EOSINOPHIL/BASOPHIL PROGENITORS: A POSSIBLE ROLE IN THE PATHOGENESIS OF ATOPIC DERMATITIS

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A Thesis Submitted to the School of Graduate Studies in Partial Fulfillment of the Requirements for the Degree Master of Science

MASTER OF SCIENCE (2018)

McMaster University, Hamilton, Ontario

Faculty of Health Sciences, Medical Sciences Graduate Program

TITLE:	EOSINOPHIL/BASOPHIL PROGENITORS: A POSSIBLE ROLE IN THE PATHOGENESIS OF ATOPIC DERMATITIS
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NUMBER OF PAGES:	vi, 73

LAY ABSTRACT

Atopic dermatitis is a common skin disease that is characterized by chronic, relapsing skin inflammation and eczematous, itchy lesions. In other allergic diseases, a cell called the "eosinophil/basophil progenitor" contributes to the accumulation of inflammatory cells in the diseased organ. We proposed that eosinophil/basophil progenitors found in the skin may be contributing to the development of local allergic inflammation. In patients with moderate-to-severe atopic dermatitis we compared acute responses to intradermal allergen and chronic skin lesions to diluent-challenged and un-affected skin, respectively. Allergen-challenged skin had more eosinophil/basophil progenitors, mature eosinophils and basophils 24 hours' post-challenge compared to unchallenged skin ($p<0.05$). Chronic skin lesions had more eosinophil/basophil progenitors than un-affected skin ($p<0.05$). The number of eosinophil/basophil progenitors positively correlated to disease severity as determined by EASI and SCORAD. Our results suggest that accumulation of eosinophil/basophil progenitors in skin of atopic dermatitis patients could support allergic inflammation and contribute to disease severity.

ABSTRACT

Atopic dermatitis (AD) is a common skin disease that is characterized by chronic, relapsing skin inflammation and eczematous, itchy lesions. In AD, systemic and local eosinophilia and basophilia is thought to contribute to disease progression in both acute and chronic lesions. It has been previously shown that in chronic allergic inflammatory diseases, tissue eosinophilia and basophilia may in part result from eosinophil/basophil (Eo/B) progenitors trafficking from the bone marrow and maturing in tissue in response to type 2 cytokines including IL-5 and IL-3. We therefore proposed that a similar mechanism could be contributing to the pathogenesis of AD. First, we compared lesional and non-lesional AD tissue, and found approximately 10-fold higher levels of Eo/B progenitors in the lesional tissue ($p<0.05$). As previous research has shown an increase in Eo/B progenitors in the airways of allergic asthmatics post inhaled allergen challenge, we next examined whether Eo/B progenitors increased locally in the acute phase of AD using the intradermal allergen challenge model. Compared to intradermal diluent challenge there was an increase in Eo/B progenitors (5.5-fold), eosinophils (18-fold) and basophils (2.5-fold) 24 hours post intradermal allergen challenge (all $p<0.05$). These increases were consistent with findings in allergic airways. Lastly, we examined the relationship between disease severity and Eo/B progenitors in inflamed lesional (chronic) and allergen-challenged (acute) tissue. We found that Eo/B progenitors in lesional tissue positively correlated with disease severity (EASI $R=0.71$, $p<0.05$ and SCORAD $R=0.65$, $p<0.05$), while in allergen-challenged tissue a trend was seen for a positive correlation between Eo/B progenitors and disease severity (EASI $R=0.48$, $p=0.07$ and SCORAD $R=0.46$,

p=0.09). These results highlight the potential involvement of Eo/B progenitors in the disease pathogenesis of AD.

ACKNOWLEDGEMENTS

I would firstly like to thank Dr. Gail Gauvreau for giving me the opportunity to be her graduate student. Gail – it has been an absolute pleasure to be your graduate student. In the past two years, your support and mentorship have been invaluable in supporting my development as a researcher. In my opinion you truly demonstrate what it means to be an excellent scientist. Thank you for hosting the most enjoyable get-togethers on your farm – my only regret is my teams devastating loss at Winter Lab Olympics 2018! Hopefully I'll be back to participate in the 2020 Summer Lab Olympics! To Dr. Roma Sehmi – thank you for your support over the past two years. I truly appreciate all the time you take with me to troubleshoot experiments and to answer my very in-depth eosinophil biology questions! To Dr. Hermenio Lima – thank you for instilling in me a life-long love of dermatology. I never thought that I would enjoy working with skin – how far I've come from the first time you dropped off a punch biopsy in the lab! I hope one day to be able to inspire in my patients the same trust and hope that you inspire in yours. To Dr. Paul O'Byrne – thank you for your unwavering support and encouragement of me in following my clinical and research dreams. I am incredibly grateful to you for exemplifying the dual roles of the clinician scientist. To the Cardio-Respiratory Lab: Karen – thank you for all your help and support with my project. Biopsy days with you and Dr. Lima were my favourite days, and getting into our dream team rhythm was the best. Thank you for

coming in early, and staying late, and always helping me find RAST results at the last minute with either the computer or the printer always breaking (I'm sorry). Sakktee – thank you for being my partner in all of this, I was really lucky to be able to go through a project and have someone else to bounce ideas off of, trouble shoot, problem solve, and lean on. Bonne chance a McGill! Caroline – I literally don't know what I would have done without you. Thank you for our Starbucks runs, Tim Hortons runs, Williams runs, big breakfast runs, 5k runs and 10k runs (there's a theme here I think….). Thank you for being the best roommate in Orlando – I can't wait to sing the office theme song with you again in Halifax. JP – another person I couldn't have lived without. My buddy – thank you for being the best mentor any new graduate student could have asked for. Thank you for helping me so so much over the past two years, and constantly supporting me and believing in me. From my first week here you were there for me, and never stopped – even from Stanford!! Thank you. Adrian, Catie O, Sue, Tara, Rick, Catie C, Dhuha, Kieran – thank you for welcoming me into the lab, helping me learn and grow, and being all around hilarious and amazing people to spend time with. Students – Nobu, Alice, Tim, Michael – I really enjoyed spending time with you, in and out of work - Kampai!

To Cat and Suby – I love you both to the moon and back. I don't think I can put into words how much I love spending time with you two and I can't wait to see what the future has in hold for both of you. Lastly, to my parents – thank you for your unwavering support and love. I couldn't have done this without you.

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LIST OF ABBREVIATIONS AND SYMBOLS

AD	Atopic dermatitis
APC	Antigen presenting cell
C/EBP	CCAAT-enhancer-binding proteins
CD	Cluster of Differentiation
CMP	Common myeloid progenitor
CXCR4	C-X-C chemokine receptor type 4
DAPI	4',6-diamidino-2-phenylindole
EASI	Eczema Area and Severity Index
ECP	Eosinophil cationic protein
ECR	Early cutaneous response
EDN	Eosinophil-derived neurotoxin
EMP	Erythroid and megakaryocytic progenitor
Eo/B	Eosinophil/basophil
EPX	Eosinophil peroxidase
FcεRI	High affinity IgE receptor
FLG	Filaggrin
GM-CSF	Granulocyte-macrophage colony-stimulating factor
GMP	Granulocytic and monocytic progenitor
H&E	Hematoxylin and Eosin
HOME	Harmonizing Outcome Measures for Eczema
HSC	Hematopoietic stem cell
IFN-γ	Interferon gamma
ID	Immunoglobulin D
IgE	Immunoglobulin E
IgG	Immunoglobulin G
IL	Interleukin
ILC2	Innate lymphoid cells
JAK	Janus Kinase
LCR	Late cutaneous response
MBP	Major basic protein
PBS	Phosphate buffered saline
RAST	Radioallergosorbent test
SCORAD	Severity Scoring of Atopic Dermatitis Index
SDF-1	Stromal cell-derived factor 1

STAT	Signal transducer and activator of transcription
TGF-β	Transforming growth factor beta
Th1	T helper 1
Th2	T helper 2
TSLP	Thymic stromal lymphopoietin
VCAM-1	Vascular cell adhesion protein 1

DECLARATION OF ACADEMIC ACHIEVEMENT

Although I was the major contributor for the work presented in this thesis, this work required collaboration from several individuals.

Dr. Gail Gauvreau, Dr. Roma Sehmi, Dr. Hermenio Lima, Dr. Paul O'Byrne, Dr. Dhuha Al-Sajee and I designed the experiments and provided technical input on the project. Dr. Hermenio Lima, Karen Howie, Caroline Munoz and I coordinated and performed the clinical procedures. Sai Sakktee Krisna and I recruited patients for the clinical procedures. I analyzed the data and prepared figures and tables for this dissertation.

CHAPTER 1: INTRODUCTION

1.1 Overview of Atopic Dermatitis

Atopic dermatitis (AD) is a common skin disease that is characterized by chronic, relapsing skin inflammation and eczematous, itchy lesions[1]. It is often associated with other atopic disorders including allergies, asthma and allergic rhinitis, and is the result of a complex interplay between the immune system, epithelial-barrier dysfunction, environmental and neurological factors[1–3]. The onset of AD occurs most commonly between 3 and 6 months of age, with most cases resolving by the end of adolescence[4]. About 10 to 30% of cases do not resolve and continue into adulthood, and a small proportion develop AD as adults[5]. The most common clinical features of the disease are pruritus, erythema, edema, excoriations, lichenification and xerosis[6]. Histologically, the most common features are epidermal spongiosis, epidermal thickness and hypertrophy, and perivascular lymphocyte and granulocyte infiltrate[1]. This section on AD focuses on the epidemiology and burden, pathogenesis and clinical diagnosis.

1.1.1 Epidemiology and Burden

The prevalence of AD has increased over the past few decades, with 15-30% of children and 2-10% of adults being affected in developed countries[7]. While most patients develop AD symptoms within the first year of life, symptoms can start in both childhood and adulthood. Patients and their families can thus be affected for much of their life, impacting quality of life and social, academic and occupational impacts[8]. Drucker et al. (2017) reviewed the current literature on the impact of AD on quality of life of both

patients and their families and found profound negative impacts on quality of life. In children, generalized AD has the second largest impact on quality of life, behind cerebral palsy[9]. The most frequent factors affecting quality of life in children were found to be sleep disruption, pruritus, sport participation interruption and embarrassment[8]. Similar impacts were noted in adults, as well as increased rates of depression, suicidal tendencies and poorer mental health than the general population[8]. Additionally, AD was ranked first by the World Health Organization 2010 Global Burden of Disease survey in the common skin disease category for both years with a disease and disability-adjusted-life-years[10]. The burden of AD on society is largely financial, due to direct costs such as medical visits and medications as well as indirect costs such as lost productivity. In the United States, this is estimated to total $4.338 billion USD per year[11].

1.1.2 Diagnosis

Atopic Dermatitis is a skin disease characterized by multiple clinical phenotypes and a high degree of heterogeneity on its presentation[12]. Diagnosis of AD relies only on clinical features, as thus far there are no specific laboratory or histological findings. Pruritus associated with eczematous lesions (can be acute, subacute or chronic) with typical morphology based on age-specific patterns, chronic or relapsing history, and personal and/or family history of atopy are considered essential features to make the diagnosis of AD[13]. Early age of onset and IgE reactivity are features to support the diagnosis. Other common associated features that suggest the diagnosis of AD are: atypical vascular responses (e.g. facial pallor, delayed blanch response), keratosis pilaris, pityriasis alba,

hyper-linearity of the palms and soles, ocular or periorbital changes (Dennie-Morgan lines and Herthoge's sign), xerosis, skin lichenification, prurigo, and ichthyosis[13]. The acute phase presents in a weeping, blistering form, or a dry and scaly papular form. The chronic phase results in thickened and lichenified skin that is hyper-pigmented from pruritus[14].

1.1.3 Clinical outcome measurements

There have been more than 20 clinical outcome measurements identified which assess the severity of atopic dermatitis[15]. Of these measurements, there is a varying level of evidence to support their use to denote clinical efficacy. Schmitt *et al*. also identified that the lack of validation of measurements causes difficulty in comparing treatments and preforming meta-analyses. To address these concerns, the Harmonizing Outcome Measures for Eczema (HOME) roadmap has been created[16]. The outcome measurements which are consistently used to evaluate AD are the Eczema Area and Severity Index (EASI), Severity Scoring of Atopic Dermatitis Index (SCORAD), the Investigators Global Assessment (IGA) and the Six Area, Six Sign Atopic Dermatitis (SASSAD) severity score[15,17]. EASI is an eczema measurement tool which has been shown to be a reliable and consistent measurement of AD severity and extent[18]. EASI combines the total body surface area with eczema over four body areas (head and neck, trunk, upper extremities and lower extremities) as well as an assessment of each area for erythema, papulation, excoriations and lichenification on a scale of 0-3 (none, mild, moderate and severe, respectively). All the scores are added together using an algorithmic formula. The

SCORAD assessment is another tool to measure eczema extent and severity[19]. SCORAD defines the extent of AD as the total percentage of the body covered (maximum score of 100%). The severity of AD is assessed through scoring 6 different symptoms from 0-3 (none, mild, moderate and severe, respectively). The symptoms assessed are: erythema, edema/papulation, oozing/crust, excoriation, lichenification, and dryness. Patients also report their assessment of pruritus and sleep loss caused by their eczema, from 0 (none) to 10 (the most possible). Both scores out of 10 are added together to give a maximum possible score of 20. Finally, all three scores are added together using an algorithmic formula. The overall evaluation of AD severity by IGA uses an ordinal scale from 0-5 (e.g., clear, almost clear, mild, moderate, severe and very severe) that is scored using points by an investigator or a physician. While not a HOME core outcome, IGA is used as the reference point for validation studies.

1.1.4 Biological markers

High serum Immunoglobulin E (IgE) levels (both total or allergen specific) are found in many AD patients (about 80%), but not in all[6]. AD has been classified in the past as extrinsic (IgE associated) and intrinsic (non-IgE-associated). Non-IgE-associated AD affects more often girls and women, is late onset, and is not associated with atopic asthma[12]. In contrast, IgE-associated AD patients develop the disease in childhood and patients have high levels of IgE[12]. Specific IgE (sIgE) can be tested to determine certain allergens which patients are particularly sensitized to.

1.1.5 Pathogenesis

The pathogenesis of AD is not well understood. It is thought that AD results from a complex interaction between the epidermal barrier and the immune system, with each factor contributing to disease initiation and progression[20]. Additionally, genetic and environmental factors impact AD and add to its complexity in understanding[10]. The epidermal barrier is crucial in protecting the underlying tissue from environmental allergens. In AD patients, the epidermal barrier breaks down causing both trans-epidermal water loss[1] and the exposure of allergens to tissue-resident immune cells. There is a strong correlation between filaggrin (FLG) mutations and AD, however up to 60% of carriers of the FLG mutation will not develop AD[10]. This indicates that epidermal barrier dysfunction is important to the development of AD, but not sufficient or necessary. Several immune cells are over-expressed in the lesions of AD patients including T helper 2 (Th2) cells, Innate lymphoid cells 2 (ILC2's), dendritic cells, eosinophils, and basophils. As the epidermal barrier breaks down in skin of AD patients, allergens are exposed to the underlying antigen presenting cells (APC's). Primed APC's migrate to local lymph nodes and activate naive T cells to Th2 cells. Th2 cells then traffic to the AD lesion and promote inflammation through release of Th2 cytokines (IL-4, IL-5, IL-13) and other cytokines including IL-25 and thymic stromal lymphopoietin (TSLP) which promote Th2 responses[7]. Immune cells such as eosinophils, mast cells, basophils, ILC2's and dendritic cells infiltrate into the AD lesion, while circulating Th2 cells cause an increase in IgE and eosinophils in serum[21]. IL-12 production by eosinophils causes the

switch from an acute Th2-like immune state to a chronic Th1-like immune state, in which cytokines such as IL-12, IL-5, GM-CSF and IL-18 are dominant[22,23].

