

**GROUP 2 INNATE LYMPHOID CELLS ARE INCREASED IN
PATIENTS WITH MODERATE-TO-SEVERE ATOPIC
DERMATITIS**

By

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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

McMaster University

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ABSTRACT

Introduction: Atopic dermatitis (AD) is characterized by chronic pruritic relapsing eczematous lesions of the skin. Eosinophilic inflammation in AD is driven by activation of type 2 inflammatory cells including CD4⁺ T cells and type 2 innate lymphoid cells (ILC2s). We have shown that type 2 cytokines, namely interleukin (IL)-5 and IL-13, stimulate migration and terminal differentiation of eosinophil progenitor cells (EoPs). We propose that these cytokines are important drivers of tissue eosinophilia in AD lesional skin. This study aimed to quantify, by flow cytometry, cells that produce type 2 cytokines in lesional skin compared to peripheral blood from moderate-severe AD patients.

Methods: In a cross-sectional study of patients with moderate-to-severe AD (n=16), type 2 inflammatory cells were enumerated in blood and cells extracted from excised skin biopsies. By flow cytometry, live, singlet CD45⁺ cells were identified as ILC2 (lin-CD127⁺CD294⁺), EoP (CD34⁺125⁺), and CD4⁺ T cells (Lin⁺CD3⁺CD4⁺). Intracellular expression of type 2 cytokines (IL-5 and IL-13) were evaluated in each cell population. In addition, we developed a protocol to enumerate ILC2s by fluorescence immune-histochemistry in lesional versus non-lesional skin samples and skin biopsies taken 24h post-intradermal challenge with allergen versus diluent. Data are expressed as median (interquartile range [IQR]) unless otherwise stated. Cross compartmental comparisons were made using the Wilcoxon rank-sum test and where applicable, correlational analyses were performed using a Spearman's rank-correlational test.

Results: There was a significantly higher number of total ILC2s in lesional skin compared to blood from AD subjects (556 [99 – 5501] vs 235 [67 – 569] cells/mL, $p=0.03$). Similarly, IL-5⁺, IL-13⁺ ILC2s, were significantly greater in skin compared to blood (6 [1 – 666] vs 1 [1 – 19] cells/mL,

$p=0.03$; 28 [1 – 1357] vs 1 [1 – 7] cells/mL, $p=0.01$, respectively). We found higher numbers of total and type 2 cytokine positive EoP in lesional skin biopsies from AD patients compared to blood (Total EoP: 815 [285 – 2794] vs 112 [46 – 247] cells/mL, $p<0.01$; IL-5+EoP: 36 [1 – 129] vs 1 [1 – 23] cells/mL, $p=0.07$; IL-13+EoP: 92 [10 – 182] vs 1 [1 – 8] cells/mL, $p<0.01$ and IL-5+IL-13+ILC2: 70 [1 – 158] vs 1 [1 – 12] cells/mL, $p=0.02$, respectively). In contrast, significantly higher numbers of total and type 2 cytokine positive CD4+ cells were found in blood compared to lesional skin biopsies from AD patients (Total CD4+: 1092 [650 – 1742] vs 58.3 [35.3– 152.4] $\times 10^3$ cells/mL, $p<0.01$ and IL-5+IL-13+CD4+ cells: 13.5×10^3 [2.1×10^3 – 42.9×10^3] vs 3.8×10^3 [1.6×10^3 – 4.9×10^3] cells/mL, $p=0.02$, respectively). For IF staining, there was a significant higher number of ILC2s in lesional compared to non-lesional skin biopsies and biopsies taken 24h post allergen- compared to diluent challenge (1 [0 – 2] vs 0 [0 - 0] cells/mm², $p=0.008$, and 2 [1 – 2] vs 0 [0 – 0] cells/mm², $p=0.0002$, respectively). Interestingly, in sex analyses we found significantly greater levels of blood ILC2 in females compared to males, but this not was found in the skin. Importantly, we found a significant correlation between lesional skin levels of ILC2 measured by flow cytometry and clinical measures of disease severity/symptoms as reported/calculated from the Patient-Oriented Eczema Measure questionnaire (POEM) score (total ILC2: $r=0.55$, $p=0.04$; IL-13+ ILC2s, $r=0.61$, $p=0.02$ and IL-5+ IL-13+ ILC2s: $r=0.75$, $p=0.002$).

Conclusions: Preferential increases in skin-resident ILC2 that produce a type 2 rich environment were found in AD subjects. These levels correlated with patient-oriented measure of disease severity. We propose that this increase may encourage recruitment of mature eosinophils and EoP and possibly drive localized differentiation of EoP into mature eosinophils that may drive the pathology of AD lesions. Furthermore, immunofluorescence staining may be a suitable alternative to flow cytometry for identification of ILC2 in the event of a low cell count. These techniques can

be used in future studies that target ILC2 biology to fully understand the role of these cells in driving AD.

ACKNOWLEDGEMENTS

First and foremost, I would like to thank God (whoever He/She/It maybe) for all the blessings throughout my life. Next, I must thank my supervisor Dr Roma Sehmi for giving me this opportunity to fly all the way from Singapore to Canada to do my master's and giving me the opportunity to dip my feet in the world of allergies and Asthma and Atopic Dermatitis, despite having a cancer background. Your constant support, confidence and advice to always “put my best foot forward” has pushed me to always give my best for these 2 years. Also, I want to thank you for the opportunity present my work at AAAAI/WAO Joint Congress 2018. I want to also thank my thesis supervisory committee for their unwavering support, and guidance. Dr Gail Gauvreau, and Dr Hermenio Lima, thank you for your input and suggestions in improving my data presentation and providing a different perspective for the study. I also would like to thank Dr Ali Ashkar, Infection and Immunity chair who sat at my first thesis committee meeting and provided great suggestions to improve my staining panel.

I'm very grateful to my lab mates in the Cardio-Respiratory Lab. The lab has real sense of family during my time here and I've definitely learned a lot from all the staff, post-docs, and students. Emma, my OSAD partner-in-crime, it was great working with you on this study and finding ways to improve the study. I wish you all the best in Halifax! Adrian, thank you for teaching me the basics of FACS staining and how to develop a staining panel. Caroline, thank you for all the laughs in the lab and for helping me wean off those Mars bars! I had so much at the Harry Potter World after AAAAI 2018 with you, Emma and Tim! JP, seriously words cannot describe how grateful I am for having met you. Thank you so much for teaching me how to think and act like a grad student and a scientist and for pushing me to always excel at whatever I am doing. Even when you're at Stanford, you're still rooting for me by texting and asking me how I

am. Dhuha, my lab “big sister”, thank you so much for your help when I was developing the ILC2 IF panel and also for treating me like I’m your little brother. I’m so happy that you got your pathology residency! Also, thank you and Imran for teaching me to always be confident. Michael, thank you for your help with the FACS staining for OSAD, especially when we had ARIA samples on the same days. Your help has been invaluable! Louis, thank you for your help with the IF optimization and staining and I’m glad to have been your student supervisor for the ILC2 IF project. Congratulations on getting into UofT’s Fundamental Immunology PhD program! I’m sure you’ll be great! Sue, thank you for being my first teacher on how to run flow. Even after I gained experience, you were always there to give support and teach me whenever I needed to troubleshoot. Karen, thank you for your help on OSAD! Catie Creighton, thank you for the constant smiles and jokes whenever I see you. Whenever I’m in a bad mood, all I need to do is to just walk to your desk and just chat for a minute and my spirits have been lifted. Also, thank you for all the goodies in your cabinet! The sugar does help although I think I gained like 5 Kg from all that eating, especially when working late nights and weekends! Jen, it was great working with you and thanks for the chats and laughs! Rick, and Tara, it was great being your patient in a couple of trials and I enjoyed the times when I was in your office, despite the allergen-challenges and sputum inductions. I really loved the photo montage in your office and I’m glad my photos are there as a legacy! Ken, thank you for teaching me how to properly gate my cells to look for ILC2s. Tim, Nobu and Alice, thank you for being my lab mates and I hoped I was a good “big brother” for you guys when you first joined the lab. Also, I’d like to thank the other undergrads who have been in the lab, Michelle, Heidi, Priya, Calab, Alex, Abi, Harry, Lulu, Shawn, Steven, Mobeen, and Matt (sorry if I forgot the rest). It was great chatting and interacting with everyone!

I also would like to thank all my friends back in Singapore for their constant friendship and love. Thank you keeping me up to speed about what's going on back home and for all the laughs on WhatsApp and Facebook. My St. Gabriel's "brothers from another mother", Aaron and Benjamin (and Brenda, and EJ) (And Emmanuel in Toronto!), my Nanyang Poly bro Franky (and Abie), my Uni of Bradford pals Jack, Cheryl, and Desmond. Jack thanks for playing co-op games with me to unwind and relax from lab work despite the time zone difference; it definitely helped! Also, my SGH pathology pals, Wai Jin, Jeffrey and Valerie. Thanks so much for all the laughs on the WhatsApp chat group and sending the Song Fa packets and emperor chicken spices over! Whenever I got homesick and craving for some local food, I just cook some Bak Kut Teh and it's like I'm back in Singapore. Also, I must thank the pathologists for always rooting for me and sending me well wishes: Dr Rafay Azar, Dr Joe Yeong and Dr Norman Chan. Special mention must go to Dr Angela Takano, who has been instrumental in my career development. Words cannot describe how grateful I am for your constant support. It was you who pushed me to give overseas studies a shot and I really wouldn't have accomplished all these things if it wasn't for you. Thank you so so much!

I also must thank the Kugathan Family for their help and hospitality. Uncle Kuga and Aunty Nirmala, thank you and Bhaskharan for picking me up at the airport and letting me stay at your home for my first couple of days in Canada and for helping me move to Hamilton with Anand. Most importantly, I wouldn't be where and who I am without my family. To my cousins/big sisters, Geetha Akka, Banu Akka, Meena Akka, Gayathri and Manoja, thank you for your support and love and well wishes on the WhatsApp chat group. My uncles Padhmanaban and Uthayachandran, thank you so much for your support, ever since I was little kid. Thank you for always keeping tabs on my health, education and career throughout my life and for supporting me going overseas to do

my grad studies. My actual big sister, Syamla, thanks for your love and support and holding down the fort at home. Finally, my parents, thank you for your love and support and thank you for letting me prioritize my education, career and pursuing my dreams and goals above everything else (And also, I must thank Skype and Apple for FaceTime!). I'll keep doing what I do to make all you guys proud back home.

Last, but not least, I must thank all the OSAD study patients and volunteers. Your dedication in supporting this study has been inspiring and for giving me the motivation for zero errors when I'm doing my experiments. There was one patient I spoke to about his decision to join the study and he said that he joined the study not because of the monetary compensation, but because he has 2 daughters and that he didn't want them to go through the same pains he went through. That was his motivation and that became my motivation to always give my best so that future patients don't have to suffer, or hopefully there won't be any future patients.

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LIST OF ABBREVIATIONS AND SYMBOLS

AD – Atopic Dermatitis
AHR – Aryl Hydrocarbon Receptor
APC – Antigen-Presenting Cells
APC – Allophycocyanin
APC-H7 – Allophycocyanin-Hilite7
ATP – Adenosine Triphosphate
BCL-11B – B-Cell Lymphoma/Leukaemia 11B
BLTR1/2 – Leukotriene B4 Receptor 1 and 2
BV421 – Brilliant Violet 421
BV510 – Brilliant Violet 510
BV605 – Brilliant Violet 605
BV650 – Brilliant Violet 650
CCL – Chemokine Ligand
CCR – Chemokine Receptor
CD – Cluster of Differentiation
CHILP – Common Helper-Like Innate Lymphoid Progenitor
CILP – Common Innate Lymphoid Progenitor
CLP – Common Lymphoid Progenitor
CRTH2 – Chemoattractant receptor-homologous molecule expressed on TH2 cells
CYSLTR1/2 – Cysteinyl Leukotriene Receptor 1 and 2
EILP – Early ILC Progenitor
ECP – Eosinophil Cationic Protein
EDN – Eosinophil-Derived Neurotoxin
ELISA - Enzyme-Linked Immunosorbent Assay
Eo/B-CFU - Eosinophil/Basophil-Colony Forming Unit
EoP – Eosinophil Progenitor Cells
EPN – Eosinophil Peroxidase
FACS – Fluorescence-Activated Cell Sorting
EPX – Eosinophil Protein X
FBS – Fetal Bovine Serum
FcεR1 – Fc Epsilon Receptor 1
FITC – Fluorescein Isothiocyanate
FFPE – Formalin-Fixed Paraffin-Embedded
FMO – Fluorescence Minus One
FOXP3 – Forkhead Box P#3
GATA3 – GATA Binding Protein 3
GFI-1 – Growth Factor Independence-1
HLA-DR – Human Leukocyte Antigen – antigen D Related
ICOS – Inducible T-Cell Costimulator
ID2 – Inhibitor of DNA-Binding Protein 2
IF – Immunofluorescence
IFN γ – Interferon- γ
IgE – Immunoglobulin E
IgE – Immunoglobulin G

IL – Interleukin
ILC – Innate Lymphoid Cells
ILCreg – Regulatory ILCs
ILC1 – Group 1 Innate Lymphoid Cells
ILC2 – Group 2 Innate Lymphoid Cells
ILC2P – ILC2 Progenitors
ILC3 – Group 3 Innate Lymphoid Cells
KLRB1 – Killer Cell Lectin-Like Receptor Subfamily B Member 1
KLRG1 – Killer Cell Lectin-Like Receptor Subfamily G Member 1
MHC-II – Major Histocompatibility Complex Class II
MBP-1 – Major Basic Protein 1
MBP-2 – Major Basic Protein 2
NK – Natural Killer
NKP – Natural Killer Progenitor
NFIL3 – Nuclear Factor, Interleukin 3
PBMC – Peripheral Blood Mononuclear Cells
PBS – Phosphate-Buffered Saline
PE – Phycoerythrin
PE-CF594 – Phycoerythrin-CF594
PE-Cy7 – Phycoerythrin-Cyanine7
PerCP-Cy5.5 - Peridinin-Chlorophyll-Protein Complex-Cyanin5.5
RAG – Recombinant Activating Gene
RAST – Radioallergosorbent Test
ROR α – RAR-Related Orphan Receptor Alpha
ROR γ t – RAR-Related Orphan Receptor Gamma
RPMI-C – RPMI Complete Medium
RUNX3 – Runt-Related Transcription Factor 3
SCFR – Stem Cell Factor Receptor
ST2 – Suppression of Tumorigenicity 2
TCF-1 – T-Cell Factor-1
TGF β 1 – Transforming Growth Factor β 1
TH0 – Naïve Helper T-Cells
TH2 – Type 2 Helper T-Cells
TNF α – Tumour Necrosis Factor- α
Treg – Regulatory T-Cells
TSLP – Thymic Stromal Lymphopoietin
 α LP – α -Lymphoid Precursor
 α – Alpha
 β – Beta
 δ – Delta
 ϵ – Epsilon
 γ – Gamma
 κ – Kappa
 μ – Micro

CHAPTER 1: INTRODUCTION

1.1: Atopic Dermatitis

Atopic dermatitis (AD) is a disorder of the skin characterized by chronic, relapsing skin inflammation, and eczematous, pruritus lesions(Bieber, 2017). The prevalence of AD has increased 2-3-fold in the past 30 years in industrialized countries, with 15-30% of children and 2-10% of adults' affected(H. Williams & Flohr, 2006). As of 2006, the cost of treating AD was CDN\$1.4 billion, with the annual expenditure per patient increasing as disease severity increases. For example, for patients with mild AD, the annual expenditure was \$282, moderate AD was \$454, and AD was \$1242. Patients with AD reported an average 3.6 visits to a government-funded clinic. Mild AD is defined as an occasional occurrence, with slight itching and scratching. Moderate AD is defined as a constant or intermittent itching and scratching, with no sleep disturbance reported. Severe AD is defined as a bothersome itching and scratching and the patient's sleep is disturbed. From these visits, 58% were to general practitioner clinics, and 28% were to dermatologists, with the remainder of patients seeing allergists, paediatricians, and other specialists(Barbeau & Lalonde, 2006). Apart from the financial burden, the patient also suffers from emotional and/or social embarrassment, resulting in a reduced quality of life. The patients, particularly adolescents, suffer from a disturbance or a loss of sleep, increased pruritus, increased anxiety and depression. It is due to this problem that patients skip school or work(Slattery et al., 2011). Currently, the gold standard of treatment is the use of systemic treatments such as corticosteroids, and cyclosporine. Due to the undesirable adverse effects caused by steroids, understanding the pathobiology of AD, as well as identifying novel biomarkers as a target for therapeutic interventions.

1.1.1: Normal Human Skin

The skin forms a physical and immunological barrier against the external environment stresses as well as pathogens such as viruses and microbiota (Di Meglio, Perera, & Nestle, 2011) (Heath & Carbone, 2013) (Grice & Segre, 2011). The skin consists of the epidermis, the dermis, and the subcutis. The epidermis consists of the stratum corneum which acts as a permeability barrier to prevent loss of moisture to allow humans to live in hot environments (Elias, 2005). The epidermis is formed by several layers of skin cells known as keratinocytes. These keratinocytes drive local inflammatory responses, as well as recruitment and activation of immune cells (Pastore, Mascia, & Girolomoni, 2006). In addition, the epidermis contains CD4⁺ and CD8⁺ T cells (Pauls et al., 2001), as well as dendritic cells (M. B M Teunissen, Haniffa, & Collin, 2012) (Langerhans cells). While the immune cells in the epidermis are typically resident and non-migratory within the epidermis, the dermis contains blood vessels which carry circulating immune cells such as eosinophils, neutrophils, memory T-cells, and monocytes. Upon activation of these circulating immune cells, they exit out of the blood vessels and enter the dermis (Gebhardt et al., 2011) (Eidsmo, Stock, Heath, Bedoui, & Carbone, 2012) (Peters et al., 2008).

1.1.2: Pathogenesis of Atopic Dermatitis

The pathogenesis of AD is proposed to be caused by the complex interaction between the epidermal barrier abnormality, the host immune system, and pruritus (Kabashima, 2013). When an allergen enters through the epidermal barrier, the epithelial cell secretes alarmins such as TSLP, IL-25 and IL-33, which then activates and matures monocytes and macrophages. Alarmins are produced by epithelial cells and they produce downstream effects on cells of the innate and adaptive immune systems. For example, they promote the induction of TH2, as well as group 2

innate lymphoid cells. In a study by Siracusa et al, it was reported that TSLP mediates the extramedullary expansion and differentiation of antigen-presenting cells (Siracusa et al., 2013). TSLP is released by injury to the epithelial cells, or upon exposure to proteases (Oyoshi, Larson, Ziegler, & Geha, 2010)(Kouzaki, O’Grady, Lawrence, & Kita, 2009). IL-25 is stored and released from cellular compartments outside the nucleus. IL-25 is constitutively expressed in cells, but upon exposure to proteases, the cell releases the cytokine (Kouzaki, Tojima, Kita, & Shimizu, 2013). In addition to the presence of proteases or allergens, IL-25 may be released in the presence of a defective skin barrier (B. E. Kim, Bin, Ye, Ramamoorthy, & Leung, 2013). IL-33 is different from IL-25 in that it is synthesized and stored in the nucleus of epithelial cells. In the context of AD, group 2 innate lymphoid cells express the receptor for IL-33, ST2, and so can respond to IL-33 and migrate to the site of inflammation (Salimi, Barlow, Saunders, Xue, Gutowska-Owsiak, et al., 2013).

There are two theories that have been proposed for AD. The “Outside-In” theory that suggests that a dysfunctional epidermal barrier, mediated by mutations in filaggrin, initiates AD by secondary immune abnormalities (Elias, Hatano, & Williams, 2008). The “Inside-Out” theory, suggests that dysregulation of the immune system including T-cell-driven abnormalities cause AD, with changes to the epidermal barrier as a secondary effect (Elias & Steinhoff, 2008). The “Outside-In” theory was proposed as an intrinsic form of AD. However, as pointed out by Brandt (B. Brandt, 2011), it contradicts the canonical definition of AD as in the “Outside-In” theory sensitization is not IgE-driven. As such, it is more suited to be considered as non-AD, as opposed to AD which is IgE-mediated. Salt et al found a correlation between elevated serum IgE levels and disease severity where in severe atopic dermatitis serum IgE level of 3922 IU/mL were detected

compared to moderate and mild AD patients who had a median serum IgE of 358 IU/mL and 44 IU/mL, respectively (Salt, Boguniewicz, & Leung, 2007).

