then dehydrated through ethanol dilutions and xylenes. Slides were mounted using Cytoseal CrystalMount and dried overnight. Likewise, human skin biopsies stained for eosinophil peroxidase (EPO) were blocked and incubated with antibody at 4°C overnight.

B. Hematoxylin and Eosin Staining and Analysis of Human and Mouse Skin

Slides were deparaffinized and rehydrated as above and then stained using hematoxylin and eosin and mounted.

C. Semi-quantitative Analysis of Immunostaining of Human Skin

40X or 60X photomicrographs of each entire skin biopsy were taken. Measurements were taken of papillary dermis, reticular dermis, hypodermis, and epidermal-dermal zone; however, only measurements of papillary dermis and epidermis/basement membrane are reported. Nerve length was calculated by calibrating each photograph to the objective with which it was taken and then

drawing a straight line between the two furthest points of a PGP 9.5-positive nerve, using Metamorph software. Nerve number was counted manually. Data were reported as average number of nerves per photo, and average length of nerve per photo.

D. Murine Skin Immunocytochemistry- epicutaneously sensitized mice

Skin sections from untreated, PBS- and OVA-treated mice were fixed in 4% formaldehyde and embedded in paraffin. Skin sections from mouse were immunostained in the same manner as human skin, using rabbit anti-mammal PGP9.5 and biotinylated goat anti-rabbit IgG. Duplicate samples were double-stained with PGP9.5 and MBP antibodies. MBP was applied overnight at 4°C, and secondary antibody, conjugated to the fluorophore Alexa Fluor-555, was applied for 2 hours at 37°C. Slides were rinsed in PBS, and mounted using Vectastain with DAPI, to stain cell nuclei.

E. Murine Skin Immunocytochemistry- K14-IL-5, K5-hTGF-β1, flaky tail mice

Slides of paraffin-embedded sections were sent from the Nancy and James J. Lee Laboratory, Mayo Clinic, Scottsdale Arizona. This mouse has high levels of IL-5 expressed under a basal keratinocyte promoter, keratin-14 (K14) and therefore, high numbers of eosinophils are recruited into the skin (unpublished data).

Slides of paraffin-embedded sections from K5-hTGF-β1 mouse skin and wild-type littermate controls were generously shared by Andrew Blauvelt at the Portland VA Hospital and OHSU Department of Dermatology (289). Slides of the flaky tail mouse skin and wild-type controls were sent by Padraic Fallon at Trinity College,

Dublin, Ireland (290). These skin sections were rehydrated and stained for PGP9.5 and/or MBP as detailed above.

F. Semi-quantitative analysis of immunostaining of mouse skin

60X photographs of entire mouse skin biopsies were taken. Slides were de-identified using an algorithm assigning random numbers to each photograph (courtesy of Gregory Scott), and measurements were taken of PGP-positive spots in the papillary dermis, reticular dermis, and epidermal-dermal zone of each blinded photograph. Nerves associated with hair follicles were not counted, because of the variability in number of hair follicles in each section. Nerve length was calculated by calibrating each photograph to the 60X objective and then drawing a straight line between the two furthest points of a PGP 9.5-positive nerve, using Metamorph software. Nerve number was counted manually. Data from each photograph were averaged to determine the mean number of nerves per photo, sum of nerve lengths per photo, and average length of nerve per photo. Random numbers were matched to each mouse sample after all measurements and calculations were completed. In sections that were double-stained for nerves and eosinophils, distance between nerves and eosinophil products was measured, using Metamorph software. If the distance was less than 8um (the diameter of an eosinophil), then they were counted as associated.

G. Dorsal Root Ganglion Immunocytochemistry

Slides were fixed in 4% paraformaldehyde for 5 minutes, rinsed with HBSS with calcium and magnesium, and stored in PBS until stained. Slides were blocked with 10% serum and stained with antibodies to PGP 9.5, ICAM-1 (courtesy of the

David Parker laboratory at OHSU), VCAM-1, eotaxin-1, or peripherin overnight at 4°C. After rinsing in PBS, a secondary antibody conjugated to Alexa Fluor-555, or biotin, was applied. Slides were rinsed and either mounted in Vector Mounting Medium with DAPI, to stain cell nuclei, or completed, using Vector Vectastain Avidin-Biotin Complex immunohistochemistry kit.

H. Dorsal Root Ganglion Imaging and Quantification

Thirty 40X photographs were taken of DRG cultures with various treatments, stained with anti-PGP 9.5 antibody. Photographs were always taken beginning in the center of each well of a 4-well chamber slide, and then every subsequent field of view was photographed, in a defined pattern for all slides. Using Metamorph or ImageJ, numbers of cell bodies, neurites, and branchpoints per cell body were counted manually. Each cell body in each photograph was quantified. In addition, neurite length was measured with the segmented line function, which allowed the nerve to be traced from end to end and measured according to the pixel per micron ratio for the 40X objective. The mean of each measurement (number of cell bodies, neurites, branchpoints and neurite length) for all cell bodies in thirty pictures was determined. Verification of this method was provided two ways: first, one group of photographs each from two experiments was quantified by a blinded observer, who knew neither the treatment groups nor the predicted outcome, and second, photographs were taken at random places in each well for one experiment, and a blinded observer then performed the measurements. Blinded observations were found to match unblinded observations. Unblinded observations are reported here.

IV. RNA AND PROTEIN ISOLATION AND QUANTIFICATION

A. mRNA isolation and quantitative real-time reverse transcriptase PCR

Clinically abnormal (lesional) and clinically normal (non-lesional) skins were obtained from C57BL/6 and K5.hTGF-β1 transgenic mice, snap frozen in liquid nitrogen, placed into Trizol (Sigma, St Louis, MO), and homogenized with a mechanical rotor for 30 seconds. RNA was isolated according to standard Trizol protocol, and was followed by further purification using an RNeasy kit (Qiagen). cDNA was prepared from 1 mg of total RNA by reverse transcription using iScript (Bio-Rad, Hercules, CA). Five reactions were pooled and diluted 5 times with water to establish the same pool of cDNA for all qRT-PCR experiments. qRT-PCR was performed with TaqMan primers and fluorescent probes for GAPDH, hTGF-β1, IL-23p19, IL-12/23p40, IL-12/35p35, IL-23R, IL-6, IL-17A, IL-22, IFN-γ, RORγt, CCR6, IL-4, foxp3, and IL-10 (Applied Biosystems, Carlsbad, CA) on the MyiQ system (Bio-Rad). Relative quantification by the delta–delta Ct method was carried out in Excel, using GAPDH as the housekeeping gene. To generate logarithmic graphs, 100 was added to all observations and the log of each was calculated. WT results were fixed at two (log 100). Gains relative to WT were shown as greater than two and losses were shown as less than two.

B. Protein Isolation and ELISA

Sera, lesional skin and non-lesional skin were obtained from C57BL/6 and K5.hTGF-β1 transgenic mice. Skin was snap frozen in liquid nitrogen, pulverized when immersed in liquid nitrogen, and subsequently re-suspended in a protein lysis

buffer (10mM Tris-HCl, pH 7.5; 0.5mM EDTA-Na2; 0.5mM EGTA; 1% Triton X-100; 0.5mM PMSF; and Protease Inhibitor Mix, diluted from 100X stock (Sigma)). The samples underwent three freeze–thaw cycles and were spun at full speed for 15 minutes to fractionate the sample and remove protein. Human TGF-β1 and murine IL-17A, IL-22, IL-1α, TGF-β1, and IL-12/23p40 ELISA kits (R&D Systems, Minneapolis, MN), the IL-23 p19/p40, IFN-γ, and IL-4 ELISA kits (eBiosciences, San Diego, CA), and the IgE ELISA kit (Biolegend, San Diego, CA) were used according to the manufacturer's instructions.

Lymph nodes from WT, K5.hTGF-β1 transgenic, or IL-17A knockout mice were harvested and cultured for 3 days in RPMI-1640, 5% FBS, 2mM L-glutamine, and 100Uml^{-1} penicillin/100 mgml^{-1} streptomycin. Cells were cultured alone or in the presence of IL-17-promoting cytokines or antibodies, including hTGF-β1, IL-6, anti-IFN-γ, and recombinant murine IL-23 (Li et al., 2007). The cells were plated at a density of 5 X10^{-6} cells per ml in six-well plates. Then, 150 ml aliquots of cell culture supernatant, to use as protein assay controls, were taken at 24-hour intervals, beginning at 12 hours. The culture medium was supplemented with 1 ml new medium on day 3. The cell culture supernatants were used in ELISA assays.

E. Statistical Analyses of Data

All data are expressed as mean +/- SEM. Comparisons between two groups were made using t-tests for unpaired data. Comparisons of nerves in lesional and non-lesional skin from the same mouse were made using paired t-tests. Multiple

comparisons used one-way ANOVA, with a Tukey's post-test to evaluate differences between groups. A p value of less than 0.05 was considered statistically significant.

Statistical analyses for qRT-PCR results were carried out using a two-tailed t-test, with four to nine degrees of freedom and 95% confidence intervals for graphing. Statistical analyses for protein results comparing WT with transgenic samples were carried out using a one-way analysis of variance with Tukey's post test, except for the serum IgE results, which were compared using an unpaired t-test.

CHAPTER THREE

Human atopic dermatitis skin contains many nerves and eosinophil granules, and they are located in similar regions.

I. INTRODUCTION

Atopic dermatitis is characterized by pruritus, or itch (1, 11). The itch sensation often begins before any lesions appear, and marks on the skin can be limited to scratches made by the patient. Estimates of the prevalence of atopic dermatitis range from 5-20% of children (15, 17-19), with 85% of patients developing the disease in the first five years of life (20).

Humans with atopic dermatitis experience itch instead of pain when tested with mechanical, electrical, low pH, or heat stimuli (240). The sensory neurons that transmit itch from the skin are primary afferents whose cell bodies reside in the dorsal root ganglia (DRG). These free nerve endings in the epidermis and upper dermis can be activated by a variety of stimuli, including proteases, neuromediators, cytokines and other small molecules (reviewed in (241, 291)) and subsequently transmit itch-specific signals to the central nervous system. It is clear that an itch-sensitive pathway of neuronal signaling exists (232, 233, 235, 238). However, the mechanisms for enhanced itch sensations in atopic dermatitis are still unclear.

One potential mechanism is an increase in itch-transmitting nerve endings in atopic dermatitis skin. Previous reports have found a higher density of nerve fibers in atopic dermatitis biopsies, using a variety of nerve markers, including the pan-

neuronal marker protein gene product 9.5 (PGP9.5)(250). Specifically, there are more PGP9.5+ fibers in the papillary and upper dermis as disease progresses from clinically normal-appearing, or *non-lesional,* skin to active disease, or *lesional,* skin (251). Particularly in lichenified lesional skin of adults with atopic dermatitis, the number and diameter of nerves are higher, due to more axons per nerve (252).

Previous data suggest that the interactions between eosinophils and nerves could lead to increased growth or arborization and branching of neurons. One report found that supernatants from activated eosinophils promote the outgrowth of neurites in a pheochromocytoma cell line in culture (191). In rats infected with helminthic parasites, expansion of nerve density in the GALT coincided with massive infiltration of eosinophils (265). In addition, interactions between eosinophils and nerves are important to the pathophysiology of other diseases. In lung sections from humans who have died from asthma attacks, as well as in antigen-challenged guinea pigs and rats with airway hyperreactivity, eosinophils are lined up along nerves (267). Treatment with a CCR3 (eotaxin receptor) antagonist redistributes eosinophils away from nerves, and reduces airway hyperreactivity significantly, indicating that eosinophil localization to nerves is an important pathological aspect of airway disease (268). This could be one mechanism by which more nerve endings appear in the skin of patients with atopic dermatitis.

In this study, we investigated the physical relationship between eosinophils and sensory nerves in the skin of humans with atopic dermatitis. We also determined the number of mast cells in each biopsy and measured the association of

mast cells and nerves, since previous reports linking mast cells to nerve arborization had indeterminate conclusions (292-296).

II. RESULTS

Human skin biopsies are representative of healthy skin and different stages of atopic dermatitis.

Skin biopsies were gathered from healthy human volunteers and subjects with atopic dermatitis. Patients with atopic dermatitis had not used topical corticosteroids for a minimum of a week. Each subject was examined by a dermatologist and confirmed to have atopic dermatitis, by clinical exam and patient and family history, as outlined in the Hanifin and Rajka criteria (see Introduction, Atopic Dermatitis Diagnosis). One biopsy was taken from each healthy control, and two were taken from each atopic dermatitis subject, for paired lesional (L) and non-lesional (NL) skin. Non-lesional skin throughout this report refers to normal-appearing skin from an atopic dermatitis patient. The attributes of the subjects are shown in Table 3.1, including the subject identifier, the type of biopsy (control, acute or chronic atopic dermatitis), and the location of the biopsy. All patients had positive family and personal history for the extrinsic, or allergic, type of atopic dermatitis, including allergies and asthma. Subject 011 was removed from further analysis due to disagreement over the additional diagnosis of ichthyosis vulgaris. H&E staining of the human skin biopsies confirmed the clinical diagnoses of healthy control, non-lesional atopic dermatitis or lesional atopic dermatitis (Figure 3.1). Control skin had thin epidermis, with a mostly acellular dermis. Non-lesional skin had thicker

epidermis, more pronounced rete pegs and dermal infiltrate and a small amount of spongiosis in the epidermis, where keratinocytes are undergoing apoptosis (297). Lesional skin had even further thickened epidermis (including acanthosis, or thickening of the stratum granulosum), many dermal cells, and predominant areas of spongiosis. In particular, the chronic samples had the most significant alterations.

Atopic dermatitis lesional skin biopsies have more nerves than non-lesional skin from the same subjects.

We used immunohistochemistry with the pan-neuronal marker PGP9.5. Photographs were taken along the length of the skin section, and nerves staining positively for PGP9.5 were counted in the papillary dermis and basement membrane zone of the skin. Representative photomicrographs of normal, non-lesional and lesional skin are shown (Figure 3.2). Quantification was performed as described in the methods (Figure 3.3).

Nerves were increased, both in number and in length, in lesional atopic dermatitis skin, compared to each non-lesional skin sample from the same patient. In addition, lesional skin samples had more nerves than all but one of the healthy controls. The data were also analyzed according to the stage of lesional skin, acute or chronic (Figure 3.4). The increase in number of nerves in lesional skin appeared to be due to the acute disease population, so the samples were analyzed alone with a paired t-test between non-lesional and acute lesional skin. This gave a highly significant p value of 0.0036. For the length of nerves, both acute and chronic

lesional skin were increased above non-lesional skin, although neither was a significant difference.

Atopic dermatitis skin biopsies have more eosinophil granule proteins, which are located around nerves in the papillary dermis.

In order to determine if eosinophil localization to nerves in the skin occurs in atopic dermatitis biopsies, we first performed single immunohistochemistry with an antibody to eosinophil peroxidase (EPO), a protein present in eosinophil granules. EPO was present in lesional atopic dermatitis skin at higher quantities than normal or non-lesional skin, and it was often located in the papillary dermis (Figures 3.5 and 3.6). This localization correlated with both the location and stage of disease in which nerves are increased (Table 3.2). In addition, double immunohistochemistry with EPO and PGP9.5 determined that eosinophils could be found around nerves in the papillary dermis, especially in lesional skin samples (Figure 3.7). This indicates that eosinophil localization to nerves could be important for the pathogenesis of disease, similar to eosinophilic asthma in humans and animals.

Mast cells are localized around nerves in non-lesional atopic dermatitis skin and are not increased in lesional skin.

Biopsies were stained with toluidine blue to examine the number of intact mast cells, and we found a trend, although no significant difference, toward more mast cells in non-lesional skin, compared to healthy controls (Figure 3.8). The number of intact mast cells in lesional skin was not increased above controls. We

also used double immunohistochemistry to determine the percent of nerves associated with mast cells, and found a significant increase in non-lesional skin over healthy control skin. We did not see the same increase in lesional skin (Figure 3.9).

III. DISCUSSION

We have shown that atopic dermatitis skin has more nerves than healthy control skin, and that the density of nerve fibers increases as the severity of disease progresses. Previous reports are in agreement with this finding (250-252). We also found that there is a correlation between number of nerves and disease stage within individuals, using paired non-lesional and lesional skin samples from the same subjects. A previous report described a progressive increase in number of PGP9.5-positive nerves as disease moves from clinically normal skin to more severe disease, (251). While we did not have significant numbers of patients with each type of acute or chronic lesional skin to draw definitive conclusions, the trend in our data was that acute skin had more nerves than chronic lesions. However, the classifications of active disease were more narrowly defined in the previous paper into categories of sub-acute, prurigo, and lichenified, so it is possible that the data agree, but have been subdivided into different categories.

We also found that the amount of eosinophil granule protein was increased in lesional skin. We hypothesize that barrier defects, such as the genetic loss of filaggrin in the epidermis in a subset of patients with atopic dermatitis (40, 298, 299), allow sensitization and activation of the nerves, followed by recruitment of eosinophils, and subsequent arborization of nerves.

Although atopic dermatitis is not dependent on histamine, previous studies have found that mast cells are increased in lesional skin compared to healthy controls (292, 293). However, most have found only slight increases in mast cells in atopic dermatitis, since mast cells are also present in normal skin. One group of authors looked in scalp, neck, elbow and buttocks, and found significant increases above healthy controls only in the neck, where they reported the most severe disease (293). In addition, they reported an increase in lesional skin mast cells, compared to non-lesional skin. However, the number of mast cells was compared between lesional skin in scalp, neck and elbow and non-lesional skin from buttocks, rather than from lesional and non-lesional skin taken from the same region of the body. Another group evaluated mast cell numbers in atopic dermatitis using specific antibodies to the enzymes tryptase and chymase. This study found a slight but non-significant increase in mast cells in atopic dermatitis skin compared to healthy controls, while nummular eczema, an entirely different and non-atopic disease, had significantly more mast cells than healthy skin (292). Some older reports found slight increases in MC in atopic dermatitis skin biopsies (294, 295), while other studies found no differences in numbers of mast cells between normal and atopic dermatitis skin (296). Finally, many of the studies on mast cells and atopic dermatitis have not used blinded analysis. In sum, it seems clear that mast cells are present in atopic dermatitis skin, and in other eczematous diseases, but that changes in mast cell number are inconsistent, and the pathological significance of these cells has not been determined.

Our data indicate that eosinophils, rather than mast cells, are specifically present in atopic dermatitis skin, in increasing numbers with severity of disease, and are often located near nerves in the papillary dermis. Because the increase in nerves is thought to be linked to the increased itch sensation in atopic dermatitis, it is possible that therapeutics that target eosinophil trafficking into skin could be beneficial for patients. Only one study has examined treatment of atopic dermatitis with anti-IL-5 monoclonal antibodies (mepolizumab, SB-240563, SCH55700, Schering-Plough), and there has not yet been a large, blinded, randomized, and placebo-controlled study conducted of this treatment.

In this small study, two single i.v. doses of anti-IL-5 monoclonal antibody (mepolizumab) one week apart were given to patients with moderate to severe atopic dermatitis (300). This study was part of a larger trial including patients with allergic rhinitis and asthma, and only 18 atopic dermatitis patients received mepolizumab. Atopic dermatitis activity was measured at days 0, 2 and 14 by a physician, and daily for pruritus by the subjects. Peripheral blood eosinophils were measured on days 0, 2, 7, 14 and 28. Eighteen out of the total patient population of 43 patients had eosinophilia (>500 eosinophils/mm^3) at the beginning of the study, which indicates that this study population could have been further delimited by presence or absence of blood eosinophilia.

Patients receiving anti-IL-5 had significantly better clinical improvement than those receiving placebo, but this was only true when the classification of "modest improvement" was included. There was no significant different in pruritus scores between groups. Mepolizumab-treated patients had significantly fewer

eosinophils in the blood, but the dose used in this study was not high enough for complete depletion from the blood. In addition, the number of eosinophils were not quantified in the skin of these patients, nor their location. This is of particular importance, as it has been shown that even prolonged depletion of circulating eosinophils failed to deplete tissue eosinophils from the airways in asthma (208). Since the clinical effects on AD were subtle, the authors discussed the possibility that eosinophil depletion from skin might not occur in the two-week time frame of the study. In addition, it is also possible that the eosinophils that remain in the blood can still chemotax to the skin, due to CCR3 receptors, indicating that perhaps dual treatment with anti-IL5 and CCR3 antagonists would be most effective. Finally, the treatment of patients with and without eosinophilia equally with anti-IL-5 suggests a major confounder that should be remedied for future studies.

In addition, two small studies were conducted on allergen patch testing in atopic patients (301, 302). This model is used to study mechanisms in the induction of dermatitis by inhaled allergens, but does not equal active atopic dermatitis (303). One study was specifically conducted on patients with atopic dermatitis and the other on patients without atopic dermatitis. In both cases, treatment with intravenous anti-IL-5 significantly reduced circulating eosinophils within 2 days after treatment. In the study of non-atopic dermatitis patients, intact and degranulating eosinophils were decreased in the skin at 6 hours after intradermal challenge with allergen. However, in the study conducted in atopic dermatitis patients, using the same dose of anti-IL-5, there was a slight *increase* in tissue eosinophils on day 2 in mepolizumab-treated patients, while there was no

significant difference in skin eosinophils between day 0 and day 16 in each treatment group, and no significant difference in skin eosinophils between placebo and anti-IL-5 treatment.

In both studies, the size of the cutaneous late-phase reaction did not differ between treatment groups, either at 6 hours or days 2, 16, and 30. Therefore, eosinophils were not responsible for the induration and swelling after LPR. However, the role of eosinophils in atopic dermatitis pruritus, nerve increases and additional pathophysiology was not determined by these studies, and in fact, the authors admitted that a limitation of their study was the smaller decrease in tissue eosinophils than blood eosinophils with anti-IL-5 treatment. Therefore, there is not yet conclusive evidence using anti-IL-5 therapy to support or contradict our hypothesis in atopic dermatitis.

Our findings are also important for other eosinophilic diseases, several of which induce itch as a predominant symptom. Itchy papular eruptions have been described in the hypereosinophilic syndrome and can be successfully treated with ultraviolet (UV) therapy (304). In prurigo nodularis, a disease defined solely by its itchiness, large deposits of eosinophil cationic protein ECP and EDN were detected by immunofluorescence. Intact eosinophils were often located near nerves, and there was an increase in the number of nerves in areas with many eosinophils (248). Finally, a disease called equine sweet itch results from sensitization to the bite of the midge and subsequent "challenge" or further bites. Eosinophils are recruited by eotaxin and monocyte chemotactic protein-1, which are expressed in the skin of sensitized horses, and horses scratch, rub and bite the lesions, which

results in skin thickening and hair loss (305). This report finally clarifies the link between eosinophils and nerves in skin and might provide insight into therapeutics targeting itch.

IV. TABLES.

Table 3.1. Characteristics of human skin biopsies.

Subject	Samples taken	Skin type	Region
001	Lesional/Non-lesional	Acute AD	Flexor knee
002	Normal	Healthy Control	Interior arm
003	Normal	Healthy Control	Interior arm
004	Lesional/Non-lesional	Chronic AD	Flexor knee
005	Lesional/Non-lesional	Acute AD	Flexor elbow
006	Lesional/Non-lesional	Chronic AD	Exterior arm
007	Normal	Healthy Control	Interior arm
008	Normal	Healthy Control	Interior arm
009	Normal	Healthy Control	Interior arm
010	Lesional/Non-lesional	Acute AD	Interior arm
011*	Lesional/Non-lesional	Chronic AD	Interior leg
012	Lesional/Non-lesional	Acute AD	Extensor elbow

* Subject 011's samples were not used for analysis, due to extensive ichthyosis vulgaris.

Table 3.2. Eosinophil peroxidase is located in the papillary dermis in non-lesional atopic dermatitis, and in all regions of the dermis in lesional skin.

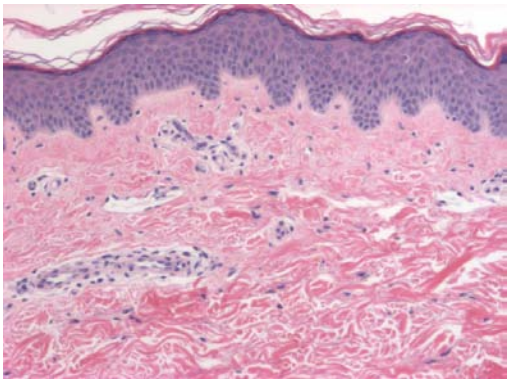
10X photographs of skin stained with anti-eosinophil peroxidase were analyzed for the region of skin in which positive staining was located. Results were compiled in a table, based on type of skin.

Name of sample	avg # EPO+ clusters	EPO+ regions of skin
002 normal	1.5	papillary dermis
003 normal	0.6	basement membrane zone
008 normal	0.25	papillary dermis
009 normal	1	reticular dermis
001 non-lesional	1.8	papillary dermis
004 non-lesional	2.43	papillary dermis
005 non-lesional	2.36	papillary dermis
006 non-lesional	3	papillary + reticular dermis
012 non-lesional	1.833	papillary dermis
001 lesional	2.5	papillary + reticular dermis
004 lesional	5.08	papillary + reticular dermis
005 lesional	2.3	papillary + reticular dermis
006 lesional	3.125	papillary + reticular dermis
010 lesional (unpaire	4.36	papillary dermis
012 lesional	2.75	papillary dermis

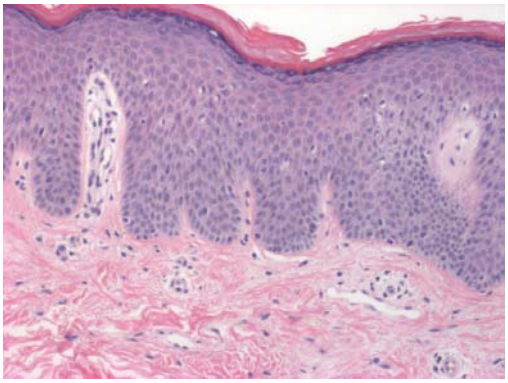
V. FIGURES.