1.2 Eosinophils

Eosinophils are granulocytes which comprise less than 5% of circulating leukocytes, but can also reside outside the vasculature in peripheral tissues. They have a half-life of approximately 18 hours in the blood[24]. During certain immune responses including helminth parasite infections and in allergic diseases including asthma, the number of eosinophils in the blood and certain tissues increases, implicating their involvement[25]. Eosinophils develop from the common CD34+/IL5Rα+ eosinophil/basophil (Eo/B) progenitor in the bone barrow. Stimulation of the Eo/B progenitor with IL-5 and granulocyte-macrophage colony-stimulating factor (GM-CSF) results in differentiation into a mature eosinophil. While this process has been traditionally thought to be confined to the bone marrow, recent evidence suggests that Eo/B progenitors can exit the bone marrow and traffic to the peripheral tissues, where they can undergo in situ hematopoiesis into mature eosinophils[26,27]. Morphologically, eosinophils can be identified histologically through staining with Hematoxylin and Eosin (H&E), in which they stain pink with a granular morphology. Functionally, eosinophils contain granules that are rich in cationic proteins including major basic protein (MBP), eosinophil cationic protein (ECP), eosinophil-derived neurotoxin (EDN) and eosinophil peroxidase (EPX). Eosinophils also release a variety of cytokines including type 2 (IL-3, IL-4 and IL-13), and type 1 (IL-2 and interferon gamma (IFN-γ)), leukotrienes and histamine. Eosinophils have been shown

to be important in steady state development, metabolic homeostasis, angiogenesis, tissue regeneration and repair and both cell and humoral immunity[28]. In immune-mediate diseases, eosinophils can contribute to disease. Specifically, eosinophils are found to play a role in Th2 diseases such as atopic dermatitis, allergic asthma and allergic rhinitis.

1.2.1 Development

The generation of granulocytes (eosinophils, basophils and neutrophils) begins from the development of hematopoietic stem cells (HSC's) in the bone marrow[29]. HSC's differentiate into the myeloid lineage through intrinsic transcription factors (PU.1 and GATA-1) as well as extrinsic factors (IL-3 and GM-CSF)[30]. The common myeloid progenitor then differentiates into a CD34+ Eo/B progenitor through intrinsic transcription factors (PU.1, C/EBPα and GATA-2) and external GM-CSF. Finally, the critical transcription factor for specifying eosinophil cell fate is GATA-1[31].

1.2.2 Activation

IL-5, IL-3, GM-CSF cytokines, and eotaxin chemokine, are the main activators of eosinophil proliferation and tissue accumulation. In the bone marrow, eosinophil precursors are stimulated to proliferate by IL-5. IL-5 and eotaxin in combination then synergistically promote the release of eosinophils from the bone marrow into circulation[32,33]. The eotaxins (eotaxin 1, eotaxin 2 and eotaxin 3) are responsible for eosinophil recruitment from the vasculature into the tissues[34]. Within the peripheral tissues, IL-5 is responsible for the activation and survival of mature eosinophils[28]. The

major sources of IL-5 are from Th2 cells and ILC2's, which are driven to produce IL-5 by the epithelial derived alarmins IL-25, IL-33 and TSLP[35,36]. Upon activation, eosinophils are able to rapidly release preformed cytotoxic mediators. Eosinophil survival is promoted by IL-3, IL-5, GM-CSF and eotaxin[37].

1.2.3 Role in allergic inflammation and allergic disease

While eosinophils have many roles in homeostasis and normal physiologic functions, they also contribute to immune-mediated diseases such as atopic dermatitis, allergic rhinitis and allergic asthma. Eosinophils in atopic dermatitis will be discussed in the following section (1.2.4). In allergic, non-allergic and chronic rhinosinusitis, local eosinophil infiltrate is consistently found[38]. Nasal allergen challenge increases tissue eosinophilia and type 2 cytokines such as IL-5[39]. Eosinophils also contribute significantly to allergic asthma, with increased eosinophils in the lungs contributing to poorer outcomes and an increase in asthma exacerbations[40]. Inhaled allergen challenge increases levels of eosinophils in blood and sputum[41,42].

1.2.4 Implications in atopic dermatitis pathogenesis

In normal physiological skin, eosinophils are not present. In contrast, atopic dermatitis patients have both tissue and peripheral eosinophilia[43]. In addition, eosinophil granule protein deposition is more pronounced with more chronic and severe AD, indicating an association between eosinophilia and disease severity[43]. Eosinophils contribute to AD pathogenesis by secreting inflammatory cytokines and chemokines. It is thought that

eosinophils may promote the switch from the acute Th2 response in AD lesions to the Th1-like immune response in chronic AD lesions[44]. The thickened skin in chronic AD lesions is thought to be due to transforming growth factor beta (TGF-β) and IL-13 released by eosinophils, which causes fibrosis of the skin tissue[45]. Current treatments for AD such as corticosteroids and tacrolimus, reduce Il-5, reduce eosinophil production and increase eosinophil apoptosis, resulting in clinical benefit[46]. Figure 1 summarizes the pathological effects of eosinophils in atopic dermatitis.

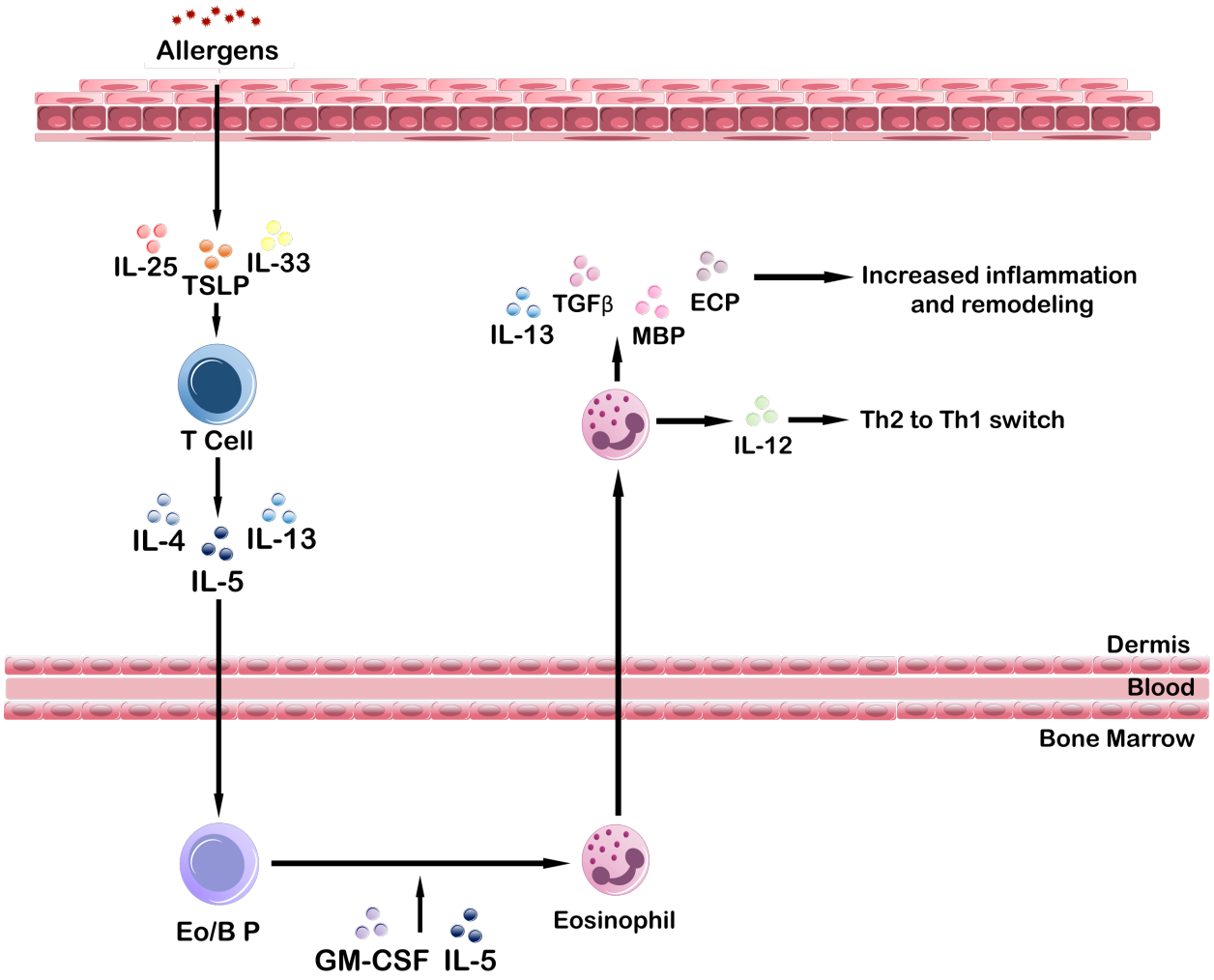

Figure 1: The pathological effects of eosinophils in atopic dermatitis. Eosinophils differentiate in the bone marrow under the influence of growth factors including GM-CSF and IL-5. Mature cells circulate in the blood, and subsequently move through endothelium into tissues under the influence of eotaxin. Within the peripheral tissues, eosinophils are activated by IL-5 and release pre-formed cytotoxic mediators and cytokines including IL-13, TGFβ, MBP, and ECP. Eosinophils also produce IL-12 which is thought to cause the switch from an acute to chronic lesion in atopic dermatitis.

1.3 Basophils

Basophils are granulocytes which are found in quantities less than 1% of peripheral blood leukocytes[47]. Basophils in humans develop from the common CD34+ Eo/B progenitor in the bone barrow, and immature basophils often present with a hybrid basophil-eosinophil phenotype[48]. There is evidence that CD34+ Eo/B progenitors can exit the bone marrow in the context of inflammation and undergo in situ differentiation into basophils in the blood or peripheral tissues[26,27]. Basophils mainly reside in circulation, with a lifespan of approximately 5 days but can also be recruited to tissues[49]. Migration of basophils into the tissue is promoted by IL-1β and IL-3, as well as chemokines such as eotaxin[50].

Morphologically, the metachromatic staining pattern of basophil cytoplasmic granules with Wright Giemsa or Toluidine Blue can differentiate basophils from other granulocytes[51]. There are also several markers which uniquely identify basophils including high expression of high affinity IgE receptor (FcεRI), CD123 (IL-3Rα), 2D7 (a granule protein) and the lack of CD117 (c-kit), which can be used to identify basophils in flow cytometry or immunofluorescence[52,53]

Functionally, basophils rapidly release histamine following crosslinking of IgE bound to their FcεRI, and release IL-4, IL-13 and IL-5 [54,55]. The potential to release Th2 cytokines implicates a pathophysiological importance for basophils in allergic diseases, including atopic dermatitis, asthma, allergies and allergic rhinitis[56]. Basophils have also been implicated in autoimmunity, infections, and malignancy[56].

1.3.1 Development

Like eosinophils, basophils develop from Eo/B progenitors in the bone marrow[30,57]. Along with IL-3 stimulation, the critical transcription factor for specifying basophil cell fate is C/EBPα[58].

1.3.2 Activation

Basophils can be activated through several different pathways, including by antibodies, and by cytokines. In antibody mediated activation, basophils release histamines and leukotrienes in response to FcεRI and IgE crosslinking[59]. IgD-antigen complexes can also activate basophils to produce antimicrobial peptides, resulting in inhibition of bacterial growth[60]. The activation of basophils by cytokines depends on if they are Il-3-elicited or TSLP-elicited during development[61].

In IL-3-elicited basophils, IL-3 binds to its receptor, IL-3R, on developing basophils, increasing proliferation in the bone marrow, as well as increasing basophil survival[62,63]. IL-3-elicited basophils are mainly activated through the cross liking of IgE with the high affinity receptor FcεRI, causing a robust response in which basophils degranulate and release mediators such as histamine, IL-4 and IL-13[59]. IL-3 elicited basophils have a higher surface expression of CD11b and CD62L compared to TSLP-elicited basophils[64]. In contrast, in TSLP-elicited basophils the binding of TSLP to its receptor (TSLPR) on CD34+ bone marrow basophil progenitors *in vivo* has been shown to increase basophil expansion, as well as increasing cellular expression of IL-5 and IL-13 in the sputum of

asthmatic patients compared to healthy controls[65–67]. TSLP-elicited basophils have a unique transcriptional profile, having higher surface expression of IL-3Rα, IL-18R and IL-33R compared to IL-3-elicited basophils[64]. They do not degranulate as robustly as IL-3-elicited basophils when cross-linking IgE, but instead respond to cytokines such as IL-18 and IL-33, resulting in the production of large volumes of IL-4[68].

In summary, TSLP-elicited basophils respond to IL-3 by producing cytokines (IL-4, IL-6) and chemokines (CCL3, CCL4, CCL12 Cxcl2). TSLP-elicited basophils can also respond to IL-33. Conversely, IL-3-elicited basophils do not respond as strongly to IL-3, and are mainly activated by IgE-medicated crosslinking and not cytokine activation [68]

1.3.3 Role in allergic inflammation and allergic diseases

Allergy and atopy are driven by a Th2 dependent mechanism, and are associated with Th2 cytokines (IL-4, IL-5, IL-13), peripheral eosinophilia and IgE production[59,61]. Basophils are potent producers of IL-4, which promotes the differentiation of naïve CD4 T cells to Th2 cells, as well as affecting other cells important to the pathogenesis of allergic disease including ILC2s[69]. ILC2's express IL-4Rα, and proliferate in response to basophil-derived IL-4[70]. Basophils have been shown to play a role in allergic diseases such as IgE-mediated chronic allergic inflammation (IgE-CAI), allergic rhinitis, and allergic asthma. In IgE-CAI, basophils have been shown to be non-redundant in delayed skin inflammation, with massive cellular infiltrate being due to a small number of basophils initiating the reaction and recruiting other inflammatory cells such as eosinophils and neutrophils[71]. In allergic rhinitis, allergen exposure stimulates mast cells

to release histamine, which activates basophil and causes the release of granule proteins and cytokines[50,72]. The depletion of basophils in mouse rhinitis models have been shown to improve symptoms of allergic rhinitis[73]. Treatment of cat-allergic allergic rhinitis patients with anti-IgE (Omalizumab) downregulates expression of FcεRI as early as 1-2 weeks after treatment, and corresponds with an associated clinical improvement in nasal symptom scores[74]. In the lungs of asthmatic patients basophils are increased, and also increase significantly after inhaled allergen challenge[42,75]. CD34+ basophil progenitor cells are also found in increased levels in the bone marrow post allergen challenge[76].

1.3.4 Implications in atopic dermatitis pathogenesis

Basophils have been found in low levels in the chronic lesions of AD patients, and have been found in peripheral blood of AD patients as well[77]. This is in marked contrast to the raised basophil levels in acute AD lesions. A study by Artis *et al*. has shown that basophils and ILC2's accumulate in the acute lesions of AD patients in close proximity and at high levels compared to control skin. This was measured by flow cytometry analysis on human skin biopsies using monoclonal antibodies. Basophils were gated on lineage+ cells and were FcεRI and CD123 positive. ILC2's were gated on lineage negative cells and were IL-33R and CD25 positive. In addition, this study showed that basophils are the primary source of IL-4 in AD lesions, and were required to promote ILC2 accumulation[78]. This was determined through the depletion of basophils in a murine model that in turn reduced the ILC2 response, which was measured using flow cytometry and histopathology. These results indicate that basophils and ILC2's have a prominent

role in promoting AD inflammation. Figure 2 summarizes the pathological effects of basophils in atopic dermatitis.