APCs, such as dendritic cells come into contact with the allergen, become primed and migrate to the draining lymph nodes where they present antigen to TH0 cells, class switching to TH2 lineage and stimulate clonal expansion of existing TH2 cells. The TH2 cells exit the draining lymph node and migrate to the skin lesion and promote inflammation via the release of type 2 cytokines (Peng & Novak, 2015). These type 2 cytokines such as IL-4, IL-5, IL-9 and IL-13, promoting B cell class switching from IgG to IgE and tissue co-localization of mast cells and recruitment of pro-inflammatory cells include basophils, eosinophils from the peripheral circulation.

One of the key cells involved in AD pathology is eosinophils. Eosinophils are derived from CD34+ hematopoietic progenitor cells in the bone marrow (J. A. Denburg et al., 1985). One to six percent of white blood cells are eosinophils and in the absence of stimulation, eosinophils survive for 8-12 hours in the circulation and persist in the tissues for 8 to 12 days (Young, O'Dowd, & Woodford, n.d.). Eosinophils, together with basophils arise from a common precursor cell which can be enumerated in *in-vitro* cultures with eosinophilopoietic cytokines as a cluster of phenotypically distinct sister cells termed Eo/B-CFU (J. A. Denburg et al., 1997). In patients with atopic diseases, Eo/B-CFU is increased which is correlated with the severity of the disease (Robinson et al., 1999). For example, in patients with Allergic Rhinitis, during the pollen season, it was found that there is a decrease in Eo/B-CFU levels in the peripheral blood, which suggests that these progenitor cells migrated to the tissues where they are differentiated and matured, a process termed in-situ differentiation (Linden et al., 1999). In addition, CD34+ progenitor cells derived from the bone marrow, there is an upregulation of the α -subunit of the IL-5 receptor (IL-

5R α or CD125) upon exposure to an allergen (Roma Sehmi et al., 1997) which then increases sensitivity to IL-5. In patients with mild allergic asthma, there is an increase in EoPs in peripheral blood, as well as the upper and lower airway mucosa, and in the sputum. In patients with severe asthma, there was a 10-fold increase in EoPs in the sputum compared to mild asthmatics suggesting an increased potential for local differentiation of progenitor cells to mature cells within the affected tissue (R. Sehmi et al., 2016).

Eosinophils are important in the elimination of parasites and pathology of allergic diseases due to the presence of granules in the cytoplasm. Eosinophils release these granules in the event of activation via cross-linking by allergen-specific-IgE, and lipid mediators and cytokines such as RANTES and eotaxin (Papadopoulos et al., 2000)(Monneret, Cossette, Gravel, Rokach, & Powell, 2003). Some of these eosinophil granules cause tissue damage as these proteins are toxic. Examples of these granule proteins are major basic protein 1 and 2 (MBP-1 and MBP-2), eosinophil cationic protein (ECP), eosinophil-derived neurotoxin/eosinophil protein X (EDN/EPX), as well as eosinophil peroxidase (EPO) (K M Leiferman, 1991). In addition to the cytotoxic nature of granules to parasites, as well as to normal tissues, these proteins are known to stimulate neutrophils, basophils, mast cells and eosinophils (Zheutlin, Ackerman, Gleich, & Thomas, 1984)(Moy, Gleich, & Thomas, 1990)(Kita, Weiler, Abu-Ghazaleh, Sanderson, & Gleich, 1992).

Under normal conditions, there are none to a few skin-resident eosinophils. However, in atopic dermatitis, eosinophils can be found in lesional skin (Kato et al., 1998). Eosinophilia is defined as more than 500 eosinophils detected per microliter of peripheral blood and a correlation of between eosinophilia and disease activity has been reported (Kägi, Joller-Jemelka, & Wüthrich, 1992). However, as there is a report showing an increase in systemic eosinophil counts may not

necessarily be due to atopic dermatitis and maybe caused by other atopic diseases, it has been proposed that blood eosinophil count as a diagnostic measure for AD may not be a feasible method (Uehara, Izukura, & Sawai, 1990). Tissue eosinophilia has been reported in both acute and chronic atopic dermatitis and that this correlates with disease severity. In chronic atopic dermatitis, tissue eosinophilia is shown to be more pronounced in skin lesions with a marked hyperplasia in the epidermis (Kiehl, Falkenberg, Vogelbruch, & Kapp, 2001). In addition, in a mouse model of atopic dermatitis, there was an increase in the thickness of both layers in the epidermis and dermis, as well as hypertrophy of the skin. This is due to the release of cytotoxic granule proteins including MBP-1, MBP-2 and ECP (Spergel et al., 1999).

Type 2 cytokines, IL-3, IL-5 and granulocyte/macrophage colony-stimulating factor (GM-CSF) are produced by a number of cells including mast cells and CD4⁺ T lymphocytes, are needed for the stimulation, maturation and differentiation of eosinophils, particularly IL-5 (Simon, Braathen, & Simon, 2004)(Nishinakamura, Miyajima, Mee, Tybulewicz, & Murray, 1996)(Yamaguchi et al., 1988). Eotaxin-1, also known as CCL11 is a chemokine that is involved in the recruitment of eosinophils. Endothelial cells, fibroblasts, eosinophils, lymphocytes, macrophages and bronchial smooth muscle cells produce Eotaxin-1 (Amerio et al., 2003). In the context of atopic dermatitis, IL-4, which is produced by TH2 cells, stimulates keratinocytes to produce Eotaxin (Nonaka et al., 2004).

IL-4 is a type 2 cytokine required for polarizing naïve t-cells(TH0) to TH2 cells, and IgE class-switching of B cells. AD mouse models have shown that overexpression of IL-4 in the epidermis developed pruritus, increased recruitment of inflammatory cells and elevated IgE levels (Chan, Robinson, & Xu, 2001). In an in-vitro study, it was found that normal human keratinocytes treated with IL-4 showed an increased expression of eotaxin-3 (CCL26), which plays a role in

eosinophils recruitment (Nishi et al., 2008). Other than eotaxin-3, another study showed that fibroblasts isolated from patient acute AD skin lesions were responsive to IL-4, and as a result, expressed eotaxin-1(CCL11) (Gahr et al., 2011).

IL-13 is a mediator in allergic inflammation (Wills-Karp et al., 1998). IL-13 is expressed in both acute and chronic AD (Hamid et al., 1996). In mouse models of AD, it was shown that IL-13 transgenic mice developed pruritus, high expression of IgE, inflammatory cell recruitment into the skin, skin fibrosis, and upregulation of TSLP and other cytokines (Zheng et al., 2009). IL-13 signals via IL-4R α and IL-13R α 1 (Khurana Hershey, 2003). Also, IL-13 binds to IL-13R α 2 which is thought to be a decoy receptor, or a dominant-negative inhibitor receptor which lacks any signalling activity. The IL-13R α 2 can regulate type 2 immune responses as a decoy receptor controlling IL-13 activity (Chiaramonte et al., 2003). In a mouse study, mice lacking IL-13R α 2 had significantly increased skin inflammation, eosinophilia, elevated IgE levels and increased transepidermal loss of water, as compared to wild type mice (Sivaprasad et al., 2010). Like IL-4, IL-13 also mediates production of eotaxin-3 (Esche, de Benedetto, & Beck, 2004). Keratinocytes treated with IL-13 increased recruitment of CD4+CCR3+ T cells by stimulating migration to the skin thus enhancing the inflammatory response (Purwar, Werfel, & Wittmann, 2006).

IL-5 is a type2 cytokine essential for eosinophils development, survival and proliferation (Simon et al., 2004). In a mouse model in which IL-5 was knocked-out, there was reduced thickening of the skin, as well as reduced skin eosinophilia (Spergel et al., 1999).

In addition, microbes such as *Staphylococcus Aureus* release serine proteases that causes the breakdown of the epidermis, as well as toxins to breakdown the proteins such as adhesion proteins in the desmosomes(M. R. Williams, Nakatsuji, Sanford, Vrbanac, & Gallo, 2017). This increases the permeability of the skin. Furthermore, the release of *Staphylococcus Aureus* toxins

activates the signalling pathways that mediate the production of pro-inflammatory mediators, as well as defensins (Huttner & Bevins, 1999) which are antimicrobial peptides, that mediate microbe killing and skin homeostasis (Kiatsurayanon et al., 2014).

1.1.3: Treatments

There is currently no cure for AD. As such there is only management of the disease, where the symptoms are treated and reduced, as well as avoiding the triggers that cause AD. The first line of treatment of AD is topical corticosteroids, where the severity of flare-up is proportionate to the dosage of the drug (Hanifin et al., 2004). The purpose of corticosteroids is to suppress allergic inflammation (Bradding et al., 1994). Corticosteroids can be applied either topically or systemically and inhibit the expression of pro-inflammatory cytokines such as IL-4, IL-5, and IFN- γ (Umland et al., 1997), thereby reducing the infiltration and survival of pro-inflammatory cells such as eosinophils (Altman, Hill, Hairfield, & Mullarkey, 1981). Consequently steroid treatment has been shown to induce eosinophil and basophils apoptosis (Walsh, Sexton, & Blaylock, 2003)(Dunsky, Zweiman, Fischler, & Levy, 1979). However, corticosteroids are associated with adverse side effects including increased susceptibility to infections and skin atrophy which were directly related to the duration and potency of the therapy. In addition, topical corticosteroids may not be effective for patients with moderate to severe AD (Barnes, Kaya, & Rollason, 2015)(Coondoo, Piske, Verma, & Lahiri, 2014).

There are rescue therapies provided to patients that suffer from moderate to severe AD that can be considered for the treatment of AD such as allergen immunotherapy (Ridolo, Martignago, Riario-Sforza, & Incorvaia, 2018)(Werfel et al., 2006), phototherapy and the use of systemic immunosuppressant therapy such as systemic oral corticosteroids (Sidbury et al., 2014). Steroid therapy is very effective at reducing inflammation in atopic diseases. However, there are potential

side effects associated with long-term treatment with oral corticosteroids including fractures, weight gain, hypertension, peptic ulcers, depression, anxiety, increased risk of infections, and hyperglycaemia (Manson, Brown, Cerulli, & Vidaurre, 2009). Interestingly, there is a paradox when using corticosteroids on *Staphylococcus Aureus*. In a study by Goggin, fluticasone, mometasone and budesonide reduced the mean biomass of *Staphylococcus Aureus* in chronic rhinosinusitis. However, the study did not investigate the effects of prednisone in AD patients (Goggin, Jardeleza, Wormald, & Vreugde, 2014).

1.2: Innate Lymphoid Cells

Innate lymphoid cells (ILCs) are a relatively novel class of innate cells that were identified a decade ago (Neill et al., 2010)(Spits et al., 2013)(Walker, Barlow, & McKenzie, 2013). These class of cells are the innate counterparts to helper T-Cells (i.e.: ILC1/TH1, ILC2/TH2, ILC3/TH17/22) and express the same cytokines. ILCs are mainly localized in mucosal tissues, such as the lung, intestines, nasopharynx, and the skin (Simoni et al., 2017). The ILCs unlike T- and B lymphocytes, do not express the recombinant activating gene (RAG) (Allan et al., 2014). Furthermore, ILCs do not express lineage-markers that are expressed on T-cells, B-cells, myeloid- and cells of the erythroid- lineages. ILCs can be divided into 2 subtypes: helper and cytotoxic. There are 3 helper ILC subtypes: group 1 innate lymphoid cells (ILC1), group 2 innate lymphoid cells (ILC2), and group 3 innate lymphoid cells (ILC3). As of writing, there is one only class of cytotoxic ILCs, termed natural killer (NK) cells that are classed under ILC1s, are considered the innate equivalent of cytotoxic CD8+ T-Cells (Lim et al., 2017).

ILCs are the innate counterparts of helper T-cells. The 3 ILC subtypes are classed by their cytokine profile, as well their transcription factors necessary for their expansion. ILC1s produce interferon- γ (IFN γ), IL-2, and tumour necrosis factor- α (TNF α) (Bernink et al., 2013)(Zhao et al.,

2018). ILC1s express the transcription factor T-bet, which is a regulator of TNF α and IFN γ effector cytokines to counter intracellular pathogens (Bernink et al., 2013). ILC2s produce Type 2 cytokines, IL-4, IL-5, IL-9 and IL-13 and express the transcription factors GATA3 and ROR α , where GATA3 is required for the regulation of effector cytokine production, mainly IL-5 and IL-13 (J. Mjösberg et al., 2012)(Wong et al., 2012). These cytokines have role in helminth expulsion, as well in allergic diseases such as allergic asthma (Smith et al., 2016), allergic rhinosinusitis (Doherty et al., 2014), as well as AD (Salimi, Barlow, Saunders, Xue, Gutowska-Owsiak, et al., 2013). In addition, ILC2s play a role in tissue repair by releasing amphiregulin(Salimi, Barlow, Saunders, Xue, Gutowska- Owsiak, et al., 2013). Interestingly, there are 2 subtypes of ILC3s: CCR6+ ILC3s, and CCR6- ILC3s. CCR6+ ILC3s produce IL-17 and IL-22 which plays a role in countering fungal infections (Sawa et al., 2010). In addition to these roles, ILC3s are required for lymphoid organogenesis in mice (Eberl et al., 2004). CCR6+ ILC3s express the transcription factor ROR γ t. Whereas, CCR6- ILC3s express T-bet, as well as the receptor NKp46. CCR6- ILC3s are termed as, “natural-cytotoxicity-receptor-positive” (NCR+) ILC3s(Chea et al., 2016). T-bet downregulates ROR γ t, which causes a similar phenotype as that to ILC1s to produce IFN γ . In this thesis, we used a combination of GATA3, CD127, CRTH2 and lineage markers to identify ILC2s via flow cytometry and immunofluorescence staining.

1.2.1: Development

ILCs, as well as adaptive lymphocytes, are derived from the common lymphoid progenitor (CLP) in the bone marrow and these give rise to the common innate lymphoid progenitor (CILP) which express α 4 β 7 integrin and CXCR6 chemokine receptor (C. H. Kim, Hashimoto-Hill, & Kim, 2016). CILP, also known as the α -lymphoid precursor (α LP) express the transcription factor

nuclear factor, interleukin 3 (NFIL3) (Seillet et al., 2014). The CILPs can form 2 distinct ILC progenitor cell lineages: the common helper-like innate lymphoid progenitor (CHILP) cells and NK progenitor (NKP) cells (Klose et al., 2014). In addition, another precursor cell has been identified: the early ILC progenitor (EILP) cells. The difference between the EILPs and CILPs is that only CILPs express CXCR6 (Yang et al., 2015) although both populations give rise CHILPs and NKPs. Whether some redundancy exists remains to be reported. CHILPs are Lin-ID2+CD127+ α 4 β 7+IL-2R α - and are GATA3-dependent for their development (Klose et al., 2014). CHILPs will then progress to either the innate lymphoid cell progenitor (ILCP) or the lymphoid-tissue inducer progenitor (LTiP), based on their transcription factor expression. CHILPs express the promyeloid leukaemia zinc finger (PLZF) to progress to ILCP downstream. ILCPs are Lin-PLZF^{high}CD127+cKIT+CXCR6- α 4 β 7^{high} (Klose et al., 2014). ILCPs commit to ILC1, ILC2 or ILC3, based on their transcription factor expression. ILCPs that express runt-related transcription factor 3 (RUNX3) and T-bet commit to ILC1 lineage, those that express RUNX3, ROR γ t and aryl hydrocarbon receptor (AHR) commit to ILC3 lineage (Ebihara et al., 2015). ILCPs that express GATA3, ROR α and B-cell lymphoma/leukaemia 11B (BCL-11B) commit to ILC2 progenitors (ILC2P) which express BCL-11B, GATA3 and TCF1 to mature into ILC2s (Califano et al., 2015).

1.2.2: Innate Lymphoid Cells Heterogeneity

In addition to the 3 subtypes of helper ILCs and the cytotoxic ILC (i.e.: NK cells) a new subtype of ILCs was recently identified: regulatory ILCs (ILCreg). ILCregs were identified in intestinal inflammation and that it played a role in an inhibitory capacity in the innate immune response. Wang et al found that ILCregs were found in both human and murine intestines (Wang et al., 2017).

ILCregs secrete IL-10 and TGF β 1, which are key cytokines involved in Treg-mediated immune suppression. Interestingly, ILCregs were found to be FOXP3 negative, which is a signature marker to identify regulatory T-Cells (Treg). This shows that ILCregs are distinct from Tregs. Although ILCregs are Lin-CD45⁺CD127⁺CD25⁺CD90⁺IL-10⁺, they do not express markers in ILC1 (NKp46 and NK1.1), ILC2 (KLRG1 and IL-33R), and ILC3 (ROR γ t and NKp46). ILCregs were found to be involved in the resolution of innate intestinal inflammation in mouse models by stimulation with IL-10 in *RAG1^{-/-}Il10^{-/-}* mice, which caused the resolution of innate colitis (Wang et al., 2017). Kim et al found ILCregs (authors called it ILC10) in spleen, axillary lymph node, inguinal lymph node and ear of oxazolone-treated in C57BL/6 mice in a study of contact hypersensitivity (H. S. Kim et al., 2016).

Another novel subtype of ILCs was identified: memory ILCs. However, it is not based on the canonical definition of memory as seen in T-cells and B-cells. Martinez-Gonzalez et al reported that in mice, papain-treated, or IL-33-treated mice increased the number of ILC2s were the highest at Day 3, as well as production of IL-5 and IL-13 (Martinez-Gonzalez et al., 2016). To determine “memory” in ILC2s, the mice were re-exposed to papain or IL-33 1 month after initial exposure. Pre-treated mice ILC2 responded much faster to the allergen upon re-exposure, compared to naïve mice, as well production of IL-5 and IL-13. A separate group of mice were treated with a fungal serine protease allergen. Their ILC2 levels dropped after 3.5 months and were exposed to papain. Compared to the control group, the fungal allergen and papain-treated mice had significantly higher numbers, which suggests that allergen-exposed ILC2s respond more efficiently to an unrelated allergen, compared to naïve ILC2s.

1.3: Group 2 Innate Lymphoid Cells

ILC2s are innate effector cells that lack the surface markers for T-cells, B-cells, and dendritic cells (J. M. Mjösberg et al., 2011). They secrete cytokines like T-cells and B-cells, and are implicated in inflammation, innate immunity, and tissue remodelling. ILC2s were originally called Nuocytes due to high levels of IL-13 production (Neill et al., 2010), Natural Helper Cells (Halim, Krauß, Sun, & Takei, 2012) and Innate Helper Type 2 (A. E. Price et al., 2010), but as these cells exhibit similar characteristics, they have been grouped to ILC2s. The development of ILC2s as detailed above requires transcription factors such as GATA binding protein 3 (GATA3) (Zhao et al., 2018), RAR-related orphan receptor Alpha (ROR α) (Halim, MacLaren, et al., 2012), T-cell factor-1 (TCF-1) (Mielke et al., 2013) and growth factor independence-1 (GFI-1) (Spooner et al., 2013). Like the other ILC subtypes, ILC2s are phenotypically identified by lineage-negative, CD45 and CD127 (IL-7R α). In addition, ILC2s also express ST2 (IL-33R) (Salimi, Barlow, Saunders, Xue, Gutowska-Owsiak, et al., 2013), Chemoattractant receptor-homologous molecule expressed on TH2 cells (CRTH2/CD294) (J. M. Mjösberg et al., 2011), inducible T-cell costimulator (ICOS) (Maazi et al., 2015), CD25 (IL-2R α) (Seehus et al., 2017), IL-25R (IL-17R β) (Salimi, Barlow, Saunders, Xue, Gutowska-Owsiak, et al., 2013), killer cell lectin-like receptor subfamily G member 1 (KLRG1) (Huang et al., 2015), killer cell lectin-like receptor subfamily B member 1 (KLRB1/CD161) (J. M. Mjösberg et al., 2011), thymic stromal lymphopoietin (TSLP) receptor (TSLPR) (Salimi, Barlow, Saunders, Xue, Gutowska-Owsiak, et al., 2013), IL-4 receptor-alpha (IL4-R α) (Brian S Kim et al., 2014), stem cell factor receptor (c-Kit/CD117) (Boyd, Ribeiro, & Nutman, 2014), cysteinyl leukotriene receptor 1 and 2 (CYSLTR1/2) (Doherty et al., 2013), and leukotriene B4 receptor 1 and 2 (BLTR1/2) (von Moltke et al., 2017).