Figure 3.1 (beginning next page). Hematoxylin and eosin stained skin sections from healthy controls and subjects with atopic dermatitis. The subjects with healthy skin have thin epidermis and mostly acellular dermis. Non-lesional atopic dermatitis skin has thicker epidermis and recruitment of immune cells into the dermis, while lesional skin has much thicker epidermis, with clear evidence of spongiosis, deeper rete pegs and many more infiltrating cells. All photographs were taken using the 20X microscope objective.

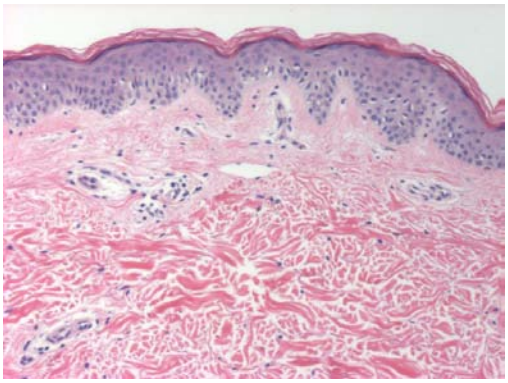
001 non-lesional



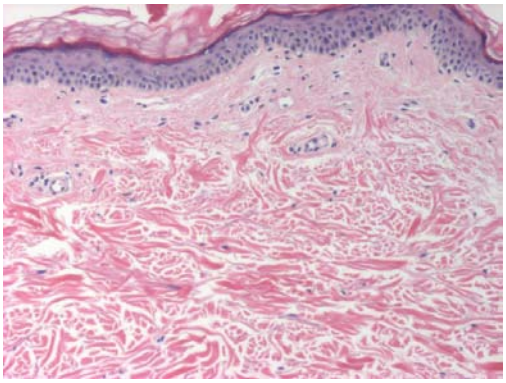
001 lesional



002 normal



003 normal



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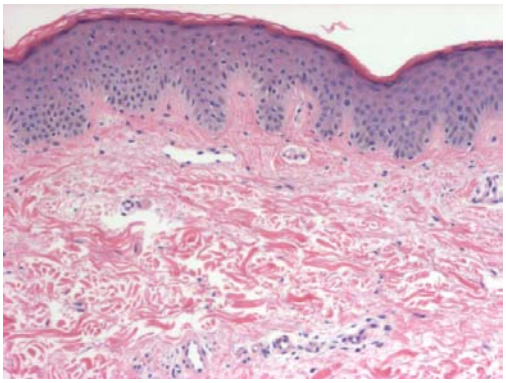

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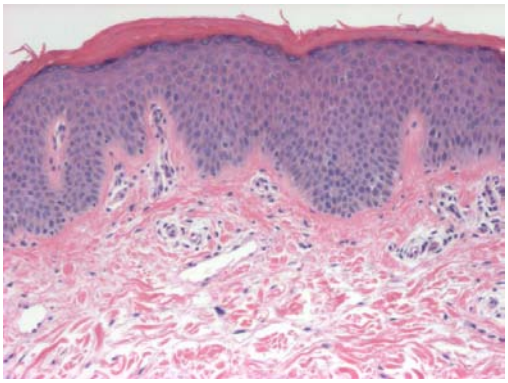

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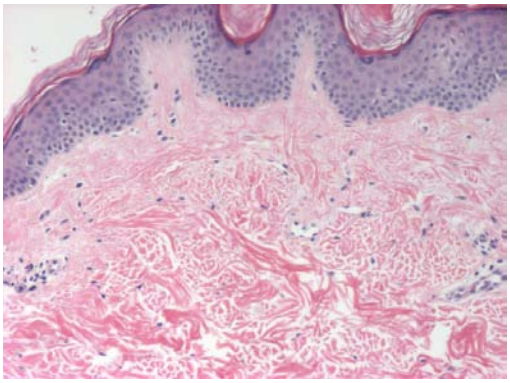

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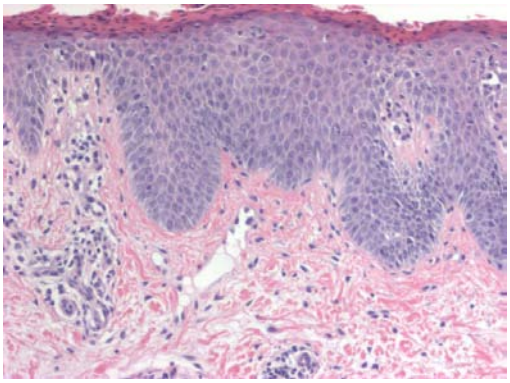

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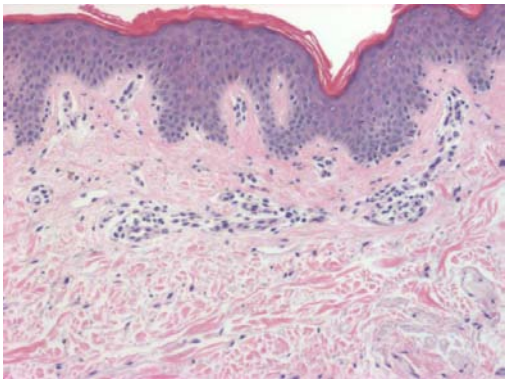

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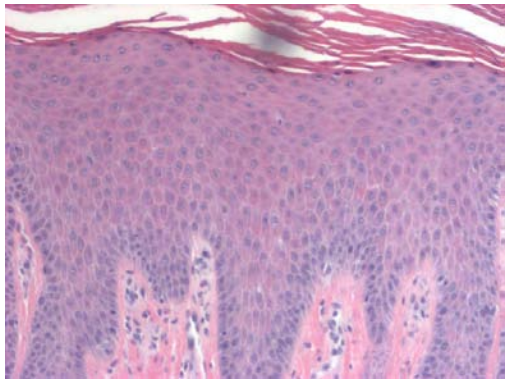

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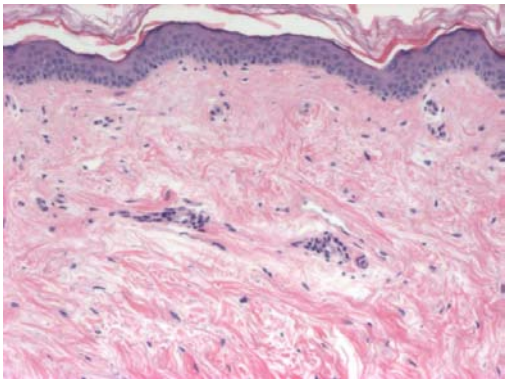

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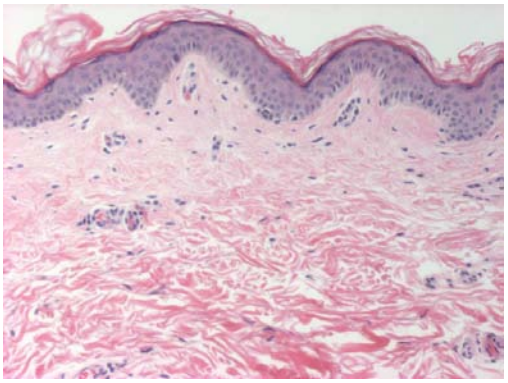

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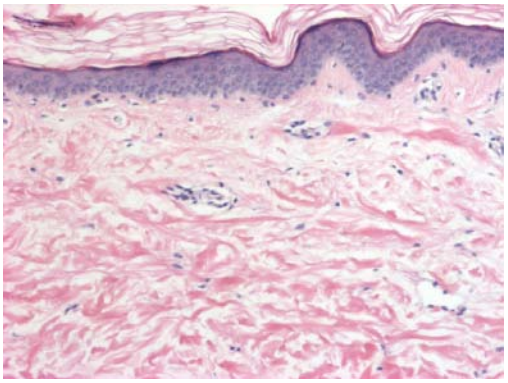

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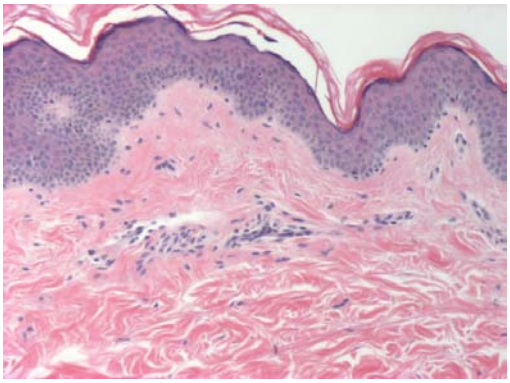

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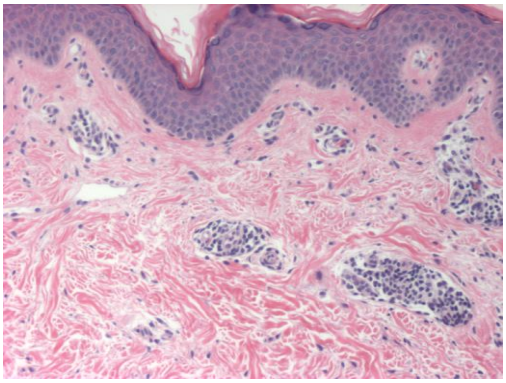

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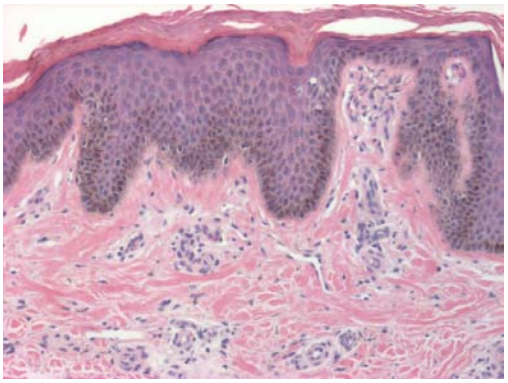

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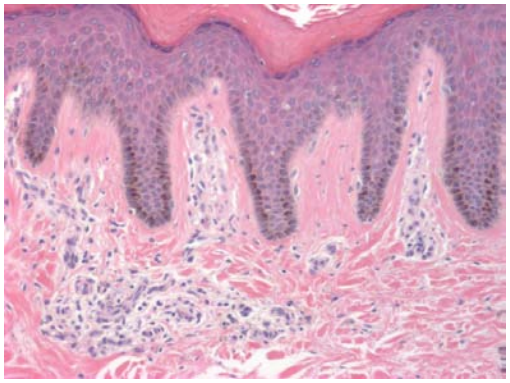

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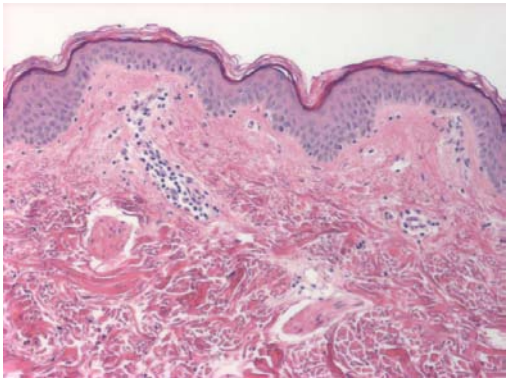

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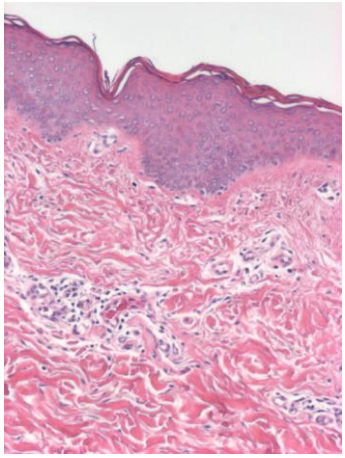

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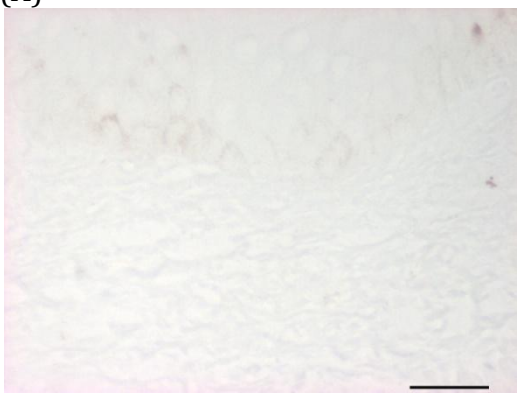

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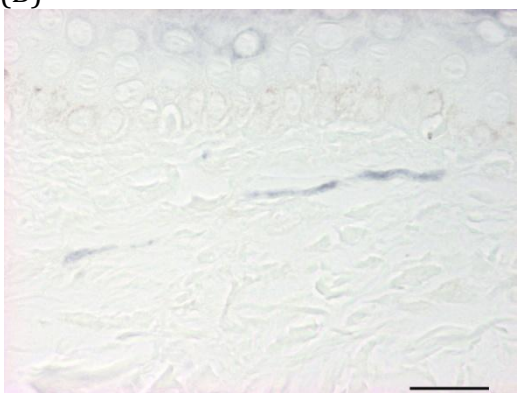

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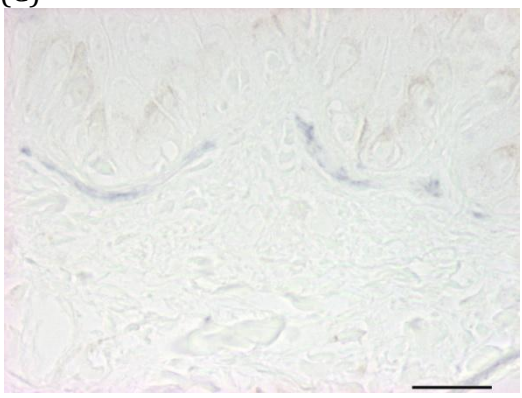


Figure 3.2. Atopic dermatitis lesional skin has more nerves, of longer length, than paired non-lesional samples. Paraffin-embedded skin biopsies were stained with anti-PGP 9.5 (gray). Representative sections are shown of healthy control skin (A), non-lesional atopic dermatitis (B), and lesional atopic dermatitis (C).
Scale bar =50um.

(A) (B)

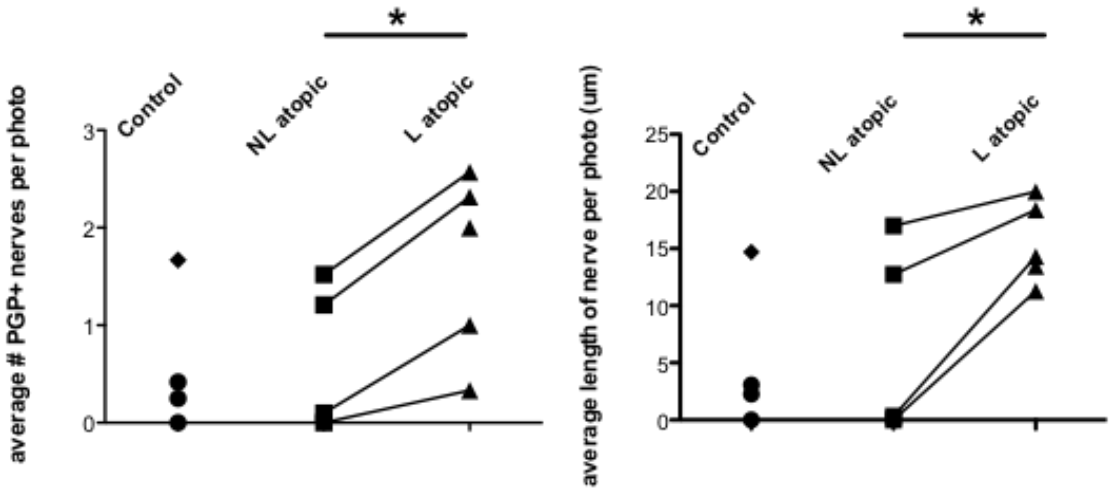


Figure 3.3. Quantification of nerve number and length in healthy control, atopic non-lesional and atopic lesional skin. Five um skin biopsies were stained with anti-PGP9.5 antibody, photographs were taken of each entire biopsy section, and nerves were quantified using Metamorph software. Control denotes healthy skin, NL atopic is non-lesional, and L atopic is lesional skin. A paired t-test was run between non-lesional and lesional skin samples from the same subjects to determine if nerves were increased. One lesional atopic sample had no paired non-lesional sample, due to use of the entire paraffin block for other experiments. (A) Average number of PGP 9.5-immunoreactive nerves per photo. (B) Average sum length of PGP 9.5-immunoreactive nerves per photo. $p<0.05$ was considered significant.

(A) (B)

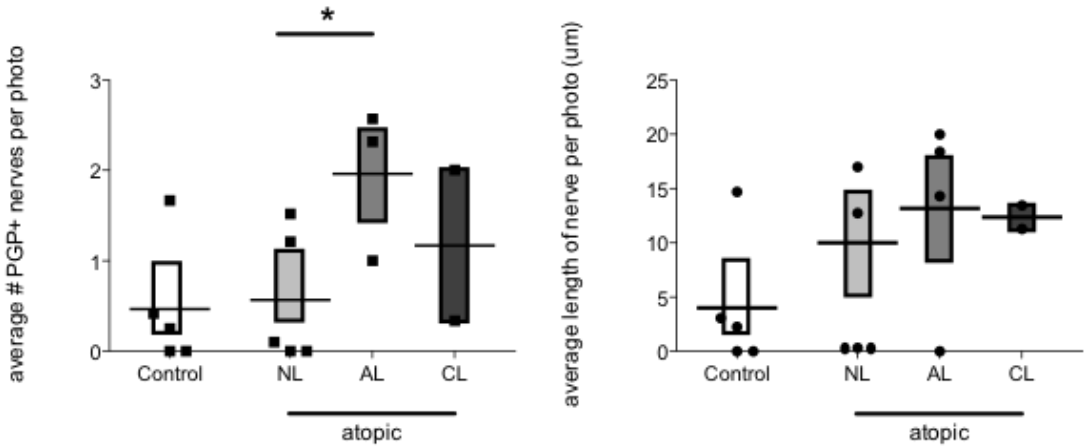
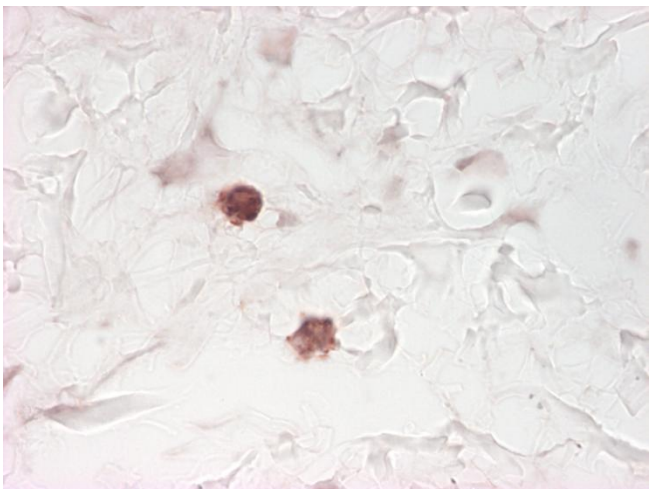


Figure 3.4. Differences in nerves based on subtype of atopic lesional skin.

Data were analyzed and graphed based on subtypes of lesional skin, either acute lesional (AL) or chronic lesional (CL), as determined by a dermatologist, compared to non-lesional skin (NL) from the same subjects or healthy control skin (control). (A) depicts average number of PGP 9.5-positive nerves per photo, and (B) shows average sum length of nerves per photo. Although there was no significance across all groups when divided this way, a paired t test between non-lesional and acute lesional skin for number of nerves gave a p value of 0.0036.

(A)



(B)

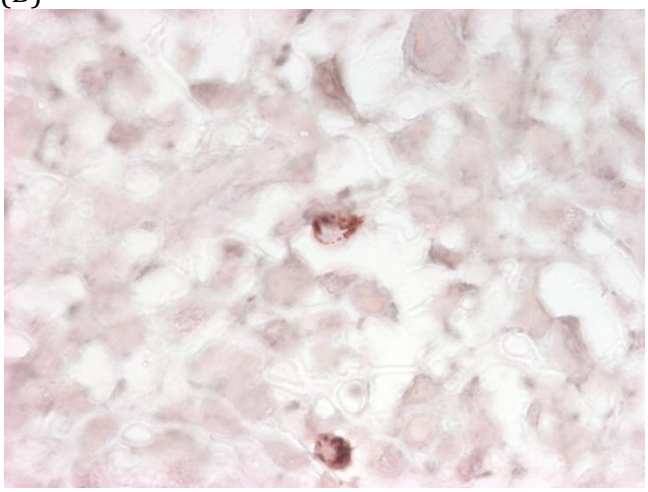


Figure 3.5. Eosinophil granule proteins are present in atopic dermatitis skin.

5 um skin sections were stained with anti-eosinophil peroxidase and photographed along their entire length, using the 60X microscope objective. (A) depicts the reticular dermis of a section of 005 lesional skin, and (B) is from 006 lesional skin. EPO is shown in dark red-brown.

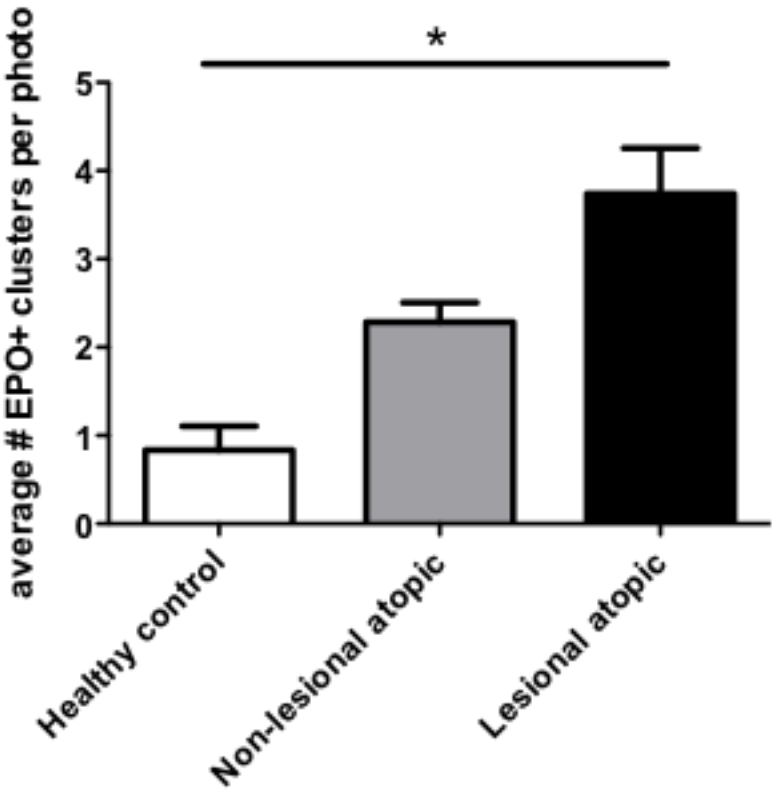
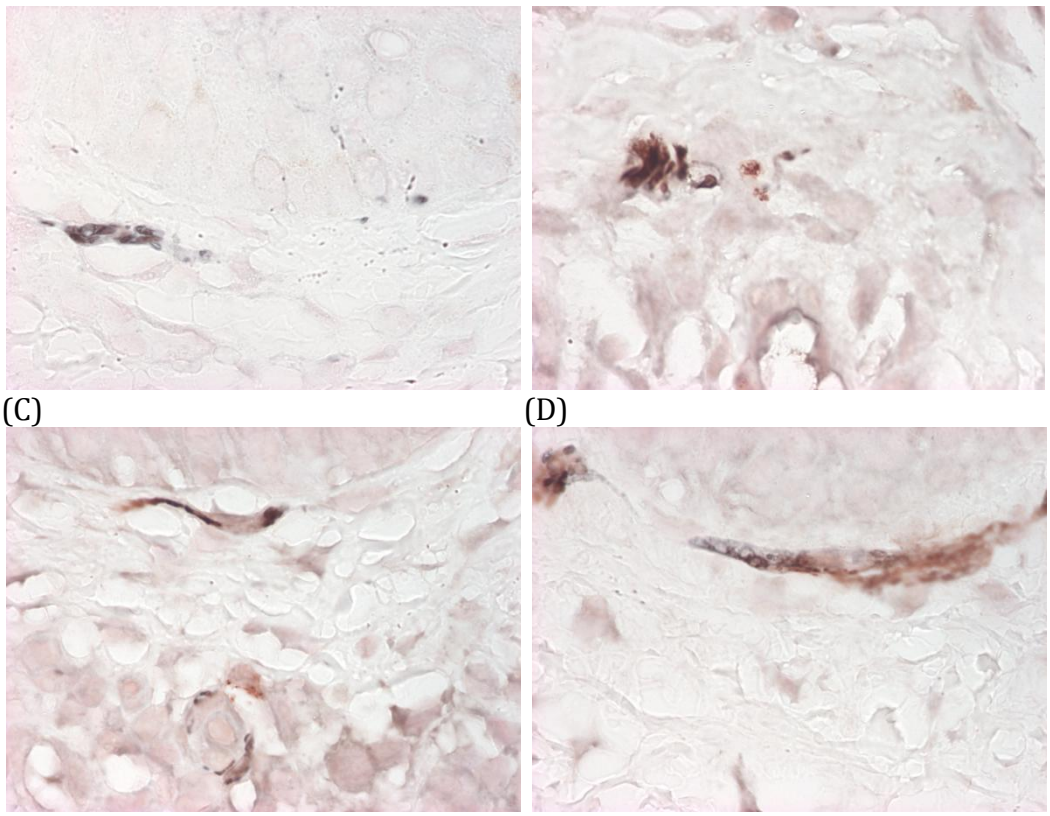


Figure 3.6. Quantification of eosinophil peroxidase in human skin.