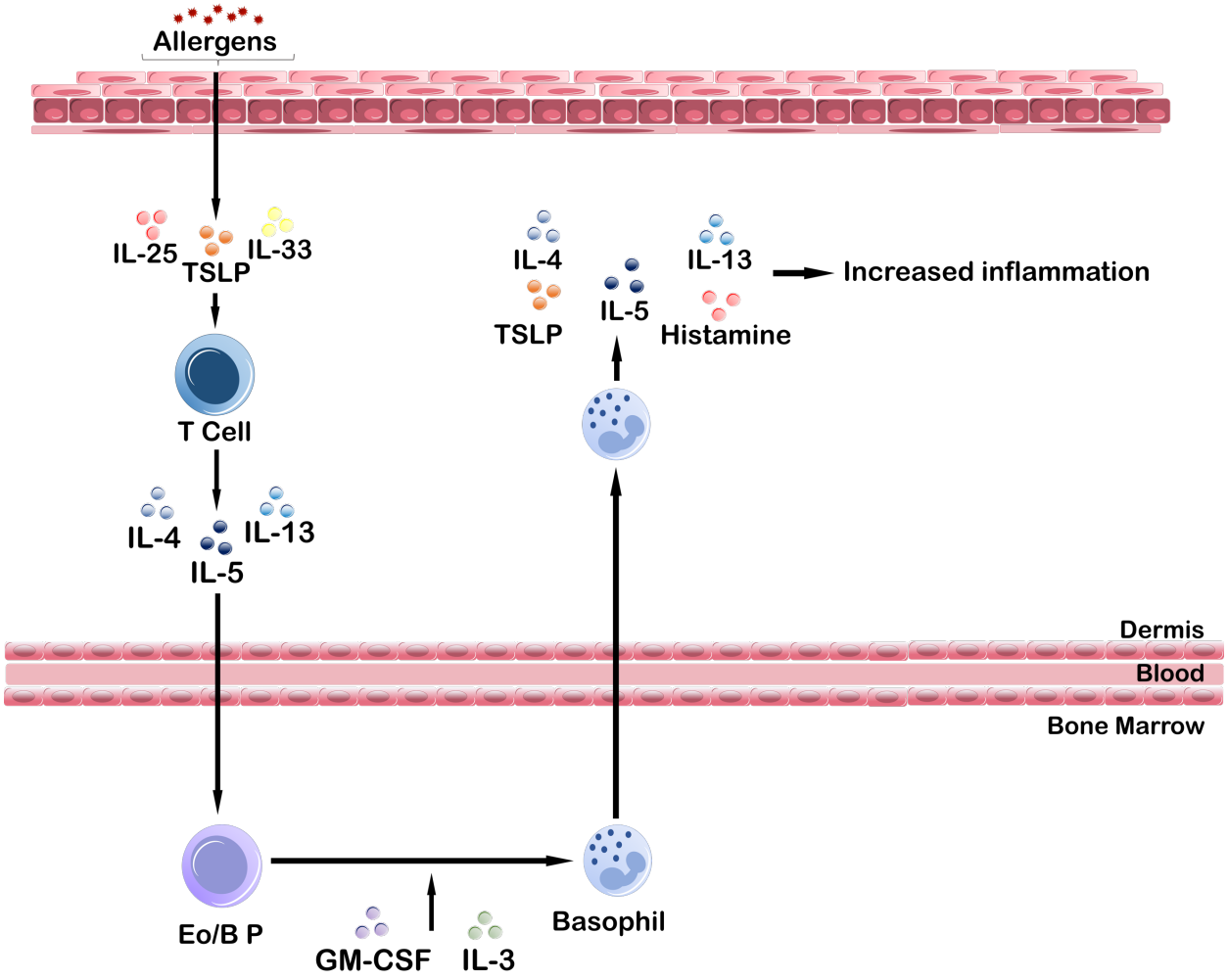

Figure 2: The pathological effects of basophils in atopic dermatitis. Basophils differentiate in the bone marrow under the influence of growth factors including GM-CSF and IL-5. Mature cells circulate in the blood, subsequently moving into the tissue, promoted by IL-1β and IL-3, as well as chemokines such as eotaxin. In the tissue, basophils release proteins and cytokines such as IL-4, TSLP, IL-5, IL-13 and histamine upon crosslinking of FcεRI and IgE, or by cytokine stimulation by IL-33 or IL-18.

1.4 Eosinophil/Basophil Progenitors

1.4.1 Myelopoiesis

The generation of granulocytes (eosinophils, basophils and neutrophils) begins from the development of hematopoietic stem cells (HSC's)[29]. HSC's differentiate into the lymphoid lineage (which includes T and B cells) and the myeloid lineage (which includes granulocytes and macrophages)[79]. The differentiation of HSC's into the common myeloid progenitor (CMP) lineage is mediated by both intracellular and extracellular factors including cell-intrinsic transcription factors such as PU.1 and GATA-1, and extrinsic factors such as cytokines produced by the stromal and haematopoietic components of the bone marrow[80,81]. The CMP then further differentiations into common precursors for granulocytic and monocytic lineages (GMPs) or common precursors for both erythroid and megakaryocytic lineages (EMPs). Dominant expression of the transcription factor PU.1 leads to the differentiation of the CMP to the GMP[81]. Finally, the differentiation of the GMP into the granulocyte precursors (which gives rise to neutrophils, basophils and eosinophils) is driven by the transcription factor C/EBPα and GATA-2[82,83]. The ratio and order of expression of C/EBPα and GATA-2 are crucial for the lineage determinant of the uncommitted GMP, and determines the eventual fate of the cell[84]. The commitment of the progenitor to an eosinophil/basophil (Eo/B) lineage is regulated by IL-3, IL-5 and granulocyte macrophage-colony stimulating factor (GM-CSF)[30,57].

1.4.2 Identification

Eo/B progenitors are identified by an IL-5Rα^{+}CD34^{+}c-Kit^{lo} phenotype[85]. IL-5Rα is the low-affinity component of the receptor for IL-5, IL-3 and GM-CSF[86] and can either be localized to the cell membrane, or in a soluble isotype form[87]. CD34 is a transmembrane glycoprotein found on hematopoietic stem and progenitor cells, whose most commonly described ligand is L-selectin[88,89]. C-kit (also known as CD117) is a transmembrane receptor tyrosine kinase that is expressed in hematopoietic stem and progenitor cells[90]. Eo/B progenitors can be identified through laboratory techniques such as flow cytometry and immunofluorescence.

1.4.3 Differentiation and Trafficking

The differentiation of Eo/B progenitors can occur either within the bone marrow environment, or distally in the peripheral tissues through a process called "in situ hemopoiesis". The commitment to either an eosinophil or basophil depends on both external stimulating factors and internal transcription factors. For commitment to eosinophil lineage, IL-5 is required and the critical transcription factor is GATA-1[31]. For commitment to the basophil lineage IL-3 is required, and the critical transcription factor is C/EBPα[58].

The mechanisms of Eo/B progenitor trafficking from the bone marrow into the blood is not fully elucidated. One study has shown that after allergen challenge there is a down-regulation of C-X-C chemokine receptor type 4 (CXCR4) expression and stromal cell-

derived factor 1 α (SDF-1α) in the bone marrow, but an increase in eotaxin in circulation[91]. SDF-1α binds to its receptor, CXCR4, retaining progenitor cells within the bone marrow[92]. Therefore, it is likely that a decrease in SDF-1α and CXCR-4 in the bone marrow allows Eo/B progenitors to be attracted into the bloodstream by eotaxin. Recruitment of Eo/B progenitors from the vasculature into the peripheral tissues is proposed to be due to the upregulation of cell surface β integrins[93]. IL-3, IL-5 and GM-CSF have been shown to upregulate β_2 integrins, thus priming transendothelial migration[94]. On the surface of endothelial cells, vascular cell adhesion protein 1 (VCAM-1) is upregulated by IL-4 and IL-13. Both β_2 integrin and α4β1integrin heterodimer are expressed on Eo/B progenitors and bind to VCAM-1, promoting extravasation into peripheral tissues[94].

1.4.4 Role in allergic diseases

Eosinophil/basophil progenitors ($CD34^+$/IL-$5R\alpha^+$) have been implicated in the pathogenesis of allergic disease such as allergic asthma and allergic rhinitis. In the bone marrow of atopic subjects there is a significantly greater number of $CD34^+$ cells compared to non-atopic subjects, and these $CD34^+$ cells exhibit enhanced responsiveness to IL-5[95]. Allergen challenge in atopic asthmatic subjects have shown an increase in Eo/B-colony forming units from blood and bone marrow[96,97]. In addition, after allergen challenge there is a higher proportion of $CD34^+$ cells in the bone marrow which express IL-5Rα [98]. Interestingly, $CD34^+$ progenitors have been found in the mucosa of the upper and lower airways[99,100]. In the sputum samples of asthmatics, $CD34^+$ progenitors are

found in increased numbers compared to healthy controls[101]. These data suggest that $CD34^+$ progenitor cells may contribute to the development of blood and tissue eosinophilia and basophilia by migrating to the tissue and differentiating after an allergic event[30]. The presence of eosinophil/basophil progenitor have not been identified in atopic dermatitis skin, and represent an attractive therapeutic target. Figure 3 summarizes the proposed role of Eo/B progenitors in atopic dermatitis.

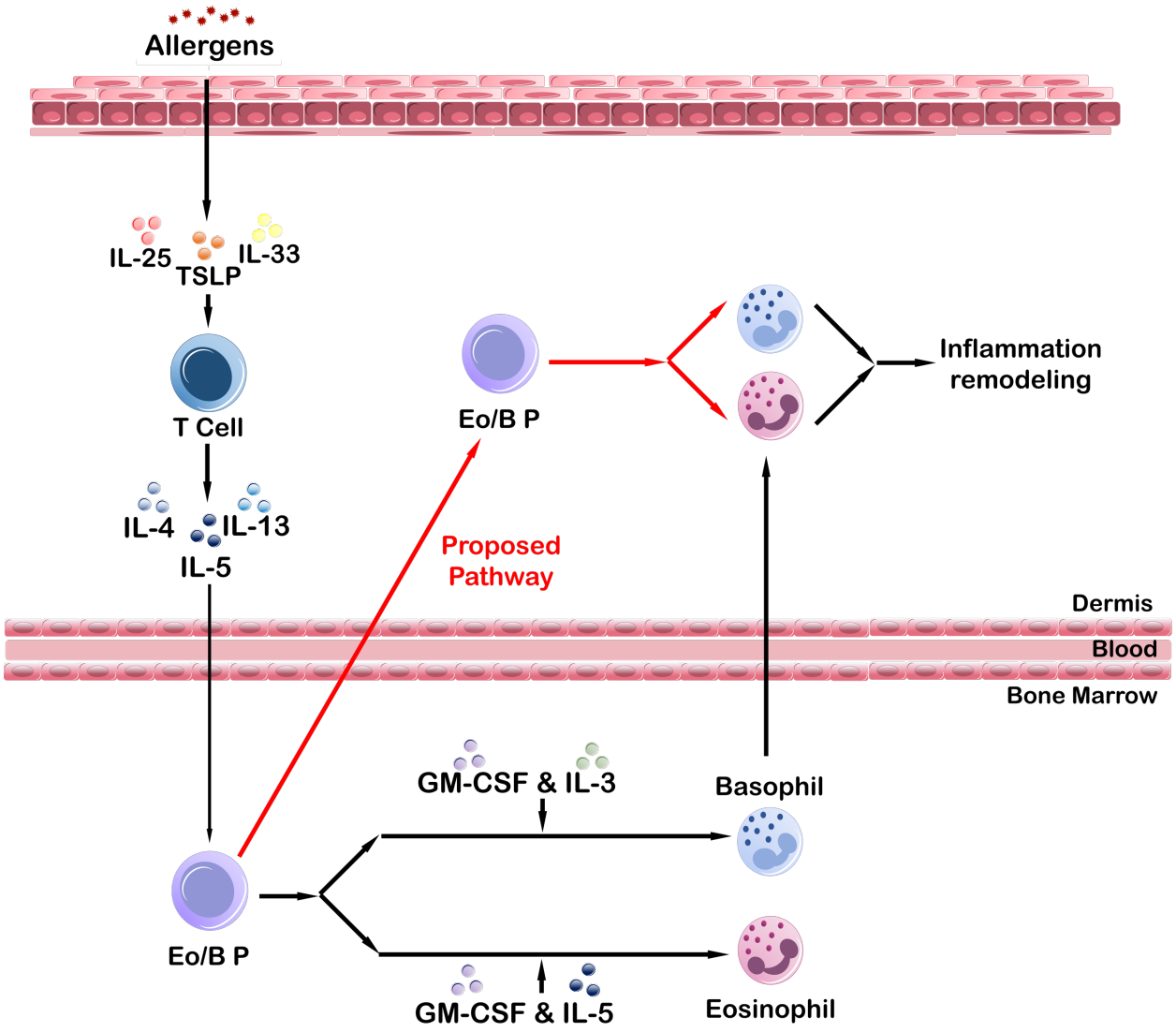

Figure 3: The proposed pathway of eosinophil/basophil progenitor egress from the bone marrow and into the peripheral tissues in atopic dermatitis patients, and the subsequent maturation into mature eosinophils and basophils in the periphery.

1.5 Treatments

1.5.1 Treatments and limitations

The basic management of atopic dermatitis is based on three principals: patient-specific trigger avoidance, antiseptic measures (dilute bleach baths), and proper skin care (skin hydration)[102]. These measures are implemented for all cases of atopic dermatitis, regardless of disease severity. For mild atopic dermatitis, basic management is supplemented with a topical anti-inflammatory applied to the acute flare-ups. The first line treatment is topical corticosteroids, with the potency tailored to the severity of the flare-up[103]. Corticosteroids inhibit the transcriptional activity of several pro-inflammatory genes, including IL-4, IL-5 and IFN-γ[104], as well as reducing the infiltration of pro-inflammatory cells such as eosinophils[105]. Steroid treatment has also been shown to induce eosinophil apoptosis, as well as basophil apoptosis[106,107]. For more moderate atopic dermatitis that is not controlled by a topical corticosteroid, a topical calcineurin inhibitor may be used. In this patient population, steroid treatment does not reduce the levels of eosinophils and basophils in the skin. Calcineurin inhibitors are macrolides that inhibits cytokine transcription in activated T cells, thus reducing inflammation by inhibiting recruitment and activation of eosinophils, basophils and mast cells[108]. For example, cyclosporine, a calcineurin inhibitor, suppresses IL-5 production by $CD4^+$ T cells, resulting in improved clinical symptoms in a study on lung function[109]. Cyclosporine also causes a reduction in primary endpoints on the United Kingdom Sickness Impact Profile and the Eczema Disability Index, demonstrating clinical efficacy in atopic dermatitis patients as well[110]. Corticosteroids and calcineurin inhibitors can also

be used proactively as opposed to retroactively in order to prevent acute flare-ups. The second line therapy for more severe atopic dermatitis patients is the use of systemic immunosuppressive drugs or phototherapy[14]. Dupilumab, a monoclonal antibody that blocks IL-4 and IL-13 is a new systemic immunosuppressive biologic drug that has recently been approved for treatment of severe atopic dermatitis in patients who have failed conventional topical and systemic medication. In phase 3 clinical trials, dupilumab was shown to improve EASI scores by at least 75%, a significant improvement compared to patients who received placebo[111].

There are many limitations to the current treatments used for atopic dermatitis. Patient compliance, especially in pediatric populations can be quite low. Patients and their caregivers are often concerned about the long-term effects of steroids, specifically atrophy of skin[112]. In addition, the time-consuming nature of the topical treatment protocols can be a deterrent to treatment adherence, as putting topical treatments on after bathing can be a long and arduous process involving many steps[113]. Probably the biggest limitation to treatment is that current protocols do not provide long term relief of symptoms, or management of patients with more severe disease[114]. New therapies are needed that focus on the specific pathways that potentiate atopic dermatitis, such as immunomodulators, as opposed to focusing on symptomatic treatment, such as steroids and anti-histamines [115].

1.5.1 Anti IL-5 and IL-5Rα Therapies

Interleukin-5 is a type 2 cytokine that is important for the survival, development, maturation and effector functions of eosinophils and basophils[116]. It is produced by cells including T cells, eosinophils, mast cells, and basophils. IL-5 binds to the IL-5 receptor which is comprised of the specific α subunit and the common β subunit[117]. The IL-5R signals through the rapid tyrosine phosphorylation of cellular proteins. The signal transducer and activator of transcription 1/5 STAT1/STAT5 and janus kinase 1/2 JAK1/JAK2 pathways are particularly important for the activation of eosinophils[117]. In atopic dermatitis patients, IL-5 plays an important role in the pathophysiology of the disease. Eosinophils, basophils and T cell levels are all elevated in AD skin compared to normal skin[7,46]. There are an increased number of IL-5Rα positive cells in the dermis of AD lesions (both acute and chronic)[118], as well as an increase in IL-5 produced by T lymphocytes[119]. An anti-IL-5 antibody, mepolizumab, has been tested on atopic dermatitis patients but achieved no statistically significant clinical improvement[120]. This result has several limitations. While peripheral blood eosinophil count was depleted significantly in the mepolizumab treated group after two weeks, clinically significant reduction of tissue eosinophils could require a longer dosing period. One study has found that 3 doses of mepolizumab over 8 weeks are required to cause a 50% reduction in lung tissue eosinophils[121]. Additionally, mepolizumab does not target other immune cells known to play a role in atopic dermatitis such as T-cells, macrophages and basophils[122]. Targeting the IL-5Rα in atopic dermatitis patients might prove to be more effective than targeting only IL-5. Benralizumab, a humanized antibody against IL-5Rα has shown

clinical efficacy in treating asthma, another disease in which the IL-5 and IL-5Rα axis is important to disease pathogenesis[123].