ILC2s can be activated by alarmins IL-25, IL-33 and TSLP to induce IL-5, and IL-13 production (Salimi, Barlow, Saunders, Xue, Gutowska-Owsiak, et al., 2013). Accumulation of ILC2 in the skin of AD patients shows that it might influence dendritic cells to further Th2 phenotypes in T cells (Halim et al., 2015). Th2 cells then release IL-4, which then causes dendritic cells to lose the ability to produce IL-10 which is a pro-inflammatory cytokine, which then leads from a self-limiting dermatitis into a chronic cutaneous inflammation (Volz et al., 2014)(Kaesler et al., 2014). In addition, ILC2s express major histocompatibility complex class II (MHC-II) (Neill et al., 2010). As such it has the potential to present antigens to T-cells for clonal expansion to TH2 cells. This was confirmed by Oliphant by showing that ILC2s expressed MHC-II, with the co-stimulatory receptors CD80 and CD86. Also, ILC2s have the ability to capture and process the antigen and present it to T-cells, albeit at an efficiency lower than dendritic cells. This then induces the ILC2s to proliferate and secrete type 2 cytokines (Oliphant et al., 2014). ILC2s are not commonly found in secondary lymphoid organs such as the spleen and lymph nodes, as compared to T-cells and B-cells (Gasteiger, Fan, Dikiy, Lee, & Rudensky, 2015).

1.3.1: ILC2 Activating Cytokines and Lipid Mediators

ILC2s are activated by various cytokines. For example, the epithelial-derived alarmin cytokines, TSLP, IL-25 and IL-33. TSLP is a member of the IL-2 cytokine family which shares a similar homology with IL-7. It binds to the TSLPR, which forms a heterodimer with IL-7R α (Pandey et al., 2000). It is expressed by mast cells, basophils, stromal cells and dendritic cells. For epithelial cells, it is expressed in mucosal tissues such as skin, intestines and lungs. ILC2s isolated from nasal polyps from patients with chronic rhinosinusitis have shown that TSLP induced the activation of STAT5, as well as GATA3, which is required for the production of IL-5 and IL-13

(J. Mjösberg et al., 2012). TSLP is involved in allergic diseases. For example, allergic asthma (Gauvreau et al., 2014), allergic rhinitis (Mou et al., 2009) and AD (Al-Sajee et al., 2018). While ILC2s are activated by IL-25 and IL-33 in the lung and intestine, Kim et al reported in a mouse model that activation of ILC2s in lesional skin in AD is independent of IL-25 and IL-33, but is dependent on TSLP (B S Kim et al., 2013). In addition, Kabata et al reported that TSLP confers resistance to corticosteroid treatment in an ovalbumin (OVA) mouse model (Kabata et al., 2013). It was reported by Walford et al, in patients in allergic rhinitis, ILC2s were found in eosinophilic nasal polyps (Walford et al., 2014). The patients were divided to the steroid and non-steroid treated groups. In the patients that took the steroids, there was a significant reduction in ILC2s. In addition, some patients have demonstrated resistance to corticosteroid therapy in atopic asthma (Leung, Spahn, & Szeffler, 2002). There is a study that showed that corticosteroids can negatively regulate ILC2s. However, when TSLP induced ILC2, it resulted in the phosphorylation of STAT5 and Bcl-xL expression. This then created a resistance to corticosteroid inhibition. However, this resistance was countered by administering the STAT5 inhibitor pimozide (Kabata et al., 2013). Despite the data showing resistance, corticosteroids are effective for a group of patients.

IL-33 is a member of the IL-1 cytokine family. Interestingly, IL-33 has 2 isoforms: a long-form isoform, and an alternatively-spliced variant. In asthmatic patients, the alternatively-spliced variant was associated with type 2 inflammation in the airways, and not the long-form (Gordon et al., 2016). IL-33 binds to ST2, which is also known as the IL-33 receptor. Furusawa et al reported stimulation of ILC2s with IL-33 in a mouse model caused the activation of p38, as well as GATA3 phosphorylation. Both are required for the production and proliferation of type 2 cytokines IL-5 and IL-13 (Furusawa et al., 2013). Other than epithelial cells, IL-33 is also released by mast cells. adenosine triphosphate (ATP) activates mast cells which then releases IL-33, which activates

ILC2s. Mohapatra et al had shown that in mouse models after exposing to chitin in the lungs, there was an increase in IL-33 and TSLP production, which activated and increased CD25 expression on ILC2s (Mohapatra et al., 2016).

IL-25, also known as IL-17E, is a member of the IL-17 cytokine family (Hurst et al., 2002) and it is involved in type 2 allergic diseases (Fallon et al., 2006)(Fort et al., 2001). IL-25 binds to IL-25 receptor (IL-17R β). Like IL-33, IL-25 is involved in helminth infections by releasing IL-13 for goblet cell hyperplasia as well as secretion of mucous. Although ILC2s respond to IL-25, interestingly, it is not as efficient as compared to IL-33, even with IL-2 co-stimulation in humans (J. M. Mjösberg et al., 2011) as well in mouse models (Moro et al., 2010).

ILC2s are also activated by gamma (γ)-chain cytokines such as IL-2, IL-4, IL-7, IL-9, IL-15 and IL-21 (Rochman, Spolski, & Leonard, 2009). These cytokines share the common γ -chain, also known as CD132 in their receptor complexes which comprise of the γ -chain and the α -chain, which is cytokine-specific. For example, for IL-2, it binds to CD25, also known as IL-2R α . The γ -chain is required as it is associated with janus kinase (JAK)-3 (JAK3) for signal transduction for downstream activation of signal transducer and activator of transcription, or STAT, which is required for transcription of certain genes for various purposes. IL-2 was originally found to be involved in NK cell development, as well as B-cell activation and proliferation, which then results in antibody production. Roediger et al found that IL-2R α is expressed on ILC2s and that treatment with IL-2 promoted expansion of ILC2s in lung tissues from mouse models and that type 2 cytokine production was increased which resulted with increased eosinophil infiltration (Roediger et al., 2015). Furthermore, IL-2 also acted as a co-factor when treated with IL-33 to allow IL-13 production, and also that CD2⁺ cells were a rich source of IL-2.

Another γ -chain cytokine is IL-7 that binds to the IL-7R α , also known as CD127. IL-7 is produced by dendritic cells and epithelial cells. Neill et al found from a microarray analysis in mouse models that CD127, ICOS and MHC-II was expressed by ILC2s (Neill et al., 2010). What the author reported as well was that IL-7 acted as a co-factor and when treated together with IL-33, there was a significant proliferation of ILC2s, but not with IL-25. In another study by Halim et al, the author confirmed Neill's result, but also reported that IL-25 was able to induce IL-5 and IL-13 production, independent, as well as dependent on IL-2 and IL-7 co-stimulation in lung ILC2s in mouse models. In addition, IL-7 may be a support in ILC2 homeostasis (Halim, Krauß, et al., 2012). In another study by Roediger et al, the author found that dermal ILC2s in mouse models required IL-7 for their survival and to produce IL-13. Furthermore, they also found that IL-2 treatment increased CD25, IL-33R and ICOS expression, as well as an increased production of IL-5, which resulted recruitment of eosinophils which then caused the mice to get spontaneous dermatitis (Roediger et al., 2013).

Another class of molecules that activate ILC2s are lipid mediators such as prostaglandins and cysteinyl leukotrienes (CysLTs). These 2 lipid mediators are the products of metabolism of arachidonic acid. prostaglandins are formed from the cyclooxygenase pathway, and CysLTs are formed from the 5-lipoxygenase pathway, both pathways are downstream of arachidonic acid. The first product of the 5-lipoxygenase pathway is leukotriene A4, which is then converted to B4, then C4, D4 and finally E4. Doherty et al reported that the CysLT1 receptor, or CysLT1R are expressed on mouse ILC2s and that LTD4 activates it (Doherty et al., 2013). Salimi et al reported that AD patients expressed CysLT1+ ILC2s and that LTC4, LTD4 and LTE4 induced the migration of ILC2s using transwell chemotaxis, with LTE4 being the most potent Leukotriene, and that LTE4 had a synergistic effect with alarmin cytokines IL-25, IL-33 and TSLP.

Furthermore, leukotrienes promoted ILC2 survival and induced the production of IL-4, IL-5 and IL-13. Finally, the author also reported that activated mast cells were the most potent producers of CysLTs (Salimi et al., 2016). Another leukotriene is LTB₄, which binds to the LTB₄R1 and LTB₄R2 receptors, but at a much higher affinity to LTB₄R1. Moltke et al reported that the *Ltb4r1* gene was detected in mouse ILC2s, but not the *Ltb4r2* gene and that LTB₄ predominantly required LTB₄R1 and it induced production of IL-13 (von Moltke et al., 2017).

Prostaglandin is a product of the cyclooxygenase pathway, downstream of arachidonic acid. An important prostaglandin in the context of ILC2s is prostaglandin D₂ (PGD₂), which is predominantly released by mast cells, as well as eosinophils. In another study by Xue et al, skin biopsies were taken from healthy human donors to investigate PGD₂ and CRTH2 in humans. What the author found was PGD₂, with CRTH2 mediated the migration of ILC2s and that CRTH2-activated ILC2s promoted production of IL-4, IL-5 and IL-13. Also, PGD₂ had a synergistic effect with IL-25 had an enhanced cytokine production, but not with IL-33 and TSLP. The study also reported that human mast cells were a potent producer of PGD₂ (Xue et al., 2014). In a letter to the editor, Chang et al investigated whether PGD₂ was involved in migration of ILC2s in atopic patients, compared to healthy controls in peripheral blood. The author reported that atopic patients had a higher dose-dependent increase in ILC2 migration compared to healthy controls and that PGD₂ binds to CRTH2. The study also confirmed Xue's findings that PGD₂ was predominantly produced by activated mast cells, but also eosinophils (Chang, Doherty, Baum, & Broide, 2014).

1.3.2: ILC2 Functions

ILC2s are involved in a myriad of functions. It is involved in the expulsion of extracellular helminth infections. ILC2s release Type 2 cytokines IL-4, IL-5, IL-9 and IL-13 to activate and

recruit eosinophils, smooth muscle contraction, goblet cell hyperplasia and increased mucus production. For example, in mouse studies by Moro et al, Neill et al, and Price et al, IL-13 was required for *Nippostrongylus brasiliensis* expulsion (Fort et al., 2001)(Neill et al., 2010)(April E Price et al., 2010). In addition to IL-13, ILC2s require TCF1 (Yang et al., 2015), GATA3 (Liang et al., 2012) and GFI-1 (Spooner et al., 2013) transcription factors, and alarmin cytokines IL-33 and IL-25 (Yasuda et al., 2012). As stated earlier, ILC2s express MHC-II which can activate TH0, which in turn releases IL-2, which in turn causes proliferation of ILC2s and release of type 2 cytokines.

ILC2s are also involved in tissue repair and healing. ILC2s release amphiregulin, which is an epidermal growth factor receptor (EGFR) ligand. Monticelli LA et al reported amphiregulin is involved in the repair of the bronchial epithelium following infection by the influenza virus (Monticelli et al., 2011). In AD, Salimi et al has shown that amphiregulin was released by ILC2 in lesion skin, following IL-33 stimulation (Salimi, Barlow, Saunders, Xue, Gutowska-Owsiak, et al., 2013).

ILC2s are also involved in homeostatic metabolism, especially in obesity. Molofsky et al reported that ILC2s are found in visceral adipose tissue and a rich source of IL-5 and IL-13, which are required for the recruitment of eosinophils and alternatively activated macrophages. These cells are implicated the homeostatic metabolism (Figures 1 and 2) (Molofsky et al., 2013).

In mouse a model by Teunissen et al, ILC2s are commonly detected in the dermis, and in close proximity to the epidermis and migration to the epidermis is mediated by IL-33 and PGD2 (Marcel B M Teunissen et al., 2014). Although there are studies showing that ILC2s reside in the skin suggesting local expansion, ILC2s in the peripheral circulation have been shown to express skin homing markers such as CLA, CCR10, suggesting that these cells may originate in the bone

marrow and with the potential to extravasate and migrate to the skin (Salimi, Barlow, Saunders, Xue, Gutowska-Owsiak, et al., 2013)(Marcel B M Teunissen et al., 2014).

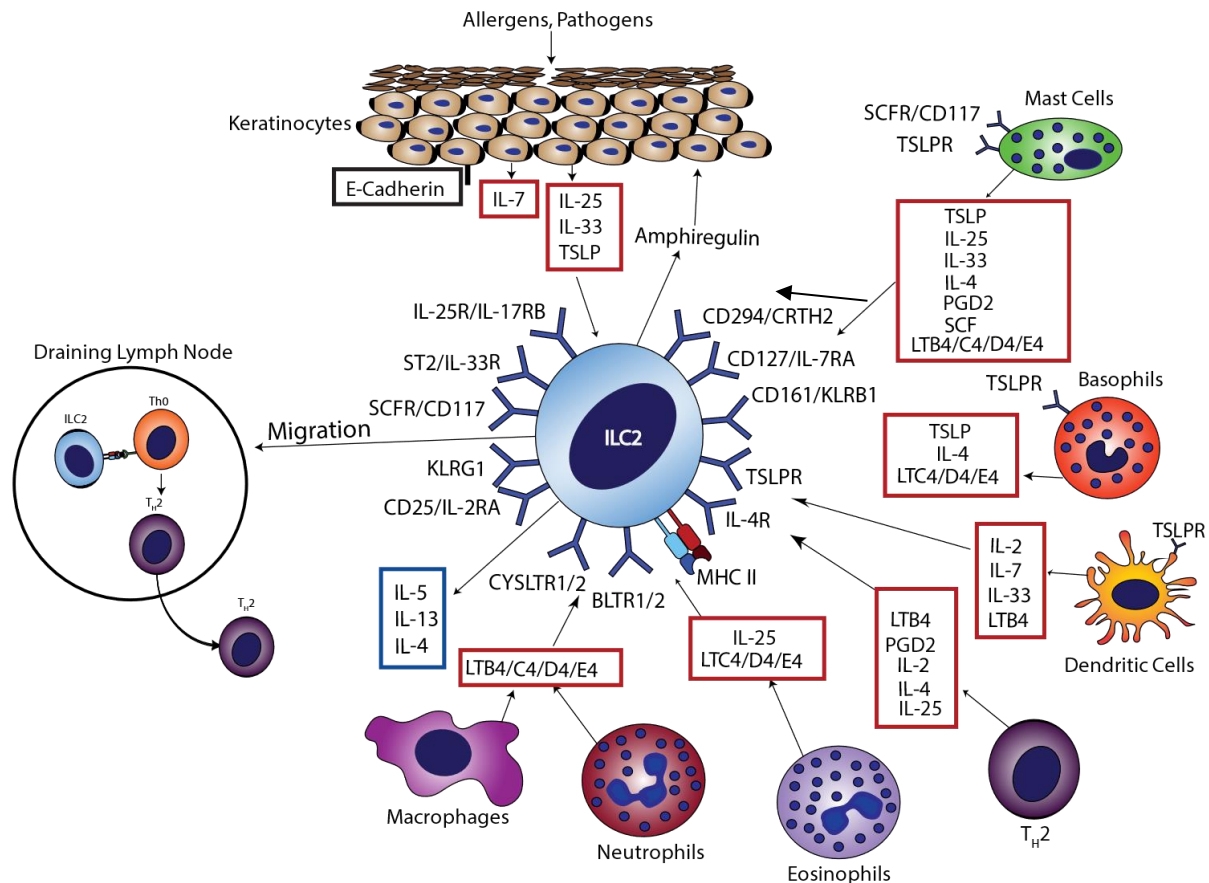


Figure 1: Activation of Type 2 Innate Lymphoid Cells (ILC2s). ILC2s are activated by alarmin cytokines TSLP, IL-25, IL-33, γ -chain cytokines IL-2, IL-4 and IL-7, lipid mediators Prostaglandin D2 and LeukotrieneB4/C4/D4/E4 (Red box). ILC2s are regulated by E-Cadherin (Black box). ILC2s express MHC II which have antigen-presenting capabilities. Activated ILC2s produce Type 2 cytokines IL-4, IL-5 and IL-13 (Blue Box).

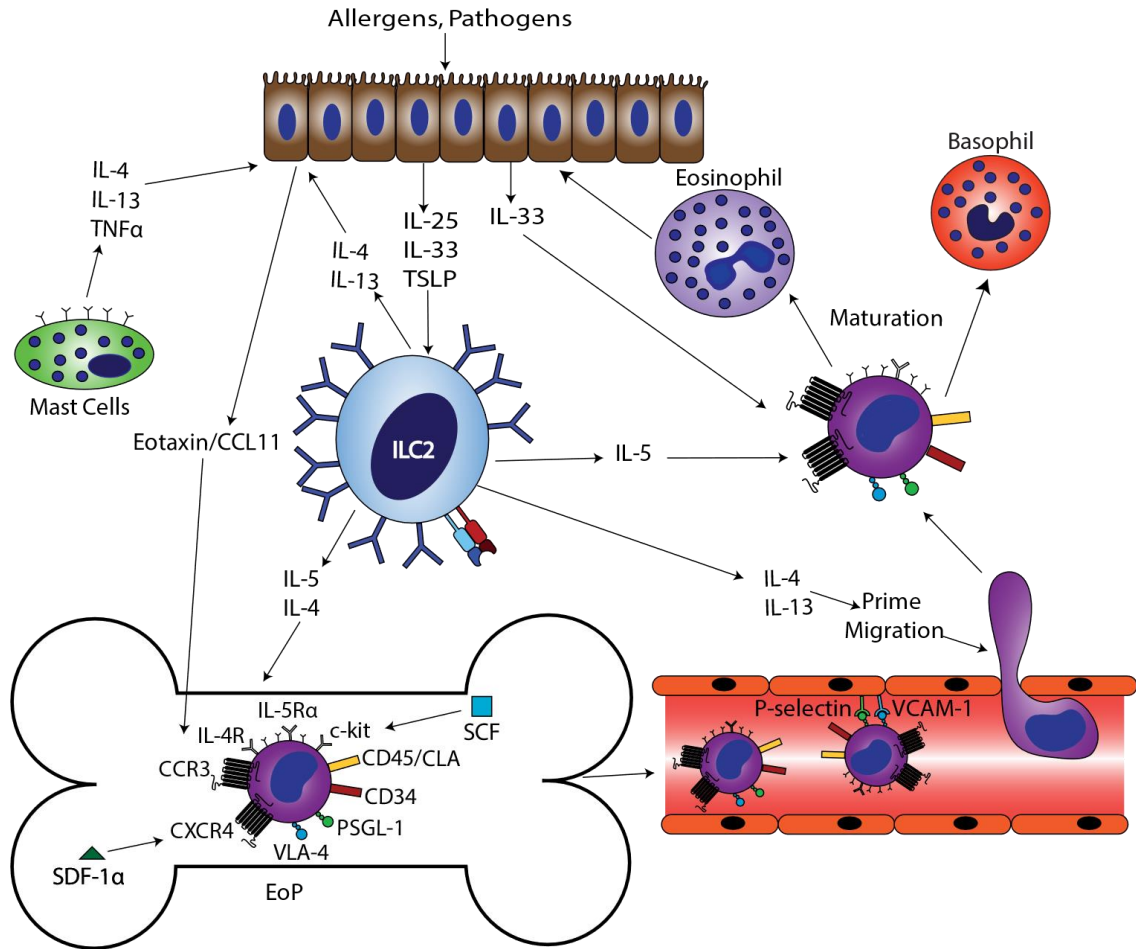


Figure 2: Activated ILC2s release Type 2 cytokines IL-4, IL-5 and IL-13 which mediates the migration of Eosinophil Progenitors (EoPs) from the bone marrow to the circulation. IL-4 and IL-13 primes the migration of EoPs out of the circulation and to the site of inflammation. IL-5 then promotes the maturation of EoPs to eosinophils and/or basophils, where eosinophils release proinflammatory mediators such as Eosinophil Basic Protein, Eosinophil Cationic Protein, Eosinophil Peroxidase and Eosinophil-Derived Neurotoxin.