60X photographs were taken of skin stained with anti-eosinophil peroxidase (EPO) and assigned random numbers for quantification. EPO+ clusters were defined as more than 5 granules, separated by at least 8um (the diameter of an eosinophil). The counting of individual granules was attempted, but was not possible, due to the prohibitively high number of individual particles. A one-way ANOVA with Tukey's post-test was run, and $p=0.0011$ for the entire data set, with $p<0.05$ for lesional atopic compared to healthy control skin.

(A) (B)



(C) (D)

Figure 3.7. Eosinophil granules can be found near nerves, especially in lesional skin. Double immunohistochemistry was performed with anti-PGP9.5 and anti-EPO antibody on 5um human skin sections. 60X photomicrographs were taken and representatives are shown below. (A) shows lesional skin as an example of a nerve (black) without EPO (red-brown) staining nearby, (B) is lesional skin, with the nerve in black and red granular EPO+ clusters next to the nerve, (C) demonstrates EPO staining near a nerve in lesional skin, and (D) shows EPO staining on top of a nerve in lesional skin.

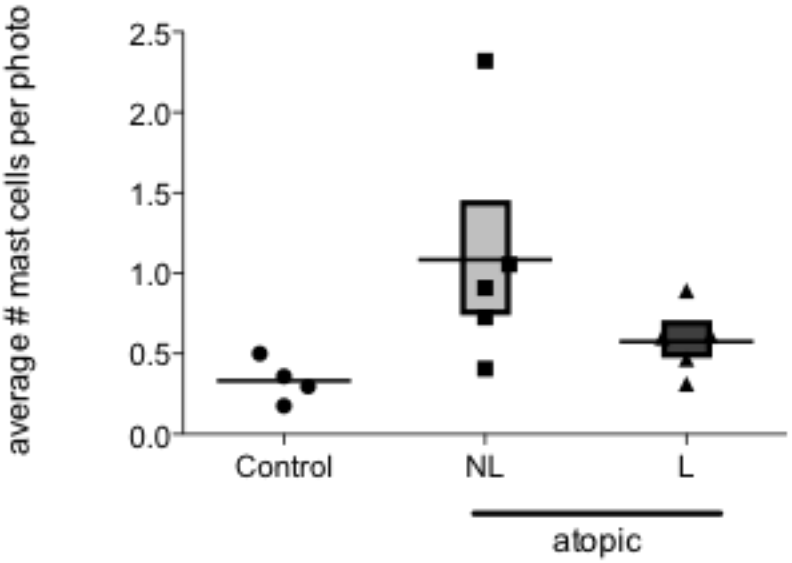


Figure 3.8. Mast cells are increased in non-lesional atopic dermatitis, although not significantly. 5um sections of human skin were stained with toluidine blue and 40X photographs were taken of each entire skin biopsy. Samples were quantified as number of mast cells per photo. A one-way ANOVA was performed with no statistical significance across all groups, and a paired t-test between non-lesional and lesional skin was also not significant.

(A) (B)

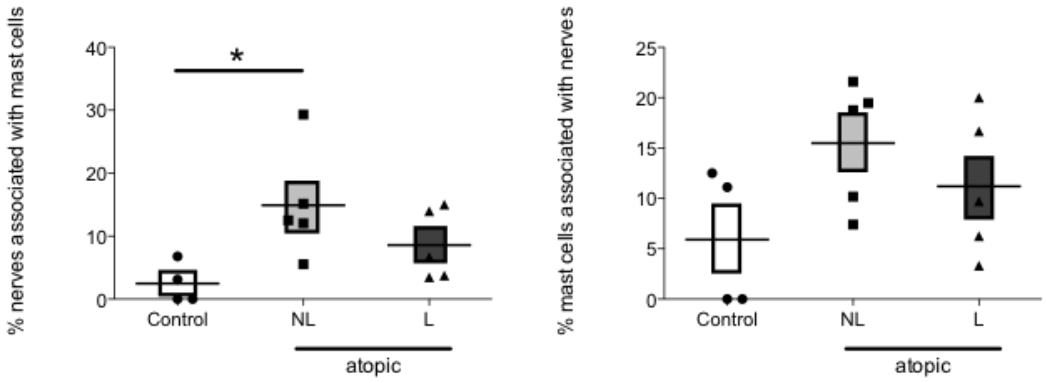


Figure 3.9. Nerves are associated with mast cells in non-lesional atopic dermatitis skin, but not in lesional skin. Double immunohistochemistry was performed with anti-PGP9.5 and toluidine blue, and 40X photographs were taken of each entire skin biopsy. (A) is the percent of nerves in each photo that are associated with mast cells within 15um, or half the diameter of a mast cell. (B) is the percent of mast cells associated with nerves. A one-way ANOVA was performed for each dataset. p=0.0469 for (A) and p=0.1492 for (B).

CHAPTER FOUR

Mouse models of atopic dermatitis and eosinophil recruitment mirror nerve increases seen in human atopic dermatitis. Nerve growth in one model depends on presence of eosinophils.

I. INTRODUCTION

A. Rationale and Hypothesis

Human atopic dermatitis skin has more nerves than healthy control skin, with degranulated eosinophils located in the same regions of the skin, near nerves. We wanted to further explore the connection between eosinophils and nerves in models that could be manipulated to determine whether recruitment of eosinophils to the skin is required for the nerve growth seen in atopic dermatitis. We tested this using a sensitization model of atopic dermatitis in mice lacking eosinophils. We also looked in other models of eosinophil recruitment to the skin to determine if nerve growth was increased above wild-type littermate controls.

B. Models Used in this Study:

i. Epicutaneous Sensitization to Ovalbumin

In mice, mild abrasion of the skin and epicutaneous exposure to ovalbumin cause inflammatory skin disease that resembles human atopic dermatitis (279). In this model, developed by Dr. Raif Geha of Children's Hospital Boston and Harvard University, mice are shaved, and then tape-stripped six times to remove the epidermal barrier, and then a gauze layer soaked with ovalbumin (OVA) is placed

for a sensitization period. This allows the high molecular weight antigen to gain access to the deeper skin layers. The model is replicable and is used frequently as a physiologically relevant model for atopic dermatitis.

Similar to atopic dermatitis in humans, the epidermis of these mice shows thickening, with focal acanthosis and spongiosis. The dermis contains neutrophils, eosinophils, mast cells and mononuclear cells. OVA-sensitized mice have a seven-fold increase in the number of eosinophils per high power field examined, compared to saline-treated mice, as well as a three-fold increase in mast cells (279). Lymphocytes are also significantly increased in OVA-sensitized skin, specifically CD3+ CD4+ and γδ-T cell receptor T cells. There are few CD8+ T cells present. mRNA for IL-4, IL-5 and IFNγ is increased in skin sections of OVA-sensitized mice over saline-treated mouse skin.

Experiments were performed in the initial characterizations of the model to determine whether the resulting skin disease is atopic dermatitis or allergic contact dermatitis (279). OVA sensitization via the skin results in increased eosinophils in bronchoalveolar lavage at 24 hours, as well as airway hyperresponsiveness to methacholine after a single inhaled ovalbumin challenge. In addition, sera from OVA-sensitized mice have OVA-specific IgE, while saline-treated mouse sera do not. Finally, OVA mice also have higher total IgE levels in serum than saline-treated mice and higher OVA-specific IgE levels (279). Because mice were systemically sensitized to antigen through the skin, as shown by airway hyperresponsiveness, the authors

determined that this model resembled the systemic sensitization of atopic dermatitis, rather than site-specific allergic contact dermatitis.

Since the initial description of this model in 1998, the same group has recreated it in numerous knockout strains to characterize the role of chemokine receptors and cytokines that are important for disease progression. They have used the epicutaneous sensitization protocol on mice lacking Th2 cytokines, chemokines, the IL-17 pathway, and epithelial-derived cytokines. Table 4.1 shows all of the cytokine knockout mice that various authors have used with this protocol. The outcomes that were most commonly measured included degree of eosinophil infiltration into the dermis, the number of lymphocytes (or specifically CD4+ T cells) present, the production of Th2 cytokines and chemokines, and whether skin immune cells differed from splenocytes in their ability to stimulate a Th2 reaction.

In this model, IL-4 and IL-5 are important for eosinophil recruitment and other pathology in the skin. Eosinophils were reduced in the skin of mice lacking IL-4, and completely absent from IL-5-deficient mice, after epicutaneous sensitization, compared to wild-type mice. Eotaxin-1 was increased in wild-type mouse skin after sensitization with OVA, but this was not similarly increased in IL-4-deficient mice. Eotaxin was not investigated in IL-5 knockouts.

In addition, dermal thickness after sensitization was reduced in mice lacking IL-5 and IFN-γ. IL-4, IL-5, and IFN-γ are present in atopic dermatitis patient skin samples (53, 59, 306), and the dependence of the model on these cytokines indicate strong parallels with human disease.

The CCR3 chemokine receptor is also required to recruit eosinophils into the skin after OVA sensitization in this model, as well as for the recruitment of eosinophils to the lung and development of airway hyperreactivity after an inhaled challenge (307). Intact eosinophils were absent from the skin of OVA-sensitized CCR3-/- mice, and no MBP was present in the skin of CCR3 knockout mice, in contrast to wild-type OVA-sensitized mouse skin. The number of eosinophils in CCR3-/- mice with saline patches was normal, and migration to the thymus, lung and lymph nodes without stimulation was not affected. There was significantly less dermal thickening on histological analysis. In private communication, the authors said that they did not assess scratching behavior changes and also found no gross clinical changes in the condition of OVA-sensitized skin.

Loss of CCR3 did not prevent the mice from mounting a normal systemic Th2 response, characterized by splenocyte production of IL-4 and IL-5 *in vitro* and elevated OVA-specific IgE in the serum. Importantly, the total number of mononuclear cells and mast cells in skin of CCR3-/- OVA-sensitized mice were the same as wild-type OVA-sensitized, indicating that CCR3 was specifically blocking eosinophil entry rather than other cell types. There were also no differences between the numbers of intact or degranulated mast cells between WT or CCR3-/- mice, even though murine mast cells can express CCR3 chemokine receptors (308, {Humbles, 2002 #415)

Finally, He et al investigated the changes in skin that occur after simply tape-stripping without OVA sensitization (309). They noted that tape stripping mouse skin, without OVA application, increases IL-6, TGF-β, and IL-10 production in skin .

Therefore, the saline-treated control mice already have changes occurring in the skin due to removal of the epidermal barrier.

The OVA sensitization model was attractive to us because it can be used on any strain of mice to recruit eosinophils to skin and generate an inflammatory dermatitis resembling atopic dermatitis. The comparison of wild-type to PHIL mice lacking eosinophils was straightforward with this tape-stripping and patching model. In addition, the originators of this model have written about some provocative unpublished data that we felt confirmed the model's applicability to human atopic dermatitis. Application of OVA to the unbroken skin of hairless mice does not result in the development of an immune response to OVA, while dendritic cells (DC) isolated from the draining lymph nodes of shaved and tape-stripped C57BL/6 mice were able to induce significantly more Th2 cytokine production by T cells from TCR transgenic D011.10 mice than DC from draining lymph nodes of mice that had only been shaved without removal of the epidermal barrier by tape-stripping. In addition, the expression of certain cytokines, especially IL-6, was dramatically increased in tape-stripped and shaved skin, over skin that had been shaved without tape-stripping. This group intends to use comparative gene expression analysis to further explore how barrier disruption and mechanical trauma might polarize DCs toward a Th2 phenotype preferentially (310). Therefore, this mouse models skin trauma, such as patients scratching the skin, and allergen sensitization in atopic dermatitis, in addition to the recruitment of eosinophils into the skin.

ii. Keratin 14-Interleukin-5 Mice (K14-IL5)

The keratin 14-IL-5 mice constitutively express IL-5 under the control of keratin 14 regulatory elements in basal keratinocytes. The mice have mild peripheral blood eosinophilia with greater than 30-fold increased numbers of eosinophils in the skin (Dr. Nancy Lee, Mayo Clinic, Scottsdale, Arizona, personal communication). Some mice develop spontaneous skin lesions and clinically apparent inflammation. Deposition of eosinophil granule proteins in the skin is also detected in these animals, using anti-MBP antibodies. These mice have exacerbated responses to application of oxazalone, a mouse model of skin inflammation.

The unique feature of these mice is the presence of released eosinophil granule proteins in the skin. Each of the other models we used contains intact eosinophils; however, these mice more closely mirror human atopic dermatitis with activated and degranulated eosinophils.

iii. Flaky Tail Mice

The flaky tail (*ft)* mice arose from a spontaneous recessive mutation at Jackson Laboratories in 1958 (46). These mice exhibit peeling tail skin beginning at 3 days of age, which resolves around 15 to 21 days of age. Constriction of the hind ankles and tail can lead to autoamputation (47). These mice do not exhibit spontaneous dermatitis or sensitization when kept in specific pathogen-free conditions (290). However, several reports found defective barrier function in homozygous flaky tail mice. While transepidermal water loss does not significantly differ between *ft/ft* mice and wild-type mice, application of various water-soluble dyes and compounds are able to penetrate more fully, both from the outside (stratum corneum) in, and from the inside (basement membrane zone) out (43,

311). The barrier defect increases with age, with slightly higher TEWL in one year old *ft/ft* animals than wild-type controls.

In addition to a defective epidermal barrier, newborn untreated flaky tail mice have definite histologic changes. The stratum granulosum of epidermis is severely attenuated and is missing the characteristic keratohyalin F-granules, which contain filaggrin (47). The stratum basale appears normal using electron microscopy. However, there is hypertrophy of the stratum corneum, called orthokeratotic hyperkeratosis, and occasional foci of acanthosis, or thickening of the stratum spinosum. Adult mice have similar changes, as well as infiltrates of inflammatory cells into the dermis (290). Younger, 6-8 week old *ft/ft* mice, also have significantly more total cells, lymphocytes, eosinophils and mononuclear cells than wild-type controls.

Flaky tail mice become systemically sensitized to antigen more easily than wild-type controls and have more extreme reactions to irritants and other haptens applied to the skin. Epicutaneous ovalbumin (OVA) sensitization without tape stripping results in significantly increased OVA-specific IgG and IgE in *ft/ft* mice, relative to PBS-treated *ft/ft* or OVA-treated wild-type mice. Splenocytes isolated from OVA-sensitized *ft/ft* mice and stimulated with OVA produce IL-4, IL-5, IL-13, IFNγ, IL-10 and IL-17, while splenocytes from wild-type and heterozygous controls do not (290). Flaky tail mice are equally sensitized to OVA as wild-type mice when it is administered via intraperitoneal injection with adjuvant. Furthermore, application of a variety of haptens and antigens results in thicker ears and more

severe dermatitis with more infiltrating cells than the same applications to wild-type controls (311).

The cause of the flaky tail phenotype was identified as a single base pair deletion in the profilaggrin gene which results in a truncated protein (290). Homozygous *ft/ft* mice do not express profilaggrin normally in the stratum granulosum, while the expression of other epidermal proteins, such as loricrin, keratin 1, keratin 14 and involucrin, does not differ from wild-type controls. Heterozygous *ft/+* mice express wild-type levels of profilaggrin and have no obvious clinical or histologic changes (47). Western blots with an antibody to either the entire profilaggrin protein or the filaggrin repeats indicate a lower molecular weight species in *ft/ft* mice, of 220 kilodaltons (kDa) compared to the normal 496 kDa species (47).

These mice are the most directly linked genetic model of human atopic dermatitis that we tested. Because truncation mutations in profilaggrin similar to the flaky tail mutation generate a predisposition to atopic dermatitis in humans (40, 298, 299) as outlined above, and because flaky tail mice have eosinophils recruited to their skin at baseline, we examined nerves in skin sections from these mice. Through colleagues at Trinity College, Dublin, Ireland, we received a small number of slides with skin sections from flaky tail mice and their wild-type littermate controls.

iv. Keratin 5- Human Transforming Growth Factor beta -1 Mice (K5.hTGF-β1)

K5.hTGF-β1 transgenic mice constitutively overexpress latent human TGF-β1 in basal keratinocytes and hair follicles under the keratin-5 promoter, which leads to the development of inflammatory skin disease (278). A recent evaluation of murine models of human psoriasis ranked *K5.hTGF-β1* transgenic mice high among numerous models, because of clinical, histological, and cellular similarities to human psoriasis (312). The creators of these mice reported clinical and cellular hallmarks of psoriasis, including erythematous scaly plaques, epidermal hyperproliferation, hyerkeratosis, acanthosis, and inflammatory cell infiltrates of CD8+ T cells and neutrophils in the epidermis and CD4+ T cells and macrophages in the upper dermis (278).

We received adult mice from the original authors in order to determine their relevance to human psoriasis, particularly regarding the IL-23/IL-17 cytokine pathway. However, in the process of our analysis, we found the clinical appearance of these mice more closely resembles atopic dermatitis than psoriasis (289). The creators of the mouse also later determined that the expression of TGF-β1 alone is not enough to create an accurate model of psoriasis (313). Therefore, we chose to further investigate this mouse in terms of nerves and eosinophils in the skin, as yet another model of eosinophil recruitment to the skin.

Whether there is a role for TGF-β1 in human atopic dermatitis is unclear. Immunohistochemistry of lesional atopic dermatitis skin demonstrated high levels of TGF-β1 compared to healthy skin. TGF-β1 was expressed throughout the epidermis and by cells infiltrating the dermis, and its receptors, TGF-βRI and TGF-

βRII, were present at high levels in both the epidermis and dermis (56). However, a survey of peripheral blood mononuclear cells from atopic dermatitis patients determined that there was significantly less TGF-β1 mRNA expression compared to healthy control cells (314). One explanation for this is that cells producing TGF-β1 have traveled to the skin and are thus removed from circulation.

There were no regulatory T cells (Tregs) in any atopic dermatitis biopsies, even though TGF-β1 is an important differentiation factor for Tregs from naïve cells (315) (reviewed in (316)). TGF-β1 in an inflammatory milieu can also contribute to the differentiation of Th17 cells (reviewed in (317)). However, there is no dramatic increase in the percent of IL-17-producing cells in acute atopic dermatitis lesional skin and no increase at all in chronic atopic dermatitis skin, compared to healthy controls (318). Compared to psoriasis, in which there is a strong upregulation of the IL-23/Th17 inflammatory pathway, one group of authors noted that the "IL-23/Th17 axis is largely absent" from atopic dermatitis skin (319).

Eosinophils are strong producers of TGF-β1. In cutaneous wound healing, the number of eosinophils in the repairing skin increases steadily until day 9 after wounding, and they are a major source of TGF-β1 (320). Eosinophils expressing TGF-β1 have been found in chronically inflamed upper airway tissues, such as nasal polyps and allergic rhinitis samples (321). Patients with eosinophilia of various etiologies have circulating eosinophils that express TGF-β1 (322). These indicate that an eosinophilic disease, such as atopic dermatitis, might feature increased levels of TGF-β1 production by eosinophils, in both blood and tissue. Interestingly,

TGF-β1 synergizes with IL-4 to increase production of eotaxin from dermal fibroblasts in culture (323). Therefore, if eosinophils or other sources of TGF-β1 and IL-4 are present, a feed forward loop would be created to induce more recruitment of eosinophils to the skin.

Therefore, the K5-hTGF-β1 mouse might be modeling eosinophils present in skin, churning out TGF-β1 and thus recruiting more eosinophils. It is clear that this transgenic mouse model is not a model of atopic dermatitis, but rather an important tool to determine the relationship between eosinophils and nerves in the skin.

One caveat to this model is that the few mechanistic studies done addressing TGF-β1 and eosinophils indicate that TGF-β1 inhibits eosinophil activation, survival effects by growth factors and can even contribute to eosinophil apoptosis (324, 325). One group determined that TGF-β1 inhibits phosphorylation of signaling proteins downstream of the IL-5 receptor, which could be a possible mechanism for such diverse effects (325). These experiments were all performed in culture. Thus, it is possible that the functions and survival of eosinophils present in the skin of K5-hTGF-β1 mice are inhibited. We did not test this possibility.

II. RESULTS

A. Epicutaneous Sensitization to Ovalbumin

As previously reported, three one-week periods of sensitization to ovalbumin separated by two 2 week periods of rest result in skin changes that resemble human atopic dermatitis (279). Figure 4.1A demonstrates the histological changes seen in

wild-type OVA-sensitized mouse skin, such as thickened epidermis and dermal infiltrate, compared to PBS-treated controls. Controls treated with PBS also undergo shaving and tape-stripping, and there are changes to the skin simply due to the removal of the epidermal barrier (Figure 4.1A, top right panel). However, eosinophil and T cell infiltration into the skin is significantly increased in OVA-sensitized skin over PBS-treated controls (Figure 2), and the most significant epidermal and dermal changes occur in OVA-treated skin (279).

In order to investigate the role of eosinophils, we used mice in which the diphtheria toxin A chain open reading frame is under the control of the eosinophil peroxidase (EPO) promoter (PHIL mice) (277). Expression of diphtheria toxin A in EPO-positive cells leads to the cell death of eosinophils and their precursors. After sensitization with OVA, these mice exhibit thickened epidermis, similar to wild-type mice, and infiltration of the skin with inflammatory cells, with the notable absence of eosinophils (Figures 4.1B, 4.2).

Among three separate experiments, epicutaneous sensitization to OVA resulted in variable recruitment of eosinophils to the skin of treated mice (Figure 4.3). Due to changes in the mouse housing that were beyond our control, each experiment was performed in a different mouse room with a different level of cleanliness. The differing results indicate that the general prevalence of antigens in a room or mouse box might affect the number of eosinophils recruited to the skin of mice after removal of their epidermal barrier. Thus, in only one of our three sets of experiments did we succeed in sensitizing the mice, as indicated by the presence of eosinophils in the treated area of the skin (Figure 4.1, 4.2, 4.3)

In wild-type mice that had increased eosinophils after OVA-sensitization, there was an increase in PGP9.5+ nerves after the sensitization period, compared to saline-treated wild-type skin (Figure 4.4). This mirrors the increase in nerves that occurs in human atopic dermatitis lesional skin. However, eosinophil-deficient PHIL mice did not show a similar increase in number of cutaneous nerves, indicating a requirement for eosinophils for nerve branching in this mouse model of atopic dermatitis (Figure 4.4). In two other experiments with no increase in eosinophils in the skin, there were no significant increases in nerves in wild-type sensitized mouse skin, and no difference in the number of nerves between wild-type and PHIL mice. These data indicate that eosinophils recruited to the skin after sensitization are important for the increases in nerves seen in skin in a model of atopic dermatitis.

B. Keratin 14-Interleukin 5 Mice (K14-IL-5)

We received paraffin-embedded skin sections on slides from James and Nancy Lee, Mayo Clinic, Scottsdale, Arizona of K14-IL-5 mice with several matched sections of wild-type littermate controls. Upon staining for eosinophil major basic protein (MBP) to determine to what extent eosinophils are recruited into the skin of these mice, we saw very bright MBP staining in transgenic mouse skin, especially in the epidermis (Figure 4.5A). In addition, double immunocytochemistry with anti-PGP9.5 indicated that much of the MBP staining in the dermis was located contiguous with nerves (Figure 4.5B). There were also many intact eosinophils in the dermis and hypodermis (data not shown). There was virtually no MBP staining in wild-type mouse skin.

Our main question is how the presence of eosinophils affects nerve numbers in the skin, so we stained K14-IL-5 sections with anti-PGP9.5 to visualize and quantify nerves (Figure 4.6). It was immediately apparent that K14-IL-5 mice had more nerves in the skin, but this phenomenon was localized to the epidermis rather than the dermis (Figure 4.6C). Since the IL-5 transgene is expressed under the control of a keratinocyte promoter, and since the majority of eosinophil MBP we saw in the skin of transgenic mice was in the epidermis as well, this indicates that the increase in nerves is linked to the location of the eosinophils. A quantification of nerves in the dermis and epidermis demonstrated what we had observed, that nerves were significantly increased in the epidermis of transgenic mice above controls (Figure 4.7).

Thus, the presence of eosinophils recruited to skin directly correlates to the number of nerves. We also performed a correlational analysis of eosinophils in nerves in all mouse models, discussed below.

C. Flaky Tail Mice

Flaky tail mice mimic atopic dermatitis found in humans with filaggrin deficiency. As these mice are not commercially available, we were fortunate to receive slides of paraffin-embedded skin sections from Padraic Fallon in Ireland. However, each *ft/ft* slide only had a single section, and there was only one slide per mouse, which meant only a single question could be addressed. For the wild-type littermate controls, each slide had one section, but there were at least two slides per mouse. This allowed us to stain the sections for eosinophil MBP to determine the levels in wild-type mouse skin in a different animal facility.

PGP 9.5 staining and quantification indicated that flaky tail mice had somewhat higher numbers of nerves in the papillary dermis and basement membrane zone than controls, except for one wild-type outlier (Figure 4.8). Upon H&E and MBP staining of this wild-type control, a significantly higher number of eosinophils were found in the skin. Excluding this outlier gave a significant difference between number of nerves in flaky tail mice and their wild-type littermate controls.

It is unclear what normal role high levels of eosinophils in the skin of wild-type mice could have. In our own mouse colony, we found great variation among C57BL/6 mice of approximately similar ages when we examined their skin for eosinophils (data not shown). Neither we nor the Department of Comparative Medicine were aware of fur mites in any of these mice. However, it is clear that the findings reported here, of wild-type mice with high numbers of eosinophils and high numbers of nerves, support our conclusions, that eosinophils influence the number of nerves in the skin.