1.6 Allergen Challenge

1.6.1 The inhaled allergen challenge model

The inhaled allergen challenge is a model that is used to study the mechanisms of allergic asthma and allergen-induced airway responses. It has been validated as a safe and highly reproducible model to study the physiology and kinetics of allergen induced asthma, as well as a disease model for the evaluation of new therapies[124]. In response to inhaled allergen challenge two distinct phases are observed, termed the early and late asthmatic response[125]. The early response is characterized by airflow obstruction (measured by FEV_1) that occurs shortly after allergen inhalation, peaks between 20-30 minutes and resolves over 2-3 hours[126]. Mechanistically, the early response is due to IgE-mediated mast cell degranulation, and subsequent release of histamines and leukotrienes causing bronchoconstriction[127]. About 3-4 hours after allergen challenge, there is another distinct episode of airflow obstruction, lasting for up to 24 hours[126]. Mechanistically, the late response is due to Th2-mediated recruitment of immune effector cells such as eosinophils, basophils and neutrophils, and the subsequent release of histamine and leukotrienes, as well as activation of B cells to produce allergen specific IgE which further activates mast cells and basophils[124].

1.6.2 The intradermal allergen challenge model

Atopic patients who are exposed to allergen injected into the dermis mount an early immune response, which is characterized by an immediate wheal and flare of the skin. This is elicited within approximately 15-30 minutes of injection of allergen and is as a result of the cross linking of IgE on mast cells and the ensuing release of histamine[128]. Subsequently, a late cutaneous response (LCR) can develop 6-24 hours later, which is caused by infiltration of immune cells such as T helper cells, eosinophils, basophils and neutrophils[128–130]. The kinetics of the late cutaneous response have been elucidated in atopic subjects, with the largest response being measure at 24 hours[130,131]. Mast cells were found to peak at 6 hours, basophils peaked at 24 hours and eosinophils peaked between 6 and 48 hours after challenge[42]. The late cutaneous response is thought to more accurately mimic clinical atopic disease such as asthma or atopic dermatitis due to the complexity of the reaction and interaction between mediators and immune cells[132]. TSLP, interleukin-2 (IL-2), interleukin-4 (IL-4) and IL-5, important cytokines found in the lesions of atopic dermatitis patients, are all present in the late cutaneous response[133–135]. In allergic rhinitis patients, prednisone treatment has been shown the reduce the size of the late cutaneous response[136,137]. A similar late phase phenotype occurs when allergen is inhaled, a model which is used extensively in asthma research[124,132,138,139]. The cutaneous late-phase response has also been used to test the effectiveness of drugs for the treatment of asthma[129,136,140]. Interestingly, the use of the late phase response as a clinical model for atopic disease has been used primarily in asthmatic or allergic rhinitis patients. It has thus been proposed that the late cutaneous response from intradermal allergen challenge would be also be a good model for atopic dermatitis[141,142].

1.7 Central Hypothesis, Specific Hypotheses and Specific Aims

1.7.1 Main Thesis Hypothesis

In situ differentiation of Eo/B P contributes to the development of eosinophilic inflammation in the skin of patients with atopic dermatitis.

1.7.2 Main Thesis Objectives

1. To determine if eosinophil/basophil progenitors, mature eosinophils and basophils are elevated in the lesional (chronic) skin of atopic dermatitis patients.
2. To determine if eosinophil/basophil progenitors, mature eosinophils and basophils are elevated in the allergen-induced (acute) late cutaneous response of atopic dermatitis patients.
3. To examine the relationship between the level of mature eosinophils and eosinophil/basophil progenitors in acute allergic skin responses and chronic lesions. To examine the relationship between eosinophil/basophil progenitors, mature eosinophils and basophils in lesional skin versus clinical scores (EASI and SCORAD).
4. To determine the relationship between eosinophil/basophil progenitors, mature eosinophils and basophils in allergen challenged skin versus clinical scores (EASI and SCORAD).

CHAPTER 2: Methods

2.1 Discussion of Study Design

After providing informed consent, patients were assessed for study eligibility at an initial screening visit approximately 28 to 14 days before the day 1/baseline visit. This initial screening visit included a full medical and medication history. Patients who met eligibility criteria underwent day 1/baseline assessments including a skin prick test and intradermal allergen challenge. After 24 hours, at visit 2, the allergen challenged site was assessed for a late cutaneous response and laboratory assessments were performed. Safety, laboratory and clinical assessments were performed at each visit (see Table A for schedule of events).

2.2 Selection of Study Population

The total target enrollment for the study was 15 evaluable subjects. The study population consisted of adults between the ages of 18-65 who had been diagnosed with moderate-to-severe atopic dermatitis.

2.2.1 Inclusion & Exclusion Criteria

Candidates were screened at visit 1 to determine if they met inclusion and exclusion criteria. To meet inclusion criteria, volunteers must be between 18-65 years of age, in good health, and with moderate to severe atopic dermatitis (as determined by IGA, EASI and SCORAD). Patients were required to provide written informed consent, and needed

to demonstrate both a positive skin-prick test to common aeroallergens (including animals, grasses, weeds, pollens and house dust mites), as well as high or positive allergen-specific IgE using RAST blood work. Lastly, subjects were required to develop a positive late cutaneous response to intradermal allergen challenge. Patients were excluded if they were treated with an investigational drug within 8 weeks or within 5 half-lives before the baseline visit, used any immunosuppressive or immunmodulating drugs within 4 weeks before the baseline visit, any treatment with biologics, initiated treatment of AD with prescription moisturizers or moisturizers containing additives such as ceramide, hyaluronic acid, urea, or filaggrin degradation products during the screening period (patients continued using stable doses of such moisturizers if initiated before the screening visit). In addition, regular use of a tanning booth within 4 weeks, precluded the subject from entry into the trial. Any active chronic or acute infection requiring treatment with systemic antibiotics, antivirals, antiparasitics, antiprotozoals, or antifungals within 2 weeks before the baseline visit, or superficial skin infections within 1 week before the baseline visit precluded subjects. Any known or suspected history of immunosuppression, including history of invasive opportunistic infections, history of human immunodeficiency virus (HIV) infection or history of hepatitis B or hepatitis C infection. Finally, the presence of any skin comorbidities or other concomitant illness(es) including psychological conditions that, in the investigator's judgment, would adversely affect the patient's participation in the study would also preclude the patient from study participation.

2.2.2 Premature Withdrawal of a Patient from the Study

Patients could withdraw their consent to participate in the study at any time without prejudice. The investigator could withdraw a subject if, in his or her clinical judgment, it is in the best interest of the subject or if the subject could not comply with the protocol. Whenever possible, the tests and evaluations listed for the termination visit were carried out. Patients who withdrew or were withdrawn from the study were not replaced.

2.4 Study Schedule of Events

Study assessments and procedures are outlined in Table 1.

Table 1: Study Schedule of Events

	Screening		
Study Day	-28 to -14	1	2
Visit	**1a**	**1b**	**2**
Baseline:			
Informed Consent	X		
Inclusion/Exclusion	X		
Medical Hx/Height/Weight	X		
RAST testing	X		
Safety:			
Vital Signs		X	X
Adverse Events		X	X
Laboratory Testing:			
Skin Prick Test and Titration		X	
Intradermal Challenge		X	
Skin Biopsy Allergen Saline Lesion Non-lesional			X X X X
Clinical Effect:			
EASI		X	
SCORAD		X	
DQLI		X	
POEM		X	

2.5 Study Flow Chart

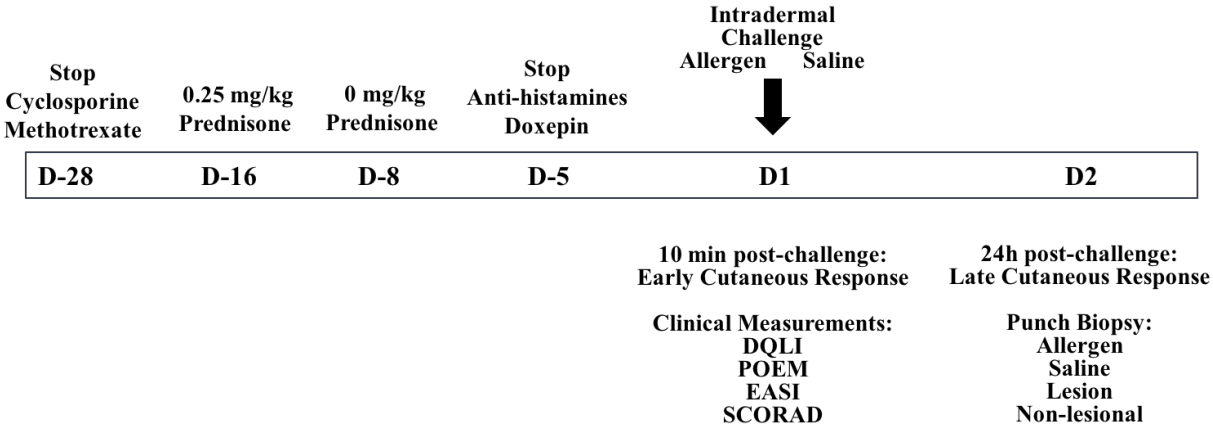

Figure 4. Study flow chart outlining run-in period, as well as study period.

2.6 Description of Procedures

2.6.1 Run-in period

Prior to the beginning of the study, patients discontinued use of systemic immunosuppressant's four weeks before visit 1. Sixteen days before visit 1, patients were given a low dose of oral prednisolone (0.25 mg/kg) for 8 days. Five days before visit 1, patients stopped any anti-histamines or doxepin.

2.6.2 Skin Prick Test

A skin prick test was conducted at visit 1. It was used to determine the allergen(s) to which each subject is sensitized. Standard allergen extracts included ragweed, trees, grass, dog, cat, horse, dust mites (*Dermatophagoides farinae* and *Dermatophagoides pteronyssinus*), alternaria, and aspergillus. Extracts were applied to the patients back by

pricking the skin with a lancet to allow exposure, and then evaluating the local reaction in the skin. A positive control (1 mg/mL histamine) and a negative control (diluent) were also applied to the skin. If an allergen provoked an allergic reaction, a raised itchy bump (wheal) developed. The size of the wheal (the raised area, not the redness) was measured and recorded with a ruler in millimeters in the horizontal and vertical directions, perpendicular to each other after approximately 10 minutes. The size of the wheal for each antigen was recorded in the source documents, along with any observed adverse reaction or event and any actions taken. A skin wheal greater than 2 × 2 mm was regarded as a positive reaction, provided that the positive and negative controls were appropriately positive (histamine) and negative (diluent), respectively.

Allergen extracts manufactured following Good Manufacturing Practice guidelines, were selected, prepared by staff and administered to the subjects.

2.6.3 Skin Prick Titration

Based on the results of the skin prick test on visit 1, the investigator chose an allergen for skin prick titration. An allergen was selected that the subject had both a positive RAST result as well as a positive skin prick test. The allergen chosen was not one that the subject was the most allergen to, nor had extremely high levels of specific IgE. This was done to minimize any risk of anaphylaxis. The selected allergen was diluted 2, 4, 8 and 16-fold with normal saline and then applied to the patients back using the same technique as described in the skin prick test. After 10 minutes the size of the wheal was measured

and recorded with a rule in millimeters in the horizontal and vertical directions, perpendicular to each other. The size of the wheal was recorded in the source documents along with an observed adverse reactions or event and any actions taken. The dilution that resulted in a wheal size of 3x3 mm was selected for intradermal allergen challenge.

2.6.4 Intradermal Allergen Challenge

The selected allergen was diluted to the concentration as chosen in in the skin prick titration (section 2.6.2) and injected in a volume of 100 µL intradermally in one standardized location on the subject's back at visit 1. A saline control was also injected in a volume of 100 µL intradermally in one locations of the subject's back. At 10 minutes' post allergen challenge the acute response wheal was measured in millimeters using a ruler and recorded in the source documents. At 24 hours after challenge, at visit 2, the size of the late cutaneous response wheal was measured using a ruler in millimeters.

2.6.5 Skin punch biopsies

At visit 2, a 4mm punch biopsy was taken from the center of 3 of the sites (1 allergen, 1 saline, 1 area of non-lesional skin). A 3mm punch biopsy was taken from the center of an active lesion on the patients back. The lesional area on the back was chosen based on meeting the criteria defined for an atopic dermatitis lesion, including lichenification, vesicles, edema and erythema. The non-lesional site was chosen on the back as well, but from an area that was unaffected by lesions.

Before a biopsy was taken, the skin was thoroughly cleaned and local anesthetic (2% lidocaine) injected to numb the skin. Punch biopsies were obtained using a sterile 3 or 4 mm skin punch (Integra LifeSciences MLT3332P/25 or MLT3334P/25) by applying and twisting until the blade of the skin punch has pierced the skin. The biopsy was removed using sterile forceps and scissors. The skin punch biopsies were used to look at histological changes in allergen compared to saline control skin, as well as in lesional and non-lesional skin.

2.6.6 Vitals

Vital signs were taken at visit 1 and include temperature (measured with WelchAllen SureTemp), blood pressure (measured with SpaceLabs HealthCare Ultraview SL) and pulse (measured with SpaceLabs HealthCare Ultraview SL). Results were recorded in the source documents.

2.6.7 Clinical Effect Assessments

Eczema Area and Severity Index (EASI)

EASI is am eczema measurement tool which has been shown to be a reliable and consistent measurement of AD severity and extent[18]. EASI scores were collected on visit 1to assess changes throughout the study. EASI combines the total body surface area with eczema over four body areas (head and neck, trunk, upper extremities and lower extremities) as well as an assessment of each area for erythema, papulation, excoriations

and lichenification on a scale of 0-3 (none, mild, moderate and severe, respectively). The EASI assessment tool is provided in the appendix (see Figure 5).

Severity Scoring of Atopic Dermatitis (SCORAD)

The SCORAD is another tool to measure eczema extent and severity[19]. SCORAD scores were collected on visit 1 to assess changes throughout the study. SCORAD defines the extent of AD as the total percentage of the body covered (maximum score of 100%). The severity of AD is assessed through scoring 6 different symptoms from 0-3 (none, mild, moderate and severe, respectively). The symptoms assessed are: erythema, edema/papulation, oozing/crust, excoriation, lichenification, and dryness. Patients also report their assessment of pruritus and sleep loss caused by their eczema, from 0 (none) to 10 (the most possible). Both scores out of 10 are added together to give a maximum possible score of 20. Finally, all three scores are added together using an algorithmic formula. The SCOARD assessment tool is provided in the appendix (see Figure 6).

Dermatology Life Quality Index (DLQI)

The DLQI is a simple questionnaire which asks patients questions related to the impact of their disease on their quality of life[143]. Patients answer 10 questions about quality of life by answering: very much, a lot, a little or not at all. These 4 answers correlated to a score of 3, 2, 1 and 0, respectively. The scores are added together to give a total score, which has a maximum possible score of 40. The DLQI is done at visit 1. The DLQI assessment tool is provided in the appendix (see Figure 7).

Patient-Oriented Eczema Measure (POEM)

The POEM is a simply and validated tool that is easily understandable and repeatable. Like DQLI, POEM measures eczema impact on quality of life. POEM asks patients how many days over the past week have they been impacted by a certain symptom of eczema including pruritus, sleep loss, bleeding, oozing, flaking and dryness. These scores are tallied to give a maximum possible score of 28. POEM is administered at visit 1. The POEM assessment tool is provided in the appendix (see Figure 8).

2.7 Description of Laboratory Procedures

2.7.1 Processing of Skin Biopsies

Skin biopsies were fixed in 10% neutral formalin for 48 hours before being transfer to 70% ethanol. Biopsies were then paraffin embedded by the McMaster Core Histology Laboratory Facility. Tissue was dehydrated in ethanol in the following sequence: 50% ethanol for 10 minutes, 70% ethanol for 10 minutes, 80% ethanol for 10 minutes, 95% ethanol for 10 minutes, 100% ethanol for 10 minutes, 100% ethanol for 10 minutes and 100% ethanol for 10 minutes. Ethanol was then exchanged with xylene in the following sequence: 2:1 ethanol: xylene for 10-15 minutes, 1:1 ethanol: xylene for 10-15 minutes, 1:2 ethanol: xylene for 10-15 minutes, 100% xylene for 10-15 minutes, 100% xylene for 10-15 minutes and 100% xylene for 10-15 minutes. xylene was then exchanged with

paraffin using the following sequence: 2:1 xylene: paraffin for 10-15 minutes, 1:1 xylene: paraffin for 10-15 minutes, 1:2 xylene: paraffin for 10-15 minutes, 100% paraffin for 1-2 hours and then 100% paraffin overnight. Tissue was then embedded in fresh paraffin and sectioned on to positively charged slides in 5 μm sections. To analyze the centre of the biopsy core, biopsies were sectioned from the centre out, and analysis was done in the centre of the core.