In addition, the frequency ILC2s are higher in skin lesional biopsies from AD patients compared to skin from healthy patients (Salimi, Barlow, Saunders, Xue, Gutowska-Owsiak, et al., 2013)(B S Kim et al., 2013). Skin homeostasis is also mediated by ILC2s as ILC2s express KLRG1 and keratinocytes or Langerhan Cells express E-Cadherin. It has been shown that the KLRG1-E-Cadherin interaction inhibits ILC2 cytokine generating activity (Salimi, Barlow, Saunders, Xue, Gutowska-Owsiak, et al., 2013). Conversely, this interaction also causes ILC2s to produce amphiregulin and it is needed in keratinocyte proliferation, apoptosis, skin remodelling and wound healing(Salimi, Barlow, Saunders, Xue, Gutowska-Owsiak, et al., 2013). In a mouse model study, mice with Rag^{-/-}, where T-cells and B-cells were knocked-out, found that ILC2s could induce AD independent of T-cells and B-cells (Salimi, Barlow, Saunders, Xue, Gutowska-Owsiak, et al., 2013), which shows that ILC2s play a role in AD. In addition, in IL-33-driven dermatitis skin lesions, ILC2s expanded and produced IL-5 and IL-13, which was inhibited by systemic IL-5 inhibition (Imai et al., 2013). This suggests that activated ILC2s have pro-inflammatory properties that could recruit immune cells. While the discovery of ILC2 exhibits a redundancy in the immune system for sources of type 2 cytokines that promote eosinophilic inflammatory diseases, it is important to understand the role of these novel cells in atopic dermatitis. This is especially highlighted from chronic inflammatory conditions such as severe prednisone dependent asthma where the gold-standard for treatment, steroid effectively attenuated T-cell activation but ILC2 mediated Il-5 and Il-13 generation was unaffected (Smith et al., 2016). Whether this indicates steroid insensitivity of the ILC2s or the inability of the steroids to control alarmin production that would stimulate local ILC2 activation remains to be investigated.

1.3.3: Detecting ILC2s

Currently, ILC2s are identified by flow cytometry staining, followed by a sequential multi-gating strategy (Salimi, Barlow, Saunders, Xue, Gutowska-Owsiak, et al., 2013)(Walford et al., 2014)(Smith et al., 2016)(Brian S Kim et al., 2014). Recently, Brügger et al reported identifying ILC2s using immunofluorescence staining on frozen sections from lesional skin from patients with AD, as well as punch biopsies from healthy controls. The authors found was that GATA3+ ILC2s were not detected in healthy control skin, but were elevated in AD lesional skin (Brügger et al., 2016). Although ILC2s were detected in lesional skin by flow cytometry (Salimi, Barlow, Saunders, Xue, Gutowska-Owsiak, et al., 2013)(Brian S Kim et al., 2014), it was also detected in healthy control skin. Salimi et al. and Kim et al. performed flow cytometry on punch biopsies collected from lesional skin, while healthy control skin was collected from routine abdominoplasty procedures from plastic surgery. Using this method of extracting cells from abdominoplasty tissues, more cells can be collected from big piece of tissue and therefore, a higher chance of quantifying ILC2s from skin of normal control patients. In addition, flow cytometry is more sensitive than IF staining, as flow cytometry employs the use of multicolour sequential gating strategies to exclude debris, doublet cells (cell clumping which the system counts as 1 cell), and dead cells. Furthermore, flow cytometry employs isotype controls to account for background noise. This allows the user to identify their cells of interest with a high confidence. There are few limitations with these studies. The healthy control skin and lesional skin are taken from 2 groups of patients (i.e.: healthy controls and AD patients). In the immunofluorescence study by Brügger et al., the author used frozen sections which may not be a good idea to preserve the tissue architecture, compared to FFPE tissues. Finally, in the same study, the author used antibodies optimized for flow cytometry, instead antibodies optimized for

immunofluorescence/immunohistochemistry staining. Here, we improved Brüggen's protocol to detect ILC2s in AD FFPE tissues by IF staining. While Kim BS et al did develop an IF staining panel to identify ILC2s in the skin, the author based it from staining the tissue with ST2 and CD3 (Brian S Kim et al., 2014). Therefore, ILC2s were identified as CD3-ST2+. The caveat however, is that ST2 is not only expressed by T-Helper Cells and ILC2s. ST2 is also expressed by basophils, CD34+ progenitor cells, epithelial cells in the bronchus, smooth muscle cells in the airway, mast cells, fibroblasts and goblet cells. Therefore, the number of ILC2s reported by Kim may be an over-estimation.

Laffont reported that in mouse asthma models, male mice had a reduced number of ILC2Ps, as well as mature ILC2s, compared to female mice. ILC2Ps were found to express androgen-receptor and androgen signalling inhibited the differentiation to mature ILC2s and restricted IL-33 mediated expansion of ILC2s in airway inflammation (Laffont et al., 2017). Cephus et al reported that female asthma patients have a higher number of ILC2s in the peripheral blood compared to males. However, the author did not report the number of ILC2s in sputum. In addition, 5 α -dihydrotestosterone inhibited type 2 IL-5 and IL-13 expression in lung ILC2s in mouse models following *Alternaria* exposure and that ILC2s in mice were negatively regulated by testosterone (Cephus et al., 2017). In another study by Warren et al, both ovalbumin and saline-treated female mice had an increased protein expression of IL-5 and IL-13 by *ex vivo* stimulation of IL-33, but no increase was seen in mice (Warren et al., 2017). Sex differences in human atopic dermatitis was assessed in this thesis.

1.4: CENTRAL HYPOTHESIS

ILC2s are preferentially increased in skin of patients with moderate-to-severe Atopic Dermatitis.

SPECIFIC HYPOTHESIS AND AIMS

Specific Hypothesis 1: ILC2s cell numbers are greater in lesional skin compared to peripheral blood from patients with moderate-to-severe Atopic Dermatitis

Aim 1: To quantify total ILC2s compared to other type 2 cells including EoPs and CD4+ cells in peripheral blood and skin by flow cytometry

Aim 2: To quantify Type-2 cytokine-producing ILC2s, Eosinophil Progenitors (EoPs) and CD4+ in peripheral blood and skin by flow cytometry

Specific Hypothesis 2: ILC2 numbers are greater in lesional skin compared to non-lesional skin, as well as allergen-challenged skin compared to diluent-challenged skin, from patients with moderate-to-severe Atopic Dermatitis

Aim 1: To quantify ILC2s in Formalin-Fixed Paraffin-Embedded (FFPE) skin biopsies (Lesion, Non-Lesion, Allergen-Challenge, and Diluent-Challenge)

Aim 2: To correlate the ILC2 counts with eosinophil counts, as well as TSLP, and IL-33 counts on the Lesion, Non-Lesion, Allergen-Challenge, and Diluent-Challenge FFPE skin biopsies.

Aim 3: To quantify CD3+ cells in Formalin-Fixed Paraffin-Embedded (FFPE) skin biopsies (Lesion, Non-Lesion, Allergen-Challenge, and Diluent-Challenge)

CHAPTER 2: MATERIALS AND METHODS

Patient Inclusion/Exclusion Criteria

Consenting patients with moderate-to-severe AD aged between 18 to 65 years were recruited from McMaster University Medical Centre. Ethics approval for this study was provided by the Hamilton Integrated Research Ethics Board (HiREB). AD patients were skin prick test positive for common allergens (e.g.: house dust mite, cat, dog, ragweed, pollen, etc.), showed a positive late cutaneous response to an intradermal allergen challenge, comparing the size of the wheal with a diluent (saline) control wheal and a positive control (histamine) wheal 24-hours post-challenge and were in generally good health. Exclusion criteria includes, pregnancy, treatment with an investigational drug, treatment with prescription moisturizers, use of tanning booths, 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, history of Human Immunodeficiency Virus (HIV) infection or Hepatitis B or C infection, any presence of skin comorbidities, immunosuppressive and immunomodulating drugs such as cyclosporine, calcineurin inhibitors and methotrexate. Patients on these immunosuppressive and immunomodulating drugs were asked to washout for 4 weeks prior to commencement of the study.

Patient Surveys and Assessments

Clinical measures of disease severity/symptoms including Eczema Area and Severity Index (EASI) score, the Dermatology Life Quality Index (DLQI) score, the Patient-Oriented Eczema Measure (POEM) score, the Investigator Global Assessment (IGA), and Severity Scoring of Atopic Dermatitis (SCORAD) scores were evaluated. Patients were asked to fill up the DLQI and POEM surveys. The purpose of the DLQI is to determine how much the patient's AD problem had

affected their Quality of Life during the week before the visit. Questions such as the severity of itching and pain, embarrassment and self-consciousness, the amount of influence AD had on the type of clothing worn, severity of disruptions during work/study and during leisure activities and social interactions. Scores were ranked from “Not At All” to “Very Much”. The scoring for each question were as follows: Very Much: 3 Points, A Lot: 2 Points, A Little: 1 Point, Not At All: 0 Points, Not Relevant: 0 Points, Yes/No: 3 Points for Yes. Scores were combined with a minimum score of 0 points and a maximum of 30 points. The quality of life was more impaired when the score increased. Scores were interpreted as follows: 0 – 1 Points: “No effect at all on patient’s life”, 2 – 5 Points: “Small effect at all on patient’s life”, 6 – 10 Points: “Moderate effect at all on patient’s life”, 11 – 20 Points: “Very large effect at all on patient’s life”, and 21 – 30 Points: “Extremely large effect at all on patient’s life”. For the POEM survey, it asked patients the duration (days), rather than severity of AD. For example, the number of days where it was itchy due to AD, sleep disruption, and bleeding and oozing. Scores were ranked as 0 for “No Days”, 1 for “1 – 2 Days”, 2 for “3 – 4 Days”, 3 for “5 – 6 Days”, and 4 for “Every Day”, with a maximum score of 28. Scores were interpreted as follows: 0 – 2: “Clear/Almost Clear”, 3 – 7: “Mild Eczema”, 8 – 16: “Moderate Eczema”, 17 – 24: “Severe Eczema”, and 25 – 28: “Very Severe Eczema”.

The EASI score was performed by a dermatologist, to evaluate the severity of AD and the area of involvement. The dermatologist evaluates the head and neck, trunk, upper extremities, and lower extremities and determines the score to represent the percentage of involvement in each respective area. The severity was then evaluated by looking at the level of erythema (redness), the papulation (skin thickness), excoriations (skin-picking), and lichenification (scratching/rubbing), and giving a score between 0 to 3, 0 being none to 3 being severe in each area as described above. The scores were then calculated with 0 as “Clear” and the maximum at 72 as “Very Severe”. 0.1

– 1.0: “Almost Clear”, “1.1 – 7.0: “Mild”, 7.1 – 21.0: “Moderate”, 21.1 to 50.0: “Severe” and 50.1 to 72.0: “Very Severe”.

The SCORAD score was performed by the dermatologist. The SCORAD was somewhat similar to the EASI (looking at areas of involvement and clinical symptoms, but with an emphasis on the severity of itching and loss of sleep). The scores were then calculated with 0 as the lowest possible score and maximum at 103. “Mild” was less than 25, “Moderate” was between 25 to 50, and “Severe” was more than 50.

The IGA was also completed by the dermatologist to determine the severity of AD by evaluating the severity of erythema, and papulation, with 0 as “Clear”, 1 as “Almost Clear”, 2 as “Mild Disease”, 3 as “Moderate Disease”, 4 as “Severe Disease”, and 5 as “Very Severe Disease” A copy of the survey and evaluation sheets attached at the Appendix section. Results of these tests were outlined in Table 1 in the results chapter.

Drug Washout and Subsequent Steroid Run-in

Systemic immunosuppressive/immunomodulating drugs were withdrawn 4 weeks before sample collection. Patients who had a skin flare up from their washouts were given topical clobetasol to provide relief, except for the lower back where the biopsies were taken. Sixteen days prior to sample collection, all patients entered a run-in period of 0.25mg/Kg body weight of prednisolone for 8 days, and an 8-day washout from the steroid. The purpose is to: 1) prevent any bad flare-ups seen in severe patients, and 2) to normalize all patients to a baseline level of steroids (Figure 1).

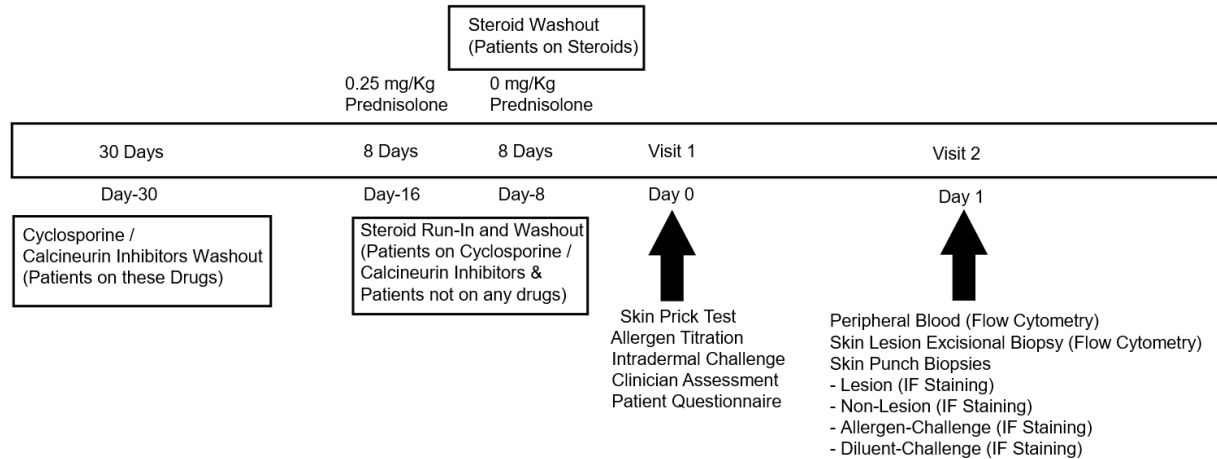


Figure 1: Flow chart detailing the study timeline for each patient. For the lesional skin, a 3 x 1 cm excisional biopsy was taken typically from the lower back for flow cytometric analyses. In addition, 4 mm punch biopsies from the lesional excised skin, non-lesional skin, allergen-challenged skin, and diluent-challenged skin were taken to be used for immunofluorescence staining. Blood was also drawn at Day 1 and was used for flow cytometry enumeration of type 2 cells.

Clinical Tests and Sampling

For this thesis, the following clinical tests and sampling was performed: skin prick test, intradermal allergen challenge, a series of punch biopsies on skin. An excisional skin biopsy was performed, as well as a blood draw.

Skin Sample Collection

Two percent lidocaine was administered to the challenge sites, as well as the lesional and non-lesional skin. Four-millimeter punch biopsies were taken from the lesional skin, non-lesional skin, allergen-challenged skin, and diluent-challenged skin. A 3 x 1 cm excisional biopsy was taken from the lesional skin. The punch biopsies were used for immunofluorescence staining, while the excisional biopsy and blood was used for flow cytometry staining.

Isolation of Immune Cells from Peripheral Blood

20mL of peripheral blood was drawn and collected in sodium heparin tubes. The blood was mixed with 1:1 McCoy's 5A (Modified) Medium (Life Technologies) and layered on Lymphoprep density gradient medium (STEMCELL Technologies). Following centrifugation, the buffy coat (i.e.: Peripheral Blood Mononuclear Cells (PBMC)) was collected and washed with McCoy's 5A and resuspended in McCoy's 5A to form a single-cell suspension and counted prior to immunofluorescence staining. The isolated PBMCs cells were normalized to number of cells resuspended in 1mL of FACS buffer.

Isolation of Immune Cells from Lesional Skin

A 3 x 1 cm excision biopsy was taken from lesional skin under 2% lidocaine. The biopsy was placed in 1X Phosphate-Buffered Saline (PBS) at 37°C with weight and dimensions taken. Hair and subcutaneous fat were trimmed and removed prior to mincing the tissue. The biopsy was minced using dissection scissors until a paste was formed and incubated in 1X PBS for 2 hours at 37°C with rocking. The minced tissue was incubated in dispase solution (Dispase (1U/mL, STEMCELL Technologies), 10% Fetal Bovine Serum (FBS) (Life Technologies), RPMI Complete Medium (RPMI-C) (In-House), and Penicillin-Streptomycin (10,000 U/mL, Life Technologies), overnight for 16-20 hours at 37°C with rocking. Collagenase P (0.8mg/mL, Sigma-Aldrich) and DNase I Solution (1mg/mL, STEMCELL Technologies) was then added to the minced tissue the following morning and incubated for 45 minutes at 37°C with rocking. The tissue and supernatant were filtered through a 50µM filter mesh, washed with RPMI-C and the cell pellet resuspended in Fluorescence-Activated Cell Sorting (FACS) buffer to form a single-cell suspension. Cell number was quantified using trypan blue (0.4%, Corning) staining of an aliquot

and a hemocytometer. The isolated single-cell suspension was then subject to immunofluorescence staining and enumerated by flow cytometry. The isolated skin cells were normalized to number of cells in 1g of tissue resuspended in 1mL of FACS buffer.

Flow Cytometry Staining

Upon isolating single-cell suspensions from peripheral blood (PBMC) and lesional skin (lymphocytes, granulocytes and keratinocytes), cells were stained for ILC2s, EoPs, CD4+, and basophils (skin only) with antibodies to the extracellular surface markers: Lineage cocktail-FITC (eBioscience), FcεR1-FITC (eBioscience), CD94-FITC (BioLegend), TCRα/β-FITC (eBioscience), TCRγ/δ-FITC (eBioscience), CD45-APC-H7 (BD Biosciences), CD4-PE-Cy7 (BD Biosciences), CD34-PerCP-Cy5.5 (BD Biosciences), CD125 (IL-5Rα)-APC (R&D Systems), Mouse IgG1κ Isotype-APC (R&D Systems), CD127 (IL-7Rα)-BV605, Mouse IgG1κ Isotype-BV605 (BD Biosciences), CRTH2-BV510 (BD Biosciences), Rat IgG2α,κ Isotype-BV510 (BD Biosciences), CD123 (IL-3Rα)-BV650 (BD Biosciences), Mouse IgG2α,κ Isotype-BV650 (BD Biosciences), HLA-DR-PE-CF594 (BD Biosciences) and Mouse IgG2α,κ Isotype-PE-CF594 (BD Biosciences), and incubated for 30 minutes at 4°C. Cells were incubated with Fixation/Permeabilization Solution (BD Biosciences) for 20 minutes at 4°C and stained for intracellular cytokine markers: IL-5-PE (BD Biosciences), Rat IgG2α Isotype-PE (BD Biosciences) and IL-13-BV421 (BD Biosciences), and Rat IgG1 Isotype-BV421 (BD Biosciences) for 30 minutes at room temperature. Cells were resuspended in FACS buffer and stored in the dark and refrigerated. Cells were acquired using a FACS LSR II flow cytometer within 2-3 days and data analyses was performed using FlowJo and cell-type specific gating strategies are shown in the Results chapter.

Intradermal Allergen Challenge

After collecting the informed consent, the patient was sent for a Radioallergosorbent Test (RAST) in the core lab at McMaster University Medical Centre to enumerate the patient's total, as well as specific IgE antibodies. A skin prick test was also performed to confirm the RAST test result and screen the patient for common allergies (e.g.: House dust mites, dog, cat, horse, tree, grass, ragweed, etc.) on the patient's upper back. The dermatologist decided which allergen to use for the intradermal allergen challenge based on the size of the wheal. A titration was done to determine a suitable dilution (from 1:1 to 1:1024) using a skin prick test on another area on the patient's upper back. An optimal dilution of allergen was one that produced a 4 x 4 mm wheal when compared to the positive control (histamine) and negative control (saline). Allergen (100 µl) was injected into the dermis and the size of the wheal was compared to the negative control (saline). The allergen wheal had to be larger than the negative control wheal. The early cutaneous response was measured after 10 minutes and the size of the wheal was recorded. The patient returned 24 hours later for the biopsies on the lesional skin, non-lesional skin, allergen-challenged skin, and diluent-challenged skin, as well as to record the late cutaneous response and wheal size.

Histopathology

The punch biopsies were individually placed in 10% neutral buffered formalin (4% formaldehyde) cups and incubated for 48 hours at room temperature. After 48 hours, the 10% formalin was disposed, and biopsies incubated with 70% ethanol. The biopsies were then sent to the core histopathology facility in the Michael DeGroot Centre for Learning and Discovery at McMaster University, where the biopsies were embedded in paraffin to form individual blocks. The blocks were collected and were sectioned using a microtome. Five micrometer sections were collected

and placed on positively-charged slides to prevent detachment of the sections during the staining process. The slides were air-dried overnight.