D. Keratin 5-Human Transforming Growth Factor-beta 1 Mice (K5.hTGF-β1)

While investigating a potential mouse model of psoriasis in the K5.hTGF-β1 mouse, we found higher than predicted numbers of eosinophils in H&E stained sections from the lesional skin of these mice (Figure 4.9). We subsequently investigated total RNA levels of several cytokines in the skin, using quantitative real-time RT-PCR (Figure 4.10). Human TGF-β1 message was elevated, as expected, but the second most dramatic change was in IL-4, a prototypical Th2 cytokine. This

cytokine is found in atopic dermatitis lesions, but not psoriasis lesions, and was a first indicator that this disease was not a model of psoriasis. In addition, there were increases in IL-17A, TNF-α, IL-6, and the p35 subunit of IL-12. We compared lesional skin to wild-type skin, non-lesional skin to wild-type, and lesional skin to non-lesional skin in each animal. IL-4 was significantly increased in both lesional and non-lesional skin compared to wild-type control skin, but there was no significant difference in IL-4 levels between lesional and non-lesional skin from the same animals. TNF-α, IL-6, IL-12p35, on the contrary, were increased in lesional skin, compared to non-lesional skin, indicating a difference after lesions had formed.

We also isolated total protein from the skin of transgenic mice and found increases in hTGF-β1, IL-4 and IFNγ, especially in lesional skin. Serum IgE was significantly elevated in transgenic mice, compared to controls. We were unable to detect any IL-23 or IL-17A protein in skin, both of which are important in the pathogenesis of human psoriasis (Figure 4.11). These data, along with antibody blockade and knockout data not discussed here, indicate that the K5.hTGF-β1 mouse is not a model of psoriasis, but might be useful in the study of eosinophil recruitment to the skin, elevated IgE and IL-4 levels, and other hallmarks of atopic dermatitis.

We examined the skin of these mice to determine nerve numbers, and we found that K5.hTGF-β1 lesional skin has more nerves than wild-type littermate control skin (Figure 4.12). We did not examine non-lesional skin samples from these mice, which would be an interesting future study.

E. All mouse models compared to each other

At the end of these studies, we wanted to understand the different correlations between eosinophils and nerves in each model. We first graphed the number of nerves found in each mouse model on the same axes, to be able to compare each model (Figure 4.13). We also correlated the number of eosinophils found in each section by H&E with the number of nerves in the skin (Figure 4.14). The Pearson R values for each model are listed in Table 4.2. The strongest correlation between eosinophils and nerves, based on the R values, was in the successful OVA sensitization experiment, followed by K5.hTGF-β1, K14-IL-5 epidermis, and the first OVA sensitization.

Clearly, the epidermal nerves in transgenic K14-IL-5 mice were double those in any other model (Figure 4.13). This indicates that the model with the strongest recruitment of eosinophils also had the largest increase in cutaneous nerves. However, the correlation of eosinophils with nerves was not perfectly linear in this model, as shown in Figure 4.14.

The models for atopic dermatitis that were used here should be considered for each individual aspect of the disease that they may or may not represent. Table 4.3 shows the qualities of human atopic lesional and non-lesional skin compared to each different mouse model. We were interested in recruitment of eosinophils and nerve growth, and we found that nerve growth happened consistently in models where there was a barrier defect, recruitment of eosinophils to the skin, and especially activation of eosinophils. Together, these data indicate that the presence

of eosinophils in the skin of mice has important effects on cutaneous nerve growth and branching.

III. DISCUSSION

In our studies, we have used previously existing mouse models of atopic dermatitis and recruitment of eosinophils into skin to try to determine the role of eosinophils in nerve branching *in vivo.* The benefits of these existing mouse models are that there are protocols in place and reagents available to assay nerves and eosinophils and the extent of skin disease in each animal. In addition, all of these mice come from inbred strains, so the variability due to genetic background within a specific model should be limited. Mouse models also provide an opportunity to manipulate the environment in a way that is enviable to scientists limited to humans in the clinic. All of the mice within a group have been housed together in the same environment, limiting the effects of different airflow, cleanliness or nutrition.

However, interestingly, while every mouse within a group lived in the same setting, every *group* of mice lived in a different environment, and this must be kept in mind for the interpretation of the studies, especially the epicutaneous sensitization to ovalbumin (OVA), discussed above. Mice used in these studies lived and died in Ireland, Arizona, the Portland VA Medical Center, and the Biomedical Research Building, in three different mouse rooms. This difference in environment could affect baseline skin inflammation, so we were careful to use wild-type littermate controls that grew up and lived with each group of mice.

Some of these studies would be impossible in humans, simply due to the existence of transgenes overexpressing cytokines. Others have been performed in humans and were simply standardized for use in mice, including epicutaneous sensitization to allergens after abrading the skin of atopic dermatitis patients (326).

Each mouse model that we used represents one or more portions of the entire disease phenotype of atopic dermatitis. Epicutaneous application of OVA and overexpression of human TGF-β1 in keratinocytes lead to systemic sensitization, indicated by elevated serum IgE and Th2 cytokines. Flaky tail mice have an impaired barrier, but no sensitization unless allergen is applied. Mice overexpressing IL-5 in keratinocytes have an enormous recruitment of eosinophils to the skin and significant activation, as indicated by released eosinophil granule proteins.

Because each model is simply a piece of the disease, it is logical that effects on nerves would differ. The most dramatic increase in nerves in skin was seen in the epidermis of K14-IL-5 transgenic mice, where eosinophils were recruited and activated. The next highest increase was in the K5.hTGF-β1 mice, which have systemic sensitization, impaired barrier, and high expression of IL-4 in the skin. Then came the OVA-sensitized mice, which also undergo systemic sensitization, but have a more mildly impaired skin barrier. Barrier removal without sensitization, as in the flaky tail mice, resulted in a significant, but not dramatic, increase in nerves in the skin. Sadly, we were not able to quantify the number of eosinophils or amount of MBP in these mice, due to the lack of samples.

It is not known what other processes might contribute to nerve growth in the skin of these mouse models. Keratinocytes and fibroblasts can synthesize nerve growth factor and, once activated by mechanical trauma or pattern recognition signaling, could independently contribute to nerve growth (255, 327). There are some reports that cytokines, such as IL-6, can induce nerve branching and might be important after mechanical trauma (328).

It is clear from our studies that eosinophils are an important cell type mediating nerve growth. Future analyses should take advantage of the PHIL mice, as well as IL-5 transgenic mice, to directly assay the role of eosinophils in each of the models used in this report.

IV. TABLES.

Table 4.1. Summary of knockout mouse experiments performed by Geha et al, in the epicutaneous sensitization mouse model of atopic dermatitis.

Mouse	Eos in dermis?	T/B cells	Th2 cytokines	Eotaxin	Paper
IL-5-/-	None	Same as WT	Less IL-4	ND	Spergel 1999
IgE-/-	Same as WT	Same as WT	Same as WT	ND	Spergel 1999
IFNγ-/-	Same as WT	Same as WT	Same as WT	ND	Spergel 1999
IL-4-/-	Fewer	More	IL-5 ok	Less*	Spergel 1999
IL-4-/- (lung)	Same-lung	Same-lung	Same-lung	ND	Herrick 2000
CCR3-/-	None	Mono same	IL4, IL5 ok	ND	Ma 2002
IL-10-/-	Fewer	Mono fewer	Less IL-4/5	Less*	Laouini 2003
TSLPR-/-	Fewer	Same as WT	Less mRNA Spleno- ok	ND	He 2008

*IL-4-/-OVA AND IL-10-/- have more MIP-1b and MIP-2 than WT-OVA

ND = not determined

Table 4.2. Correlation of nerves with eosinophils in various mouse models of eosinophil recruitment to skin. Number of nerves and number of eosinophils were analyzed for correlation using Prism 5 for Mac OSX, since each measurement was made independently. Pearson's correlation was used, which assumes the values had a Gaussian distribution. Based on the R values, it is clear that the successful OVA sensitization experiment had the strongest correlation between intact eosinophils and nerves (closest to 1), followed by K5.hTGF-β1, K14-IL-5 epidermis, and the first OVA sensitization.

Name of model	**R value**
OVA-Sensitization Experiment #1	0.2123
OVA-Sensitization Experiment #2 (eosinophils infiltrated, increase in nerves)	0.4038
OVA-Sensitization Experiment #3	0.0045
K14-IL-5 Dermis	0.0014
K14-IL-5 Epidermis	0.1921
K5.hTGF-β1	0.3437

Table 4.3. Summary of mouse models and representation of human atopic dermatitis.

Model	Barrier defect?	Sensitization? (i.e., high IgE?)	Eos in skin?	Eos activated?	More nerves?
Human lesional AD	yes	yes	yes	yes	yes
Human non-lesional AD	yes/no	yes	few	yes	no
OVA-sensitized wild-type	yes	yes-published	yes	?	yes
PBS- wild-type	yes	no	few	?	no
OVA-sensitized PHIL	yes	?	no	no	no
K14-IL-5	?	?	yes	yes-degranulated	yes
Flaky tail	yes	no	yes	?	yes
K5.hTGFβ1	yes	yes	yes	?	yes

V. FIGURES.

(A)

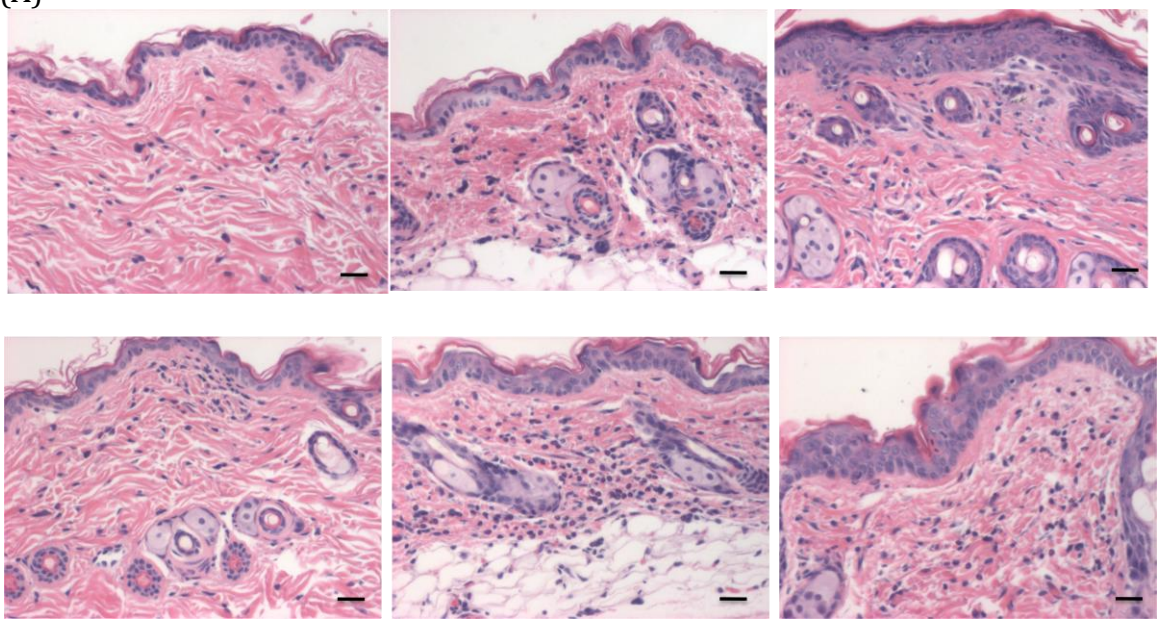


Figure 4.1A. Epicutaneous sensitization to ovalbumin of wild-type mice results in thicker epidermis and dermal infiltration of inflammatory cells.

Hematoxylin and eosin (H&E) of mouse skin samples from PBS-treated controls (top panel) and OVA-sensitized mice (bottom panel). Scale bars = 50um.

(B)

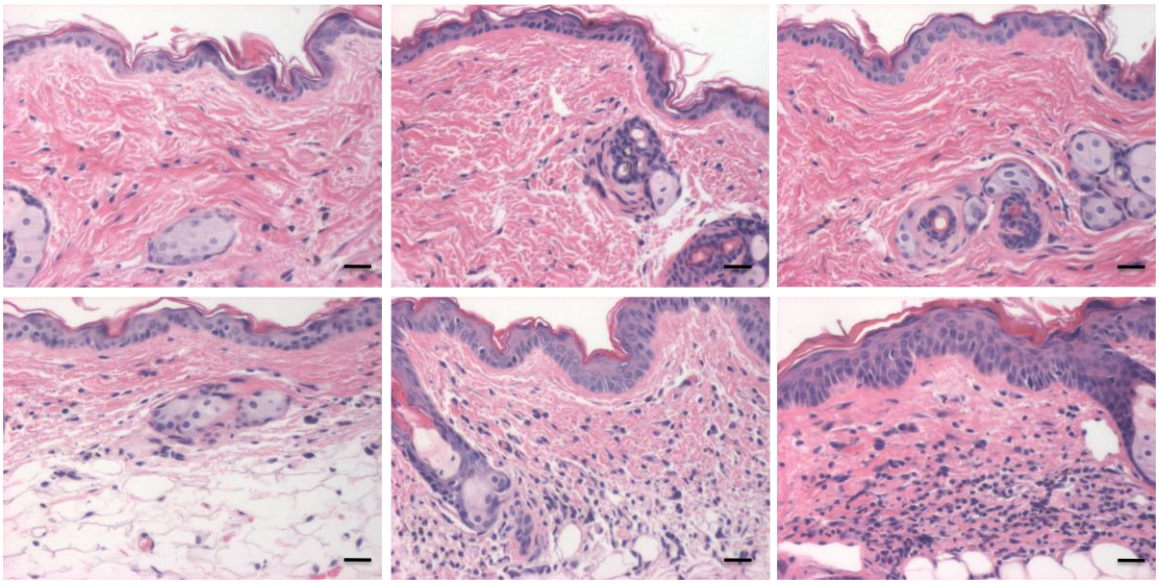


Figure 4.1B. Epicutaneous sensitization to OVA results in epidermal thickening and infiltration of immune cells into the dermis of eosinophil-deficient PHIL mice, but no eosinophils. H&E stained sections of 6 different PHIL mice. PBS-treated controls are in top panel, while OVA-sensitized PHIL are in the bottom panel. Scale bars = 50um.

PBS-treated skin **OVA-treated skin**

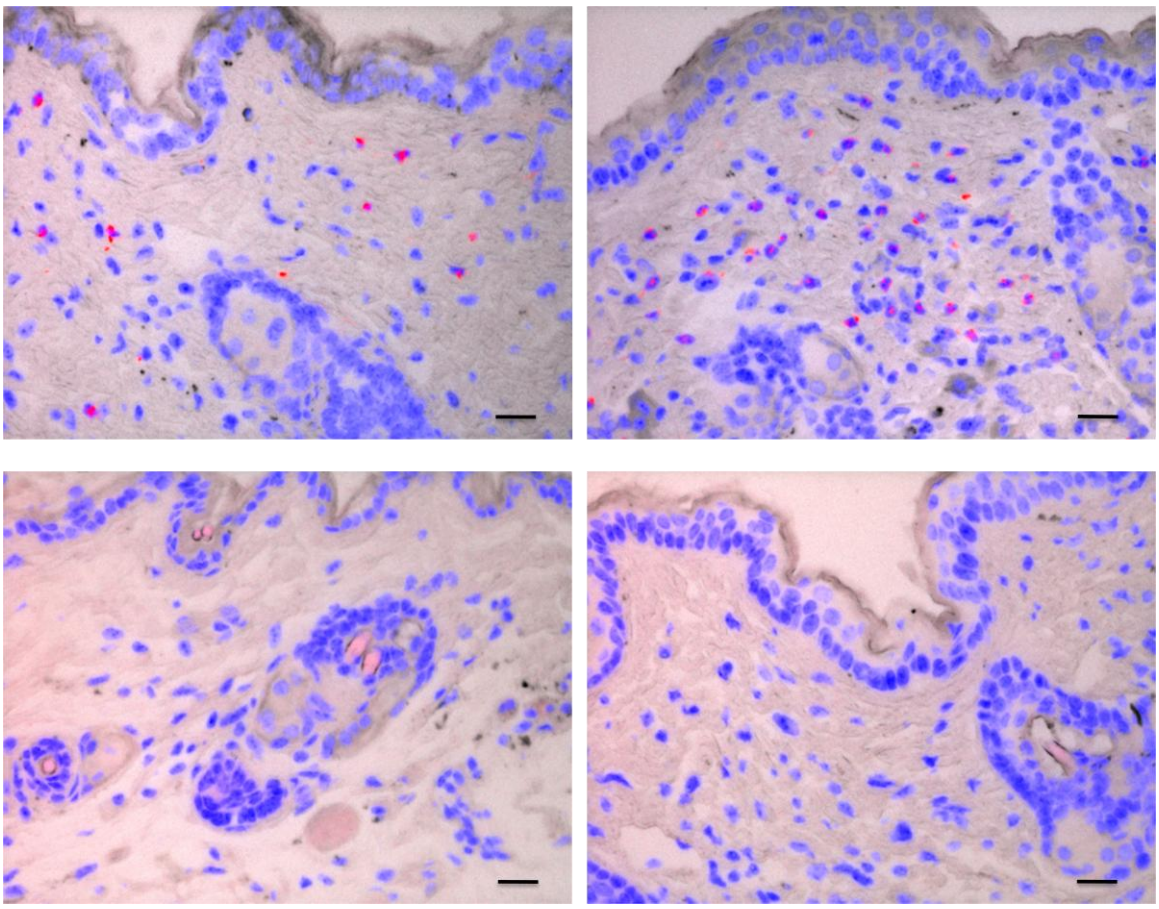


Figure 4.2. Eosinophils infiltrate into wild-type mouse skin sensitized to OVA, but not PHIL skin. Sensitized mouse skin was fixed in 4% paraformaldehyde and stained for eosinophil major basic protein (MBP) (pink), and counterstained with DAPI for nuclei (blue). Nerves are stained using PGP9.5 antibody (black).
Top row: wild-type, bottom row: eosinophil-deficient PHIL. Left panels: PBS-treated skin, right panels: OVA-treated skin. Scale bars = 50um.

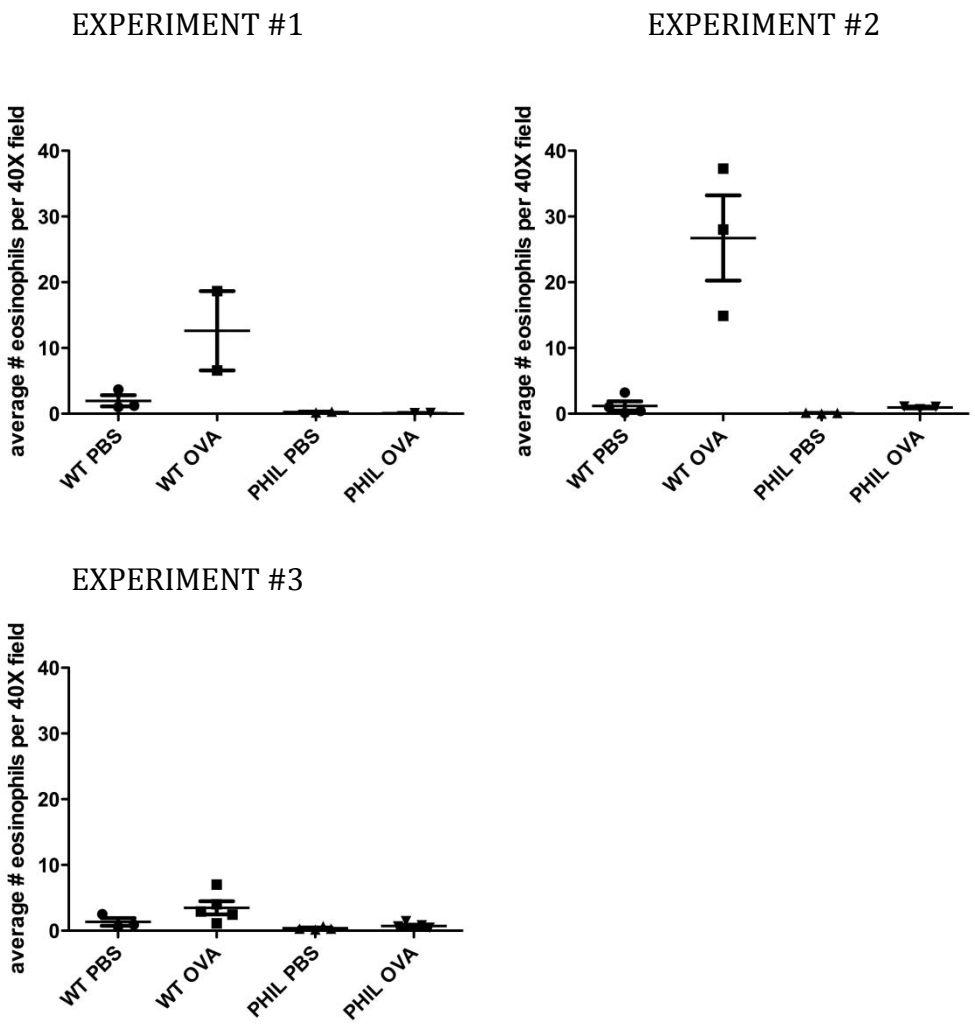


Figure 4.3. Epicutaneous sensitization results in variable recruitment of eosinophils to the skin of mice. Eosinophils were counted in H&E stained samples using the 40X objective. Data are shown as average number of eosinophils per 40X field. Each graph depicts an independent experiment, each of which occurred in a different animal housing room.

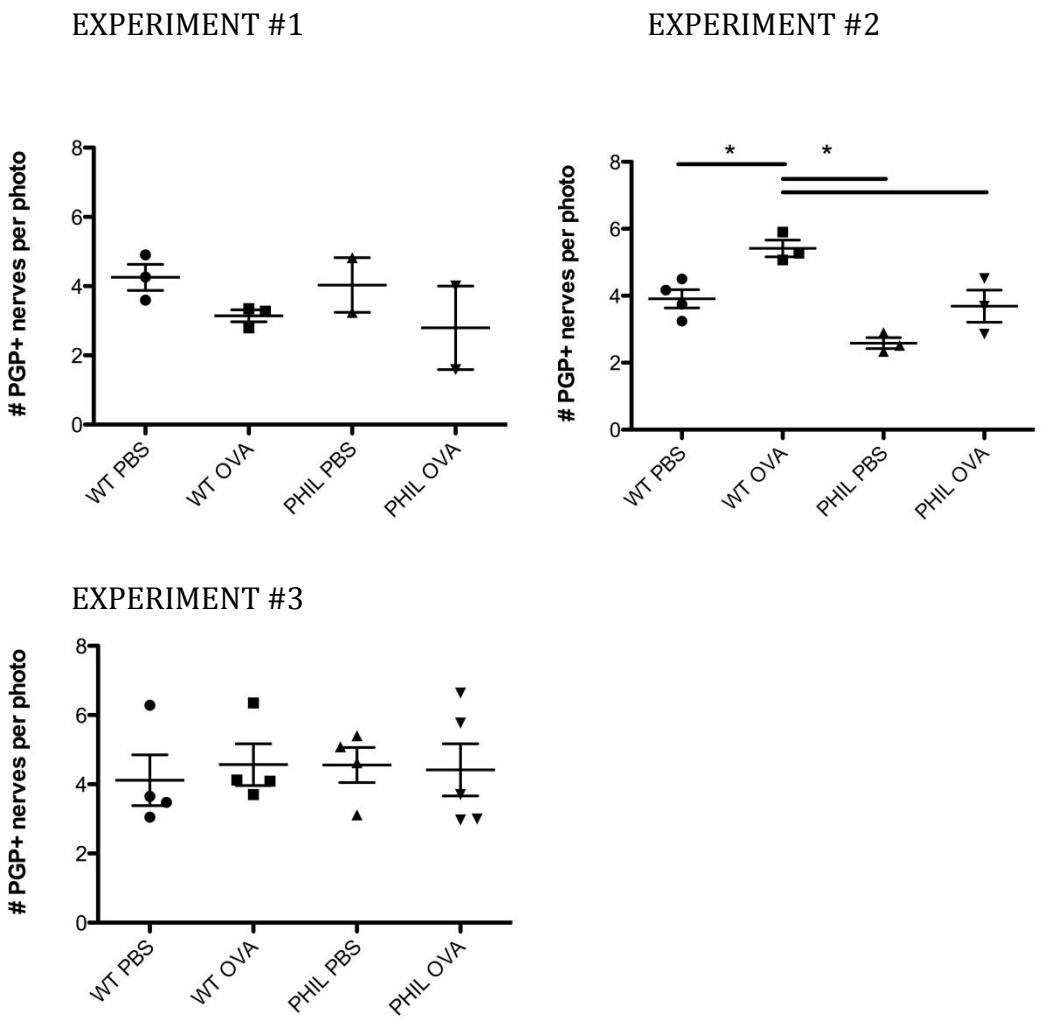
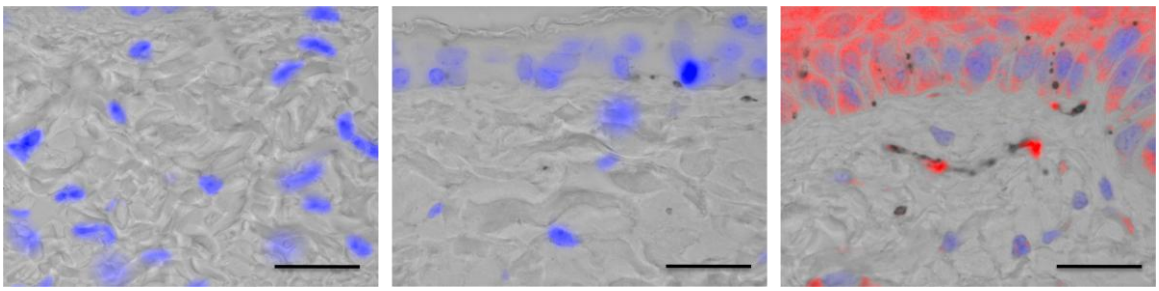


Figure 4.4. Cutaneous nerve numbers are increased in mice that have increased eosinophils. Skin sections from epicutaneously sensitized mice and controls were fixed and stained using anti-PGP9.5 to visualize nerves. Each section was photographed along its length. Random numbers were assigned to each photograph and the number of nerves present in the papillary dermis and basement membrane zone was quantified by an observer blinded to the treatment group. Data are shown as average number of PGP+ nerves per photo and are in the same order as Figure 3. * denotes $p<0.05$.