2.7.2 Hematoxylin and Eosin Staining for Eosinophils

Sections were hematoxylin and eosin stained by the McMaster Core Histology Laboratory Facility. Formalin-fixed paraffin-embedded tissue sections of human skin punch biopsies were deparaffinized using xylene and descending concentrations of alcohol. Sections were then stained with hematoxylin, de-stained with acid ethanol, stained with eosin, and then cover-slipped. The stain results in collagen being stained pale pink, acidophilic cytoplasm (such as eosinophils) being stained red, basophilic cytoplasm (such as basophils) being stained purple, nuclei being stained blue, and erythrocytes being stained cherry red. See Figure 9A for a representative eosinophil.

2.7.3 Basophil Staining

Formalin-fixed paraffin-embedded tissue sections of human skin punch biopsies were deparaffinized using xylene (9 minutes) and descending concentrations of alcohol (100% ethanol for 6 minutes, 95% ethanol for 3 minutes, 70% ethanol for 3 minutes, 50% ethanol for 3 minutes and finally dH_2O for 5 minutes). Next, heat-induced epitope

retrieval with citrate buffer (pH6) was performed for 3 minutes using a pressure cooker, followed by 5 minutes in PBS 1x. Sections were incubated with 3% normal horse serum for 30 minutes, and then incubated with rabbit anti-human mast cell tryptase (Abcam; ab2378) 1:250 dilution overnight at 4°C. Goat anti-rabbit Alexa Fluor 657 (Abcam; ab150079) 1:250 dilution was used for detection and applied for 1 hour at room temperature. Sections were further incubated with mouse anti-human 2D7 antibody (Abcam; ab155577) 1:250 dilution overnight at 4°C. Donkey anti-mouse IgG Alexa Fluor 488 (Abcam; ab150113) 1:250 dilution was used for detection and applied for 1 hour at room temperature. Sections were then stained with DAPI and cover-slipped. See Figure 9B for a representative basophil.

2.7.4 Eosinophil/Basophil Progenitor Staining

Formalin-fixed paraffin-embedded tissue sections of human skin punch biopsies were deparaffinized using xylene (9 minutes) and descending concentrations of ethanol (100% ethanol for 6 minutes, 95% ethanol for 3 minutes, 70% ethanol for 3 minutes, 50% ethanol for 3 minutes and finally dH_2O for 5 minutes). Next, heat-induced epitope retrieval with citrate buffer (pH6) was performed for 3 minutes using a pressure cooker, followed by 5 minutes in phosphate buffered saline (PBS) 1x. Sections were then incubated with 1.5% normal horse and 1.5% normal goat serum for 20 minutes, and then incubated with rabbit anti-human IL-5Rα (Abcam; ab198808) 1:100 overnight at 4°C. Isotype and negative control slides were also stained using rabbit IgG and PBS, respectively. Goat anti-rabbit FITC (Abcam; ab150077) 1:200 was used for detection and

applied for 1 hour at room temperature. Sections were further incubated with mouse anti-human CD34 antibody (Abcam; ab8536) 1:100 dilution overnight at 4°C. Isotype and negative control slides were also stained using mouse IgG and PBS, respectively. Sheep anti-mouse Alexa Flour 594 (Abcam; ab150113) 1:200 dilution was used for detection and applied for 1 hour at room temperature. Sections were then stained with DAPI and cover-slipped. See Figure 9C for a representative eosinophil/basophil progenitor.

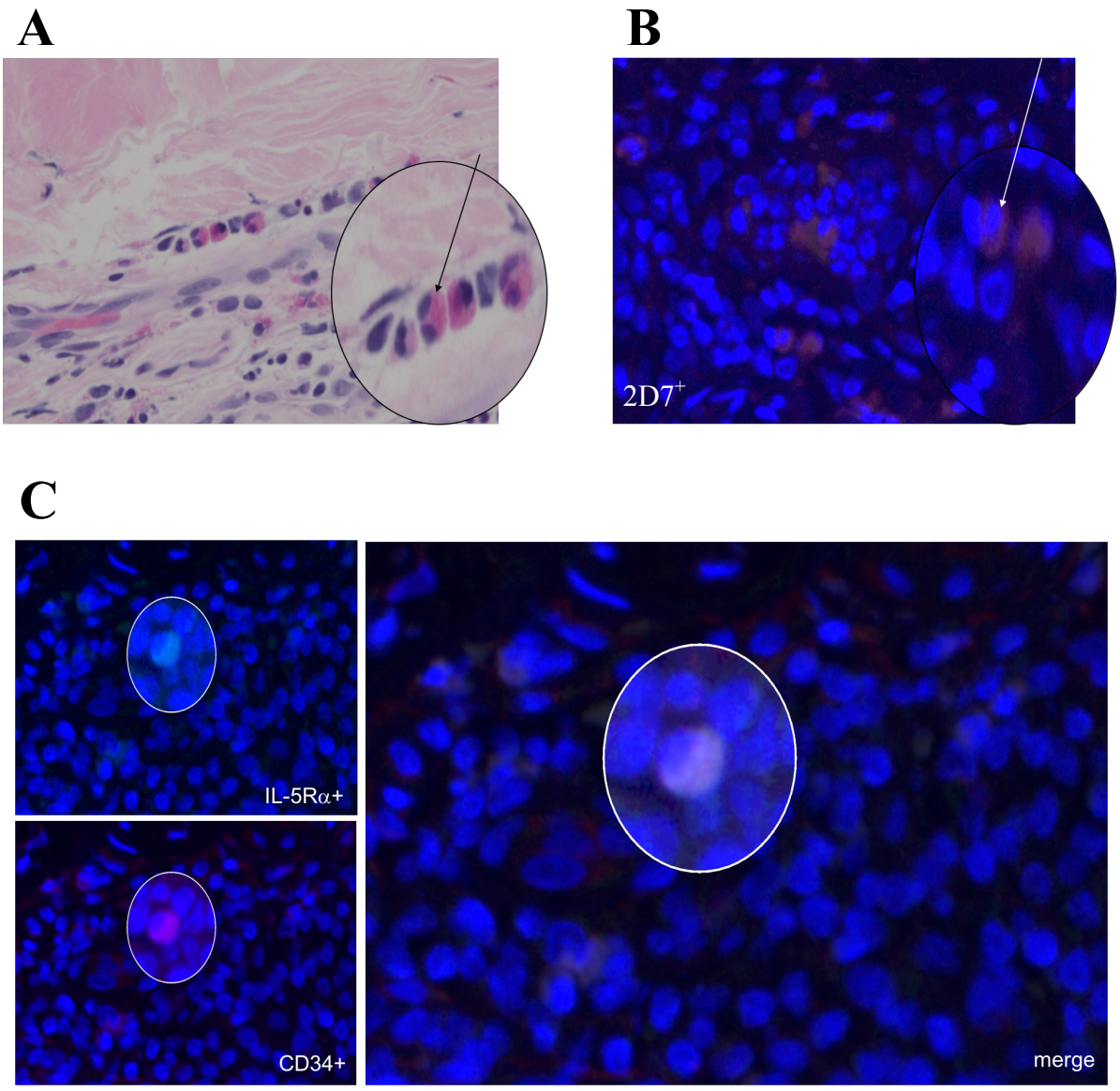

Figure 9. Representative images of H&E (A) and IF (B and C) stained punch biopsy sections.
A) Enlarged section shows eosinophils. Mature eosinophils are identified as cells with a segmented nucleus, granules and stain reddish-pink in a hematoxylin and eosin stain.
B) Enlarged section shows a mature basophil. Basophils are identified as 2D7-TRITC positive and mast cell tryptase-FITC negative.
C) Enlarged section shows an eosinophil progenitor. Eosinophil progenitors are identified as CD34 (TRITC-red) and IL-5Rα (FITC-green) positive and Von Willebrand factor (Cy5-violet) negative.

2.7.5 Image processing and cell counting

Sections were visualized and scanned using the Nikon Imaging Software Elements software. A negative slide was used to set voltages. Eosinophil/basophil progenitors were enumerated as CD34/IL5Ralpha double positive and Von Willebrand negative, per mm^2 in the papillary dermis. Mature eosinophils were enumerated as eosin positive and bi-lobed nucleus positive per mm^2 in the papillary dermis. Mature basophils were enumerated as 2D7 positive, mast cell tryptase negative per mm^2 in the papillary dermis. The size of the papillary dermis is between 0.8 and 1.5 mm^2, depending on the patient and the size of the punch biopsy. Please refer to Figure 10 for a diagram of the papillary dermis and the areas which were counted in the analysis.

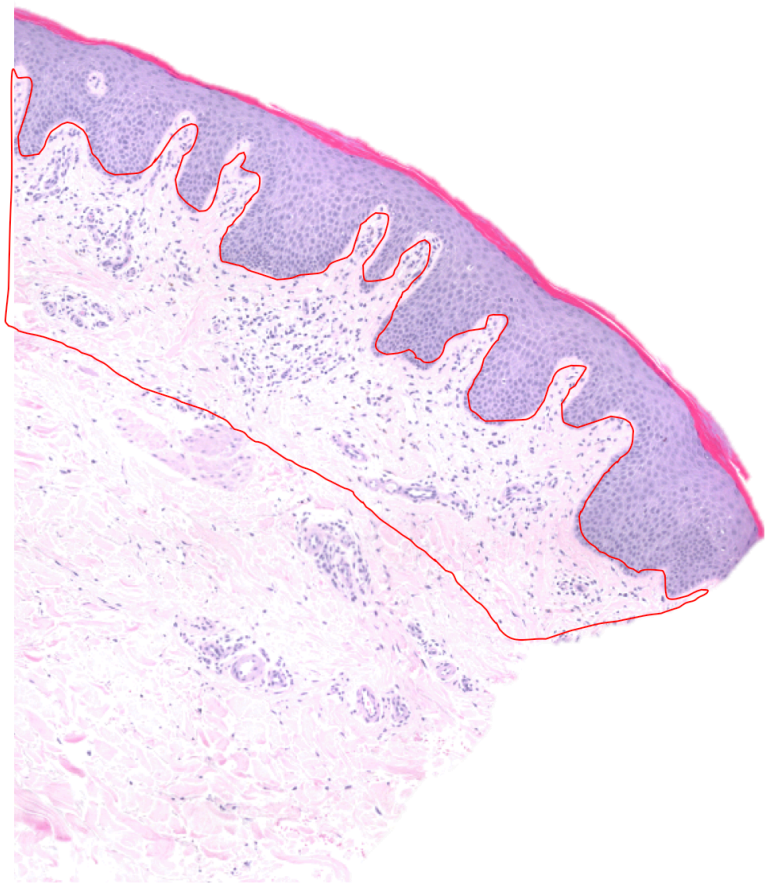

Figure 10. Representative image of an H&E stained skin section from an atopic dermatitis patient. The papillary dermis is outlined in red.

2.8 Statistics

2.8.1 Determination of Sample Size

The determination of sample size was based on Gaga et al 1991, who looked at the increase in eosinophils in allergen challenged compared to saline challenged skin in atopic subjects[144]. Using these values, it was determined that for a 95% increase in eosinophils, at a power level of 80% and an alpha level of 0.05, a sample size of 10 subjects was needed. Therefore, with 10 subjects we are adequately powered to detect differences in cellular infiltrate in allergen challenged biopsies.

2.8.2 Statistical Methods

Continuous data were summarized using means, standard deviation, minimums, mediums and maximums. Categorical data were summarized using counts and percentages. Missing data was not included in the summaries.

2.8.2.1 Demographics and Baseline Data

Demographic and baseline data were summarized descriptively. Continuous variables (age, height, weight) were summarized with mean, median, SD, minimum and maximum. Categorical variables (sex) were summarized with frequency and percentage.

2.8.2.2 Clinical Effect Data

Data comparing wheal size from the early cutaneous response in allergen compared to saline conditions were compared using a non-parametric Wilcoxon paired t-test. A test of normality (D'Agostino & Pearson) was performed.

Similarly, data comparing wheal size from the late cutaneous response were compared between allergen and saline conditions using a non-parametric Wilcoxon paired t-test. A test of normality (D'Agostino & Pearson) was performed.

2.8.2.2 Laboratory Data

Cellular levels (eosinophils, basophils and eosinophil/basophil progenitors) were compared between allergen and saline conditions using a non-parametric Wilcoxon paired t-test. A test of normality (D'Agostino & Pearson) was performed prior to analysis.

Cellular levels (eosinophils, basophils and eosinophil/basophil progenitors) were compared between lesional and non-lesional conditions using a non-parametric Wilcoxon paired t-test. A test of normality (D'Agostino & Pearson) was performed prior to analysis.

Cellular levels (eosinophils, basophils and eosinophil/basophil progenitors) were correlated to EASI and SCORAD scores using a Spearman rank correlation test. A test of normality (D'Agostino & Pearson) was performed prior to analysis. An adjustment for multiple correlations was performed using the Holm-Bonferroni method.

2.9 Data Management

Clinical data were kept locked in the McMaster Cardio-respiratory lab, and did not leave the building. Electronic clinical data did not contain patient identifiers and were kept on a secure computer. Records will be kept for 10 years as per the GCP guidelines.

2.10 Ethical Considerations

This study was conducted in accordance with the ethical principles of the Declaration of Helsinki, and consistent with International Conference on Harmonization (ICH)/Good Clinical Practice (GCP) and applicable regulatory requirements. Approval was obtained from the local ethics committee (the Hamilton Integrated Research Ethics Board), and all patients provided written and oral informed consent.

2.10.1 Informed Consent

The ICF used was reviewed and approved by the Hamilton Integrated Research Ethics Board. Patients were provided with a copy of the consent form. The purpose, methods, objectives, and potentials risks of the study were explained clearly and any questions were answered before obtaining consent. The ICF was then signed by the study participant, the investigator reviewing the ICF with the study participant as well as the physician. The original informed consent was retained in the study records for the patient.

2.10.2 Patient Confidentiality

All appropriate measures were taken to ensure anonymity of the patient. Patients were identified only by their patient identification number. Patients were informed of their right to have their data removed from the study records if they so wished.

CHAPTER 3: RESULTS

The demographic data from the study is illustrated in **Table 2**. The gender division was 40.0% female and 60.0% male. Patients had an average age of 39.0 [18.0 – 64.0] years, a median POEM of 22 [6.0 – 28.0], a median DLQI score of 14 [4.0 – 30.0], a median EASI score of 18 [2.7 – 48.0] and a median SCORAD of 62,5 [18.0 – 80.5]. These scores place all patient in the moderate to severe category for atopic dermatitis severity. For all patients, the allergen used for the intradermal allergen challenge was part of the house dust mite and mold family.

Table 2. Demographic Data from 15 patients..

	Sex	Age	POEM	DLQI	EASI	SCORAD	Allergen for IAC
OSAD-001	F	18	10	10	7.7	47.7	HDMDF
OSAD-002	M	51	28	27	48	80.5	Alternaria
OSAD-003	M	64	28	17	39.9	57.5	HDMDP
OSAD-005	F	28	25	4	32.6	64.7	HDMDF
OSAD-006	M	51	26	14	18	49.6	HDMDP
OSAD-007	F	28	26	18	12.2	37.6	HDMDP
OSAD-008	F	59	17	9	10.4	29	HDMDF
OSAD-009	M	54	25	30	43.5	80.2	HDMDF
OSAD-010	F	40	13	8	2.7	18	HDMDF
OSAD-011	M	22	6	4	40.4	61.47	HDMDF
OSAD-012	F	22	22	16	30.9	56.5	HDMDF
OSAD-013	M	39	28	21	21	57.9	HDMDP
OSAD-014	M	27	15	16	3.2	21.1	HDMDP
OSAD-015	M	51	15	9	7.5	30.76	HDMDP
OSAD-016	M	24	10	6	7.8	27.57	HDMDP
n (frequency%)	F: 6 (40.0) M: 9 (60.0)						
Mean (SD)		28.53 (14.85)	19.60 (7.39)	13.93 (7.63)	21.72 (15.44)	48.01 (19.36)	
Median		39.0	22	14	18	49.6	
Range (min, max)		46.0 (18.0, 64.0)	22 (6.0, 28.0)	26 (4.0, 30.0)	45.3 (2.7, 48.0)	62.5 (18.0, 80.5)	

In the lesional skin of atopic dermatitis patients, the presence of mature eosinophils and mature basophils was largely patient dependent (see **Figure 11**). There was no significant increase in eosinophils in lesional vs non-lesional skin (0 [0 – 0] to 0 [0 – 0] cells per mm^2, $p>0.05$). There was also no significant difference between basophils in non-lesional skin compared to lesional skin (0 [0 – 1.944] to 0 [0 – 0.2252] cells per mm^2, $p>0.05$). However, the lesional skin showed a marked and significant increase in eosinophil/basophil progenitors in lesional compared to non lesional skin (6.48 [2.86 – 10.79] to 0 [0 – 0.73] cells per mm^2, $p<0.001$).