ILC2 and CD3 Immunofluorescence Staining

For the immunofluorescence (IF) staining for ILC2s and CD3⁺ cells, a 4-day multicolour sequential staining strategy was developed. On Day 1, the air-dried slides were deparaffinized in xylene and rehydrated in decreasing concentrations of ethanol (100%, 95%, 75%, and 50%). Negative and isotype control slides were included. Cytospins of lineage-negative and lineage-positive cells isolated from PBMCs and fixed with cold methanol on positively-charged slides were used as a positive control. The slides underwent heat-induced antigen retrieval using a pressure cooker for 4 minutes in 10mM pH 6.0 citrate buffer. Slides were cooled and placed in distilled water and 1X PBS. The sections were permeabilized with 0.2% Triton X-100 (Sigma-Aldrich) for 10 minutes at room temperature and washed in 1X PBS for 5 minutes. The sections were blocked with 2% normal goat serum for 20 minutes at room temperature. The sections were washed in 1X PBS 3 times for 5 minutes each. The sections were stained for GATA3 (rabbit anti-human, 1:50 dilution, Invitrogen), with the isotype control slide stained with rabbit Immunoglobulin G (IgG) isotype antibody (1:200 dilution, Novus Biologicals) and negative control slide with 1X PBS and incubated at 4°C in the dark overnight. As keratinocytes express GATA3, it serves as an additional positive control.

On Day 2, slides were washed in 1X PBS 3 times for 5 minutes each. Sections were stained with a GATA3 secondary antibody (Goat anti-rabbit Alexa Fluor 594, 1:250 dilution, Invitrogen) and incubated for 1 hour at room temperature. Slides were washed in 1X PBS 3 times for 5 minutes each. ILC2 slides were stained for CRTH2 (Rabbit-anti-human, 1:50, Invitrogen), while the CD3

slides were stained for CD3 (Mouse anti-human, 1:200, Invitrogen) with the isotype control slide stained with mouse IgG isotype antibody (1:200 dilution, Novus Biologicals) and negative control slide with 1X PBS and incubated at 4°C in the dark overnight. As keratinocytes express CRTH2, it serves as an additional positive control.

On Day 3, slides were washed in 1X PBS 3 times for 5 minutes each. Sections were stained with a CRTH2 secondary antibody (Goat anti-rabbit Alexa Fluor 647, 1:250 dilution, Invitrogen), or CD3 secondary antibody (Goat anti-mouse Alexa Fluor 647, 1:250 dilution, Invitrogen), and incubated for 1 hour at room temperature. Slides were washed in 1X PBS 3 times for 5 minutes each. ILC2 slides were stained with a lineage cocktail, with the CD3 slides were stained with the same lineage cocktail, excluding CD3 and CD4, with the isotype control slide stained with mouse IgG isotype antibody (1:200 dilution, Novus Biologicals) and negative control slide with 1X PBS and incubated at 4°C in the dark overnight.

On Day 4, slides were washed in 1X PBS 3 times for 5 minutes each. Sections were stained with a lineage cocktail secondary antibody (Goat anti-mouse FITC, 1:250 dilution, Abcam), and incubated for 1 hour at room temperature. Slides were washed in 1X PBS 3 times for 5 minutes each. Slides were mounted with Fluoroshield with DAPI (Sigma-Aldrich) and a coverslip and slides were scanned and analyzed with a fluorescence microscope (Table 1).

Eosinophil Counting

After the technicians from the core histopathology facility produce the FFPE blocks, they sectioned 1 slide for hematoxylin and eosin (H&E) staining. The H&E slide was viewed under a light microscope to count the number of eosinophils. The purpose was to perform a Spearman Correlation to determine if ILC2s contributed to tissue eosinophilia.

Marker	Dilution	Company
CD1a	1:20	BD Biosciences
CD3	1:200	Invitrogen
CD4	1:100	Novus Biologicals
CD11c	1:100	Invitrogen
CD14	1:25	eBioscience
CD16	1:100	Invitrogen
CD19	1:50	Invitrogen
CD20	1:200	Invitrogen
CD34	1:100	Invitrogen
CD94	1:20	LSBio
CD123	1:100	LSBio
CD203c	1:20	BioLegend
FcεR1	1:50	Novus Biologicals
GATA3	1:50	Invitrogen
CRTH2	1:50	Invitrogen
Goat anti-Rabbit IgG Secondary Antibody, Alexa Fluor 594	1:250	Invitrogen
Goat anti-Rabbit IgG Secondary Antibody, Alexa Fluor 647	1:250	Invitrogen
Goat anti-Mouse IgG Secondary Antibody, Alexa Fluor 488	1:250	Abcam

Table 1: List of primary and secondary antibodies for immunofluorescence staining

TSLP and IL-33 Immunofluorescence Staining

The immunofluorescence (IF) staining for TSLP and IL-33, a 2-day multicolour sequential staining strategy was developed by Al-Sajee et al (Al-Sajee et al., 2018). The deparaffinization is similar to the ILC2/CD3 staining protocol, while the antigen retrieval time for this staining protocol was set to 5 minutes in 10mM pH 6.0 citrate buffer. Slides were cooled and placed in distilled water and 1X PBS. The sections were permeabilized with 0.2% Triton X-100 (Sigma-Aldrich) for 10 minutes at room temperature and washed in 1X PBS for 5 minutes. The sections were blocked with 2% normal goat serum for 30 minutes at room temperature. The sections were washed in 1X PBS 3 times for 5 minutes each. The sections were blocked with 3% normal goat serum for 40 minutes (TSLP) and 3% normal horse serum for 40 minutes (IL-33) at room temperature. The sections were washed in 1X PBS 3 times for 5 minutes each. For the TSLP slide, a TSLP rabbit anti-human (1:200, Abcam), and for the IL-33 slide, an IL-33 goat anti-human (1:40, ThermoFisher Scientific) was added and 1X PBS was added to both TSLP and IL-33 negative control slide. All slides were incubated overnight at 4°C.

On Day 2, slides were washed in 1X PBS 3 times for 5 minutes each. For the TSLP slide and negative control slide, a mouse anti-rabbit secondary antibody (Alexa Fluor 647 (Cy5) 1:250 dilution, ThermoFisher Scientific) was added, and for the IL-33 slide and negative control slide, a donkey anti-goat secondary antibody (Alexa Fluor 594 (TRITC) 1:20 dilution, ThermoFisher Scientific) was added and incubated for 1 hour at room temperature. Slides were washed in 1X PBS 3 times for 5 minutes each. Slides were mounted with Fluoroshield with DAPI (Sigma-Aldrich) and a coverslip and slides were scanned and analyzed with a fluorescence microscope.

Statistical Analyses

The total skin extracted cell number was adjusted to cells per gram of tissue weight and multiplied by the proportion of population specific events expressed as a percentage of total CD45+ cells to calculate the absolute number of population specific cells in 1 ml of FACS buffer. Similarly, population specific events in the PBMC fraction were expressed as a percent of total CD45+ cells and multiplied by the total MNC cell counts from whole blood to provide the number of cells per mL of blood. A Wilcoxon rank-sum test was used for within group analyses of ILC2, EoP, CD4+ cells enumerated by flow cytometry. Similar analyses were performed for ILC2 and CD3 cells counted in lesional and non-lesional biopsies and from allergen and diluent -challenged skin. In addition, correlation tests were performed with the patients' clinical disease severity scores and TSLP/IL-33 IF staining using non-parametric Spearman rank-correlation with Bonferroni correction. All statistics were performed using GraphPad Prism 6. The *p*-value was set to 0.05.

CHAPTER 3: RESULTS

Patient Demographics

Sixteen patients with moderate-to-severe AD were recruited for this study (n=16; 10 male, 6 female). Thirteen patients were Caucasians, 2 were East Indian, and 1 was East Asian. Eight patients were challenged with House Dust Mites *Dermatophagoides pteronyssinus*, 7 patients were challenged with House Dust Mites *Dermatophagoides farinae*, and 1 to *Alternaria*. Looking at the patient surveys and assessments, the average DLQI score was 13, which was “Very large effect on patient’s life”, the average POEM score was 19, which was “Severe eczema”, the average EASI score was 22, which was “Severe”, the average IGA score was 3.6, which was “Moderate Disease”, and the average SCORAD score was 48, which was “Moderate”, but very close to “Severe”. Baseline demographics are summarized in Table 1.

Identification of ILC2s, EoPs, CD4+ Cells and Basophils in Lesional Skin and Peripheral Blood by Flow Cytometry

ILC2s were identified by sequential gating flow cytometry as Lin-FcεR1-CD94-TCR-CD45+CD127+CD294+ in lesional skin, as well as peripheral blood in patients with moderate-to-severe AD. EoPs were identified as CD45^{dim}+CD34+CD125+ and CD4+ cells were identified as CD45+Lin+CD4+. Intracellular IL-5 and IL-13 cytokine staining of activated ILC2s, EoPs, and CD4+ cells was performed on freshly isolated cells without any further stimulation *either in vitro* or *ex vivo*. We also identified basophils as CD45+Lin+HLA-DR-CD123+. Figures 1-4 shows the gating strategies for ILC2s, EoPs, CD4+ cells and basophils respectively.

Subject	Age (Years)	Sex	Race	Allergen	DLQI	POEM	EASI	IGA	SCORAD
1	18	Female	Caucasian	HD MDF	10	10	7.7	5	47.7
2	51	Male	Caucasian	Alternaria	27	28	48	5	80.8
3	64	Male	Caucasian	HD MDP	17	28	39.9	4	57.5
4	28	Female	Caucasian	HD MDF	4	25	32.6	4	64.7
5	51	Male	Caucasian	HD MDP	14	26	18	4	49.6
6	28	Female	Caucasian	HD MDP	18	26	12.2	3	37.6
7	59	Female	E.I	HD MDF	9	17	10.4	3	29.0
8	54	Male	Caucasian	HD MDF	30	25	43.5	5	80.2
9	40	Female	Caucasian	HD MDF	8	13	7.2	3	29.6
10	22	Male	Caucasian	HD MDF	4	6	40.4	4	61.47
11	22	Female	Caucasian	HD MDF	16	22	30.9	4	56.5
12	39	Male	Caucasian	HD MDP	21	28	21	4	57.90
13	27	Male	Caucasian	HD MDP	16	15	3.2	3	21.1
14	51	Male	Caucasian	HD MDP	9	15	7.5	3	30.76
15	24	Male	E.I.	HD MDP	6	10	7.8	3	27.57
16	18	Male	E.A.	HD MDP	1	12	26.3	4	52.95

Table 1: Patient demographics and clinical characteristics (n=16); E.I – East Indian, E.A – East Asian, HD MDF – House Dust Mite *Dermatophagoides farinae*, HD MDP - *Dermatophagoides pteronyssinus*, DLQI - Dermatology Life Quality Index, POEM - Patient Oriented Eczema Measure, EASI - Eczema Area and Severity Index, IGA - Investigator Global Assessment, SCORAD - Scoring Atopic Dermatitis

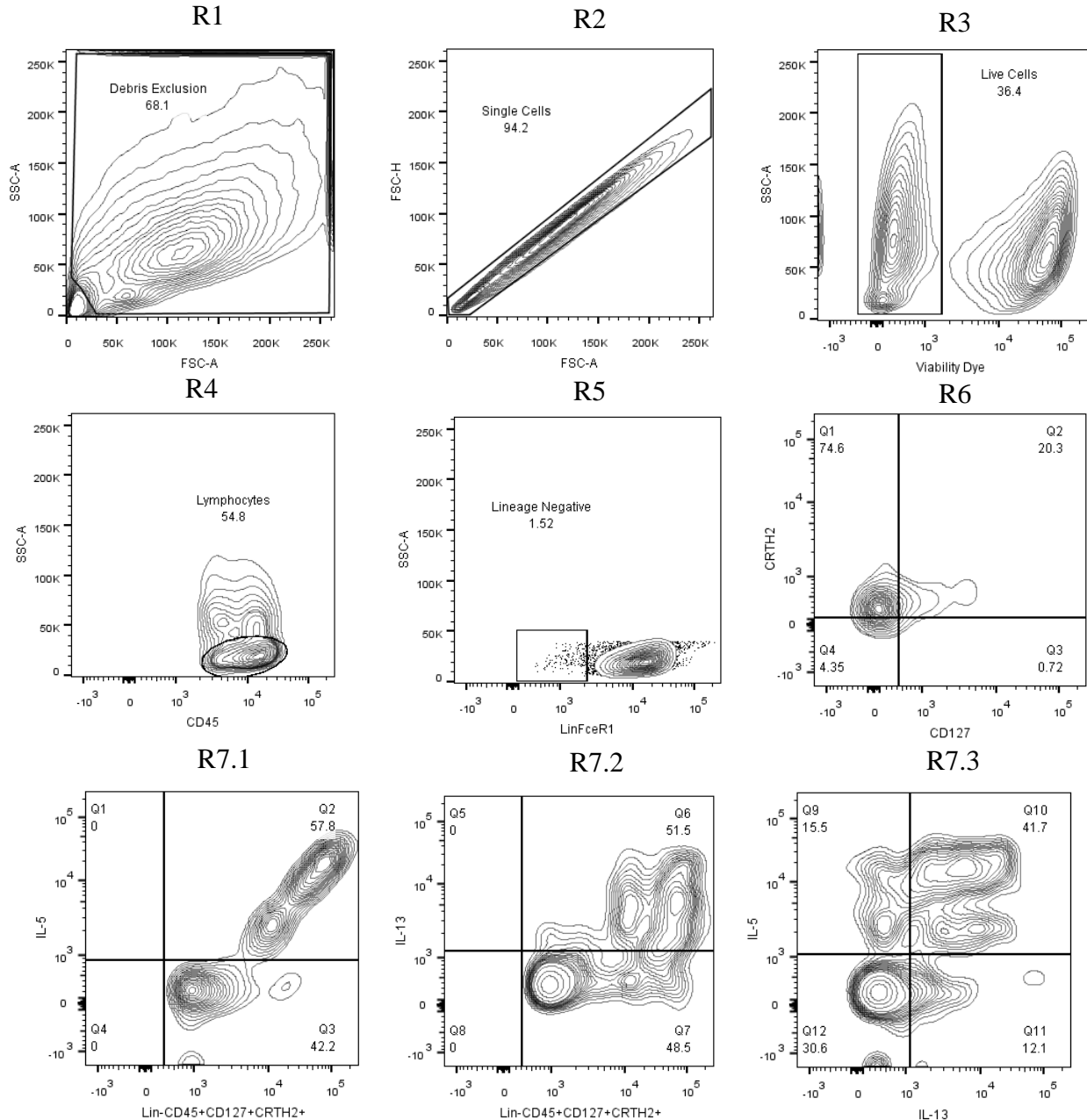
ILC2 Gating Strategy

Figure 1: Multicolor sequential gating strategy for ILC2s as described in Materials and Methods chapter. Cells were gated to exclude debris (R1), identification of singlet cells (R2), and live cells using a viability dye (R3). Lymphocytes were gated on CD45+ cells (R4) and ILC2s were gated as the lineage negative (lin-) population where lin- events were identified using an FMO (R5). Total ILC2s were logically gated as CRTH2+CD127+ events (R6 Q2). Activated ILC2s were identified as IL-5+ (R7.1), IL-13+ (R7.2), and IL-5+IL-13+ (R7.3) events.

EoP Gating Strategy

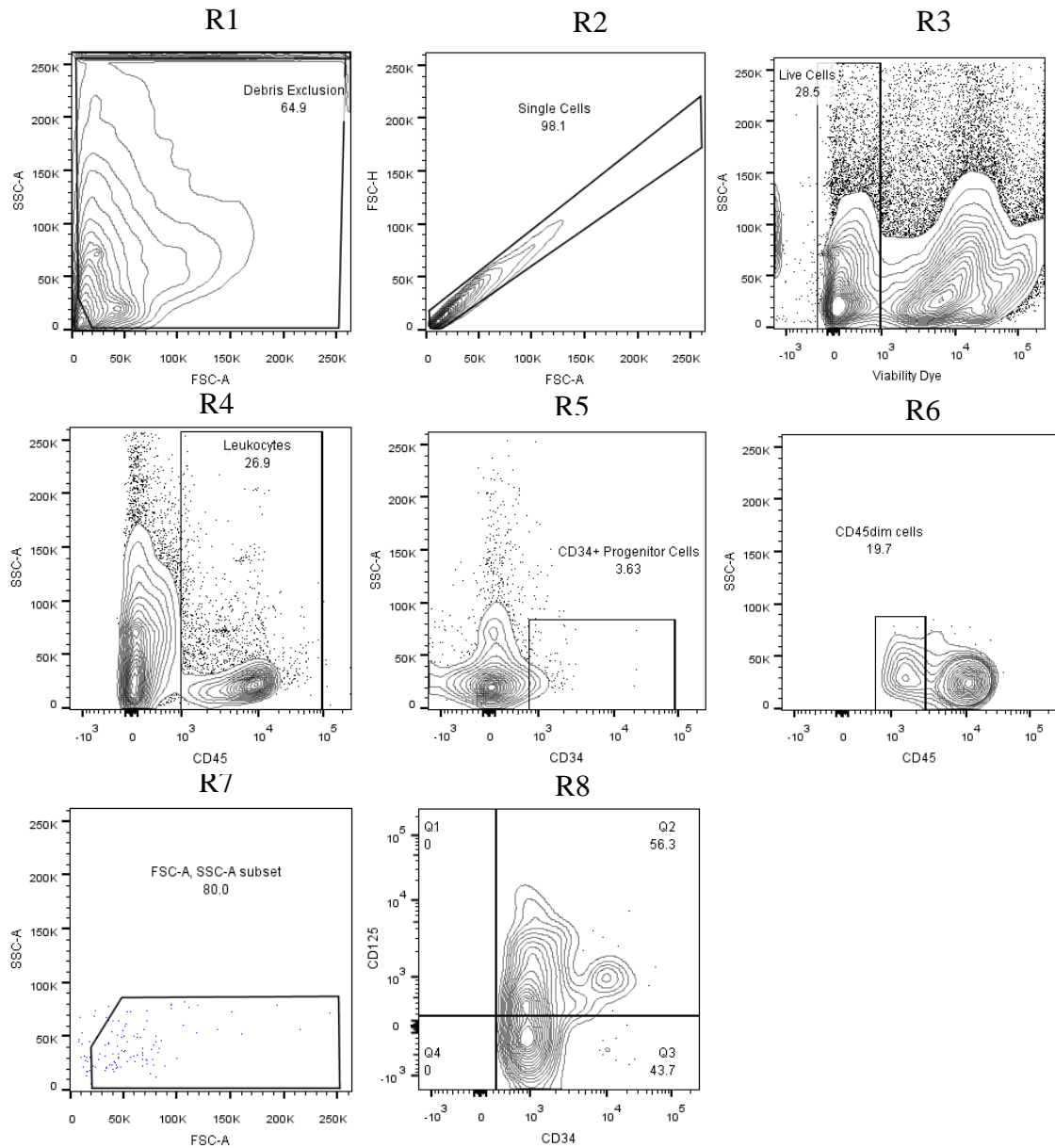


Figure 2: Multicolor sequential gating strategy for EoPs as described in Materials and Methods chapter. Cells were gated to exclude debris (R1), singlet cells (R2), and live cells determined by exclusion of viability dye (R3). Lymphocytes were gated as CD45+ SSC^{low} events (R4) and EoP as CD34+ events (R5) with CD45dim and low granularity (R6 and R7). Total EoPs were identified CD45+CD34+CD125+ (R8)

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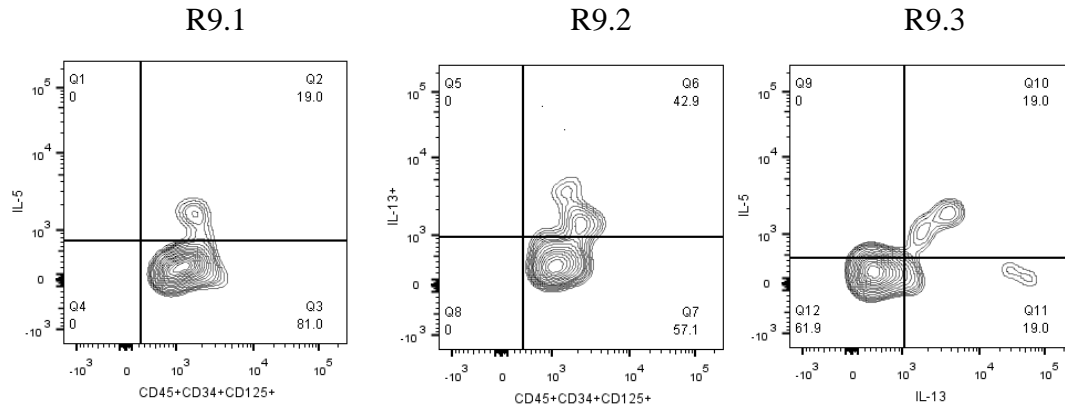


Figure 2 cont.: Activated EoPs were identified as IL-5+ (R9.1), IL-13+ (R9.2), and IL-5+IL-13+ cells (R9.3).