(A)



(B)

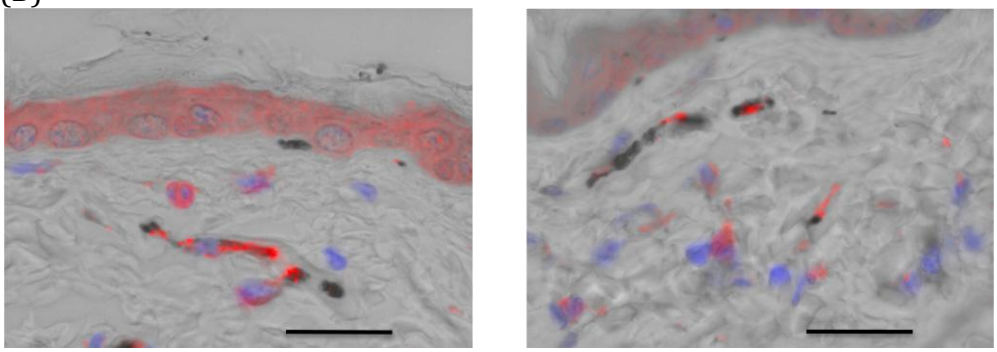
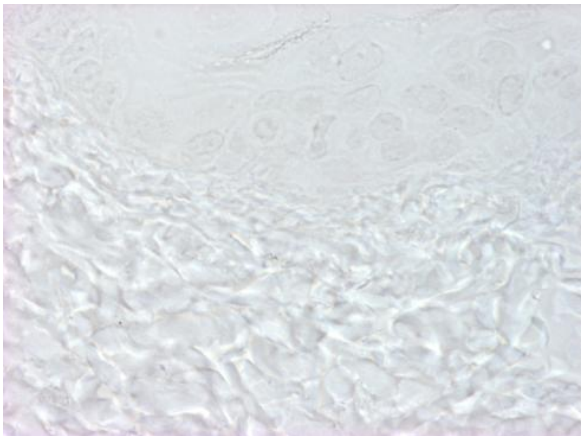
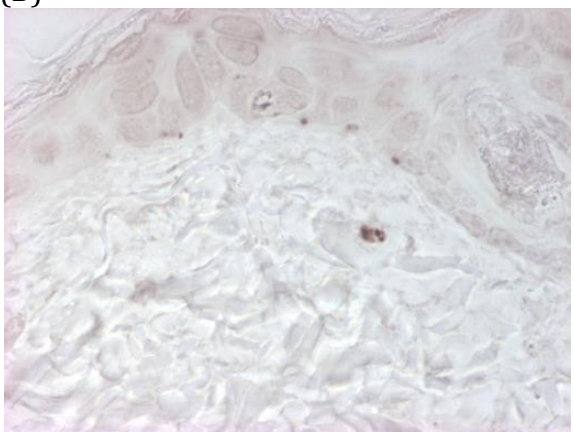


Figure 4.5. Keratin 14-Interleukin 5 mice have high levels of eosinophil granule protein in skin. Slides from paraffin-embedded skin sections were shipped from the Mayo Clinic, Scottsdale, AZ, and were stained with anti-MBP antibody (pink), anti-PGP9.5 antibody (black), and counterstained with DAPI (blue). (A) Left: IgG negative control, middle: wild-type mouse skin, right: K14-IL-5 transgenic mouse skin. (B) Anti-MBP staining (pink) often occurred in close proximity to nerves (black). Scale bars = 50um.

(A)



(B)



(C)

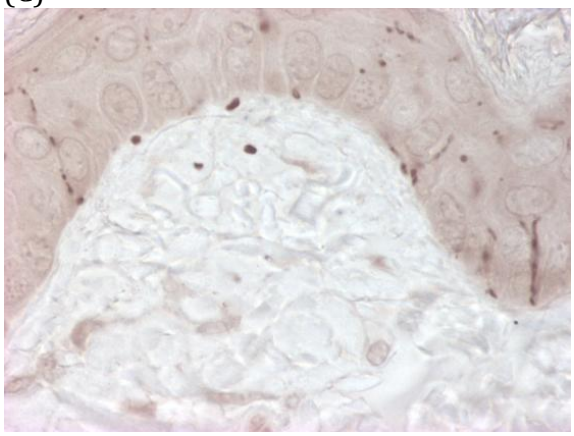
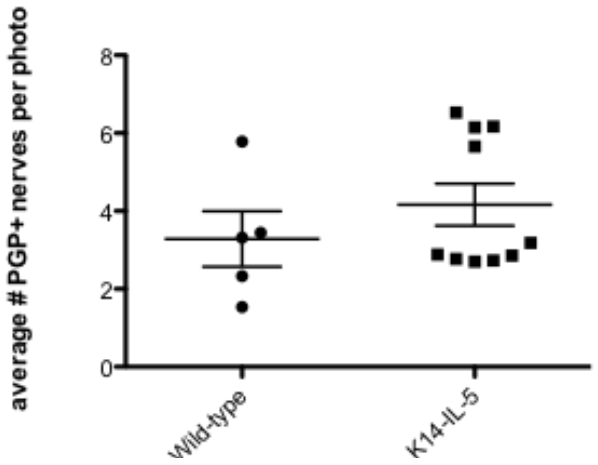


Figure 4.6. Keratin 14-Interleukin 5 mice have more nerves in epidermis and basement membrane zone than wild-type controls, but similar numbers in the dermis. 5um skin sections on slides were stained using anti-PGP9.5 to visualize nerves (brown). Photographs of each entire section were taken, and random numbers assigned to each for quantification by an observer blinded to the genotype. Quantification is demonstrated in Figure 7. (A) IgG negative control, (B) representative photomicrograph of PGP9.5 staining of a wild-type mouse section, (C) PGP9.5 stained K14-IL-5 skin section. Note the extensive linear nerves from the basement membrane zone through the layers of the epidermis.

(A)



(B)

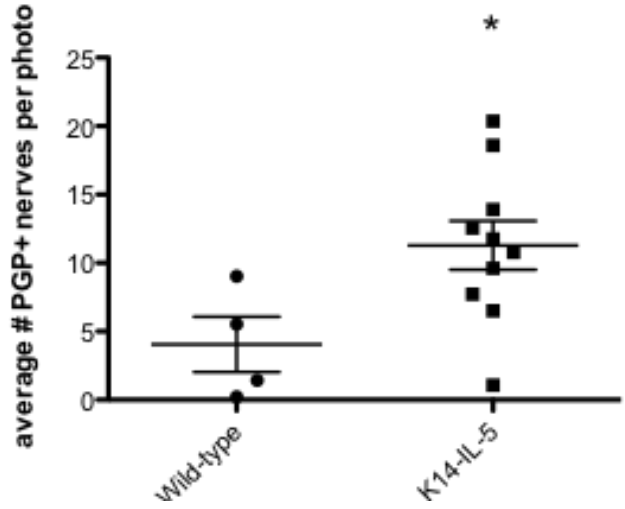
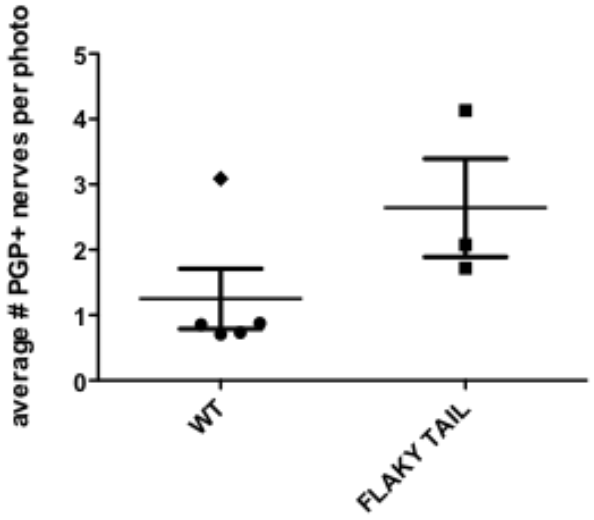


Figure 4.7. K14-IL-5 mice have more nerves in epidermis, compared to wild-type, but similar numbers in the dermis. Paraffin-embedded skin sections were stained with anti-PGP 9.5, assigned random numbers and quantified.

(A) Quantification of nerves in papillary dermis in K14-IL-5 mice and wild-type littermate controls. (B) Quantification of nerves in epidermis and basement membrane zone of K14-IL-5 mice and wild-type littermate controls. * denotes $p < 0.05$ using an unpaired t-test.

(A)



(B)

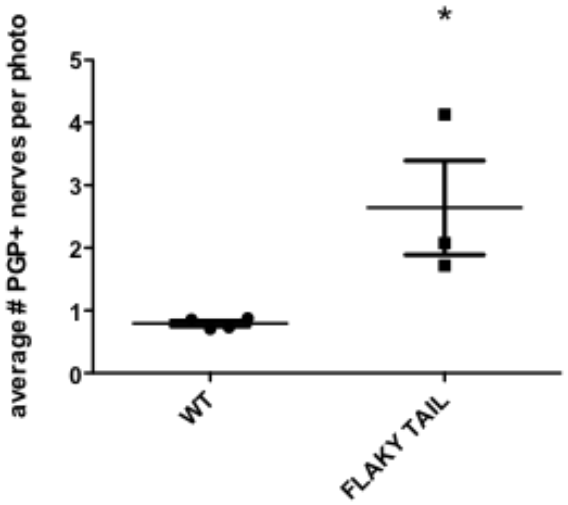


Figure 4.8. Flaky tail mouse has increased nerves compared to wild-type littermate controls. Skin sections from filaggrin-mutant flaky tail mice were stained using anti-PGP9.5, photographs were taken and random numbers assigned. Nerves were quantified using Metamorph by an observer blinded to the genotype. (A) and (B) show quantification of same samples, except that (A) includes the wild-type outlier (diamond) that had significantly higher numbers of eosinophils than any of the other controls. * denotes significantly different than control.

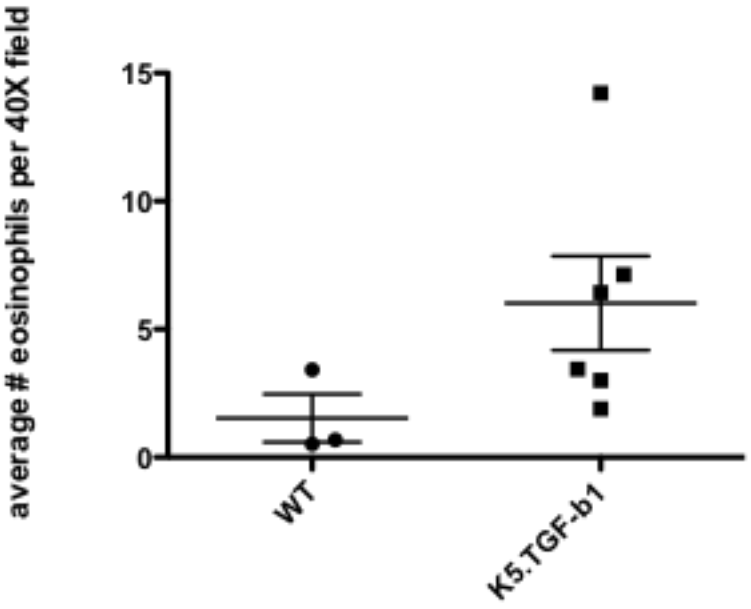


Figure 4.9. K5.hTGF-β1 mice have eosinophils present in skin.

Intact eosinophils were counted in sequential H&E fields using the 40X microscope objective. p=0.150 using t-test.

Figure 4.10 (next page). RNA from K5.hTGF-β1 mice indicate a disease resembling atopic dermatitis more than psoriasis. Total RNA was isolated from skin of wild-type or lesional/non-lesional skin of K5.hTGF-β1 mice, and cDNA was synthesized and amplified with primers for each listed gene. Graphs on left show fold change over GAPDH housekeeping gene. Graphs on right show direct comparisons between gene expression in different types of skin. (a) K5.hTGF-β1 lesional skin compared with wild-type; (b) K5.hTGF-β1 non-lesional skin compared with wild-type; (c) K5.hTGF-β1 lesional skin compared with K5.hTGF-β1 non-lesional skin.

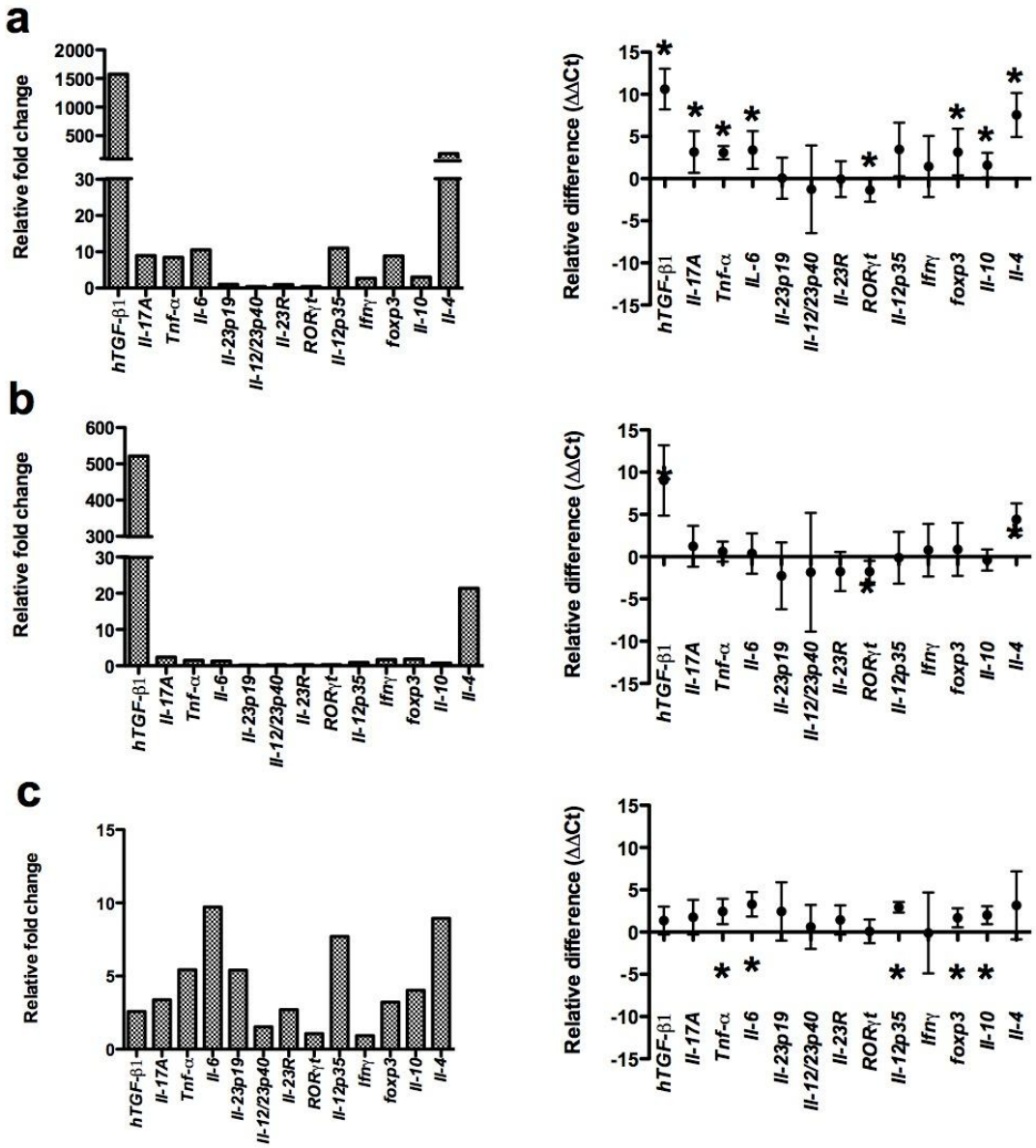
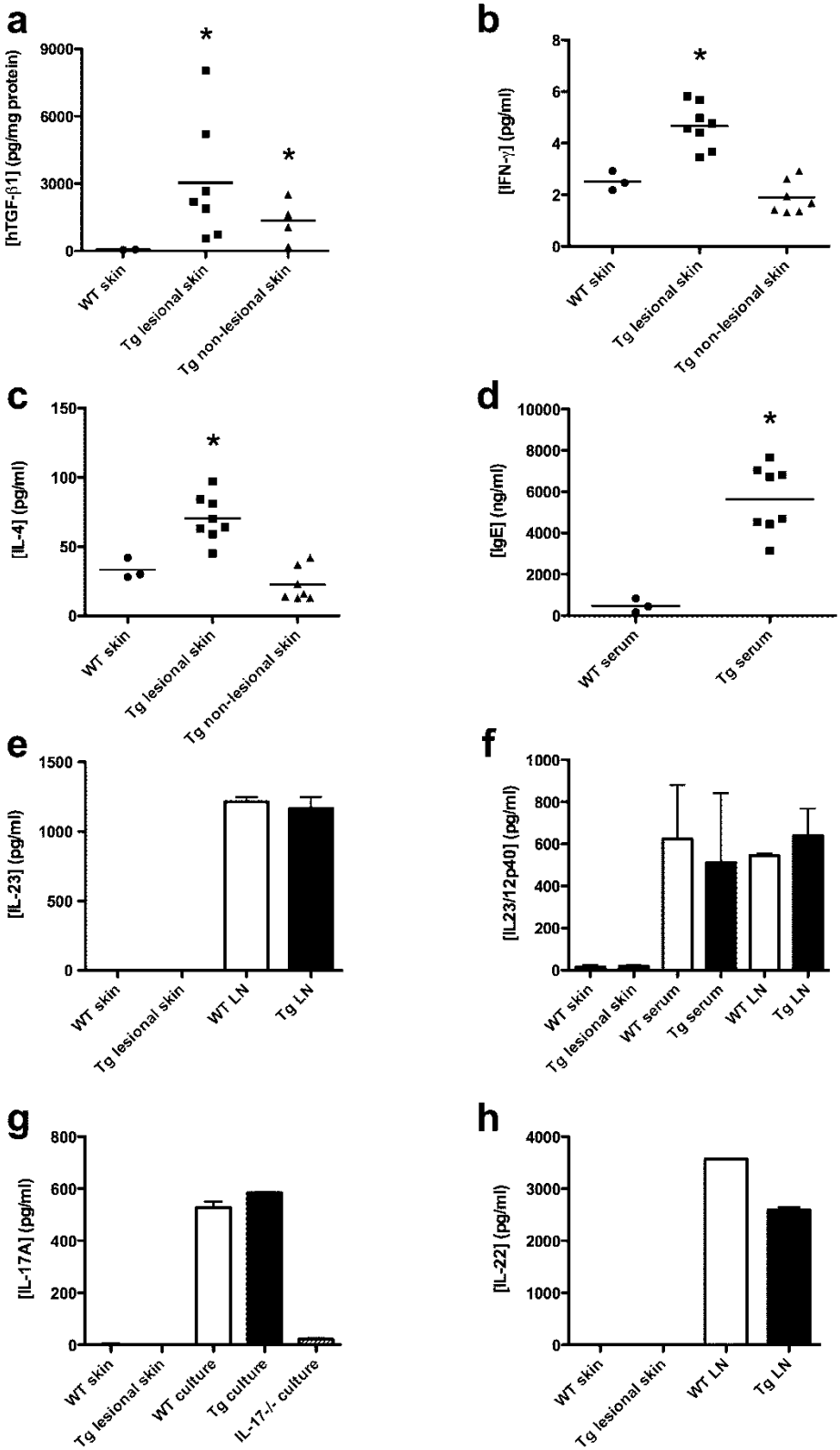

a
b
c
Relative fold change
Relative difference (ΔΔCt)
hTGF-β1
Il-17A
Tnf-α
Il-6
Il-23p19
Il-12/23p40
Il-23R
RORγt
Il-12p35
Ifnγ
foxp3
Il-10
Il-4

Figure 4.11 (next page). Protein profile from the skin and sera of K5.hTGF-β1 mice indicates a disease resembling atopic dermatitis more than psoriasis. Total protein was isolated from the skin (a-c, e,f) or sera (d) of wild-type or K5.hTGF-β1 mice. Both lesional and non-lesional skin were taken from K5.hTGF-β1 mice. Protein was assayed by ELISA. * denotes $p < 0.05$, significantly different than control.


a
[hTGF-β1] (pg/mg protein)
9000
6000
3000
0
*
*
WT skin
Tg lesional skin
Tg non-lesional skin
b
[IFN-γ] (pg/ml)
8
6
4
2
0
*
WT skin
Tg lesional skin
Tg non-lesional skin
c
[IL-4] (pg/ml)
150
100
50
0
*
WT skin
Tg lesional skin
Tg non-lesional skin
d
[IgE] (ng/ml)
10000
8000
6000
4000
2000
0
*
WT serum
Tg serum
e
[IL-23] (pg/ml)
1500
1000
500
0
WT skin
Tg lesional skin
WT LN
Tg LN
f
[IL23/12p40] (pg/ml)
1000
800
600
400
200
0
WT skin
Tg lesional skin
WT serum
Tg serum
WT LN
Tg LN
g
[IL-17A] (pg/ml)
800
600
400
200
0
WT skin
Tg lesional skin
WT culture
Tg culture
IL-17-/- culture
h
[IL-22] (pg/ml)
4000
3000
2000
1000
0
WT skin
Tg lesional skin
WT LN
Tg LN

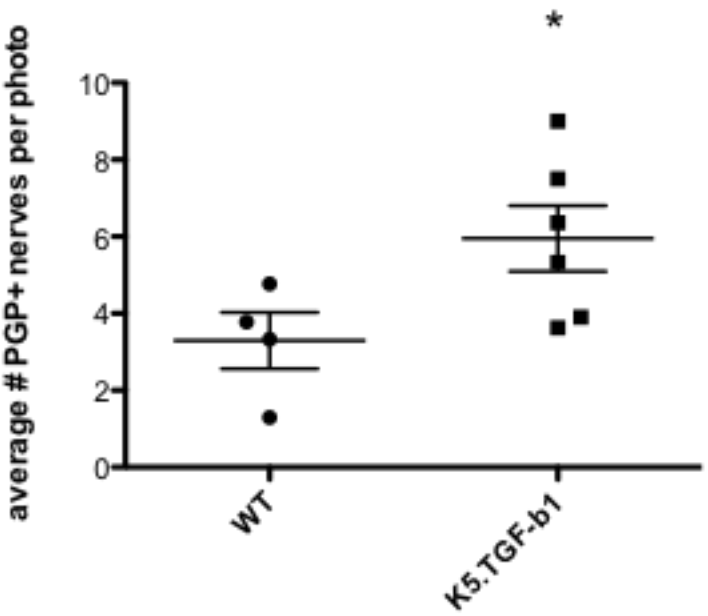


Figure 4.12. K5.hTGF-β1 mice have more nerves than wild-type littermate controls. Lesional skin sections from K5.hTGF-β1 were stained with anti-PGP9.5 and quantified in a blinded manner as described above. All mouse samples assayed had a disease severity score of 3 or above, on a scale of 0 to 4, with 4 being the worst disease. * denotes $p < 0.05$ and significantly different from control.