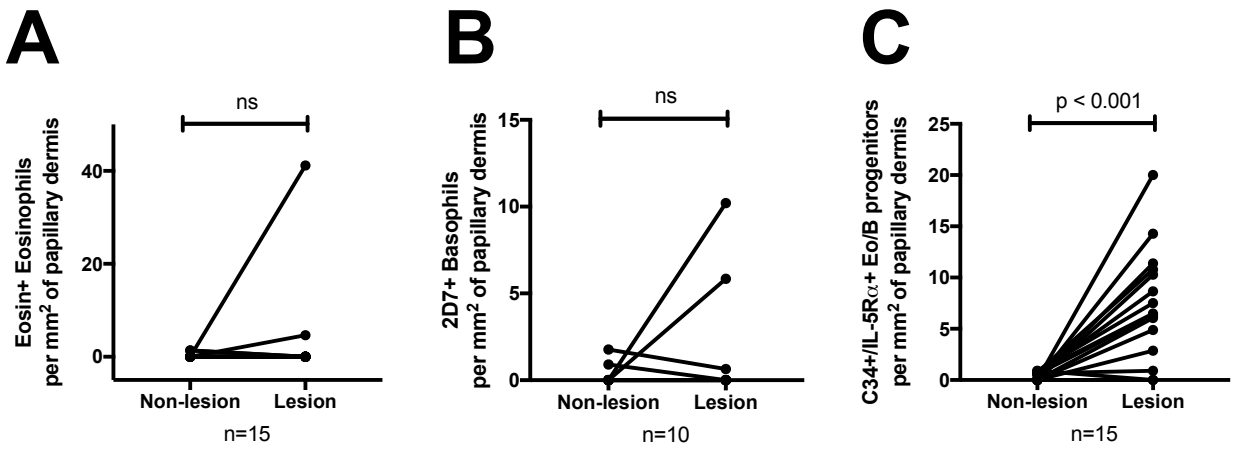

Figure 11: Comparison of cell levels between lesional and non-lesional skin. Eosinophils (A, n=15) were measured through H&E staining as eosin positive and bi-lobed nucleus positive, per mm^2 in the papillary dermis. Basophils (B, n=10) were measured through IF staining as 2D7 positive, per mm^2 in the papillary dermis. Eosinophil/basophil progenitors (C, n=15) were measured through IF staining as CD34/IL5Rα double positive and Von Willebrand factor negative, per mm^2 in the papillary dermis.

The wheal sizes of the early cutaneous (measured at 10 minutes post allergen challenge) and late cutaneous (measured at 24 hours post allergen challenge) responses are shown in **Figure 12.** The early cutaneous response caused by allergen was significantly larger than the wheal size caused by saline challenge (2.76 [0.64 – 5.4] to 0 [0 – 0] cm^2, $p<0.0001$). Likewise, at 24 hours post-challenge, all subjects developed a late cutaneous response. The late cutaneous response caused by allergen was significantly larger than the wheal size caused by saline challenge (1.41 [0.9 – 2.58] to 0 [0 – 0] cm^2, $p<0.0001$).

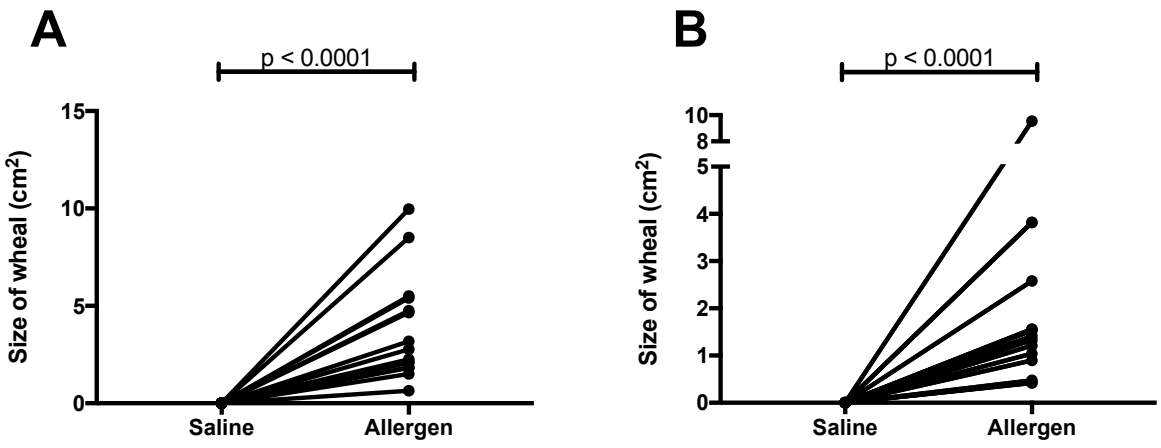

Figure 12: Wheal size measured at 10 minutes post allergen and diluent challenge (A, n=15) and 24 hours post allergen and diluent challenge (B, n=15).

In unaffected skin challenged with allergen, there was a significant increase in mature eosinophils at 24 hours post allergen challenge compared to saline control (20.31 [10.06 – 33.77] to (0 [0 – 0] cells per mm^2, $p<0.001$). There was a trend toward a significant increase in basophils at 24 hours post allergen challenge compared to saline control (3.952 [0 – 12.3] to 0.5155 [0 – 2.449] cells per mm^2, $p=0.0781$). Eosinophil/basophil progenitors in the allergen challenged skin compared to saline control were also significantly increased at 24 hours post challenge (6.67 [2.6 – 13.27] to 0 [0 – 1.89] cells per mm^2, $p<0.05$) (**Figure 13)**

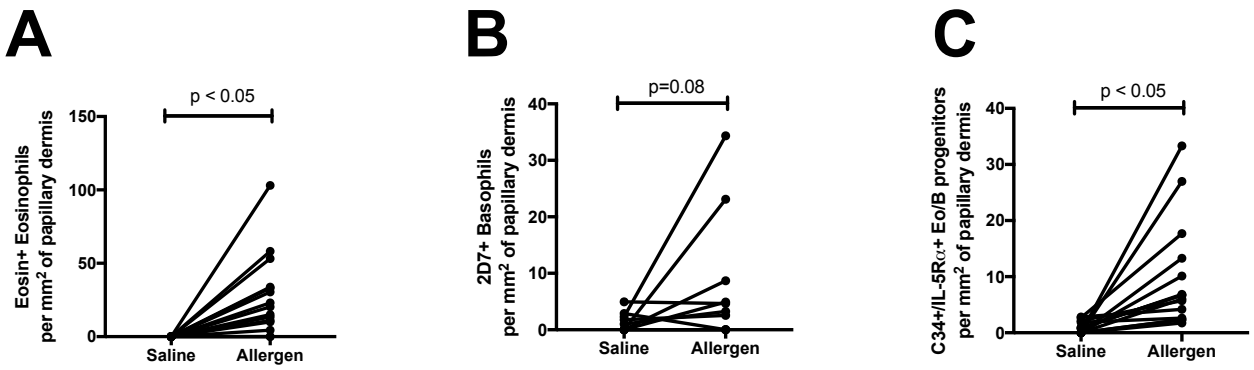

Figure 13: Comparison of cell levels between allergen and saline challenged skin 24 hours' post challenge. Eosinophils (n=15) were measured through H&E staining as eosin positive and bi-lobed nucleus positive, per mm^2 in the papillary dermis. Basophils (n=10) were measured through IF staining as 2D7 positive, per mm^2 in the papillary dermis. Eosinophil/basophil progenitors (n=15) were measured through IF staining as CD34/IL5Rα double positive and Von Willebrand factor negative, per mm^2 in the papillary dermis.

There was no relationship found between eosinophils and Eo/B progenitors in allergen challenged (r=0.2073, p>0.05) or lesional (r=0.2477, p>0.05) skin (**Figure 14)**. Likewise, there was no relationship found between basophils and Eo/B progenitors in allergen challenged (r=0.3374, p>0.05) or lesional (r=0.2461, p>0.05) skin (**Figure 14)**.

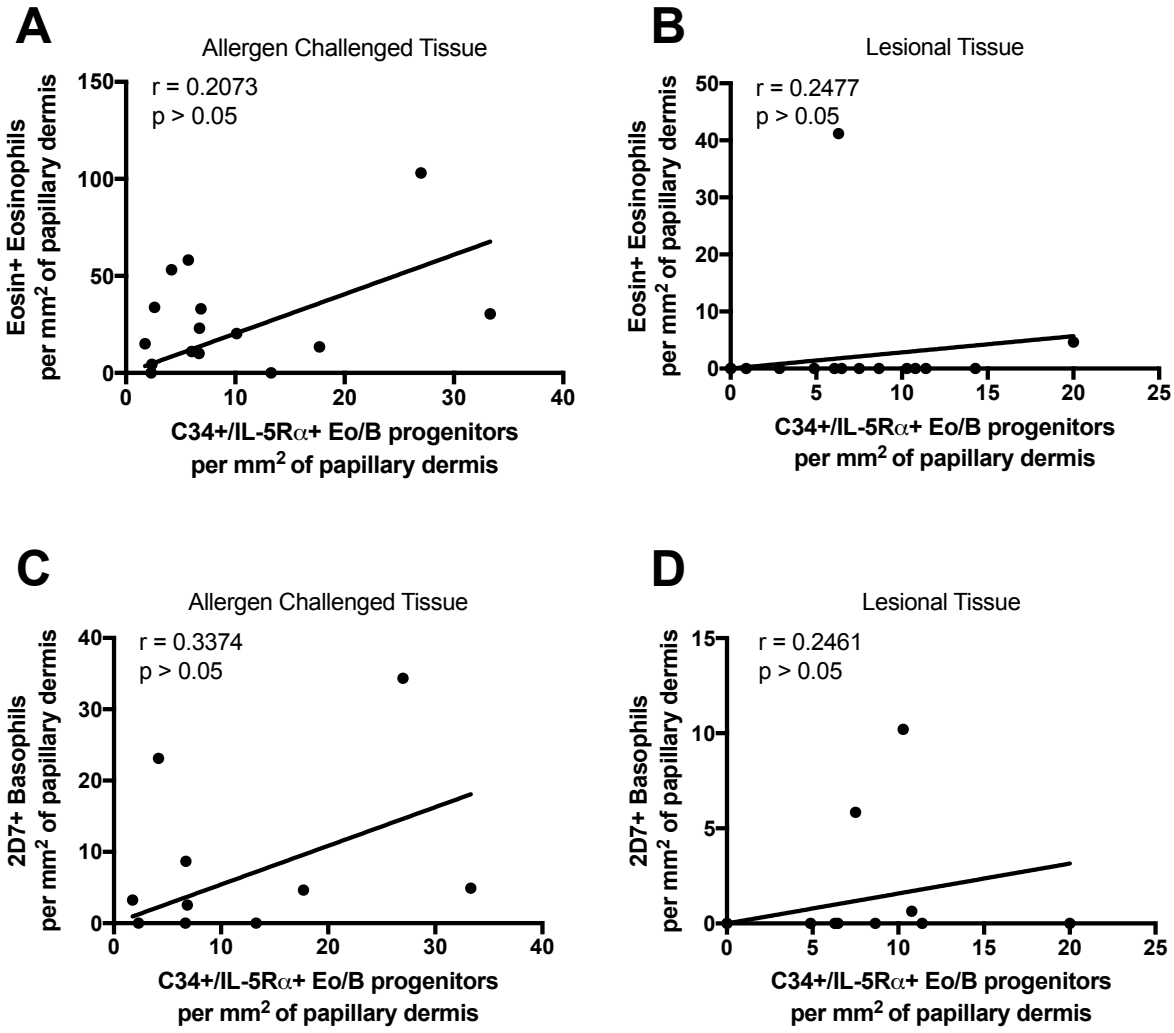

Figure 14: Spearman correlations between eosinophil (n=15) and basophil (n=10) levels per mm^2 and eosinophil/basophil progenitors in either allergen challenged or lesional skin.

We examined the relationship between eosinophils, basophils and Eo/B progenitors in allergen challenged tissue to both EASI and SCORAD (**Figure 15)**. There was a trend toward a positive correlation between Eo/B P's to both EASI (r=0.4821, p=0.0711) and SCORAD (r=0.4571, p=0.0888) scores. Eosinophils trended toward a positive correlation with SCORAD (r=0.5058, p=0.0565) but not EASI (r=0.3843, p>0.05). There were no correlations between basophils and either of the clinical outcome measurements. A Holm-Bonferroni correction was applied to account for multiple comparisons.

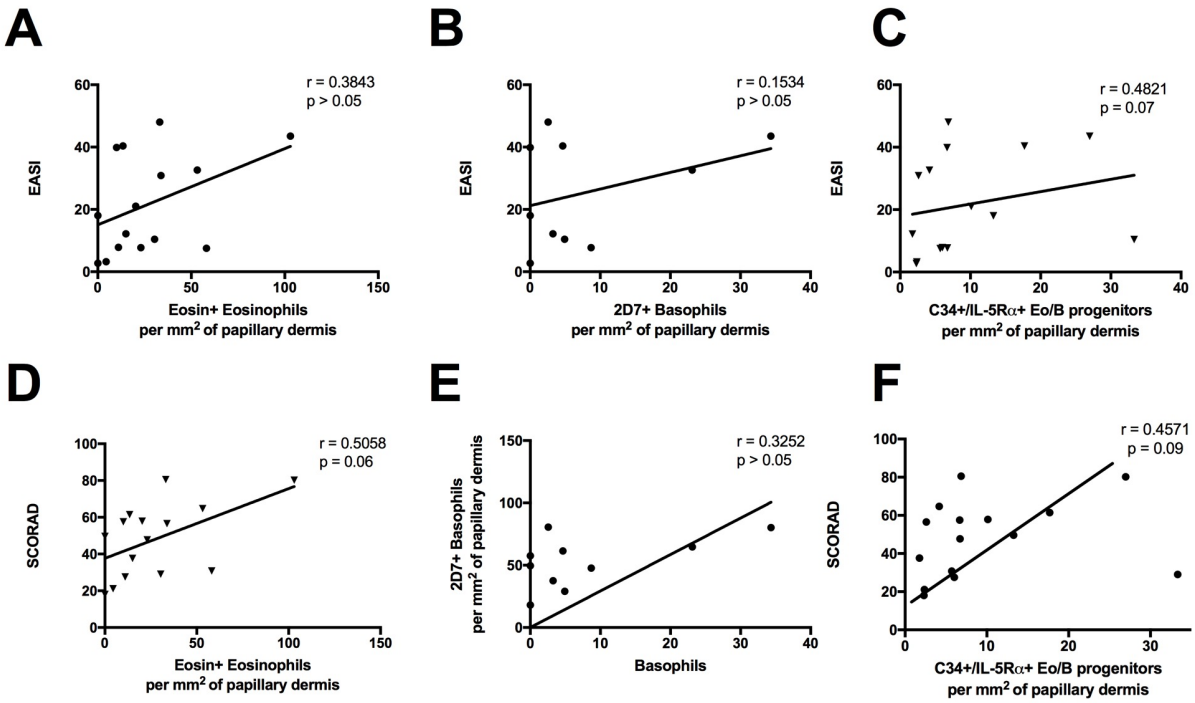

Figure 15: Spearman correlations between eosinophil (n=15), basophil (n=10) and eosinophil/basophil progenitor (n=15) cell levels per mm^2 in allergen challenged tissue and clinical scores (EASI and SCORAD).