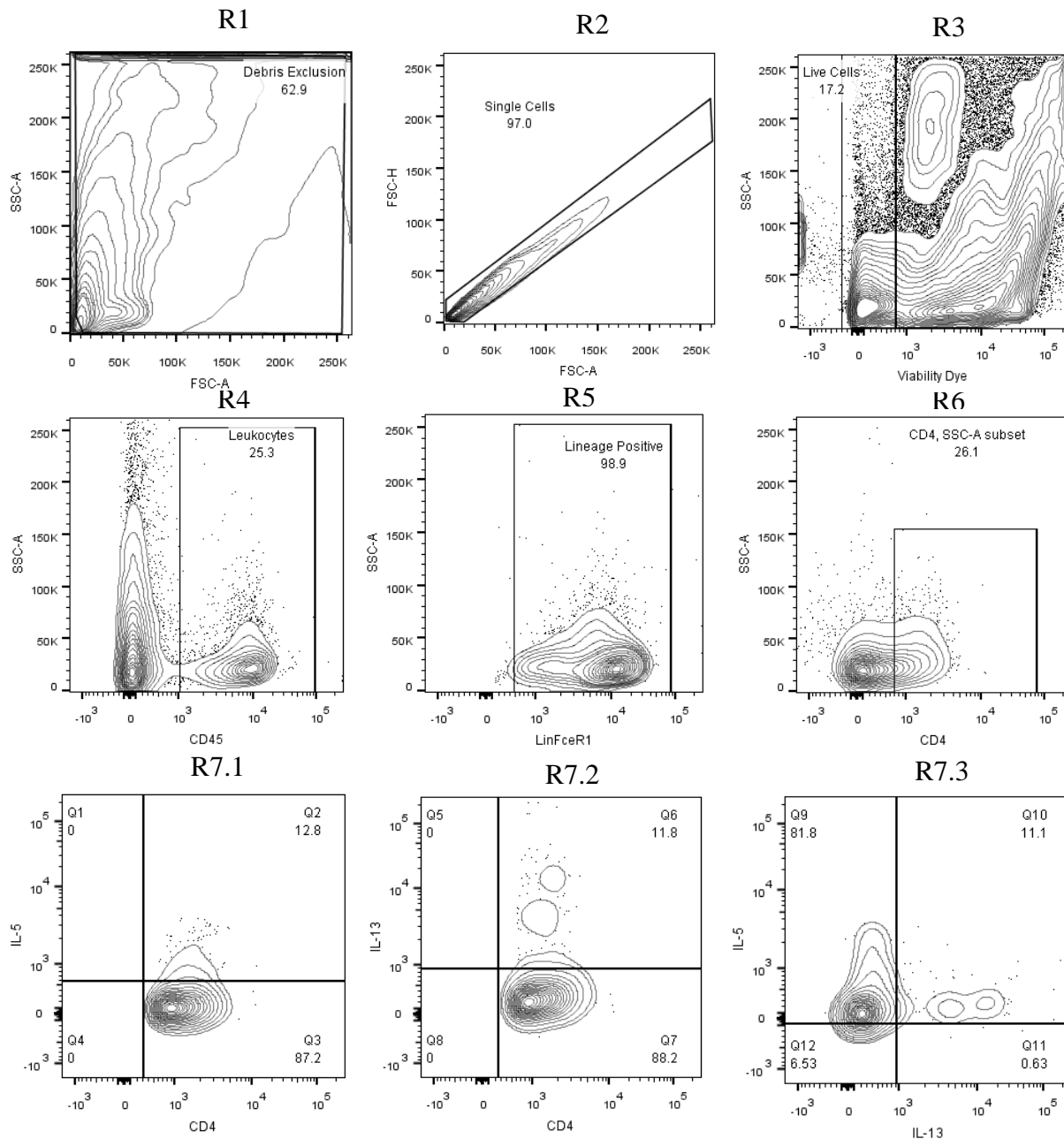
CD4+ Cell Gating Strategy

Figure 3: Multicolor sequential gating strategy for CD4⁺ cells as described in Materials and Methods chapter. Cells were gated to exclude debris (R1), followed by isolation of singlet cells (R2), and live cells were isolated using a viability dye (R3). Lymphocytes were gated from CD45⁺ cells (R4). CD4⁺ cells were defined as lineage positive (lin⁺) and lin⁺ were identified using an FMO (R5). From there, total CD4⁺ cells were identified (R6). Activated CD4⁺ cells were identified as IL-5⁺ (R7.1), IL-13⁺ (R7.2), and IL-5⁺IL-13⁺ (R7.3).

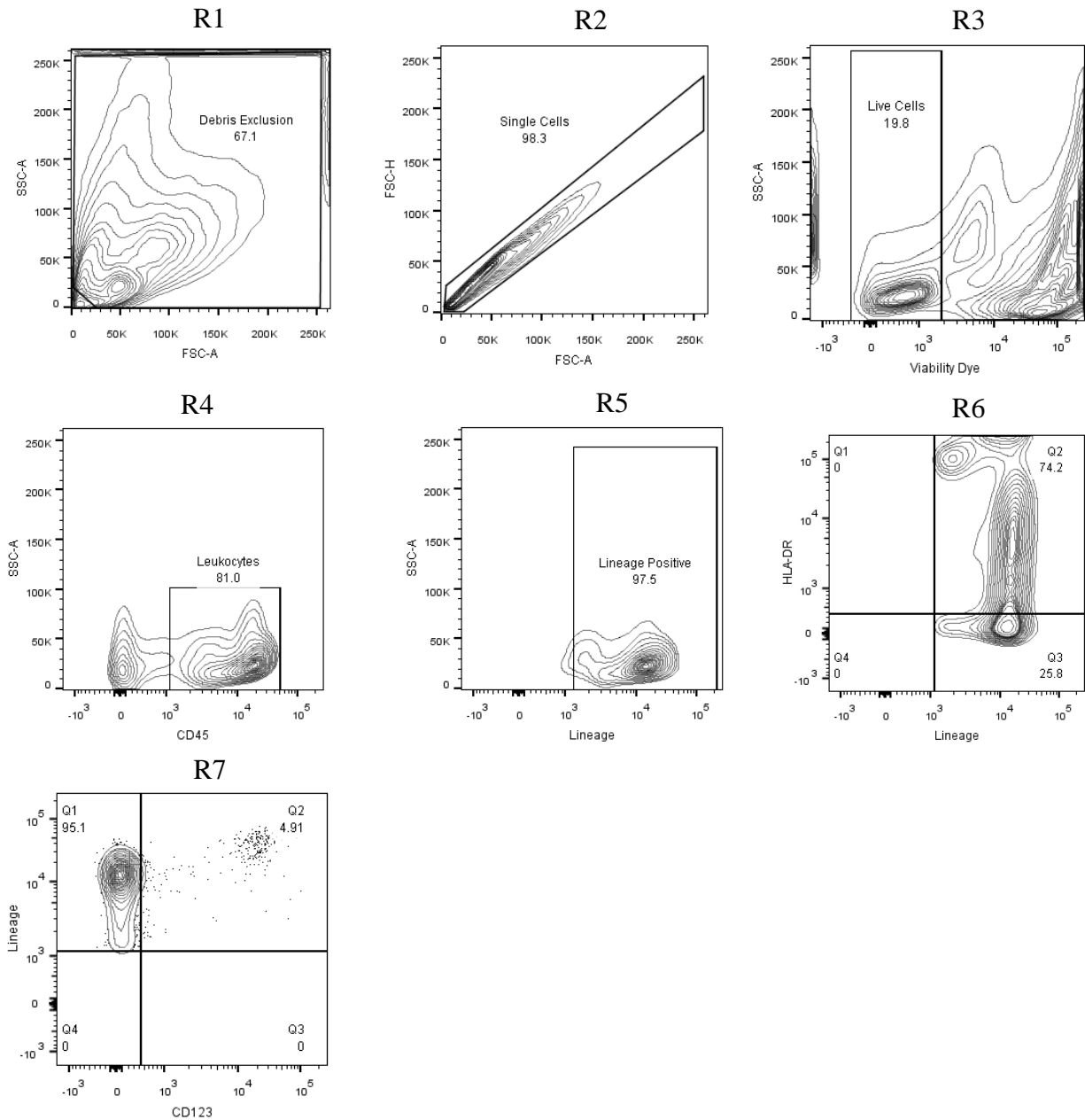
Basophil Gating Strategy

Figure 4: Multicolor sequential gating strategy for Basophils as described in Materials and Methods chapter. Cells were gated to exclude debris (R1), followed by isolation of singlet cells (R2), and live cells were isolated using a viability dye (R3). Lymphocytes were gated from CD45+ cells (R4). Basophils cells were defined as lineage positive (lin+) and lin+ were identified using an FMO (R5). From there, total basophils were identified as Lin+HLA-DR-CD123+ (R6 and R7).

Enumeration of ILC2s in Lesional Skin and Peripheral Blood

We measured ILC2 levels in lesional skin and peripheral blood of all patients with AD. The absolute number of ILC2s identified by flow cytometry were a significantly greater in skin compared to blood (556 [99 – 5501] vs 235 [67 – 569] cells/mL, $p=0.03$). Similarly, for cytokine-producing activated-ILC2s, there were significantly greater numbers of IL-5+ILC2s (6 [1 – 666] vs 1 [1 – 19] cells/mL, $p=0.03$), and IL-13+ ILC2s (28 [1 – 1357] vs 1 [1 – 7] cell/mL, $p=0.01$) in the skin compared to blood. There was a higher number of IL-5+IL-13+ ILC2s in skin compared to blood, this was not significant (5 [1 – 470] vs 1 [1 – 14] cells/mL, $p=0.11$) (Fig 5). When expressed as a percentage of CD45+ cells, they were a significantly greater percentage of total ILC2s in skin compared to blood (0.04 [0.003 – 0.181] vs 0.006 [0.003 – 0.018] %, $p=0.04$). Similarly, for cytokine-producing activated-ILC2s, there were significantly greater percentage of IL-5+ILC2s (0.0 [0.0 – 0.00102] vs 0.0 [0.0 – 0.0004] %, $p=0.03$), and IL-13+ ILC2s (0.001 [0.0 – 0.02] vs 0.0 [0.0 – 0.004] %, $p=0.02$) in the skin compared to blood. There was a higher percentage of IL-5+IL-13+ ILC2s in skin compared to blood, this was not significant (0.0 [0.0 – 0.01] vs 0.0 [0.0 – 0.001] %, $p=0.1289$).

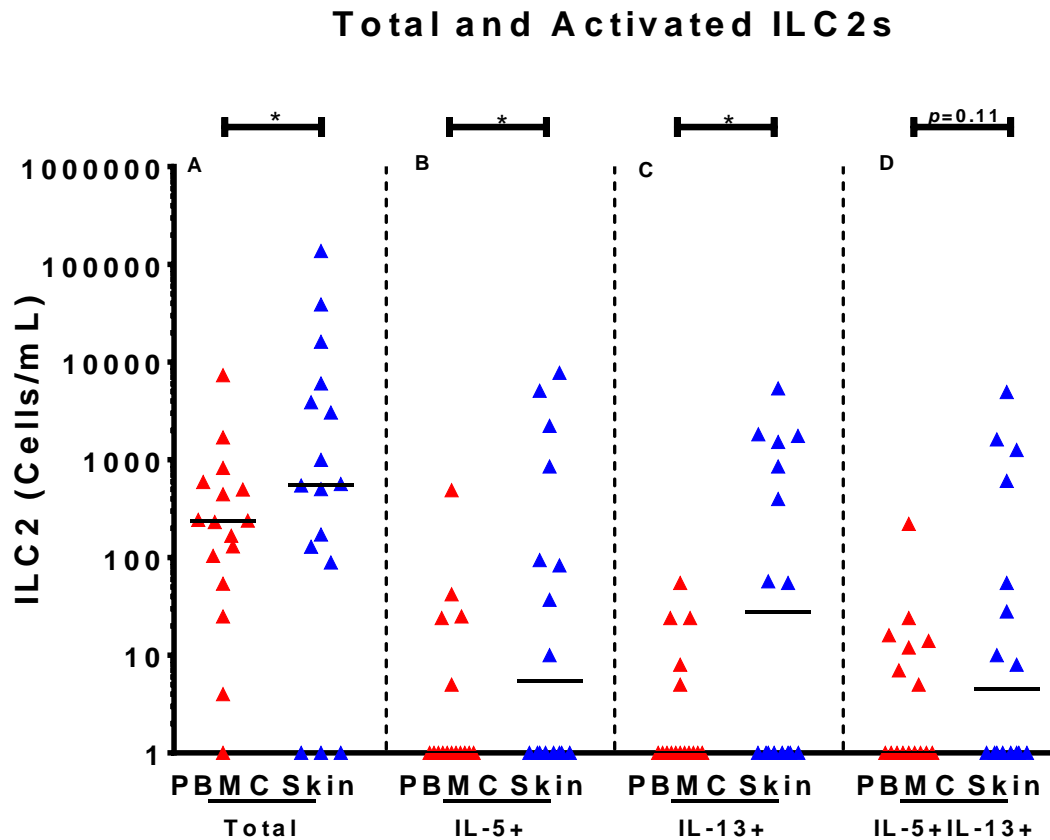


Figure 5: Comparison of total and intracellular cytokine expression levels of ILC2s. **(A)** A significant increase seen in total ILC2s in the skin compared to blood (n=16) **(B-C)** A significantly higher number of IL-5+ and IL-13+ producing ILC2s in the skin, compared to blood (n=16) (* $p < 0.05$). **(D)** There was no significant difference seen between IL-5+ and IL-13+ producing ILC2s in the blood and skin (n=16); (* $p < 0.05$). (Note: Cells that were zero were changed to 1 to account for log scale)

Enumeration of EoPs in Lesional Skin and Peripheral Blood

We measured EoP levels in lesional skin and peripheral blood of all patients with AD. The absolute number of EoPs identified by flow cytometry were significantly greater in skin compared to blood (815 [285 – 2794] vs 112 [46 – 247] cells/mL, $p<0.01$). For cytokine-producing activated-EoPs, there was a trend for higher number of IL-5+ EoPs in the blood compared to the skin (36 [1 – 129] vs 1 [1 – 23] cells/mL, $p=0.07$) and significantly higher number of IL-13+ EoPs (92 [10 – 182] vs 1 [1 – 8] cells/mL, $p<0.01$), and IL-5+IL-13+ EoPs in the skin compared to blood (70 [1 – 158] vs 1 [1 – 12] cells/mL, $p=0.02$) (Figure 6). When expressed as a percentage of CD45+ cells, they were a significantly greater percentage of total EoPs in skin compared to blood (0.06 [0.004 – 0.228] vs 0.003 [0.001 – 0.005] %, $p=0.002$). Similarly, for cytokine-producing activated-ILC2s, there were significantly greater percentage of IL-5+ILC2s (0.0004 [0.0 – 0.0072] vs 0.0 [0.0 – 0.0003] %, $p=0.014$), and IL-13+ ILC2s (0.002 [0.0002 – 0.03] vs 0.0 [0.0 – 0.0004] %, $p=0.005$) in the skin compared to blood. There was a significantly higher percentage of IL-5+IL-13+ ILC2s in skin compared to blood (0.001 [0.0 – 0.007] vs 0.0 [0.0 – 0.006] %, $p=0.02$).

Total and Activated Eosinophil Progenitors

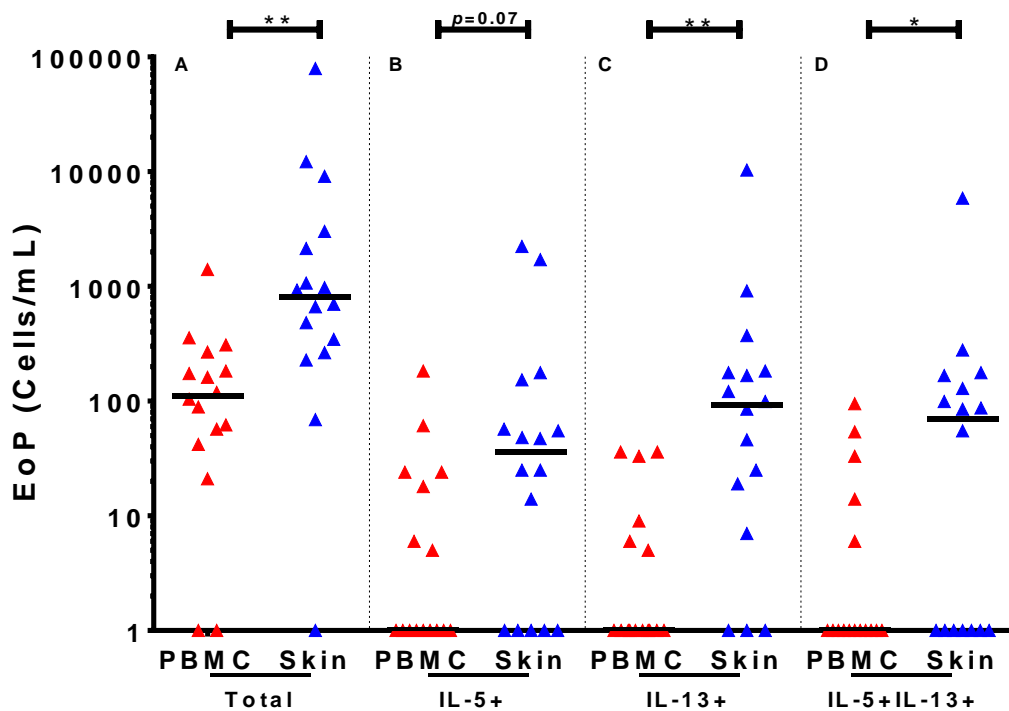


Figure 6: Comparison of total and intracellular cytokine expression levels of EoPs. (A) A significantly higher number of total EoPs in the skin compared to blood (n=16) (B-D) A significantly higher number of IL-5+ and IL-13+ and IL-5+IL-13+ producing EoPs in the skin, compared to blood (n=16); (* $p < 0.05$, ** $p < 0.01$). (Note: Cells that were zero were changed to 1 to account for log scale)

Enumeration of CD4+ Cells in Lesional Skin and Peripheral Blood

We measured CD4+ cell levels in lesional skin and peripheral blood of all patients with AD. When evaluating total CD4+ cells in absolute numbers, there was a statistically significantly higher number in blood compared to skin (1092×10^3 [$650 \times 10^3 - 1742 \times 10^3$] vs 58.3×10^3 [$35.3 \times 10^3 - 152.4 \times 10^3$] cells/mL, $p < 0.01$). For cytokine-producing activated-CD4+ cells, there was a numerically higher number of IL-5-producing CD4+ cells in the skin, compared to blood but not statistically significant (1.3×10^3 [$0.13 \times 10^3 - 42.7 \times 10^3$] vs 2.5×10^3 [$0.4 \times 10^3 - 5.7 \times 10^3$] cells/mL, $p = 0.50$) in the blood and skin respectively. There was a numerically higher number of IL-13-producing CD4+ cells in the blood compared to skin but not statistically significant (9.9×10^3 [$1.6 \times 10^3 - 35.2 \times 10^3$] vs 2.9×10^3 [$1.9 \times 10^3 - 15.0 \times 10^3$] cells/mL, $p = 0.09$). There was a statistically significant higher number of IL-5 and IL-13-producing CD4+ cells in the blood compared to skin (13.5×10^3 [$2.1 \times 10^3 - 42.9 \times 10^3$] vs 3.8×10^3 [$1.6 \times 10^3 - 4.9 \times 10^3$] cells/mL, $p = 0.02$) (Figure 7). When expressed as a percentage of CD45+ cells, they were a significantly greater total CD4+ cells in blood compared to skin (33.5 [$26.29 - 41.87$] vs 1.3 [$0.37 - 4.19$] %, $p < 0.0001$). Similarly, for cytokine-producing activated-IILC2s, there were significantly greater percentage of IL-5+IILC2s (0.1 [$0.02 - 0.31$] vs 0.02 [$0.003 - 0.97$] %, $p < 0.0001$) in the skin compared to blood. For IL-13+ IILC2s (0.46 [$0.07 - 0.80$] vs 0.21 [$0.03 - 18.88$] %, $p = 0.89$) it was greater in the blood compared to skin. There was a higher percentage of IL-5+IL-13+ IILC2s in skin compared to blood, this was not significant (0.45 [$0.13 - 1.07$] vs 0.12 [$0.03 - 0.25$] %, $p = 0.09$).