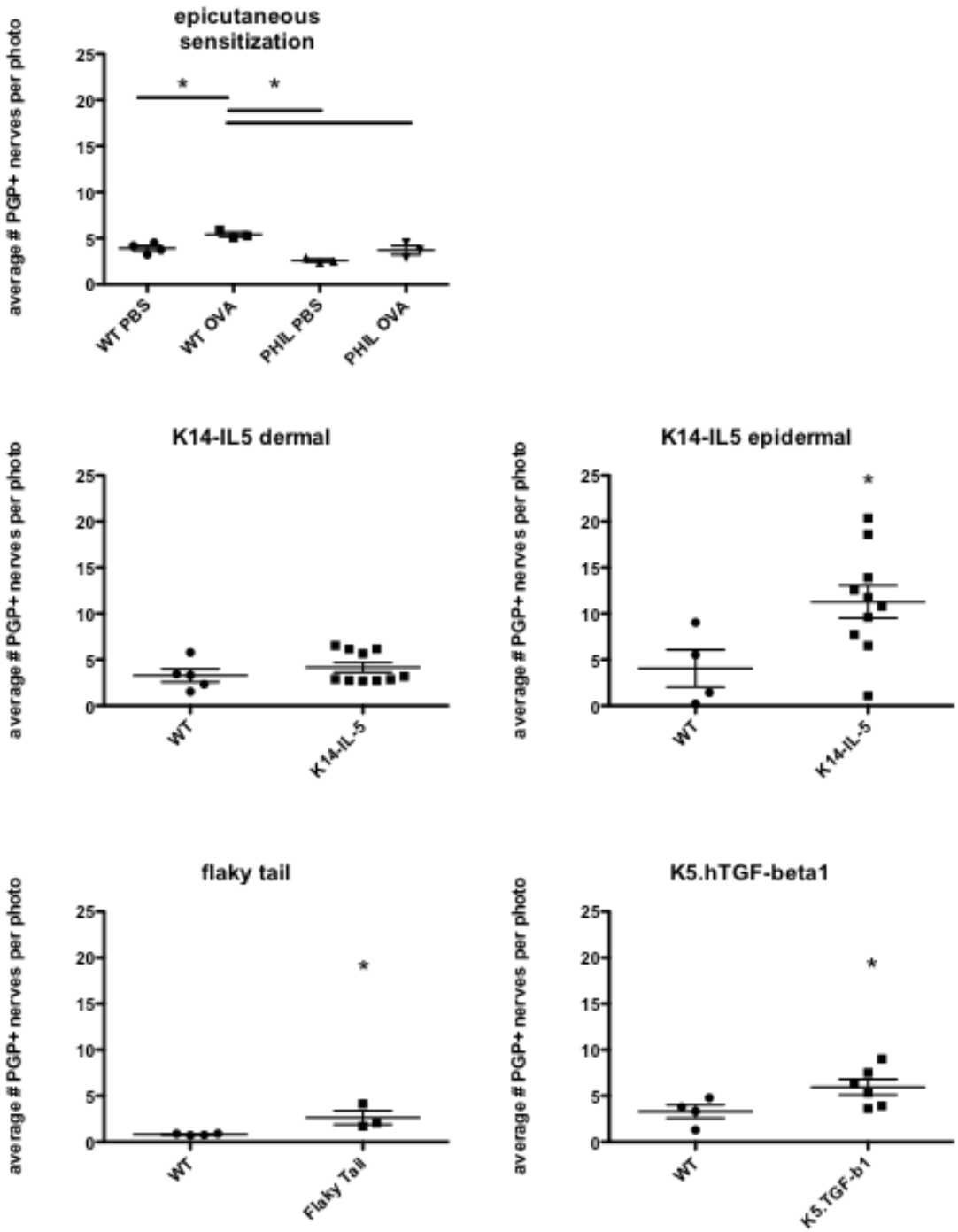
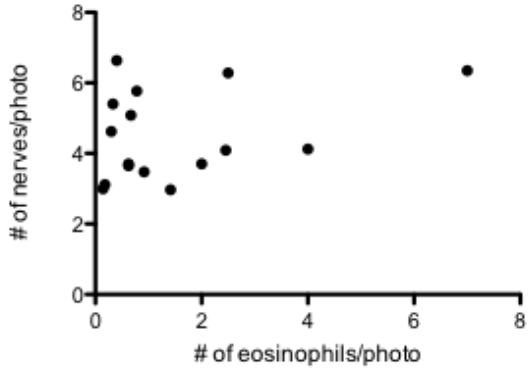


Figure 4.13. Number of nerves in all mouse models, graphed on the same axes.

Figure 4.14. Correlation of eosinophils in skin with number of nerves in various mouse models (next page). Intact eosinophils were counted in H&E sections using the 40X objective and graphed along the x axis, while nerve counts from 40X fields of sections stained for PGP9.5 were graphed along the y axis. (A), (B) and (C) are three separate epicutaneous ovalbumin sensitization experiments, (D) is K14-IL-5 mouse dermis, (E) is K14-IL-5 mouse epidermis, and (F) represents K5.hTGF-β1 mice.

(A)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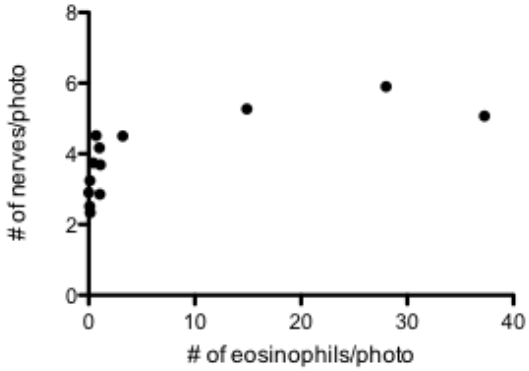
# of eosinophils/photo
0
2
4
6
8

(B)


# of nerves/photo
# of eosinophils/photo
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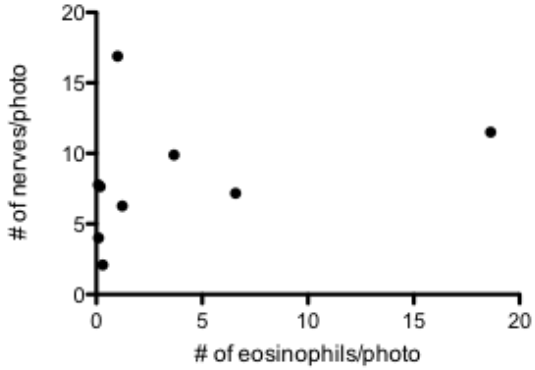
10
20
30
40

(C)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# of eosinophils/photo
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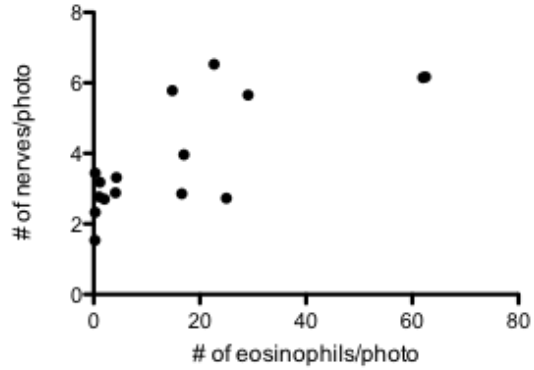
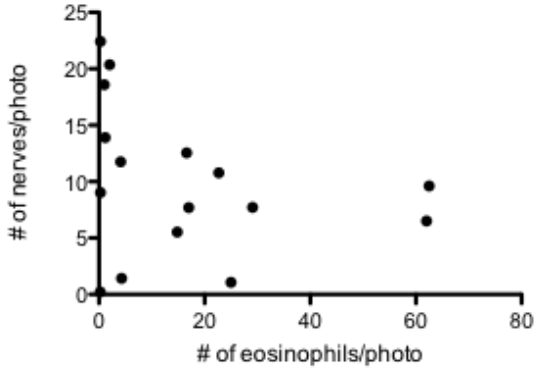
5
10
15
20

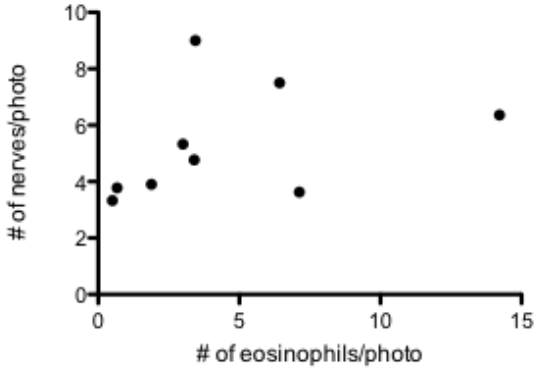
(D)


# of nerves/photo
# of eosinophils/photo
0
20
40
60
80

(E)


# of nerves/photo
# of eosinophils/photo
0
20
40
60
80

(F)


# of nerves/photo
# of eosinophils/photo
0
5
10
15

CHAPTER FIVE

Eosinophils increase sensory neuron branching through a secreted mediator which is not Nerve Growth Factor (NGF).

I. INTRODUCTION

Skin innervation is similar in humans, mice, rats, guinea pigs, rabbits and other mammals (220). A nerve plexus resides in the deep reticular layer of the dermis, and individual nerves branch upwards to supply the dermis, hair follicle and epidermis. Unmyelinated and lightly myelinated afferent nerve fibers terminate in the epidermis, upper hair follicles and upper dermis (329), and relay information from each skin region back to the spinal cord.

Sensory neurons that innervate the skin have their cell bodies located in dorsal root ganglia (DRG) that lie outside the spinal cord. DRG neurons each have one axon that branches into two processes, central and peripheral, which innervate targets in the central nervous system and periphery, respectively. Cutaneous sensory neurons grow directly to target skin regions and then progressively arborize, or add fine axonal branches, in a specific and regulated manner (217). During fetal development, there is hyperinnervation of the epidermis and then gradual retraction to concentrate sensory terminals at the dermal-epidermal junction and the papillary dermis (330). Over the course of development in the mouse embryo, epidermal innervation by all different subtypes of sensory neurons retracts to a nerve plexus that is beneath the epidermis (330). After a wound, skin

becomes reinnervated over time, with a similar progression of small fibers, then increased density and subsequent retraction, as in embryonic development (331).

Each region of skin innervated by a DRG sensory neuron is called a dermatome and has been determined using electrophysiological testing after stimulation of the skin (reviewed in (217)). The precise signals directing axons to each region of skin are unclear. One previous hypothesis was that neurons were recruited to their targets by neurotrophic factors, such as NGF, and then survived or retracted, based on competition for such factors (332). However, correlation of neuron cell death, survival and proliferation with innervation of the skin in the hindlimb determined that cell death peaks before axons reach their target sites, with no time to compete for trophic factors and relay life and death signals retrogradely (333). In addition, a series of experiments in sensory neurons that innervate the whisker pad of mice (the most densely innervated skin region) demonstrated that NGF receptors did not appear on the surface of neurons until after they reached their targets, and that NGF levels in the skin target were low until after the nerve fibers had arrived (334). Therefore, NGF was ruled out as the guidance molecule for sensory neuron growth to the skin.

However, NGF has other roles in nerve growth, besides directing development and initial outgrowth of axons to the skin. Embryonic and neonatal DRG grown in culture are dependent on NGF for survival (283), although adult DRG survive and project neurites without it (335). In a transgenic mouse that overexpresses NGF in basal keratinocytes, using the keratin-14 promoter, the authors founds hyperinnervation in the epidermis, dermis and around hair follicles,

hypertrophy in sensory and sympathetic ganglia, and more large nerve bundles in the reticular layers of the dermis (336). Subsequent studies of these mice determined that the increase in size and number of nerves in the dermal plexus was due to an increase in axons, rather than number of nerve endings (329).

Sensory neurons can be removed from laboratory animals and kept alive in culture for long periods of time, while retaining the morphological and electrophysiological properties of neurons *in vivo* (283, 335, 337-339). However, neurons in culture are not perfect models of neurons *in vivo*. The main caveat to studies of isolated DRG sensory neurons is that they have properties of regenerating cells, including expression of molecules that are present in successfully regenerating axons, such as growth-associated protein 43 (340). Isolation of DRG requires axotomy of both central and peripheral processes, and initiates the program of regeneration that occurs in a live mammal after nerve crush or axotomy.

The central and peripheral axons have fundamentally different responses to injury, mostly due to their different environments. Central processes are inhibited from regrowth by myelin-associated molecules, while macrophages and Schwann cells clear away myelin in the periphery to allow immediate regeneration (reviewed in(341)). However, peripheral axon injury can stimulate the regeneration program in the entire cell, conditioning the central axon to be able to regrow as well (reviewed in(341)), and this presumably occurs in DRG isolated and placed in culture, since neurite outgrowth in cultured DRG neurons is not limited to one axonal process.

Neurite outgrowth from DRG sensory neurons depends on appropriate expression of receptors for guidance cues, as well as their correct localization to the tips of extending axons, known as growth cones (reviewed in (342)). One potential mechanism for neurite outgrowth after peripheral nerve injury is an intracellular increase in cAMP, which activates protein kinase A, thereby activating CREB-dependent transcription, as well as inhibiting Rho kinases which modulate actin cytoskeleton assembly. Other observations, such as the increase in IL-6 in regenerating neurons and the importance of integrins to neurite outgrowth, are beyond the scope of this discussion but will be relevant for future studies (reviewed in (341)). In cultured adult DRG neurons, NGF also greatly increases the initial rate of axonal regeneration, although the same number of control, NGF-treated, and anti-NGF treated neurons have neurites after 17 days of culture (335).

Eosinophils constitutively synthesize specific neurotrophins, including NGF, brain-derived neurotrophic factor (BDNF), and neurotrophin-3 (NT-3), and they can be triggered to release these factors upon stimulation (191, 261). In contrast to this, several clinical case reports describe interactions of eosinophils with nerves in human patients, often with pathological consequences. In patients with hypereosinophilic syndrome, symptoms can include multifocal neuropathy, diffuse and chronic itching, rash, and peripheral and central nervous system dysfunctions (270-273). Eosinophil granules are cytotoxic when applied at high concentrations to a variety of cells in culture (99-101). However, the release of granule proteins, preformed cytokines and other regulatory molecules from eosinophils is tightly controlled and can occur through regulated vesicle secretion, called piecemeal

degranulation, or whole cell degradation, called cytolysis (94). After release from the cell, eosinophil granules continue to be regulated by receptor-ligand interactions and can activate signal transduction to secrete cytokines or any of the granule proteins (98).

Previously, we have shown that eosinophils interact with nerves in the airways of patients with asthma, in animal models of asthma, and in culture. Eosinophils are found adjacent to nerves in airway biopsies of humans who died from asthma attacks, a histological finding that is recapitulated in antigen-challenged guinea pigs and rats (267). Primary cultures of parasympathetic neurons from guinea pig and human airways express eotaxin-1, as well as ICAM-1 and VCAM-1, and these participate in binding of eosinophils to these neurons (164, 268). In addition, the association of eosinophils with airway nerves is important in the pathophysiological changes that lead to airway hyperreactivity (267, 269)

However, our previous studies have focused on parasympathetic neurons, and the interactions between eosinophils and nerves in the airways. In this report, we investigated the interactions between sensory neurons from the DRG and eosinophils, which are present and activated in atopic dermatitis skin ((343) and Chapter 3). We found that eosinophils directly cause branching of sensory neurons in culture, through a mechanism that does not involve NGF. This effect was specific to eosinophils, as mast cells did not replicate the effect. We believe this is a new mechanism of neurite outgrowth that could be important in diseases with high numbers of eosinophils in the skin.

II. RESULTS

Eosinophils increase sensory neuron branching in vitro.

The cell bodies of the sensory neurons that innervate the skin are located in the dorsal root ganglia (DRG), and these were dissected from wild-type mice, enzymatically dissociated and plated on Matrigel. Blood was taken from NJ1638 mice, in which the IL-5 gene is expressed under the control of the CD3δ promoter, leading to high levels of circulating eosinophils (121). Eosinophils were isolated using density centrifugation and fluorescence-activated cell sorting (FACS) based on size and granularity. Cells obtained in this way were 75-99% eosinophils, and five out of six experiments used >90% pure eosinophils. Eosinophils were added to DRG cell cultures for twenty-four hours. The cultures were then fixed, stained with PGP9.5, and the number of cell bodies, length of neurites, number of neurites per cell body, and number of branchpoints per neurite were quantified. Neurons grown with eosinophils had dramatically increased neurite branching, compared to neurons grown alone (Figures 5.1 and 5.2). The number of cell bodies, number of neurites per cell body and the length of the longest neurite was not significantly different between neurons alone or neurons with eosinophils (Figure 5.3). There was also no difference in DRG cell survival with or without eosinophils (data not shown).

Eosinophils must be alive to increase neuron branching

In order to determine whether the eosinophils needed to be alive to have this effect on neurite branching, we freeze-fractured eosinophils and added them to neuronal cultures for twenty-four hours. Eosinophils killed in this way did not increase neurite branching (Figure 5.4A).

Mast cells do not increase neuron branching in vitro

Since mast cells were localized around nerves in human skin biopsies, we isolated mast cells from the peritonea of wild-type mice and added them, in the same numbers as eosinophils, to the neuron cultures for twenty-four hours. Mast cells did not increase neurite branching (Figure 5.4).

Physical contact between eosinophils and neurons is not required for increase in branching

Adding culture medium from eosinophils plated on Matrigel for twenty-four hours to DRG cultures also increased neuron branching, indicating that the morphological changes do not require cell contact, and adding culture medium from co-cultures of DRG neurons with eosinophils to fresh DRG cultures further increased the amount of neurite branching (Figure 5.5). In addition, this ruled out the effects of an increased concentration of cells in the culture on neurite branching.

Blocking nerve growth factor (NGF) does not inhibit eosinophil-induced neurite branching

Adding exogenous murine NGF (40 ng/ml) to DRG cultures increased neurite branching, and this effect was blocked by an antibody to NGF (20 μg/ml) (Figure 5.6). However, adding the same concentration of anti-NGF to DRG cultures co-cultured with eosinophils (Figure 5.6) or treated with eosinophil culture medium (data not shown) did not prevent the increase in neurite branching.

Neurons from dorsal root ganglia produce eotaxin-1, ICAM-1 and VCAM-1.

DRG neurons were cultured alone for twenty-four hours and stained for eotaxin-1, a chemokine that acts through the CCR3 chemokine receptor on eosinophils (29, 141). Neurons synthesized eotaxin-1, both in the presence or absence of exogenous NGF (Figure 5.7A). In addition, neurons also produced the adhesion molecules ICAM-1 and VCAM-1, but only when exogenous NGF was added to the cultures (Figure 5.7B,C). This indicates a regulated expression of adhesion molecules that could be affected by the presence and activation of eosinophils, fibroblasts, or other cells that express NGF (191, 255).

III. DISCUSSION

We have shown that eosinophils induce neurite outgrowth from DRG sensory neurons in culture, using a mechanism that is not dependent on NGF. Previous studies have concluded that eosinophil granule proteins are toxic to nerves. Early experiments by Gordon 1933, in which homogenized lymph nodes from Hodgkin's disease patients injected into animals resulted in paralysis, were later discovered to be due to toxicity from eosinophils in the lymph nodes. In 1979, Durack described

the isolation of eosinophil granules from the blood of hypereosinophilic patients and subsequent intrathecal or intracerebral injection into animals (259). Guinea pigs and rabbits developed progressive ataxia and muscle weakness and wasting, with pathologic changes upon histologic examination, including apparent myelin degradation. The injection of mast cell or neutrophil suspensions did not cause the same type of neurologic damage. Similar experiments with isolated eosinophil granule-derived cationic proteins were performed and physiologic measurements of sensory fibers were performed (260). ECP and MBP evoked strong responses from pulmonary C fibers when administered via the trachea, similar to several other cationic proteins, including synthetic poly-L-lysine.

However, in all of the previous experiments, isolated granules or fractured eosinophil supernatants were used, often at supraphysiological concentrations. In this study, we used live eosinophils, isolated from the blood of mice, at a concentration of 1 X 10^6 cells/ml, which is 10-fold lower than the Durack study of fractured eosinophils. In addition, there are alternative interpretations of the prior data. First, degradation of myelin is an important first step in the regeneration of DRG neurons, and it is possible that what was observed *in vivo* was the first step in a process of neuron regeneration. In addition, stimulation of C fiber action potentials by eosinophil granule protein does not necessarily indicate neuron damage, but could signify neuron activation. Finally, intact eosinophils have extremely regulated release of their granule proteins and interact with other cells in many other subtle ways that would not be approximated by the experiments listed above (96). The difference, therefore, between adding isolated granule proteins and intact, healthy

eosinophils to a culture is similar to the vast chasm between experiments using isolated perforin or granzyme compared to healthy CD8+ T cells.

Our findings support the previous literature on NGF-dependent neurite outgrowth, and the anti-NGF experiments indicate that it is not the factor released by eosinophils into supernatant to induce neurite growth. This leaves the obvious question of the identity of the soluble mediator. Studies on neurite outgrowth suggest that signaling through STAT-3, increasing intracellular cAMP or otherwise stimulating protein kinase A activity are two common mechanisms to begin signal transduction that will alter the dynamic equilibrium of actin assembly and depolymerization (341). Eosinophils are capable of synthesizing IL-6, which signals through STAT-3 (344) They also produce many ligands for Gs-protein-coupled receptors, which stimulates adenylyl cyclase and results in an increase in intracellular cAMP. Potential experiments to determine the mediator could include biochemical purification from supernatants and isolation by mass spectrometry or testing additional blocking antibodies against likely candidates. Besides IL-6 and other cAMP inducers, BDNF, NT-3 and NT-4 are all known neurotrophins that are synthesized by eosinophils and can influence neurite outgrowth (191, 261).

We did not determine the mechanism of regulation of eotaxin-1 production in isolated DRG sensory neurons. It is likely to resemble signal transduction pathways in other cell types, such as fibroblasts. Eotaxin-1 expression is stimulated by ligands which signal through nuclear factor (NF)-κB or signal transducer activator of transcription(STAT)-6 (345). Specifically, signaling through the IL-4Rα,

either by IL-4 or IL-13, or activation of cells by tumor necrosis factor (TNF)-α, induces eotaxin-1 mRNA and protein production in dermal fibroblasts, human airway epithelial cells, and human airway fibroblasts (345-347). Other cytokines can synergize with IL-4, IL-13 or TNF-α to further increase eotaxin-1 production. These include TGF-β and oncostatin M (348, 349). However, dermal fibroblasts constitutively synthesize low levels of eotaxin-1 without stimulation (346).

In addition, although dermal fibroblasts constitutively produce eotaxin-1, the presence of IL-4 and IL-13 in the skin of people with atopic dermatitis would induce higher expression levels of eotaxin-1. This, in turn, would increase the recruitment of cells bearing CCR3 receptors, including eosinophils. It is possible that this creates a feedforward loop for further inflammation and cell recruitment. Therapeutics targeting eotaxin-1 and CCR3 might be clinical useful in atopic dermatitis.

In addition, treatments targeting ICAM-1 have already been tested in pilot trials of patients with severe atopic dermatitis. Efalizumab is a humanized monoclonal antibody generated against CD11a, which is one subunit of LFA-1, the receptor for ICAM-1. Clinical case reports indicate that efalizumab can successfully treat severe atopic dermatitis (350, 351). The biologic basis for disease improvement has only been investigated in one paper, which examined the phenotypes of circulating T cells in the blood of treated patients. Besides a decrease in CD11 expressed on T cells, these authors also found an increase in T cells expressing cutaneous lymphocyte antigen, a skin-homing marker. The paper's

conclusion was that disease improved due to the inhibition of T cell entry into the skin (352).

However, LFA-1 is expressed on many other cell types besides T cells, including eosinophils. It is possible that efalizumab treatment inhibited eosinophil entry into the skin and migration to the nerves. This possibility was not investigated. Unfortunately, the development of adverse reactions to efalizumab after extended treatment (3 months to 2 years) includes acute autoimmune thrombocytopenia, so this treatment may be best for short-term control of severe exacerbations (353).

There are several limitations to this study. First, as mentioned above, DRG neurons in culture resemble regenerating neurons, rather than adult neurons *in vivo.* The relevance to this study is that they will more readily arborize, potentially amplifying the branching effect of eosinophils. In addition, the co-cultures only include two types of cells, rather than the total skin milieu of fibroblasts, epithelial and endothelial cells, which will undoubtedly affect neuron growth by production of guidance cues, neuromodulators, and inflammatory molecules. Using reconstructed human epidermis would be helpful here; indeed, experiments have been performed with DRG explants grown on human and rodent skin explants that indicate direction of neurite outgrowth is preferentially toward skin (354, 355). The addition of eosinophils to either skin explants or reconstructed human epidermis co-cultured with DRG and measurement of directional outgrowth of neurites could be informative. In addition, mouse models of neuron growth and relative dependence on eosinophils are an important addition to these *in vitro* findings (Chapter 4).

Finally, the eosinophils added to the DRG cultures were isolated from IL-5 transgenic mice using FACS, and thus could be activated, either from the IL-5 stimulation or the mechanical process of cell separation. It will be important to understand whether the activation state of the eosinophils matters for DRG neurite outgrowth, and this could be tested using the derivation of eosinophils from bone marrow precursors. Given some of the data from mouse models above, we hypothesize that activation of eosinophils will be crucial to neuron outgrowth in the skin.

However, we believe this study elucidates an important new mechanism for neurite outgrowth of primary sensory neurons by eosinophils, which could be important for pathogenesis of atopic dermatitis and other hypereosinophilic disorders. Determining the specific mediator released by eosinophils will be an important step forward in understanding this process.

IV. FIGURES

(A) (B)

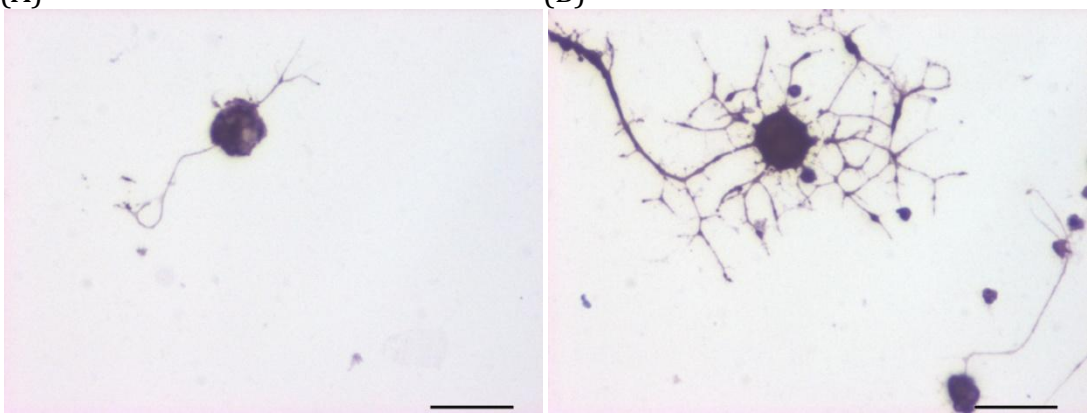


Figure 5.1. Eosinophils increase nerve branching after co-culture for 24 hours. DRG neurons were isolated and pre-plated overnight to remove adherent cells then co-cultured with eosinophils for 24 hours in four-well chamber slides. Slides were fixed with 4% paraformaldehyde and stained with anti-PGP9.5 antibody to visualize nerves (black) and chromotrope 2R for eosinophils (red). (A) Photomicrograph of a single DRG neuron cultured alone for 24 hours. The large circular figure is the cell body, and there are 2 long neurites and one shorter neurite extending from it. (B) Photomicrograph of 2 DRG neurons cultured with eosinophils, one in the middle of the figure and the other in the bottom right corner. Eosinophils can be seen near the larger cell body and along the single neurite of the neuron with the smaller cell body. Scale bars = 50μM.