We examined the relationship between eosinophils, basophils and Eo/B P's in lesional tissue to both EASI and SCORAD (**Figure 16)**. Neither basophils nor eosinophils correlated with either clinical scores. However, Eo/B P's were found to correlate significantly with both EASI (r=0.706, p=0.0043) and SCORAD (r=0.6452, p=0.0111). A Holm-Bonferroni correction was applied to account for multiple comparisons.

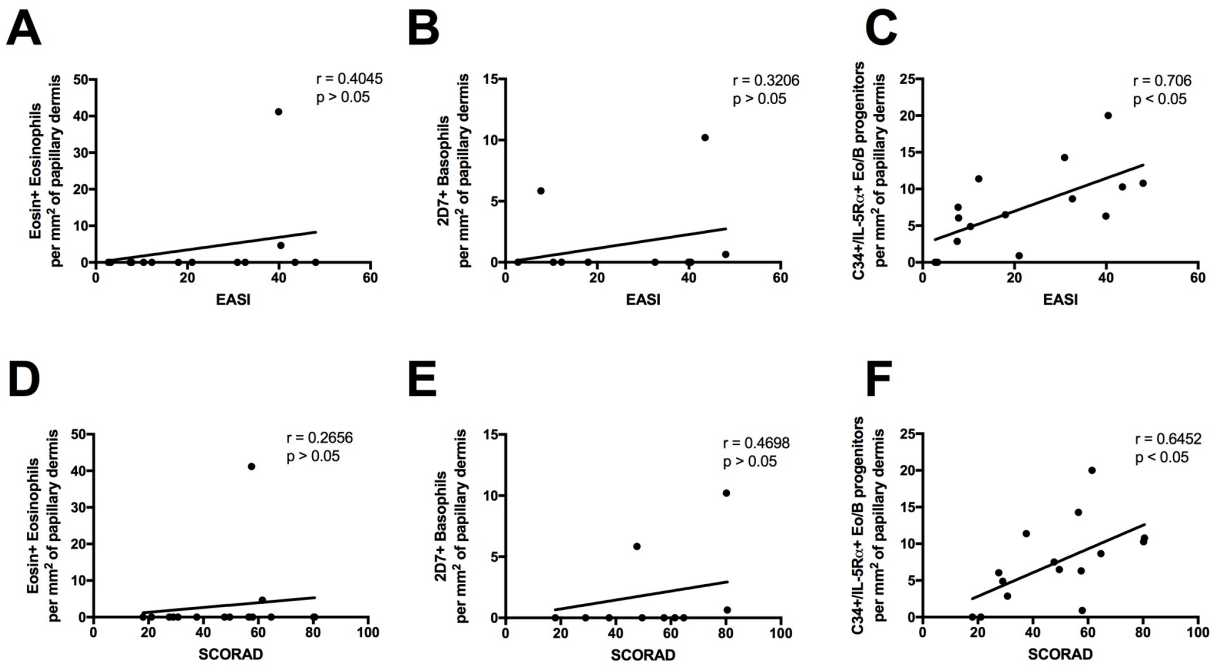

Figure 16: Spearman correlations between eosinophil (n=15), basophil (n=10) and eosinophil/basophil progenitor (n=15) cell levels per mm^2 in lesional tissue and clinical scores (EASI and SCORAD).

CHAPTER 4: DISCUSSION

4.1 Eosinophil/basophil progenitors: implicated in the pathogenesis of atopic dermatitis

For the first time, this study has demonstrated that eosinophil/basophil progenitors are elevated in the skin of patients with atopic dermatitis. This elevation in eosinophil/basophil progenitors is consistent with the pathogenesis of other allergic diseases, such as allergic asthma and allergic rhinitis. In the steady state, the bone marrow releases a small number of $CD34^+$ progenitor cells, which traffic throughout the bloodstream where they have the potential to migrate into tissues and differentiate into mature effector cells such as eosinophils and basophils[145]. However, in atopic patients, the number of progenitors released by the bone marrow is higher, and within the tissues these cells can act as pro-inflammatory effector cells, contributing to localized inflammation[67]. $CD34^+$ progenitors have been found in the mucosa of the upper and lower airways in asthmatics, the sputum of asthmatics, and have been shown to increase in the bone marrow and blood after allergen challenge[96,97,99,100]. Our data suggest that eosinophil/basophil progenitors could be playing a role in maintaining the chronicity and inflammation in atopic dermatitis lesions, similarly to the pathogenesis seen in allergic asthma[67].

Although we observed elevated levels of eosinophil/basophil progenitors, we did not see an associated increase in eosinophils in lesional tissue. This is consistent with literature

that has shown that dermal eosinophils undergo cytolytic degranulation when activated, and thus infrequently identified in histological analysis due to lack of granules[146]. A previous study by Kiehl et al has shown that eosinophil granule proteins are present in almost 100% of chronic lesions, and suggests that the absence of formed eosinophils does not indicate an absence of tissue eosinophilia[43].

There is limited data available in the literature on basophils in chronic lesions[77]. Our study used the 2D7 antibody as a marker for basophils, which is an antigen found in the secretory granules of basophils. Interestingly, when basophils degranulate the amount of 2D7 is decreased and thus activated or degranulated basophils may not be captured by a 2D7 stain[147]. Therefore, similar to eosinophils, we are proposing that basophils are present but degranulated, in the chronic lesions of atopic dermatitis patients. This would explain why high levels of eosinophil/basophil progenitors are found in the lesional tissue, but there is not an associated increase in basophils or eosinophils.

After finding the presence of eosinophil/basophil progenitors in the lesions of atopic dermatitis patients, we looked to see if an intradermal allergen challenge of non-lesional skin increases the numbers of eosinophil/basophil progenitors, along with an associated increase in eosinophils and basophils. Previous work has shown that after inhaled allergen challenge, $CD34^+$ progenitors increase in the sputum of asthmatics, so we were therefore expecting to see an increase in $CD34^+$/IL-5Rα^+ Eo/B P's after allergen challenge[97]. It has also been reported that 24 hours post intradermal allergen challenge there is a significant

increase in both eosinophils and basophils in the late cutaneous response of atopic subjects[42,130]. Our patient population with moderate to severe AD, and not using systemic anti-inflammatory drugs or anti-histamines all developed a late cutaneous response, and both the early and the late cutaneous responses were significantly larger than saline control (which for all patients was consistently 0 cm^2). Eosinophil/basophil progenitors were significantly increased in allergen challenged tissue compared to saline control, as expected based on previously reported data from allergic asthmatics. Likewise, both eosinophils and basophils were significantly elevated post allergen challenge.

There has been no previously reported literature that looks at correlations between eosinophil/basophil progenitors and eosinophils or basophils after allergen challenge in other allergic diseases. We had hypothesized that there would be a correlation between the number of eosinophil/basophil progenitors and eosinophils or basophils due to their lineage relationship, however we did not find a correlation between the levels of eosinophil/basophil progenitors and eosinophils, or a correlation between eosinophil/basophil progenitors and basophils. Eosinophil/basophil progenitors have been shown to have both proliferative and differentiative capabilities, and thus individual differences in proliferative and differentiative capabilities could potentially explain why a correlation does not exist between the number of eosinophil/basophil progenitors and eosinophils and basophils after allergen challenge[30]. The kinetics for eosinophil and basophil infiltration into the late cutaneous response have been previously studied. At 24 hours, the size of the late cutaneous response is the largest, and $CD4^+$ T cells, Th2

cytokines, eosinophils and basophils are all significantly elevated[42]. In contrast, the kinetics of eosinophil/basophil progenitor trafficking from the bone marrow into the lungs of mice after inhaled allergen challenge has demonstrated that eosinophil/basophil progenitors significantly increase at 6 hours post inhaled allergen challenge before declining to pre-allergen challenge levels by 12 hours[148]. Therefore, it is possible that an increase in Eo/B progenitors precedes the increase in eosinophils and basophils, suggesting that a positive or negative correlation would not occur at a single temporal moment. Alternately, the fact that H&E staining does not capture activated/degranulated eosinophils, and 2D7 staining does not capture degranulated basophils could indicate that in the patients who had higher levels of eosinophil/basophil progenitors, there could be an increase in degranulated eosinophils and basophils. Further investigation is warranted to understand why a correlation does not exist between the precursor and terminally differentiated cells.

Previous work has looked at the relationship between eosinophil/basophil progenitors and atopic disease severity in patients with asthma. Robinson et al compared bronchial biopsies from patients with allergic asthma to healthy controls. Firstly, they demonstrated that $CD3^+$/IL-$5R\alpha^+$ $mRNA^+$ cell numbers were increased in bronchial biopsies from asthmatic subjects compared to healthy controls. Secondly, they showed that among the asthmatic patients there was a significant negative correlation between $CD3^+$/IL-$5R\alpha^+$ $mRNA^+$ cell numbers and percent predicted FEV_1 using a Spearman correlation ($r= -0.71$, $p<0.02$)[149]. Another study by Makowska et al looked at a group of mild allergic

asthmatics compared to severe asthmatics, finding increased levels of $CD34^+/IL\text{-}5R\alpha^+$ cells in the blood of severe asthmatics. Makowska et al found that blood $CD34^+/IL\text{-}5R\alpha^+$ cell numbers negatively correlated with percent predicted FEV_1. In addition, they looked at the dose of inhaled steroid used to control asthma and found a significant positive correlation with $CD34^+/IL\text{-}5R\alpha^+$ cell numbers[150]. Our work shows that levels of eosinophil/basophil progenitors are positively correlated with both EASI and SCORAD clinical scores in patients with atopic dermatitis, with greater numbers of eosinophil/basophil progenitors found in patients with higher severity of disease. EASI scores algorithmically measure the area of the body covered by eczema, as well as the severity of the eczema based on redness, papulation, excoriation and lichenification. A formula is then used to calculate the total EASI score, and a higher score correlates to more severe disease. Likewise, SCORAD measures body surface area covered by eczema as well as intensity based on redness, swelling, oozing/crust, excoriations, lichenification and xerosis. SCORAD also considers patient reported subjective symptoms on sleeplessness and itchiness. The body surface score, intensity score and patient reported scores are added together in a formula to give the total SCORAD score. Both clinical scores reflect the severity of disease. In the lesions of moderate to severe AD patients, there is a significant correlation between both EASI and SCORAD and eosinophil/basophil progenitor levels ($r=0.706$, $p<0.05$ and $r=0.645$, $p<0.05$). This suggests that eosinophil/basophil progenitors could be contributing to sustaining the chronic inflammation in atopic dermatitis. A possible biological mechanism could be that the presence of eosinophil/basophil progenitors in the tissue either contribute to

worsening of disease by differentiating into mature effectors (eosinophils/basophils). Eosinophils and basophils then drive allergic inflammation and remodelling by releasing proteins such as MBP and histamine respectively[43,72].

In asthma, it has been shown that eosinophil/basophil progenitors themselves can act as potent effectors of allergic inflammation through the release of Th2 cytokines such as IL-5, IL-13, and GM-CSF[65,67,151]. Interestingly, the release of Th2 cytokines by eosinophil/basophil progenitors in allergic asthma is triggered by TSLP. In atopic dermatitis, TSLP is thought to be a possible candidate protein involved in the initiation, development and progression of disease, and is produced by epithelial cells in the skin[152]. Therefore, it is possible that TSLP is also activating eosinophil basophil progenitors in the skin of atopic dermatitis patients, and contributing to sustained chronicity of disease. Of note, the r values (being r=0.706, and r=0.645 for EASI and SCORAD respectively) show that not all the clinical severity can be explained by the number of eosinophil progenitors in lesions. Atopic dermatitis is a complex disease, the immune system, the epidermal barrier, genetic factors and environmental factors all thought to contribute to disease pathogenesis[10,20]. Therefore, it is likely that other factors are influencing overall disease severity in addition to levels of eosinophil/basophil progenitors.

In allergen challenged skin we observed a trend towards a positive relationship between eosinophils/basophil progenitors versus EASI and SCORAD. This is surprising as EASI and SCORAD reflect whole body scores, whereas the allergen challenged skin represents

a small fraction of total skin area. In addition, the allergen challenge is performed on an area of non-lesional/unaffected skin, which we have shown to have very low levels of eosinophil/basophil progenitors. When challenged with allergen, there is an average of a 5.5-fold increase in progenitor levels, and the magnitude of the increase for each patient correlates with their disease severity (as denoted by EASI and SCORAD). This evidence implies that the non-lesional skin of patients with more severe disease is primed for supporting allergen-induced increases in eosinophil/basophil progenitors. Furthermore, this primed tissue could be an integral component of developing acute lesions, and not just important to sustaining inflammation in chronic lesions.

4.2 Clinical Implications

Clinically, the finding that eosinophil/basophil progenitors are elevated in lesions, are elevated after allergen challenge and correlate with disease severity suggest that eosinophil/basophil progenitors could be a therapeutic target for the treatment of atopic dermatitis. One of the most significant conclusions from this data is the strong positive correlations between eosinophil/basophil progenitors and disease severity.

In allergic asthma, there has been clinical success using drugs that target the IL-5 and IL-5Rα axis. Mepolizumab is an anti-IL-5 antibody that has been testing on atopic dermatitis patients but achieved no statistically significant clinical improvement[120]. However, the dosing period used in this clinical trial was likely to short to cause a clinically significant reduction in tissue eosinophils. Reslizumab is another anti-IL-5 monoclonal antibody. It

has not been tested in atopic dermatitis, however it has shown clinical efficacy in treating uncontrolled eosinophilic asthma, reporting a significant reduction in the frequency of asthma exacerbations compared to placebo control[153]. Further investigation of reslizumab for the treatment of AD and effects on progenitor levels would be interesting. Dupilumab targets the IL-4 and IL-13 axis, and has shown clinical benefit in trials for both allergic asthma and atopic dermatitis[154]. Interestingly, eosinophil/basophil progenitors also express the receptor for IL-4 and IL-13, IL-4Rα. While a reduction in eosinophil//basophil progenitors after dupilumab treatment has not been explicitly measured, it is possible that dupilumab could be reducing progenitor numbers. Benralizumab, a humanized antibody against IL-5Rα, which reduces the amount of eosinophil/basophil progenitors, eosinophils and basophils has shown clinical efficacy in treating asthma[123]. It would be pertinent to investigate the effect of benralizumab to see if it translates to a clinical benefit for atopic dermatitis patients as well.

4.3 Limitations

This study has a few limitations. Although eosinophil/basophil progenitors have been shown to increase in lesions, increase after allergen challenge, and are positively correlated with disease severity, a mechanistic study would be required to definitively say that they are contributing to disease severity. A study could be done which looks at current treatments of atopic dermatitis such as cyclosporine or steroid, and could determine if a reduction in eosinophil/basophil progenitors occurs after a treatment period, and the reduction correlates with the reduction in disease severity. Additional

studies could look at *in situ* hematopoiesis in atopic dermatitis lesions or a time course of progenitor activity after allergen challenge. Additional studies are discussed in detail in section 4.4 Future Directions.

In addition, as this is a preliminary study, data does not exist to determine an appropriate sample size for any eosinophil/basophil progenitor calculations. The sample size calculations were based on eosinophil data in late cutaneous responses in atopic dermatitis patients, which we believe to be an appropriate surrogate[42,141,144]. It is possible that stronger relationships would be observed with a larger sample size. Another limitation in this study is that different methodologies and skin sections were used to stain for the three cellular outcomes. There exists an inherent variability in skin sections, which could explain some of the variability between patients. For all patients, we stained 2 skin sections with H&E. Eosinophil/basophil progenitors and basophils were stained using immunofluorescence while eosinophils were stained using H&E. This makes direct correlations difficult as the cells are on different slides and the methodologies are different, and there exists variability around each of the measurements. Immunofluorescence itself has limitations, including auto-fluorescence and non-specific fluorescence. To control for these factors, both a negative and an isotype control were used for the staining.

4.4 Future Directions

Further investigation is required to better understand the relationship between eosinophil/basophil progenitors and their implications in atopic dermatitis disease severity. While we can extrapolate from other studies done in asthma and allergic rhinitis, without further work no concrete conclusion can be made. We have proposed the following for possible future work:

1) To confirm other findings in the literature, as well as to address the lack of correlation between eosinophil/basophil progenitors and eosinophils or basophils in lesional skin, we are proposing to stain tissue sections for eosinophil granule proteins such as EG2, MBP and EPO. In addition, we are proposing to stain for a basophil granule marker such as pro major basic protein 1 (proMBP1). Evidence of degranulated/activated eosinophils/basophils could help to explain the high number of progenitors seen in atopic dermatitis lesions.