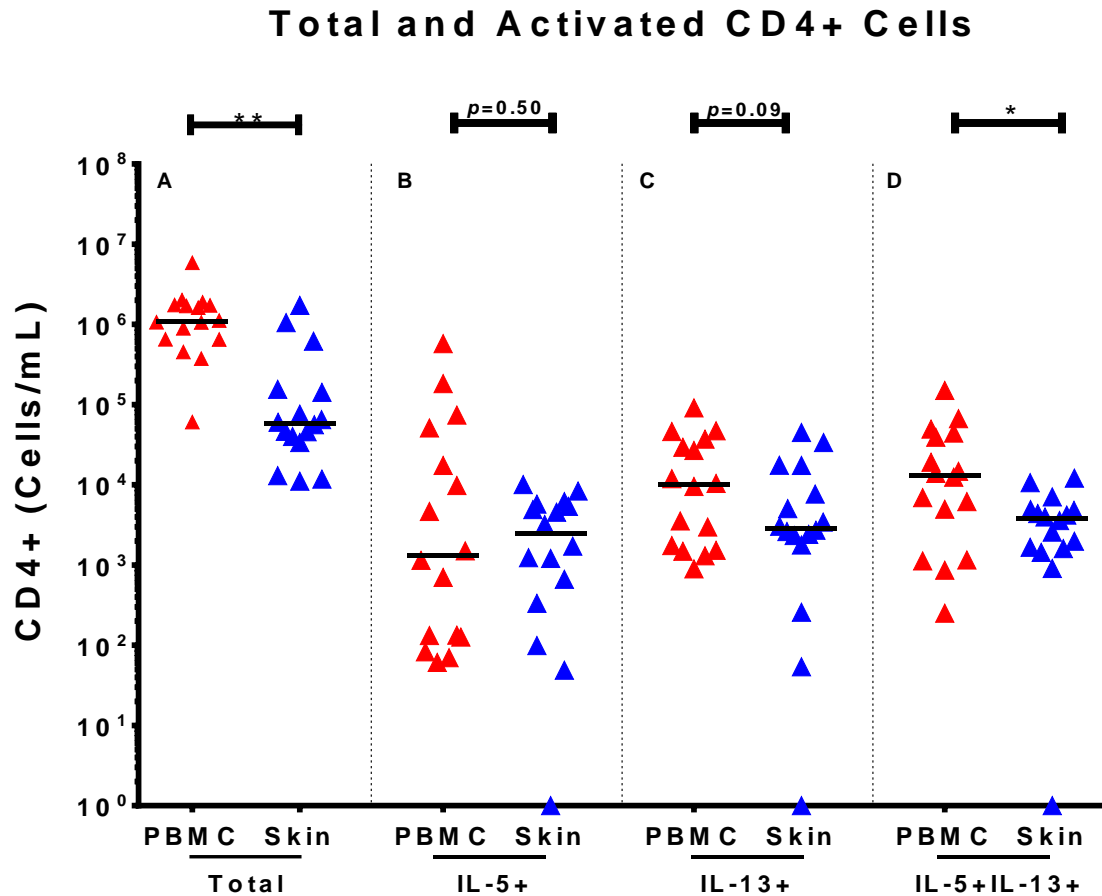


Figure 7: Comparison of total and intracellular cytokine expression levels of CD4+ cells. (A & D) A significantly higher number of total CD4+ cells and IL-5+IL-13+ CD4+ cells in the blood, compared to skin (B) There is a numerically higher number of IL-5+ ILC2s in the skin compared to blood, but not statistically significant (C) There is a numerically higher number of IL-5+ ILC2s in the blood compared to skin, but not statistically significant (n=16).; (* $p < 0.05$, ** $p < 0.01$). (Note: Cells that were zero were changed to 1 to account for log scale)

Enumeration of Basophils in Lesional Skin and Peripheral Blood

We measured basophil levels in lesional skin and peripheral blood of all patients with AD. When evaluating total basophils in the lesional skin and peripheral blood, there was a higher number of basophils in the blood compared to the skin (100×10^3 [$0.001 \times 10^3 - 100 \times 10^3$] vs 277.3×10^3 [$4.2 \times 10^3 - 66.4 \times 10^3$] cells/mL, $p=NA$). However, caution should be taken as different methodologies were done to enumerate basophils. Blood basophils were differentially counted by the McMaster University Medical Centre Core Laboratory, while the skin basophils were enumerated by flow cytometry (Figure 8). When comparing all the cell types in lesional skin and peripheral blood, CD4+ cells were the highest, while there was an equal expression of ILC2s and EoPs (Figure 9).

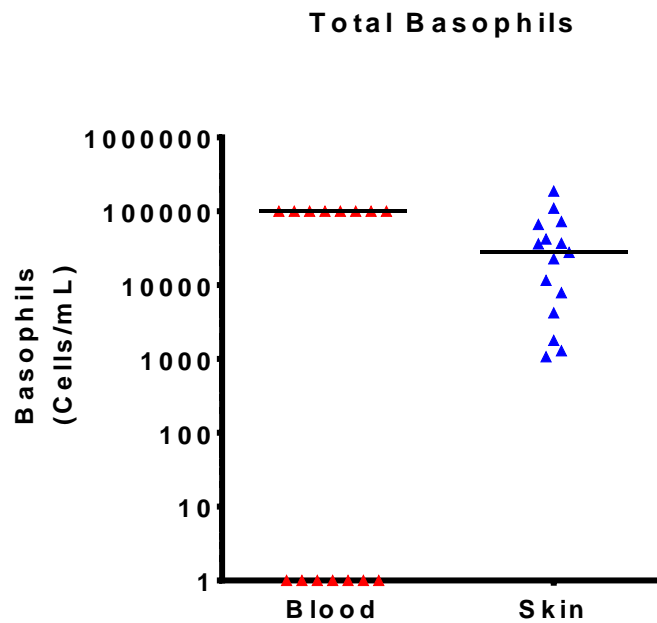


Figure 8: Comparison of total levels of basophils. No statistics was done as skin basophils were enumerated by flow cytometry and blood basophils were counted by McMaster University Medical Centre Core Laboratory by differential blood counts. Skin basophils were normalized to number of cells in 1 gram of tissue per mL of FACS buffer. (Note: Cells that were zero were changed to 1 to account for log scale)

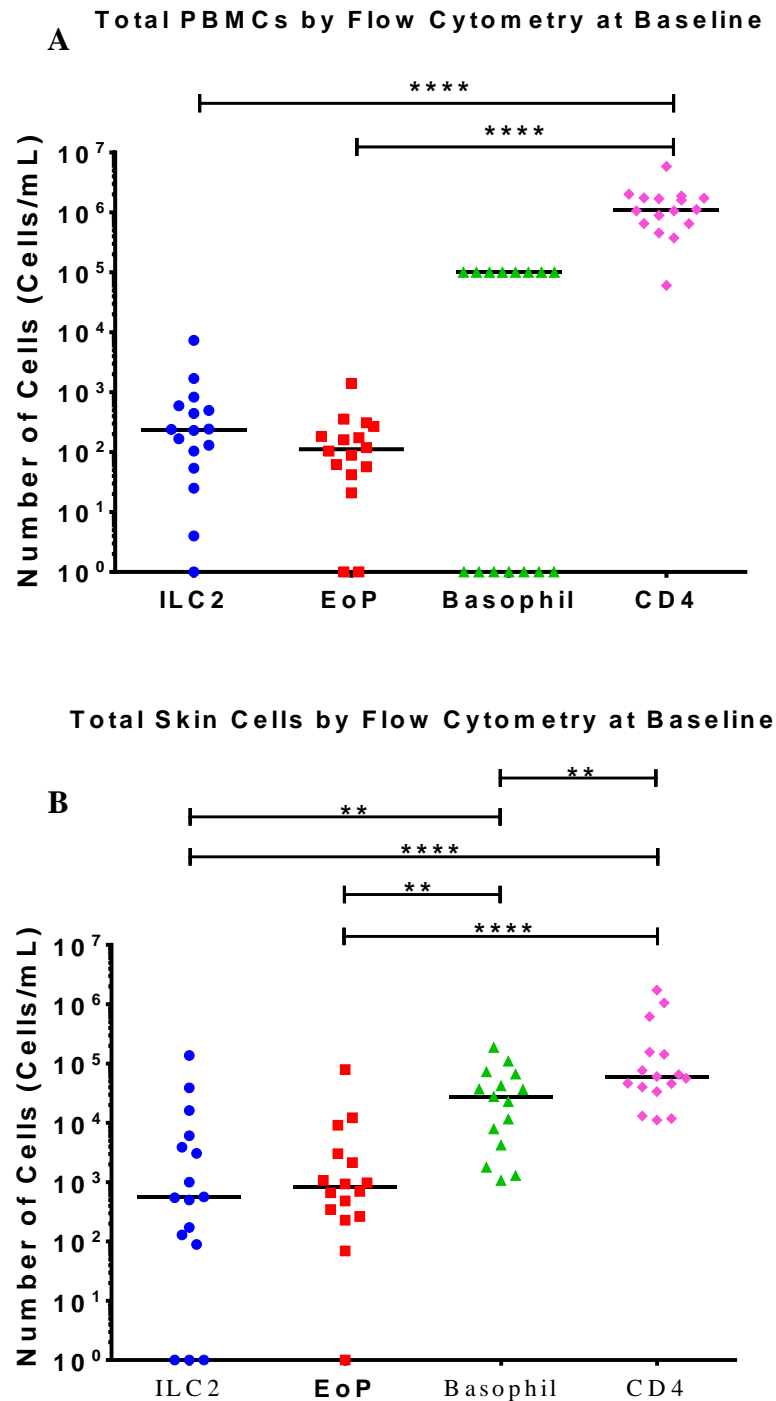


Figure 9: Comparison of total ILC2s, EoPs, basophils and CD4+ cells in peripheral blood (**A**) and lesional skin (**B**). Compared to total ILC2, EoP and basophils, there was a significantly higher number of total CD4+ cells in skin and there was a significantly higher number of total CD4+ cells compared to total ILC2s and EoPs in blood. There is an equal number of total ILC2s and EoPs in both blood and skin (n=16); (** $p < 0.01$, **** $p < 0.0001$). (Note: Cells that were zero were changed to 1 to account for log scale)

Total and Activated ILC2s are lower in Males compared to Females

We compared the number of total and activated ILC2s in males and females to determine if there are any sex differences in AD patients. In a study by Ebata et al, free testosterone was significantly lower in male patients with AD, compared to healthy controls. We investigated sex differences in ILC2s in AD patients. Our data showed total ILC2s in blood were significantly higher in number in females, compared to males with AD (0.5×10^3 [$0.2 \times 10^3 - 2.5 \times 10^3$] vs 0.1×10^3 [$0.02 \times 10^3 - 0.3 \times 10^3$] cells/mL, $p=0.03$). In the lesional skin, there was a numerically higher number of total ILC2s in females, compared to males, but this was not statistically significant (1.8×10^3 [$0.1 \times 10^3 - 7.0 \times 10^3$] vs 0.5×10^3 [$0.07 \times 10^3 - 14.3 \times 10^3$] cells/mL, $p=0.79$). For activated IL-5+ ILC2s, there was a numerically higher number of ILC2s in females, compared to males in both blood and skin, but not statistically significant (Blood IL-5+ ILC2: 0.003×10^3 [$0.001 \times 10^3 - 0.14 \times 10^3$] vs 0.001×10^3 [$0.01 \times 10^3 - 0.007 \times 10^3$] cells/mL, $p=0.37$) and Skin IL-5+ ILC2: 0.07×10^3 [$0.001 \times 10^3 - 3.6 \times 10^3$] vs 0.001×10^3 [$0.001 \times 10^3 - 0.28 \times 10^3$] cells/mL, $p=0.33$, respectively). For activated IL-13+ ILC2s, there was a numerically higher number of ILC2s in females, compared to males in blood and numerically higher number of ILC2s in females compared to males in skin, but not statistically significant (Blood IL-13+ ILC2: 0.006×10^3 [$0.001 \times 10^3 - 0.02 \times 10^3$] vs 0.001×10^3 [$0.001 \times 10^3 - 0.001 \times 10^3$] cells/mL, $p=0.09$ and Skin IL-13+ ILC2: (0.2×10^3 [$0.001 \times 10^3 - 1.6 \times 10^3$] vs 0.028×10^3 [$0.001 \times 10^3 - 1.1 \times 10^3$] cells/mL, $p=0.99$, respectively). For activated IL-5+IL-13+ ILC2s, there was a numerically higher number of ILC2s in females, compared to males in both blood and skin, but not statistically significant (Blood IL-5+IL-13+ ILC2: 0.004×10^3 [$0.001 \times 10^3 - 0.02 \times 10^3$] vs 0.001×10^3 [$0.001 \times 10^3 - 0.01 \times 10^3$] cells/mL, $p=0.70$ and Skin IL-5+IL-13+ ILC2: (0.03×10^3 [$0.001 \times 10^3 - 1.3 \times 10^3$] vs 0.005×10^3 [$0.001 \times 10^3 - 0.2 \times 10^3$] cells/mL, $p=0.72$, respectively) (Figure 10).

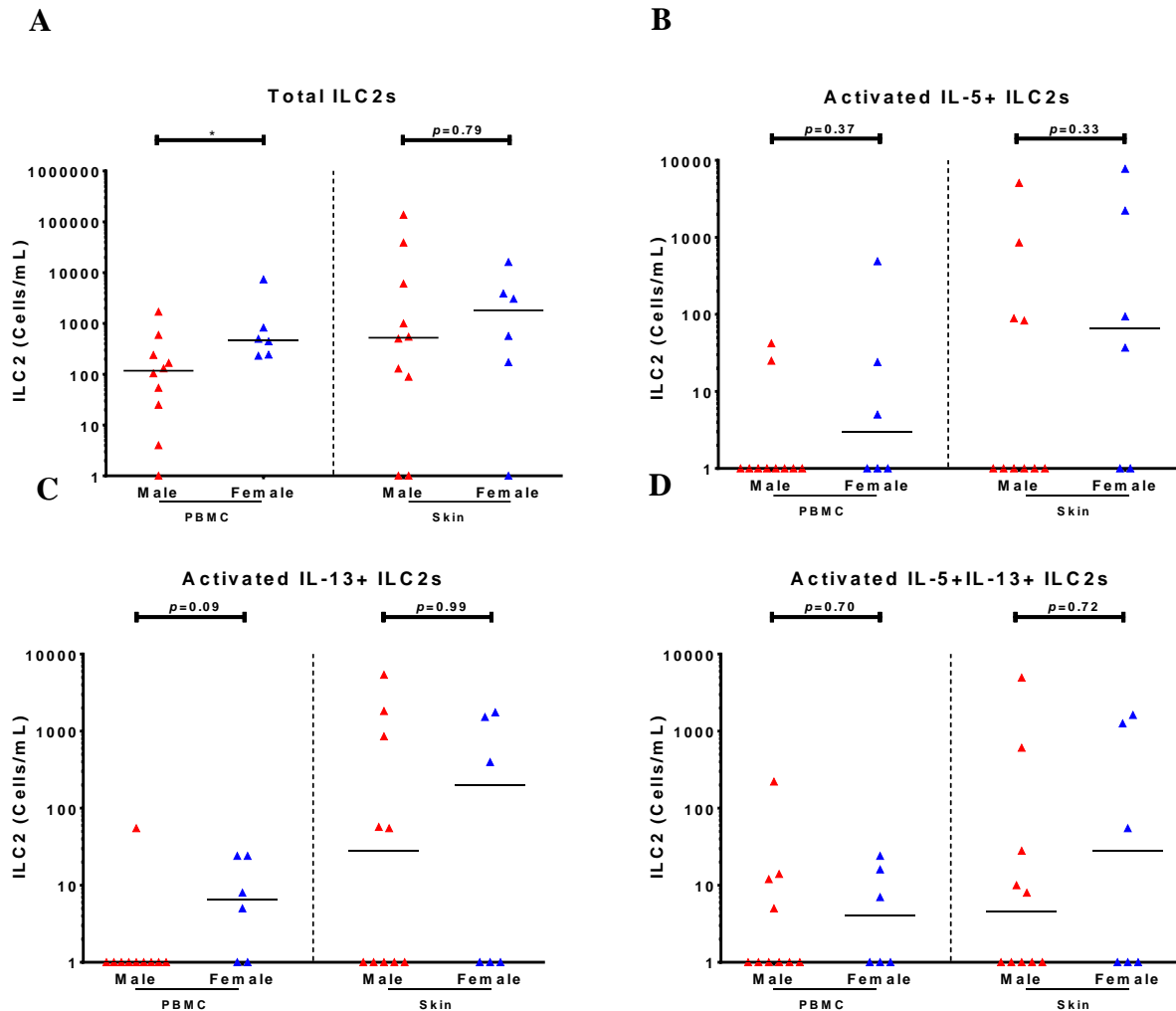


Figure 10: Sex differences in total and activated ILC2s in blood and skin. **(A)** Median total ILC2s in blood was significantly higher in females than males. Median total ILC2s in skin was higher in females than males, but not statistically significant. **(B – D)** Median activated ILC2s in both blood and skin were higher in females than males, but not statistically significant.

ILC2s are preferentially located at the dermal-epidermal junction

ILC2s by immunofluorescence staining was identified by Lin⁻ (Green), GATA3⁺ (Red) and CRTH2⁺ (Purple), with DAPI (Blue) to identify live cells (Figures 11 and 12). Cells that were Lin⁺GATA3⁺CRTH2⁺ were thought to be CD4⁺ cells, or a subset of Regulatory T-Cells (Tregs) that is GATA3⁺. From our immunofluorescence staining, we found that ILC2s were mainly localized in the dermal-epidermal junction, with sporadic ILC2s located in other parts of the dermis. No ILC2s were found in the epidermis (Figure 13).

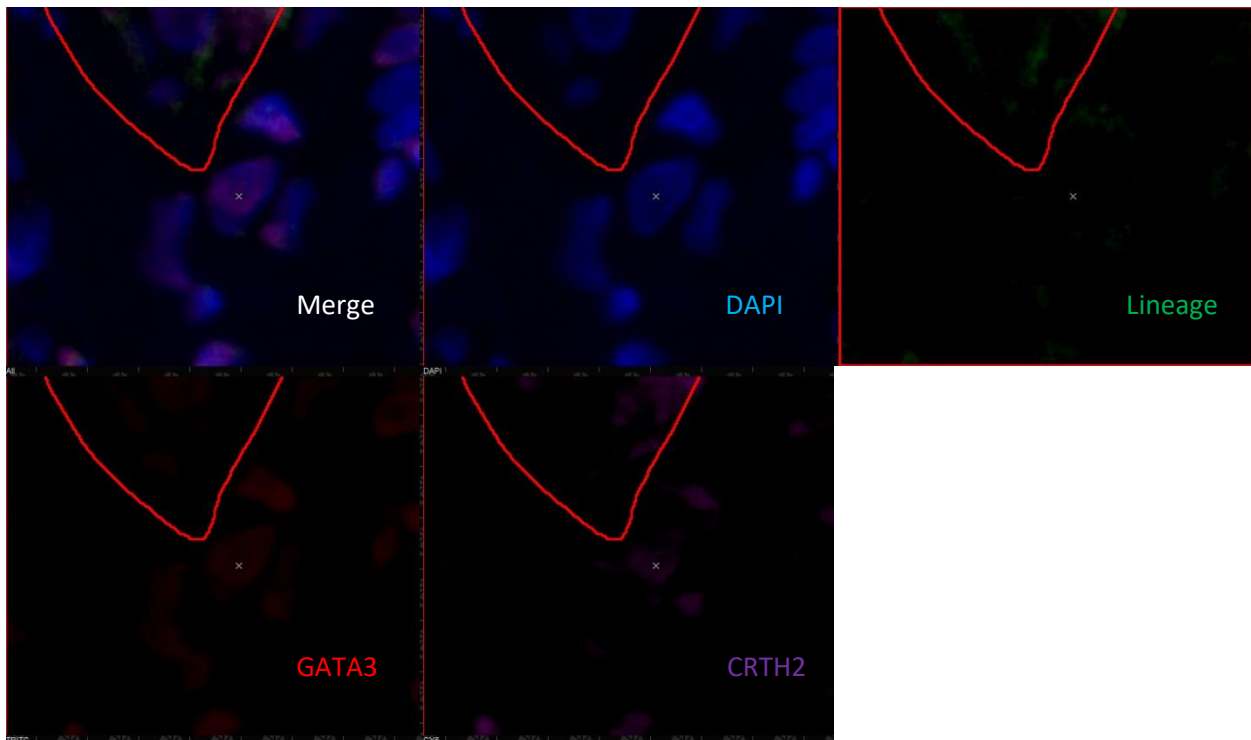


Figure 11: Identification of ILC2s. ILC2s were identified by a lack of Lineage staining, and positive for GATA3 and CRTH2.