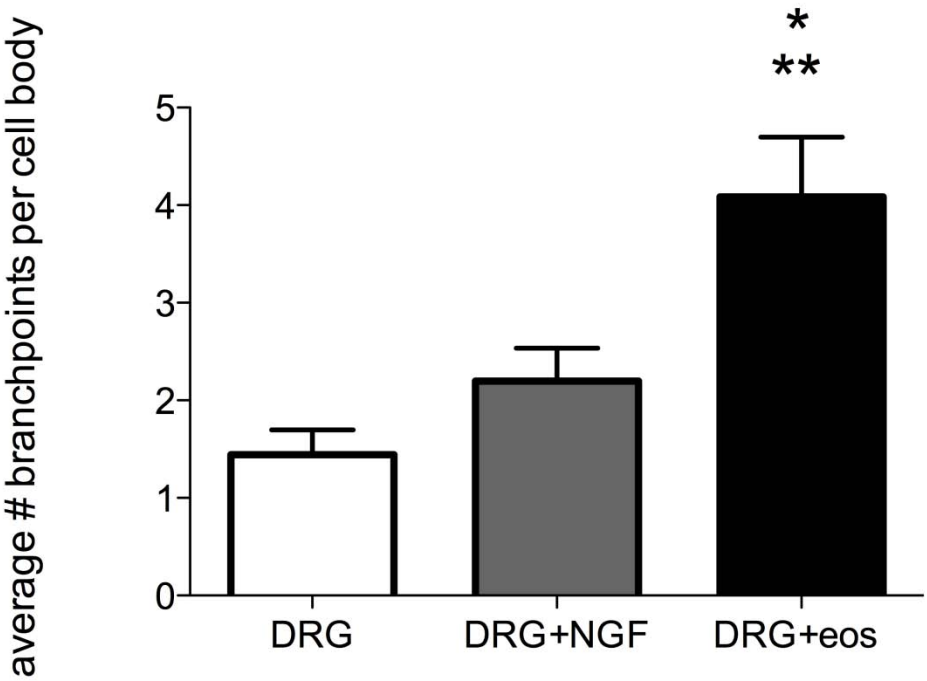


Figure 5.2. Eosinophils increase nerve branching after co-culture for 24 hours. Quantification of eosinophil-induced neuron branching. DRG neurons were isolated, cultured, and stained as described above, with an overnight pre-plating step followed by 24 hours of culture alone (white bar), with 40ng/ml NGF (gray bar), or with eosinophils (black bar). Thirty photographs were taken with the 40X microscope objective using the same pattern in each of three wells on a slide and the number of branchpoints on each neurite per cell body was counted by hand.
* denotes significantly different than DRG alone; ** denotes significantly different than DRG+NGF. n= 7 independent experiments.

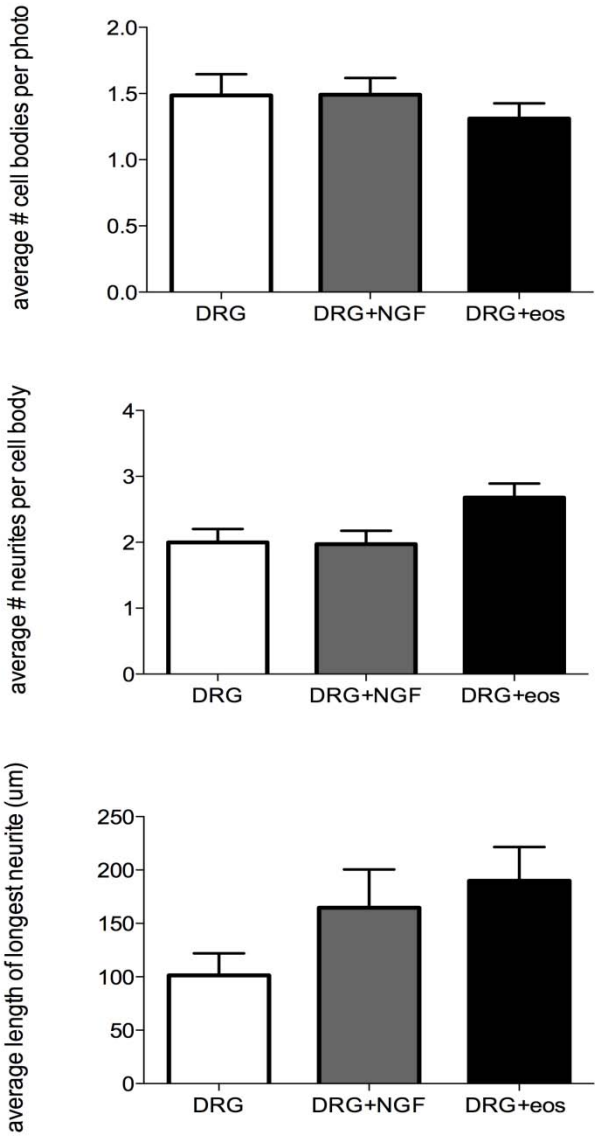
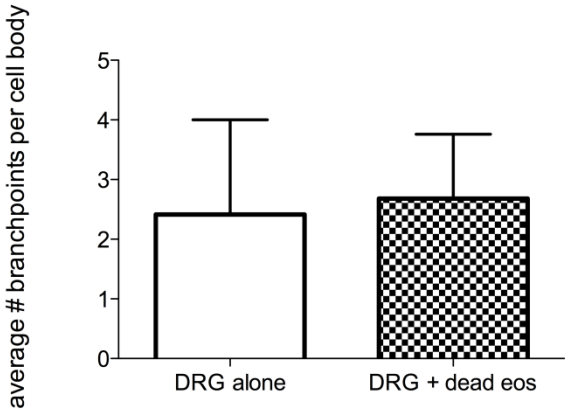


Figure 5.3. Co-culture with eosinophils does not change the number of neuron cell bodies, number of neurites, or length of the longest neurites. DRG neurons were cultured, stained and photographed as above. Metamorph software was used to measure the length of neurites, and cell bodies and number of neurites were counted by hand. n= 7 experiments.

(A)



(B)

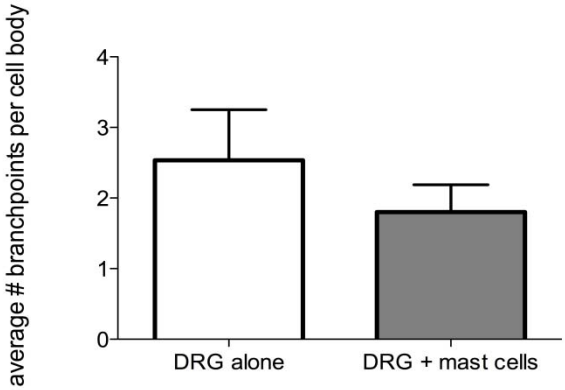


Figure 5.4. Eosinophils must be alive to have branching effect on neurons, and mast cells do not increase branching of neurons. (A) Eosinophils were isolated from NJ.1638 mice as described and then freeze-thawed in deionized water twice, then resuspended in culture medium and plated with DRG neurons overnight. Slides were fixed and stained as above, and the average number of branchpoints per cell body was counted by hand using 40X photographs with Metamorph. n= 2 experiments. (B) Mast cells were isolated from the peritonea of wild-type mice and added to DRG neuron cultures for 24 hours. Slides were stained and quantified as above. n= 4 experiments.

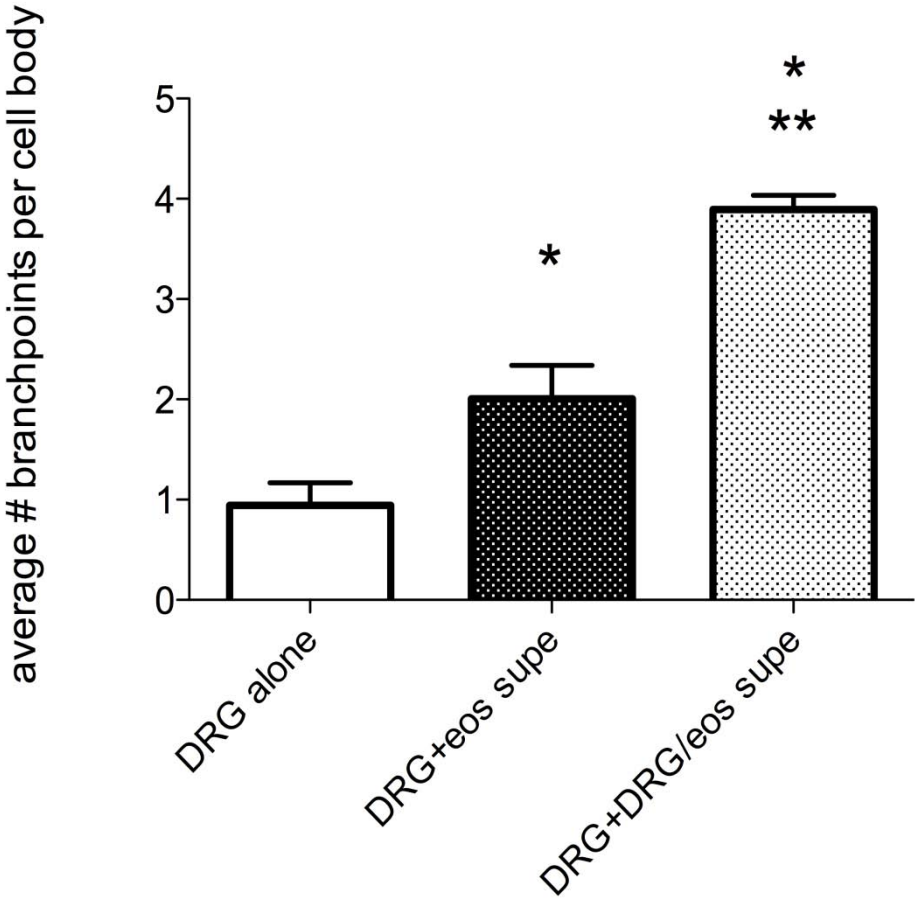


Figure 5.5. Culture medium from eosinophils alone or eosinophils and neurons causes increase in neuron branching when applied to new DRG cultures. Culture medium from eosinophils cultured alone (middle bar) or eosinophils with DRG (right bar) for 24 hours was applied to new DRG cultures for 24 hours, ad cells were fixed, stained, photographed and quantified as above. * denotes significantly different than DRG alone; ** denotes significantly different than DRG with eosinophil only culture medium. n= 4 experiments.

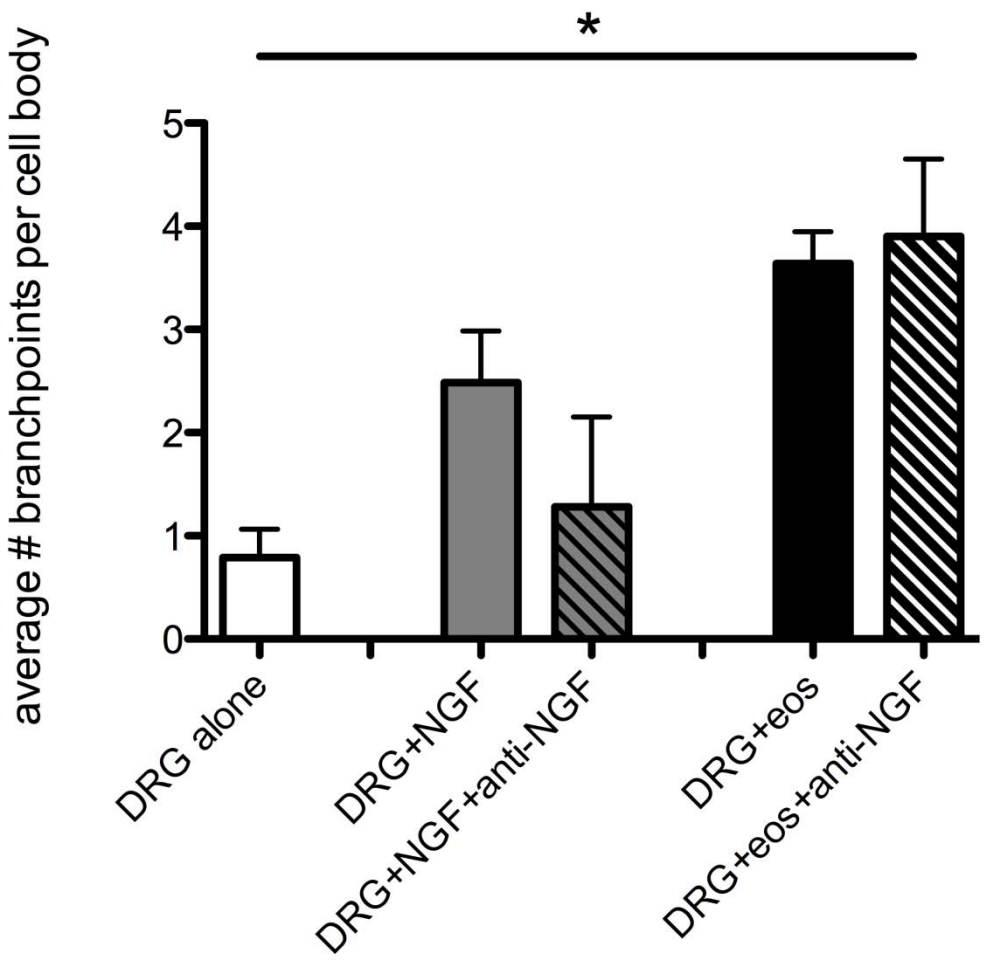
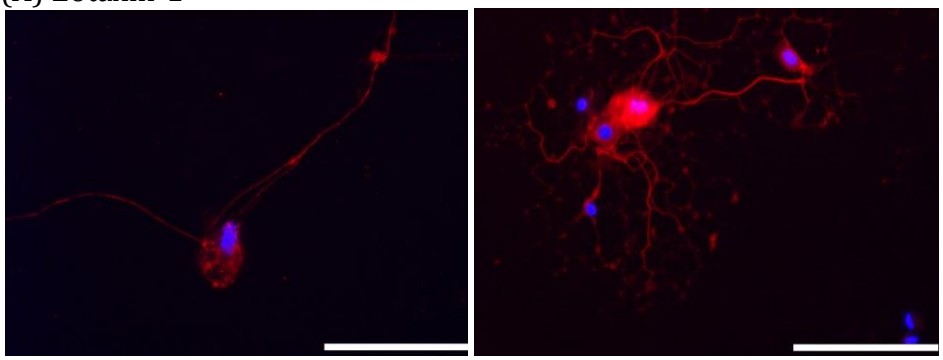


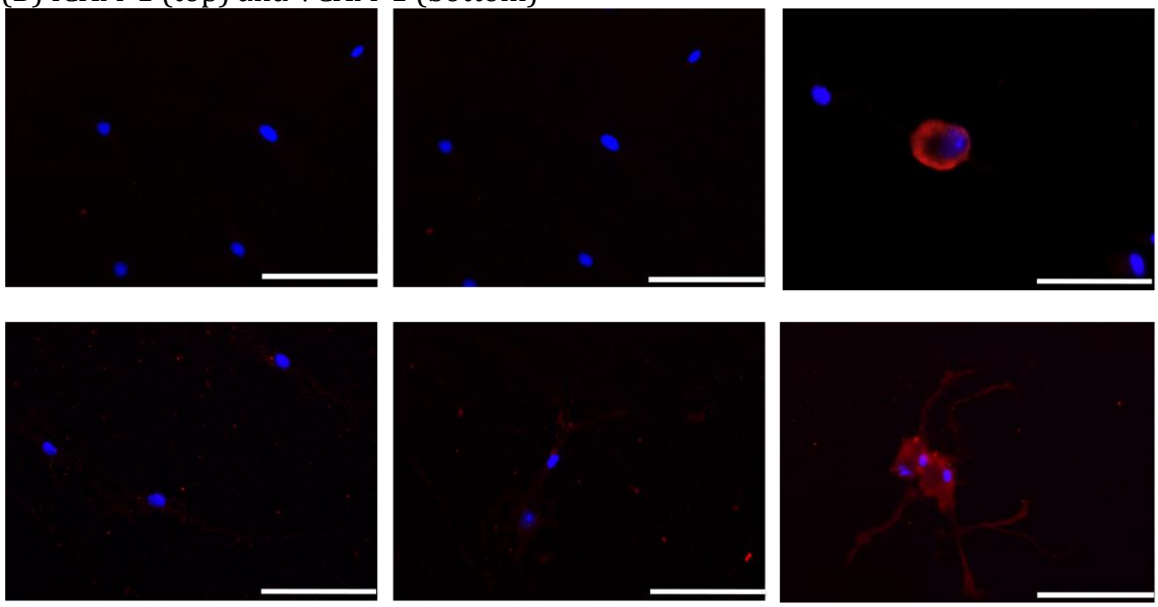
Figure 5.6. Blockade of NGF inhibits NGF-induced nerve branching, but not eosinophil-induced nerve branching. DRG neurons were plated and incubated alone (white bar), with 40 ng/ml NGF (gray solid bar), with NGF and 20 μg/ml anti-NGF (gray hatched bar), with eosinophils (black bar), or with eosinophils and 20 μg/ml anti-NGF (black hatched bar) for 24 hours. n=2. * denotes significant one-way ANOVA across all groups.

Figure 5.7 (following page). DRG neurons produce eotaxin-1, ICAM-1 and VCAM-1. (A) DRG neurons were isolated and cultured alone for 24 hours, then fixed and stained with anti-eotaxin-1 (red). Cell nuclei were counterstained using DAPI (blue). Two representative photomicrographs are shown. (B) Scale bars = 50μM. (B) DRG neurons were cultured alone or with 40ng/ml NGF for 24 hours, then fixed and stained with anti-ICAM-1 (top) or anti-VCAM-1 (bottom). Left, no primary with secondary negative control; center, DRG stained with antibody in cultures without exogenous NGF; right, DRG stained with antibody in cultures with NGF added. (C) Quantification of relative fluorescence units, compared to background and to negative controls, was performed using Metamorph.

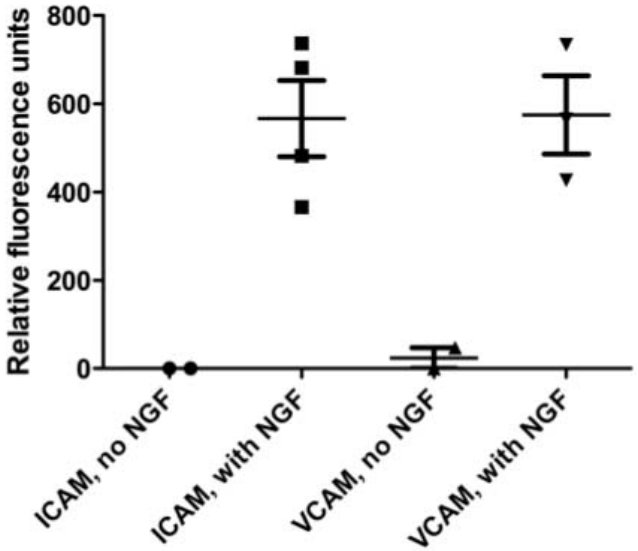
(A) Eotaxin-1



(B) ICAM-1 (top) and VCAM-1 (bottom)



(C)



CHAPTER 6: GENERAL DISCUSSION

I. SUMMARY OF FINDINGS

We have found that eosinophils increase the branching of sensory neurons. We have shown that this is a direct effect of a secreted factor made specifically by eosinophils while they are alive in co-cultures with isolated neurons. This counters previous reports of eosinophil neurotoxicity (100, 101, 260), but confirms other findings that eosinophils can positively influence the survival and function of neurons (356, 357). Based on strong evidence that the eosinophil's environment tightly regulates release of granule proteins and other mediators, as well as data suggesting that granules are additionally regulated by receptor-ligand interactions (94, 96, 176, 358, 359), it is likely that the eosinophil's role is not only brute destruction but modulation of the immune milieu (360). The production of eosinophil-recruiting molecules, such as eotaxin-1, and adhesion molecules, suggests that nerves are active participants in the interaction, while the increased growth of neurons in culture with mixed neuron-eosinophil supernatant indicates that neurons are also releasing growth mediators.

Eosinophils are required for the nerve increase in a skin sensitization mouse model of atopic dermatitis. However, this model did not consistently result in increased eosinophils in the skin. We believe the housing of mice in different levels of containment could have affected their eosinophilia after ovalbumin sensitization, as the mice living in the lowest level of precaution, and therefore the dirtiest, had the greatest increase in eosinophils in their skin.

We investigated another mouse model of atopic dermatitis, the flaky tail mouse. These mice, like atopic dermatitis patients, have increased permeability of the epidermal barrier to antigens, due to their lack of filaggrin in the stratum corneum. Filaggrin is the strongest genetic contributor to human atopic dermatitis yet identified.

We also investigated mouse models of eosinophil recruitment to the skin, and we found that these mice also had increases in the number of cutaneous nerve terminals. The most dramatic effect was in the K14-IL-5 mouse, which expresses IL-5 in the epidermis and had huge increases in nerves, specifically in the epidermis. Humans with atopic dermatitis also have elevated levels of IL-5 in their lesional skin and increases in nerves. Our original purpose for studying the K5-TGFβ-1 mouse was related to psoriasis, but to our surprise, the mice had elevated serum IgE, high tissue IL-4 levels and eosinophils present in all regions of the skin. These mice also had more nerves in the skin than their wild-type littermates, in yet another model of eosinophil-mediated nerve increases. The role of TGFβ-1 in atopic dermatitis is not clear, but eosinophils in allergic diseases produce large quantities of TGFβ-1, so this model could mimic having activated eosinophils in the skin.

Finally, we have localized eosinophil granules in human atopic dermatitis skin to the same regions where nerves are increased. This proximity supports our contention that these two cell types might engage in a functional interaction that could influence the morphology of neurons. Intact eosinophils are only rarely visible in lesional and non-lesional skin. We have shown in allergic asthma models that the

number of eosinophils in tissue is not related to pathology; rather, it is the number of eosinophils located near nerves that leads to airway hyperreactivity (267). In a similar manner, it appears that the rare eosinophils in atopic dermatitis skin preferentially locate in areas where sensory nerve terminals exist, and deposit granule proteins near nerves, potentially having effects on sensory neuron function, as well as morphology.

Our original hypothesis was ambitious and included experiments to evaluate nerve morphology, electrophysiology, as well as release of mediators. However, the characterization of the increase in neurite branching won our attention for this particular project. It will be important in the future to further characterize the possible sensitization, functional changes, or modified neurotransmitter release of neurons cultured with eosinophils. It is likely that neurite branching is not the only neural outcome induced by eosinophils.

Our study identifies several new targets for future potential therapeutics for the treatment of atopic dermatitis. By inhibiting eosinophil migration from the bone marrow, using anti-IL-5 monoclonal antibodies, or by preventing their entry into skin, using CCR3 antagonists, eosinophils would not be able to influence nerve branching. Blockade of adhesion molecule interactions, using anti-ICAM-1 or VCAM-1, might also decrease eosinophil and other immune cell entry into skin and prevent the inflammation associated with atopic dermatitis.

II. FUTURE DIRECTIONS

Many questions remain unanswered. We observed the direct effect of eosinophils on sensory nerves in co-cultures through an unknown mediator. To identify the mediator, biochemical fractionation of the eosinophil supernatant could be performed. Fractions could be isolated and applied to DRG neurons, in order to see which fraction induces branching. Mass spectrometry analysis could help identify the causative molecule.

We also did not identify whether a particular subset of sensory neurons was preferentially affected. We grouped neurons into small or large, and darkly or lightly staining cell body populations (Figure 6.1). We quantified their numbers after culturing for 24 hours under different conditions and saw no change in the number of cell bodies in each population (Figure 6.2). Using this same morphological analysis of small and large cell bodies, there was also no obvious increase in branching in one group above the other (data not shown). We also did experiments using peripherin antibodies, which recognize C fibers only and did not see a difference between peripherin-positive and –negative cells in terms of their branching after exposure to eosinophil culture medium (Figure 6.3). In the future, it will be important to use antibody staining against a panel of markers for A and C fibers, including CGRP, Substance P, neurofilaments. These can be correlated to the immunohistochemical findings in atopic dermatitis and potentially one subset of neurons will be affected above the others.

We used NGF blocking antibodies to investigate the mechanism of enhanced neurite growth *in vitro*, but found no decrease in branching. We also tried to block

branching by using a homolog of cAMP that binds protein kinase A and renders it unavailable to bind cAMP and perform its normal functions in neurite extension. We also did not see any decrease in neurite branching in these experiments, but it is difficult to say if this was because of blockade of branching, or interference with a separate protein kinase A-dependent function. Further experiments using blockers of neurite extension should be performed, in order to determine which cellular pathway is induced by eosinophils.

In terms of the mouse models, it is not known whether nerves increase in all models that recruit eosinophils to the skin. The several that we examined had nerve increases, but there are always more models. We did not examine other potential mechanisms for nerve increases, perhaps by other cells. Keratinocytes can produce NGF, and nerves cultured with primary human epidermal cells send neurites preferentially toward the skin cells (258, 354). Therefore, it is possible that eosinophils and keratinocytes, or other cell populations, cooperate to enhance neuron growth *in vivo*.