2) In allergen challenged tissue, it would be interesting to again stain for eosinophil and basophil granule protein markers, to see if the addition of counting activated cells increases a correlation with eosinophil/basophil progenitors. This would help to either prove or disprove the hypothesis that eosinophil and basophil numbers directly correlate with eosinophil/basophil progenitors.

3) Mechanistically, there are several experiments that could be done to determine how eosinophil/basophil progenitors are contributing to disease pathogenesis:

a) To determine if eosinophil/basophil progenitors are differentiating *in situ* in atopic dermatitis lesions: a lesional biopsy could be taken and digested with collagenase before being stimulated with IL-5 and control, and then stained for mature eosinophils, basophils and eosinophil/basophil progenitors using flow cytometry.

b) To determine if atopic dermatitis patients have higher tissue eosinophil/basophil progenitors than non-atopic subjects: skin punch biopsies from normal subjects compared to skin punch biopsies from atopic dermatitis lesions could be stained using IF for eosinophil/basophil progenitors and levels compared between the two.

c) To determine if a reduction in eosinophil/basophil progenitors correlates with improvement in SCORAD and EASI clinical scores: treatment of patients with either prednisone or a targeted therapy such as Benralizumab could help us determine if a reduction in eosinophil/basophil progenitors correlates with disease severity improvement.

d) To determine the kinetics of eosinophil/basophil progenitor migration and/or in situ differentiation: an intradermal challenge could be repeated on 6 areas of an

atopic subjects back. A punch biopsy could then be taken at 0, 4, 6, 12, 24 and 48 hour's post challenge. This might help to elucidate the temporality of recruitment of eosinophil/basophil progenitors as well as eosinophils, basophils, and Th2 cells.

4.5 Summary

In conclusion, the observed increases in eosinophil/basophil progenitors post allergen challenge and in the lesional tissue is a novel finding which could potentially lead to greater understanding of the sustained chronicity in moderate to severe atopic dermatitis patients. The correlations with disease severity add further evidence for a role for eosinophil/basophil progenitors in atopic dermatitis. However, the precise role of eosinophil/basophil progenitors within the complex pathogenesis of atopic dermatitis remains to be elucidated.

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APPENDIX

ECZEMA AREA AND SEVERITY INDEX (EASI) RATING ASSESSMENT

Area of Involvement (0-6):

Head/Neck:	Trunk	Upper Extremities	Lower Extremities
/9	/36	/18	/36

Use the following scoring criteria for area of involvement

0 No eruption
1 < 10% of the respective body region
2 10-29% of the respective body region
3 30-49% of the respective body region
4 50-69% of the respective body region
5 70-89% of the respective body region
6 90-100% of the respective body region

Severity of AD Signs (0-3, including half-steps):

	Head/Neck	Trunk	Upper Extremities	Lower Extremities
Erythema				
Infiltration/Papulation				
Excoriations				
Lichenification				

Erythma	**(E)**
0 – None	
1 – Mild	Faintly detectable erythema: very light pink
2 – Moderate	Full red, clearly distinguishable
3 – Severe	Deep/dark red
Infiltration/Papulation	**(I)**
0 – None	
1 – Mild	Barely perceptible elevation
2 – Moderate	Clearly perceptible elevations but not extensive
3 – Severe	Marked and extensive elevation
Excoriations	**(Ex)**
0 – None	
1 – Mild	Scant evidence of exorciations with no signs of deeper skin damage (erosion, crust)
2 – Moderate	Several linear marks of skin with some showing evidence of deeper skin injury (erosion, crust)
3 – Severe	Many erosive or crusty lesions
Lichenification	**(L)**
0 – None	
1 – Mild	Slight thickening of the skin discernible only by touch and with skin markings minimally exaggerated
2 – Moderate	Definite thickening of skin with skin markings exaggerated so that they form a visible criss-cross pattern
3 – Severe	Thickened indurated skin with skin markings only visibly portraying an exaggerated criss-cross pattern

Scoring

Please perform mathematical equations vertically (i.e. in each column)

	Head/Neck	**Trunk**	**Upper Extremities**	**Lower Extremities**	
Erythema	(_____	(_____	(_____	(_____	
Infiltration/Papulation	+_____	+_____	+_____	+_____	
Excoriations	+_____	+_____	+_____	+_____	
Lichenification	+_____)	+_____)	+_____)	+_____)	
Area of involvement	**X**_____	**X**_____	**X**_____	**X**_____	
Multiplier	**X 0.1**	**X 0.3**	**X 0.2**	**X 0.4**	
Score	(_____+	_____+	_____+	_____)	Total Score =

_____ _____

Signature of Individual Completing Date

Figure 5. The EASI assessment tool used to give a numerical total score for atopic dermatitis, based on both the area of involvement and 4 key symptoms: erythema, infiltration/papulation, excoriations, and lichenification.

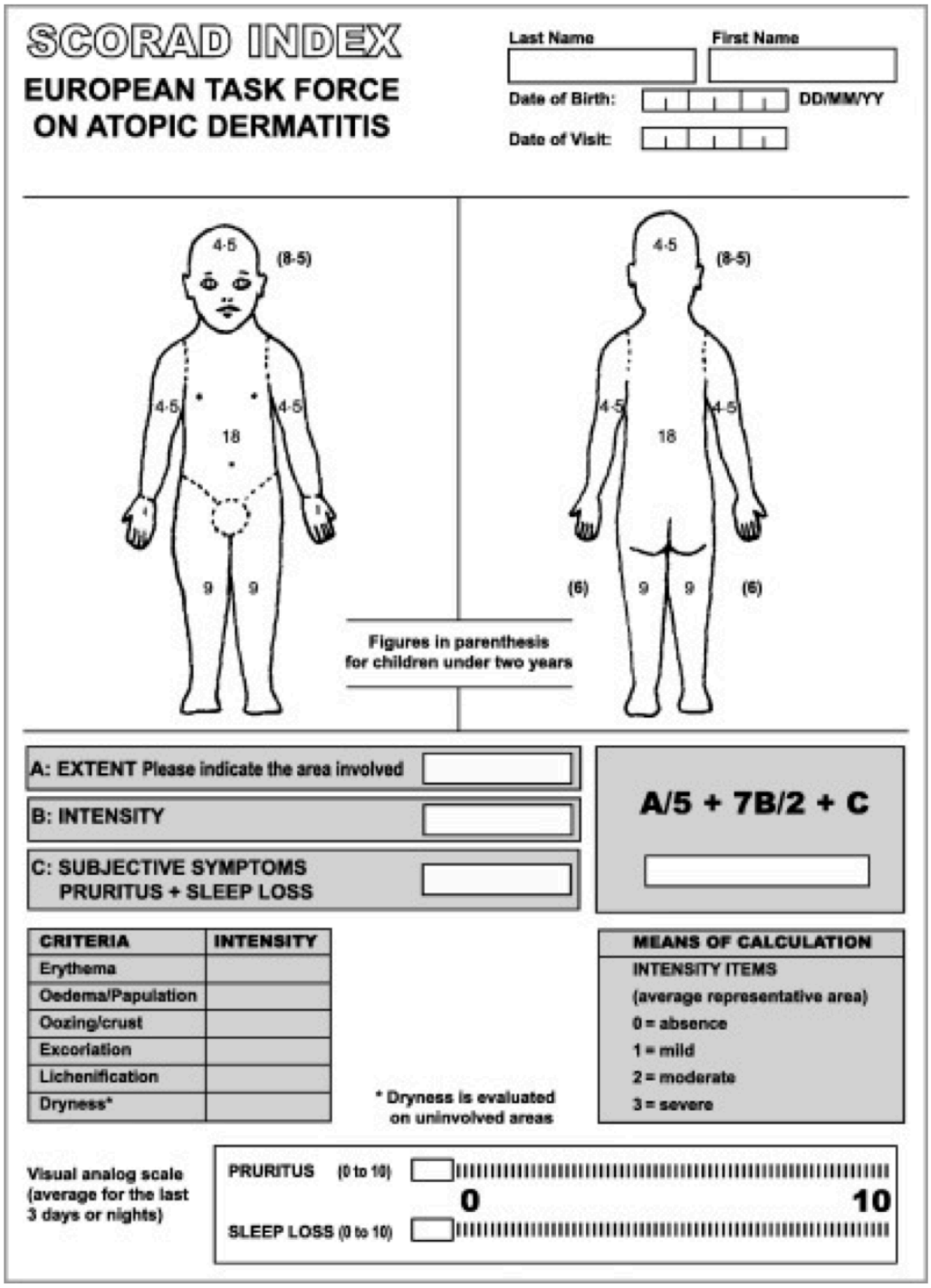


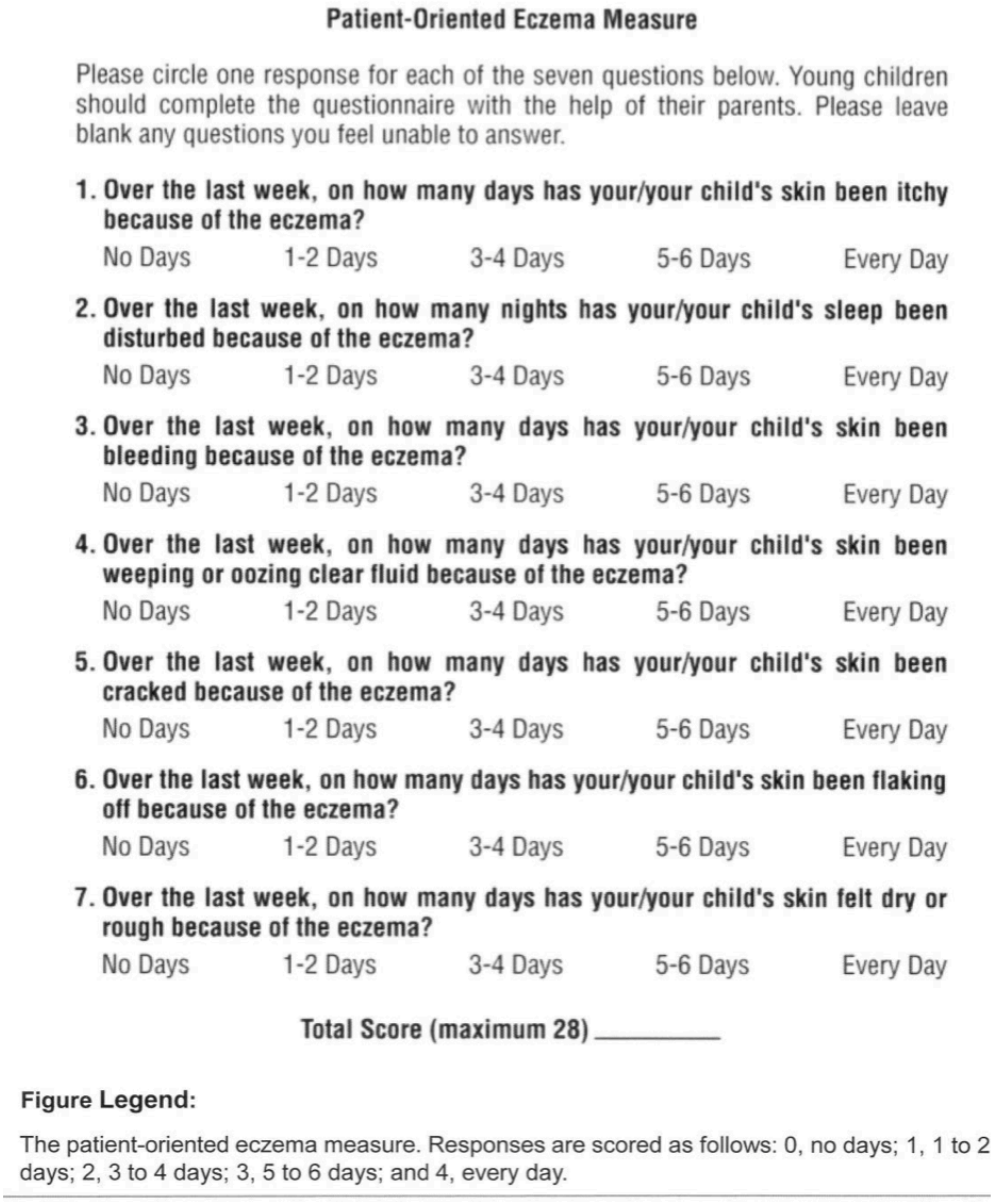
Figure 6. The SCORAD assessment tool used to give a numerical total score for atopic dermatitis, based on both the area of involvement, symptoms (including erythema, infiltration/papulation, oozing/crust, excoriations, lichenification and dryness) as well as patient symptom scores (pruritus and sleep loss).

DERMATOLOGY LIFE QUALITY INDEX

The aim of this questionnaire is to measure how much your skin problem has affected your life over the last week.

1.	Over the last week, how itchy, sore, painful or stinging has your skin been?	Very much A lot A little Not at all	□ □ □ □	
2.	Over the last week, how embarrassed or self-conscious have you been because of your skin?	Very much A lot A little Not at all	□ □ □ □	
3.	Over the last week, how much has your skin interfered with you going shopping or looking after your home or yard?	Very much A lot A little Not at all	□ □ □ □	Not relevant □
4.	Over the last week, how much has your skin influenced the clothes you wear?	Very much A lot A little Not at all	□ □ □ □	Not relevant □
5.	Over the last week, how much has your skin affected any social or leisure activities?	Very much A lot A little Not at all	□ □ □ □	Not relevant □
6.	Over the last week, how much has your skin made it difficult for you to do any sport?	Very much A lot A little Not at all	□ □ □ □	Not relevant □
7.	Over the last week, has your skin prevented you from working or studying?	Yes No	□ □	Not relevant □
	If "No", over the last week how much has your skin been a problem at work or studying?	A lot A little Not at all	□ □ □	
8.	Over the last week, how much has your skin created problems with your partner or any of your close friends or relatives?	Very much A lot A little Not at all	□ □ □ □	Not relevant □
9.	Over the last week, how much has your skin caused any sexual difficulties?	Very much A lot A little Not at all	□ □ □ □	Not relevant □
10.	Over the last week, how much of a problem has the treatment for your skin been, for example by making your home messy, or by taking up time?	Very much A lot A little Not at all	□ □ □ □	Not relevant □

Figure 7. The DLQI assessment tool used to give a numerical total score for the impact that atopic dermatitis has on patient quality of life. This is a patient reported outcome.



Patient-Oriented Eczema Measure

Please circle one response for each of the seven questions below. Young children should complete the questionnaire with the help of their parents. Please leave blank any questions you feel unable to answer.

1. **Over the last week, on how many days has your/your child's skin been itchy because of the eczema?**
   No Days 1-2 Days 3-4 Days 5-6 Days Every Day
2. **Over the last week, on how many nights has your/your child's sleep been disturbed because of the eczema?**
   No Days 1-2 Days 3-4 Days 5-6 Days Every Day
3. **Over the last week, on how many days has your/your child's skin been bleeding because of the eczema?**
   No Days 1-2 Days 3-4 Days 5-6 Days Every Day
4. **Over the last week, on how many days has your/your child's skin been weeping or oozing clear fluid because of the eczema?**
   No Days 1-2 Days 3-4 Days 5-6 Days Every Day
5. **Over the last week, on how many days has your/your child's skin been cracked because of the eczema?**
   No Days 1-2 Days 3-4 Days 5-6 Days Every Day
6. **Over the last week, on how many days has your/your child's skin been flaking off because of the eczema?**
   No Days 1-2 Days 3-4 Days 5-6 Days Every Day
7. **Over the last week, on how many days has your/your child's skin felt dry or rough because of the eczema?**
   No Days 1-2 Days 3-4 Days 5-6 Days Every Day

Total Score (maximum 28) _____

Figure Legend:

The patient-oriented eczema measure. Responses are scored as follows: 0, no days; 1, 1 to 2 days; 2, 3 to 4 days; 3, 5 to 6 days; and 4, every day.

Figure 8. The POEM assessment tool used to give a numerical total score for the impact that atopic dermatitis has on patient quality of life. This is a patient reported outcome.