In addition, the role of scratching in inducing nerve fiber increases was not separated from eosinophil recruitment in these studies. Scratching can induce mast cell degranulation and increase the number of substance P-immunoreactive fibers in mouse skin (361). In a spontaneous mouse model of atopic dermatitis, disease does not develop if sensory nerves are ablated at birth by capsaicin and scratching is prevented (362). In fact, many clinicians agree that the worst lesions in atopic dermatitis are produced by scratching.

Finally, itch was not directly measured in the mouse models. Preliminary data from ongoing studies with collaborators indicate that mice lacking eosinophils scratch less when topical dermatitis inducers are applied to ear skin. This was measured by counting the number of scratches each mouse performed in a defined time window. We did not perform behavioral studies such as these, and so the role of eosinophils in itch is still undefined until that work is completed.

III. IMPLICATIONS

Our results give functional significance to previous reports of increased eosinophil proteins in atopic dermatitis lesions and high numbers of eosinophils in the blood of atopic dermatitis patients: one consequence of the presence of eosinophils in the skin is an increase in nerve terminals. These findings are important for several reasons.

First, we showed that eosinophils lead to increased neuron branching, which might be responsible for increased sensation of itch in the skin. If this hypothesis is correct, targeting eosinophils with therapeutics (such as anti-IL-5, anti-CCR3, anti-ICAM-1) could eliminate the main clinical complaint in atopic dermatitis: itch. This could be a breakthrough in treatment, to prevent the disease lesions from forming after scratching, rather than simply reacting to them once they have formed.

Second, we have determined that mast cells are not responsible for the nerve terminal increases. Previous studies have tried to associate blood mast cell products or numbers with atopic dermatitis and have consistently shown no correlation with disease. Plasma levels of the mast cell mediators histamine, tryptase or

prostaglandin D2 are not increased in atopic dermatitis patients (363). Serum levels of mast cell tryptase are not correlated to severity of atopic dermatitis (364), and there is no difference in the number or function of mast cell progenitors circulating in blood of patients with atopic dermatitis, compared to healthy subjects (365).

However, reports of mast cell localization near nerves in disease continue to convince many researchers that they were crucial for disease development. Mast cells are often located near nerves, in the skin and in ganglia, and this occurs under both normal and pathological conditions (366-370). Sugiura and colleagues reported that mast cells had invaded nerves in 9 out of 10 atopic dermatitis biopsies that they examined, over a range of disease stages (371). However, they did not examine healthy skin in their study. In addition, they reported that many of the mast cells were degranulated. Our study only determined the number of intact mast cells associated with nerves, and it is possible that this could be the source of difference between the findings. A subsequent study found slight increases in dermal nerve-mast cell contacts in atopic dermatitis, with lesional skin reaching statistical significance (253). However, nerves and mast cells in the basement membrane and papillary dermis were not significantly associated, except in nummular eczema. Lesional atopic dermatitis skin had significantly more mast cell-nerve interactions, but only in the regions below the papillary dermis. In addition, these authors did not use a pan-neuronal marker, such as PGP9.5, but nerve subtype-specific markers. They found increases in nerve fiber numbers, but the mast cell association was not convincing.

Third, we have linked seemingly unrelated findings into a more complete model of the disease (Figure 6.4). In our hypothesis, barrier dysfunction allows access to keratinocytes and fibroblasts, activates them and induces them to produce eotaxins and other chemokines to recruit eosinophils. It is possible that keratinocyte-released mediators also act on nerves, inducing their production of eotaxin, ICAM-1, and VCAM-1. Eosinophils infiltrate the skin and are selectively localized around nerves in the papillary dermis, perhaps due to changes in eotaxin and adhesion molecule expression. Eosinophils become activated and degranulate, potentially through cell adhesion molecule-mediated mechanisms, and induce a program of neurite extension near the basement membrane zone between the dermis and epidermis. The patient has more nerves in the skin, feels a sensation of itch, scratches, and lesions develop. This cycle is an ongoing one, with a feed-forward component, since eosinophil-produced IL-4 and TGF-β-1 enhance eotaxin's ability to recruit other eosinophils. This cycle only abates when topical or systemic therapies intervene, decreasing eotaxin levels and inhibiting eosinophils from entering the skin.

IV. LIMITATIONS

There were several limitations to this study. For the human skin portion, we were able to obtain interesting and important data from the limited number of biopsies. In the future, a larger number of samples will allow for refinement of our hypothesis. In particular, more subjects would have helped the analysis of

eosinophil proteins in the skin, as we, and others, have found that not all atopic dermatitis patients have eosinophils. In addition, one of our normal subjects had high eosinophils as well as a higher number of nerves. While this is interesting and supports our hypothesis, it would have been better to have a larger number of normal subjects, in order to confirm that this participant was definitely outside of the normal range.

The mouse study also had its limitations, as the epicutaneous sensitization model proved to be inconsistent. When the ovalbumin sensitization worked, the eosinophils that were recruited to the skin led to increased nerve terminals. The major drawback to the mouse protocol was determining eosinophil infiltration of the skin only after the three rounds of sensitization were completed. Adding a mid-term assay for OVA-specific IgE would be helpful in knowing earlier whether the sensitization had occurred.

The other mouse limitations mostly involved small numbers of available mice from other sources, and we are grateful for the slides that our collaborators shared with us. This allowed us to generate provocative preliminary data for future studies.

The sensory neuron and eosinophil co-culture experiments also had limitations, which were mostly based on the experimental design and could not be modified. DRG neurons isolated and placed into culture have many aspects of regenerating neurons, so it is possible that their response to eosinophils *in vitro* do not exactly model neurons *in vivo.* Because of this, we used mouse models to confirm our *in vitro* findings. In addition, the mouse eosinophils used for co-culture

were isolated from the blood of transgenic mice overexpressing IL-5. As discussed in chapter 1, IL-5 can recruit eosinophils from the bone marrow and affect their terminal maturation, but they can also activate eosinophils. We also used FACS to purify eosinophils. It is possible, through either of these mechanisms, that the eosinophils used in this study were activated and had enhanced, or simply different, effects on neurons. If the activation status of eosinophils in these cultures becomes important, it might be useful to use eosinophils differentiated from bone marrow (123) or from parasite-infected mice, to determine the different potential capabilities of differentially activated eosinophils to cause neurite outgrowth. Parallel to this, the mast cells isolated from the peritonea of wild-type mice were not activated by any exogenous agents in any way prior to co-culture with neurons. It is possible that this is the reason for their lack of stimulation of DRG neurons to grow or branch.

V. FIGURES

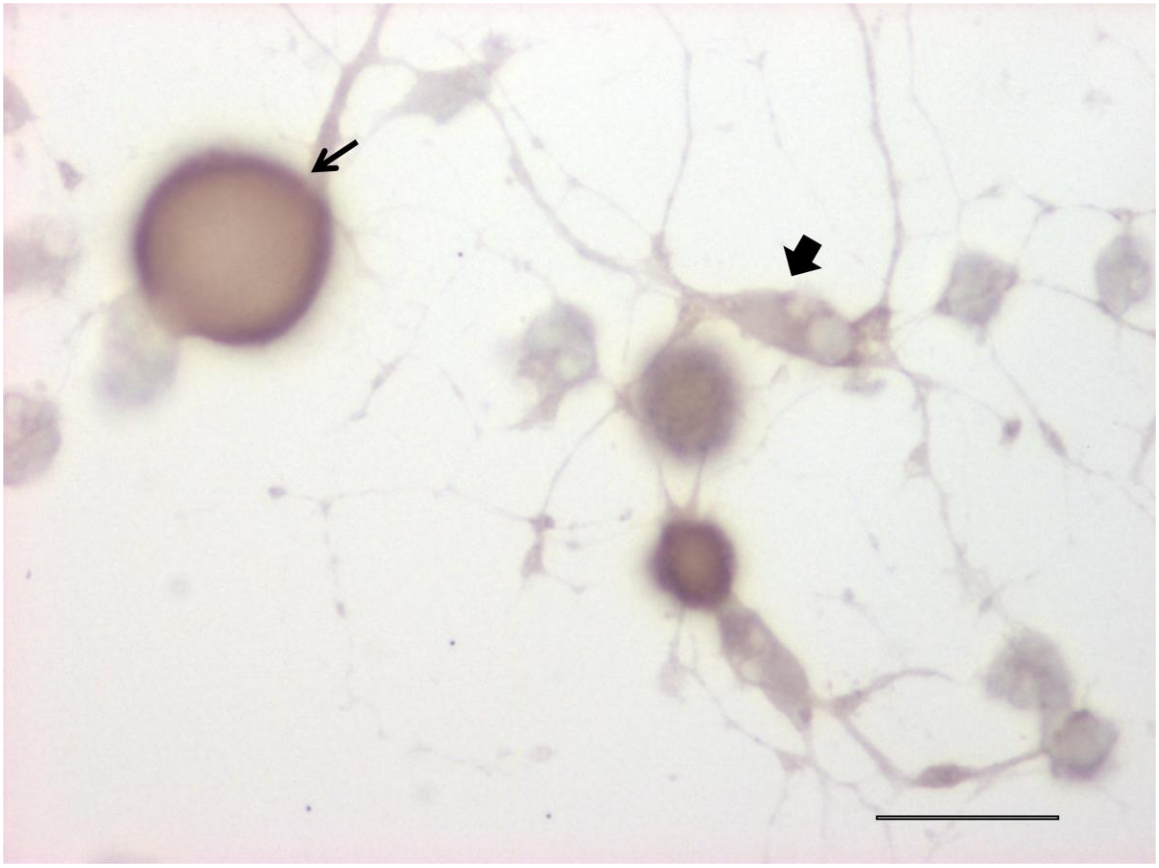


Figure 6.1. Neuron cultures stained with anti-PGP 9.5 can be divided into populations of large or small, and darkly or lightly staining cell bodies. DRG neurons were isolated and cultured for 24 hours under various conditions, and then fixed and stained with anti-PGP 9.5. Photographs were taken using the 40X objective, and cell bodies in each photo were grouped as small (< 50 um) or large (> 50 um), or lightly or darkly staining. The arrow on the left is pointing to a large, dark neuron, while the right arrow indicates a small, light cell body.

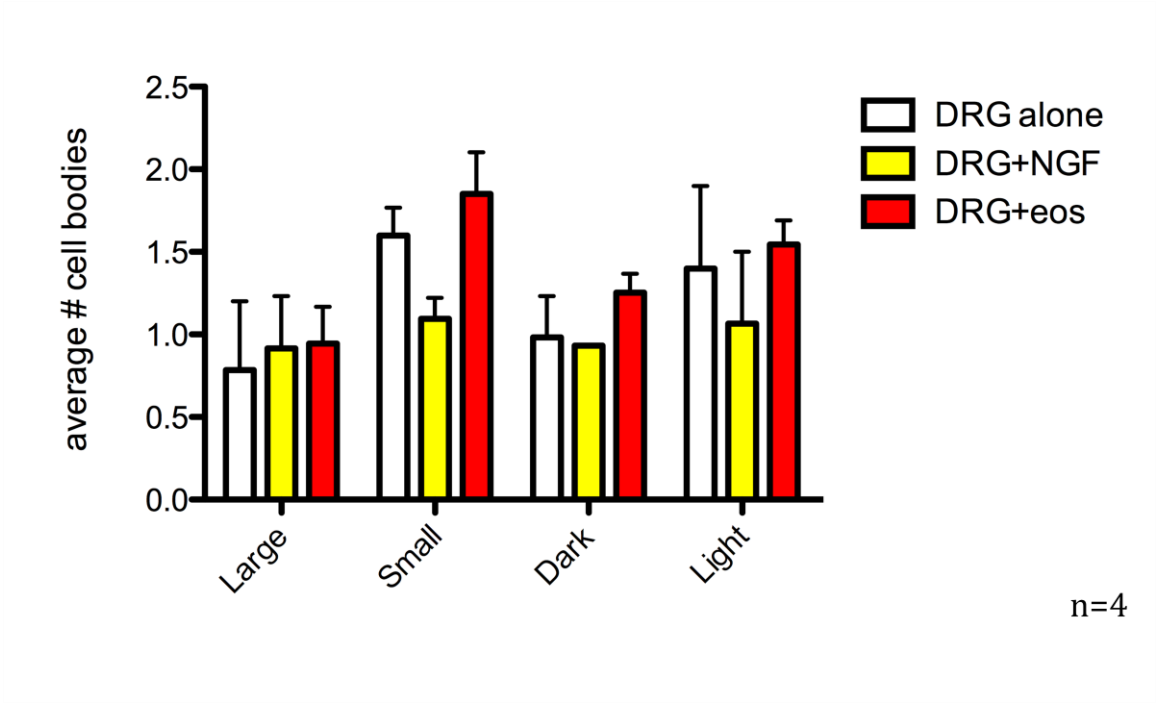
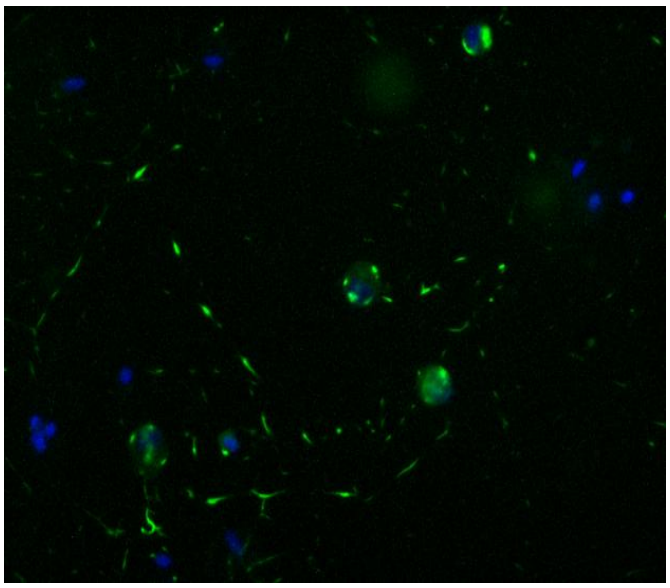


Figure 6.2. Culture with eosinophils does not alter the relative number of DRG neurons with small or large cell bodies. DRG neurons cultured alone (white), with NGF (yellow), or with eosinophils (red) for 24 hours were stained with anti-PGP 9.5 and quantified according to large or small cell bodies, and darkly or lightly staining for PGP 9.5.

(A)



(B)

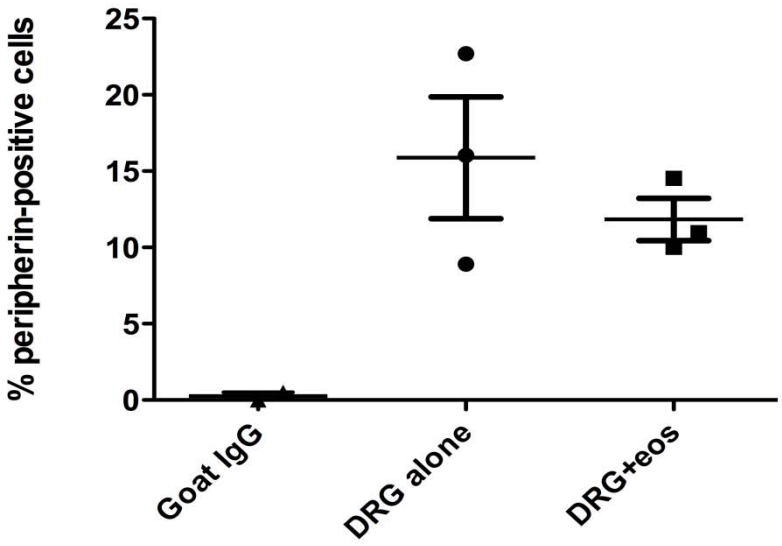


Figure 6.3. Eosinophil-induced neurite branching does not occur preferentially in peripherin-immunoreactive neurons. Cultures of neurons were stained with anti-peripherin antibody. Representative photomicrograph shown in (A). Quantification is shown in (B). n=3 experiments

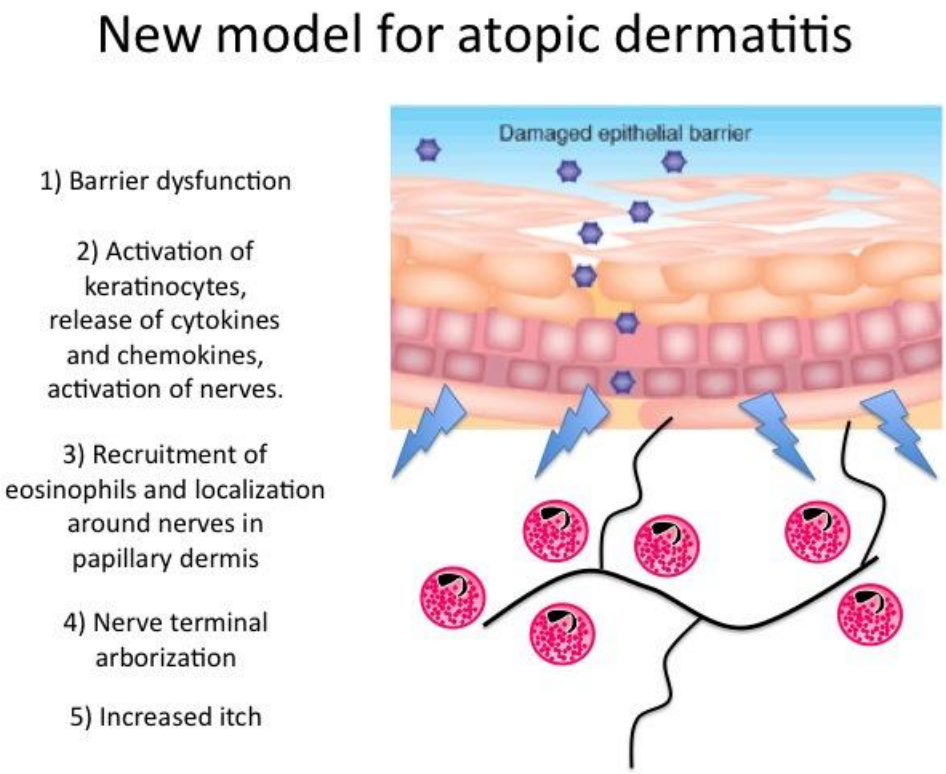


Figure 6.4. Model of atopic dermatitis development.

Barrier dysfunction activates keratinocytes and fibroblasts, which recruit eosinophils through increased production of eotaxin and other chemokines. Eosinophils infiltrate into skin, localize preferentially around nerves, and become activated, possibly through cell adhesion molecule interactions. Eosinophils release granules, which stimulate neurons to arborize in the papillary dermis and basement membrane zone. The person (or mouse) feels an increased sensation of itch, scratches the skin, and a lesion forms.

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APPENDICES

Appendix A: Synonyms for atopic dermatitis.

Synonyms for atopic dermatitis: atopic eczema, infantile eczema, prurigo Besnier, lichen Vidal, endogenous eczema, Spatexudatives Ekzematoid, neurodermatitis (constitutionalis)

[Hanifin and Rajka, 1980]

Appendix B: Diagnostic criteria for atopic dermatitis.

I. 1977: Hanifin J and Lobitz W, Arch Dermatol Vol 113, May 1977, pg 663-670

Proposed Diagnostic Criteria for AD:

Must have each of the following:

1. Pruritus

2. Typical morphology and distribution:

    a. flexural lichenification in adults

    b. facial and extensor involvement in infancy

3. Tendency toward chronic or chronically-relapsing dermatitis.

plus

Two or more of the following features:

1. personal or family history of atopic disease

2. immediate skin test reactivity

3. white dermatographism and/or delayed blanch to cholinergic agents

4. anterior subcapsular cataracts

or

four or more of the following features:

1. xerosis/ichthyosis/hyperlinear palms

2. pityriasis alba

3. keratosis pilaris

4. facial pallor/infraorbital darkening

5. Dennie-Morgan infraorbital fold

6. elevated serum IgE

7. keratoconus

8. tendency toward non-specific hand dermatitis

9. tendency toward repeated cutaneous infections

II. 1980: Hanifin J and Rajka G, Acta Dermatovener (Stockholm) Suppl 92:44-47, 1980

"firm diagnosis of atopic dermatitis would require the presence of at least 3 basic features:

1) Pruritus. history of itching. Jacquet suggested that all cutaneous changes are secondary to itch-induced scratching. (Jacquet, L. In La Pratique Dermatologique, ed E Besnier, L Brocq, L Jaquet, vol 5, Masson Paris, 1904.)

2) Lichenification.

3) Chronically relapsing course. AD is remarkable for its chronicity and for flares and relapses

4) Atopic history a) personal [manifestations of allergic respiratory disease are present in roughly 50% of patients, varies with age of population; infants may develop respiratory symptoms much later] or b) family members [approx 70% of

pts with AD are aware of other family members who have one or more manifestations of atopy]

Minor features: 1) Xerosis, 2) Ichthyosis, 3) Immediate (type I) skin test reactions, 4) Elevated serum IgE, 5) early age of onset, 6) cutaneous infections, 7) non-specific hand dermatitis, 8) nipple eczema, 9) cheilitis- chronic desquamation of the upper lip, both lips or perioral areas, 10) recurrent conjunctivitis, 11) Dennie-Morgan infraorbital fold, 12) Keratoconus (not often seen in AD pts), 13) anterior subcapsular cataracts, 14) orbital darkening, 15) facial pallor and facial erythema, 16) pityriasis alba- mild, post-inflammatory hypopigmentation, 17) anterior neck folds, 18) itch when sweating, 19) intolerance to wool and lipid solvents, 20) periofollicular accentuation, 21) food intolerance, 22) course influenced by envtl and emotional factors, 23) white dermatographism and delayed blanch.

III. 1994: Williams HC, Br J Dermatol 131(3), 406-416, 1994. UK Working Party's diagnostic criteria for AD. Indep hospital validation.

Evidence of a pruritic skin condition plus 3 or more of the following:

1. history of flexural (skin crease) involvement
2. history of dry skin
3. onset under the age of 2 years
4. personal history of asthma or allergic rhinitis
5. visible flexural dermatitis as seen by a clinician

Appendix C: List of reagents

Penicillin/Streptomycin	HyClone SV30010	Pen 100 U/ml Strep 100 ug/mL
Dulbecco's Modified Eagle Medium		
Fetal Bovine Serum		
Hank's Buffered Salt Solution		
Ketamine		100 mg/kg
Xylazine		5 mg/kg
Grade V ovalbumin	Sigma	100 ug/ml
Pentobarbital		300 mg/kg
Trypsin-EDTA		1.25%
Transwell plates		
Matrigel	BD Biosciences	
Cytosine arabinoside		
Paraformaldehyde		4%
ITS	CellGro 25-800-CR	
L-glutamine		
Bovine serum albumin		
Hemacolor	EMD Chemicals	
Percoll		
Toluidine blue		
Trypan blue		
Antigen unmasking solution		

Mouse anti-human PGP 9.5	Serotec	1:500, 1:1000
Rabbit anti-mammal PGP 9.5	Serotec	1:500, 1:1000
Biotinylated goat anti-mouse		
Biotinylated goat anti-rabbit		
Alexa 555 goat anti-rat		
Normal goat serum	Vector	
Avidin-Biotin Conjugation kit	Vector	
DAB or SG kit	Vector	
Mounting Medium with DAPI	Vector	
Cytoseal Crystal Mount		

Mice:

Wild-type, C57BL/6	Jackson Laboratories	
PHIL	Nancy and James J Lee Lab	Scottsdale, AZ
NJ1638 (CD3-IL-5 transgenic)	Nancy and James J Lee Lab	Scottsdale, AZ
K14-IL-5 (IL-5 transgenic)	Nancy and James J Lee Lab	Scottsdale, AZ

Medium recipes:

C2 nerve medium

95ml DMEM (with pyruvate and L-glut)

5ml 2% BSA in DMEM

2ml 100X stock L-glut in DMEM

2ml 100X ITS

100ml F12 medium.

PMC culture medium

462.5ml DMEM

25ml FBS

5ml 200mM L-glutamine

2.5ml gentamicin (10mg/ml)

5ml 1M HEPES (20mM final, to give a pH of 7.4)

Isotonic Percoll solution, 70%

7ml Percoll, sterile

1ml 10X HBSS

0.1ml FBS, sterile

1.9ml ddH2O, sterile

Appendix D: Commonly used dermatologic terms

acanthosis: thickening of granular layer of skin

eczema: any spongiotic dermatitis

edema: swelling due to fluid leakage

filaggrin: cleaved from pro-filaggrin; a major contributor to the epidermal barrier

hyperkeratosis: thickening of the entire epidermis

hyperplasia: proliferation of cells, specifically keratinocytes

lesional: clinically abnormal appearing skin from an atopic dermatitis patient

non-lesional: clinically-normal appearing skin from an atopic dermatitis patient

papillary dermis: upper region of dermis abutting the dermal-epidermal junction

pruritus: itch

rete pegs: epidermal hyperplasia that forms incursions into the papillary dermis

reticular dermis: lower region of dermis abutting the hypodermis

spongiosis: intercellular edema due to keratinocyte apoptosis

stratum corneum: top layer of skin that sloughs off; forms main epidermal barrier

hypodermis: deepest layer of skin containing adipocytes, blood vessels and nerve plexi

xerosis: dry skin

Appendix E: Patient Intake Form for Human Biopsy study

DATE:

Patient Number _____

Name of Patient _____

Date of Birth _____

Family History of Atopic Dermatitis/Eczema:

Personal History of Atopic Dermatitis/Eczema:

Approximate age when disease appeared:

Time without topical treatment for disease (days):

Current medication list:

Patient-rated *itch* on a scale of 1-5 (1 = no itch, 5 = unbearable, keep up at night):

Physical Exam:

Samples taken:

Circle one or more: Normal skin Lesional skin Non-lesional skin