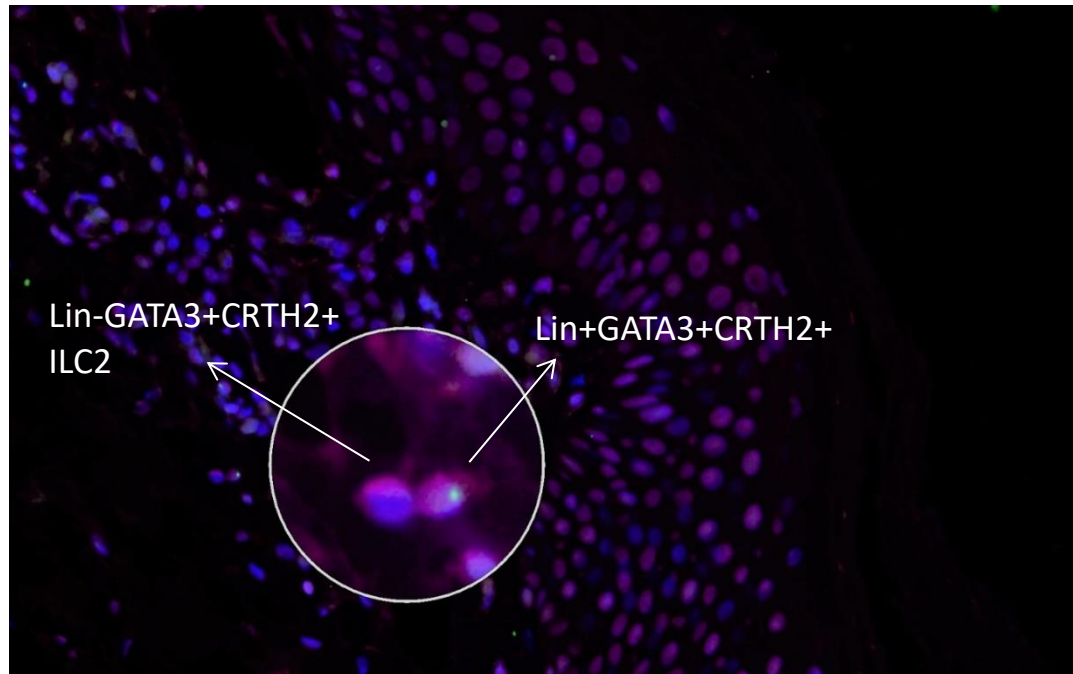


Figure 12: Identification of ILC2s. ILC2s were identified by a lack of Lineage staining, and positive for GATA3 and CRTH2. Other cells that were positive for Lineage, GATA3, and CRTH2 were possible CD4+ cells, or a subset of GATA3-positive Regulatory T-Cells.

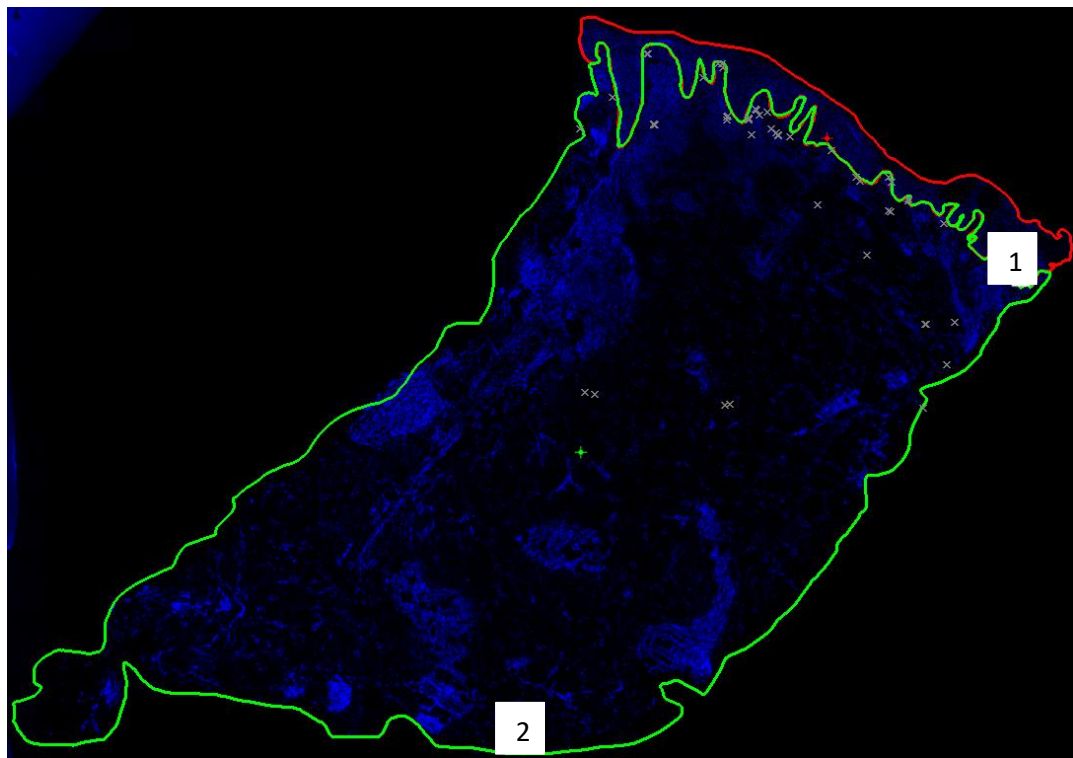


Figure 13: Localization of ILC2s. ILC2s were mainly localized dermis part of the dermal-epidermal junction, although there were sporadic ILC2s located in other parts of the dermis (green zone). White cross denotes 1 ILC2. (Red zone 1: Epidermis, Green zone 2: Dermis)

When comparing allergen-challenged skin with saline-challenged skin, there was a significantly higher number of ILC2s in allergen-challenged skin sampled 24h post challenge (2 [1 – 2] vs 0 [0 – 0] cells/mm², $p=0.0002$). When comparing lesional skin with non-lesional skin, there was a significantly higher number of ILC2 in the lesional skin (1 [0 – 2] vs 0 [0 – 0] cells/mm², $p=0.008$) (Figure 14).

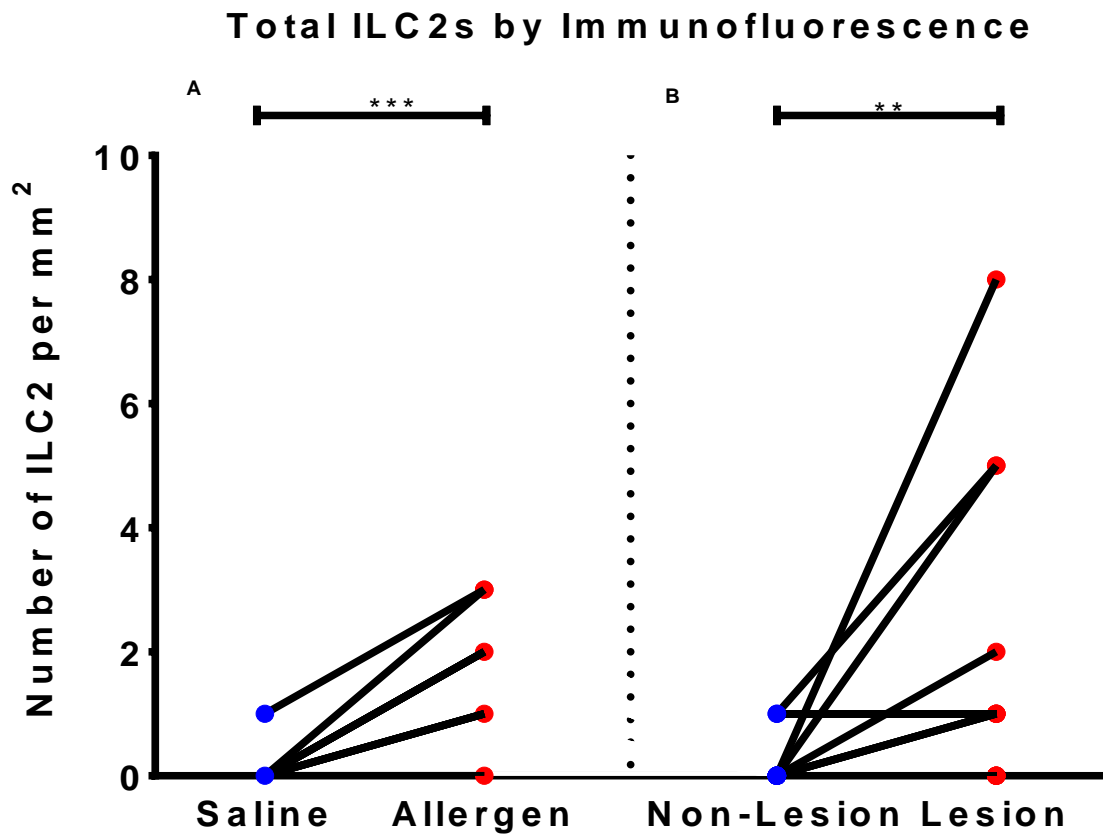


Figure 14: Comparison of ILC2 counts in allergen-challenged, saline-challenged skin, lesion and non-lesion skin biopsies. (A) A significantly higher number of ILC2s in the allergen-challenged skin compared to saline-challenged skin (n=15) (B) A significantly higher number of ILC2s in the lesion skin compared to non-lesion skin (n=15); (** $p<0.01$, *** $p<0.001$).

We then stained the biopsies with CD3 to count the number of CD3+ cells positive for Lin and negative for CRTH2. When expressed as cells per mm², there was a significantly higher number of CD3+ cells in the allergen-challenged skin, compared to the saline-challenged skin (7575±9687 vs 3666±4800 cells/mm², $p=0.02$). There was a numerically higher number of CD3+ cells in the non-lesional skin, compared to the lesional skin, but this was not statistically significant (5085±11594 vs 4706±8788 cells/mm², $p=0.39$). We also correlated the number of ILC2s with the number of CD3+ cells in lesional skin, non-lesional skin, allergen-challenged skin and diluent-challenged skin. No correlation was found between ILC2s and CD3+ cells in lesional skin, non-lesional skin, allergen-challenged skin and diluent-challenged skin.

Correlation of ILC2s with Clinical Data

In AD, there are no studies that showing the correlation of clinical data with the number of ILC2s. Here, we report for the first time, the correlation of ILC2s with clinical data such as AD patient surveys and clinician assessments such as DLQI, POEM, SCORAD, EASI, IGA. We found significant correlations with total skin ILC2s, but not blood ILC2 levels and POEM scores ($r=0.55$, $p=0.04$), IL-13+ ILC2s ($r=0.61$, $p=0.02$) and IL-5+ IL-13+ ILC2s ($r=0.75$, $p=0.002$) (Figure 15). In contrast, we found no correlation of skin or blood ILC2 and DLQI, SCORAD, EASI and IGA scores. We also found no correlation of blood and skin ILC2s with blood and skin EoPs.

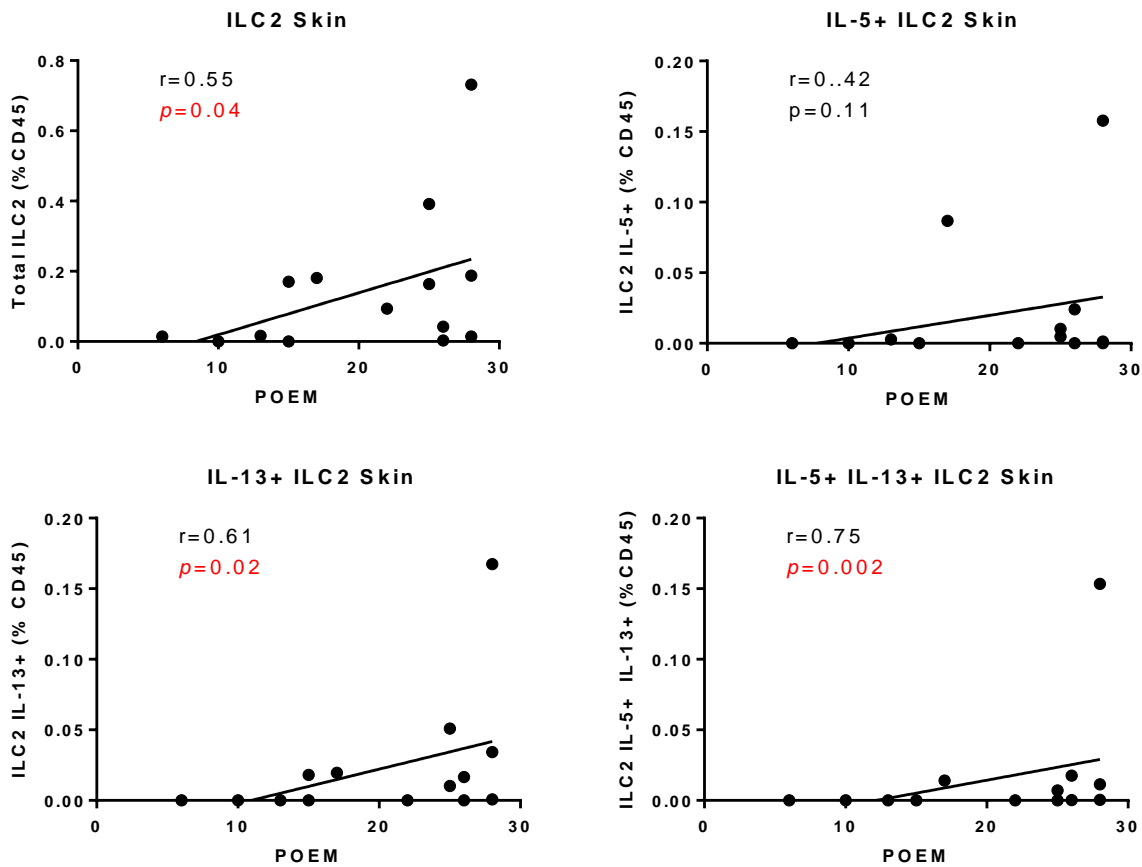


Figure 15: Spearman correlation of total and activated skin ILC2s expressed as % CD45 with POEM scores (n=16). A Holm-Bonferroni correction was applied to account for multiple comparisons; POEM - Patient Oriented Eczema Measure

We also correlated ILC2 counts by IF with TSLP and IL-33 staining. There was no correlation in lesional skin, non-lesional skin, allergen-challenged skin and diluent-challenged skin between ILC2s and either IL-33 or TSLP immune-positive levels in skin (Figure 16).

Additionally, we investigated a relationship between ILC2s and tissue eosinophilia as determined by H&E stains on our lesional skin, non-lesional skin, allergen-challenged skin, and diluent-challenged skin biopsies. We found no correlation in any of the ILC2s in our all our skin biopsies with the mature eosinophil counts. We also found no correlations with ILC2s and CD4+ cells with EoPs separately. Interestingly, when we combined our ILC2s and CD4+ cell counts expressed as a percentage of CD45+ cells, there was a significant correlation with EoPs ($r=0.58$, $p=0.03$) (Figure 17).

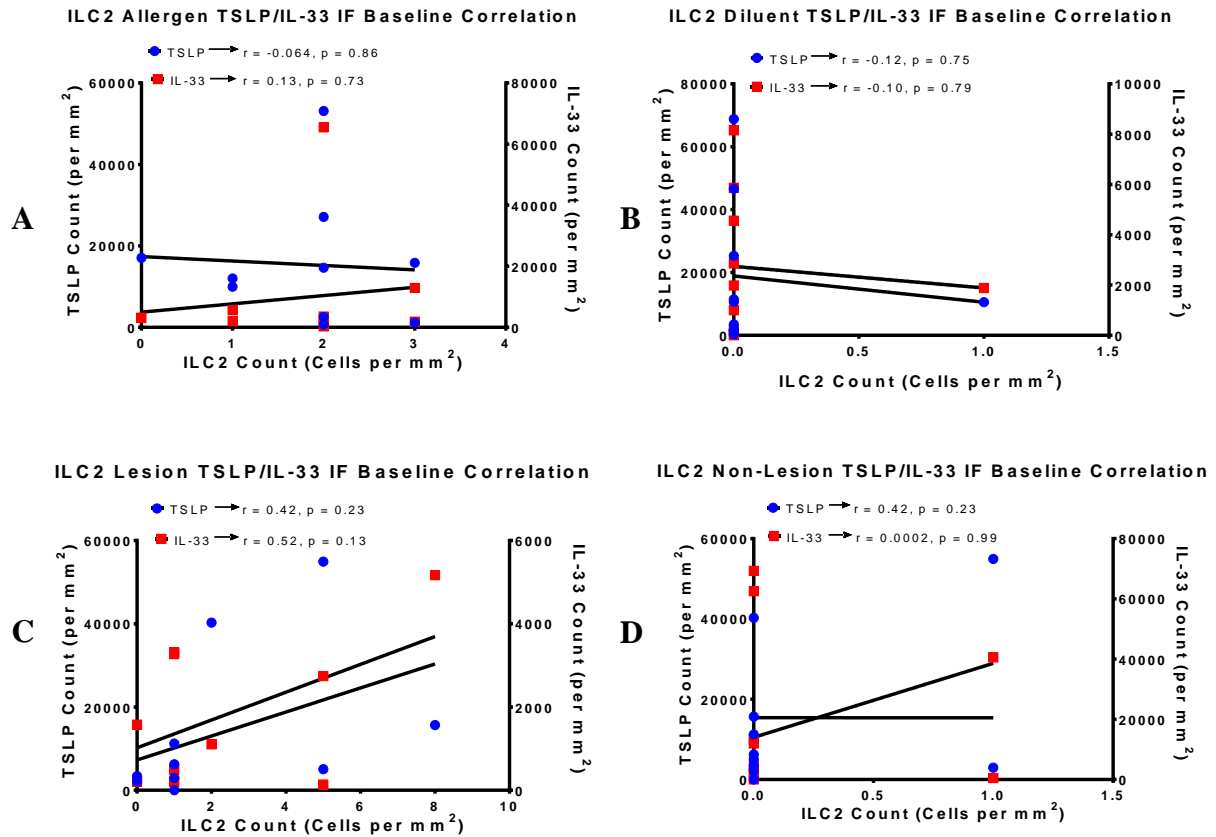


Figure 16: Spearman correlation of ILC2s with TSLP and IL-33 in allergen-challenged skin (A), diluent-challenged skin (B), lesional skin (C) and non-lesional skin (D) (n=10).

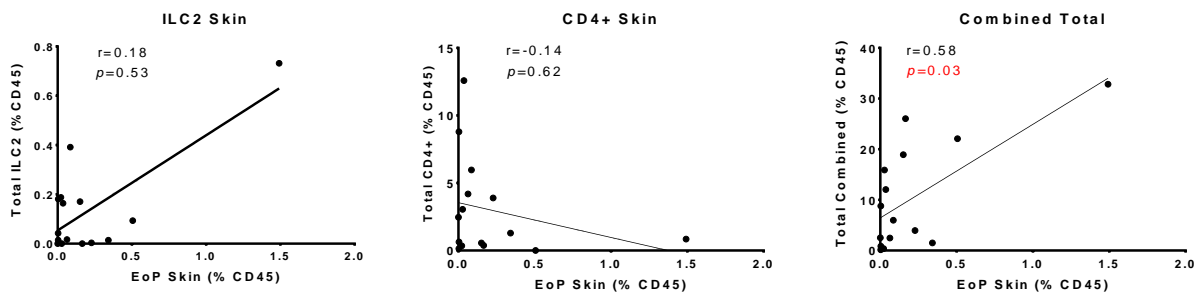


Figure 17: Correlation of ILC2s (A), CD4+ cells (B) and combined cells (C) with EoPs. There was no correlation seen with ILC2s and CD4+ cells separately but there was a correlation seen with combined cells and EoPs (n=16).

CHAPTER 4: DISCUSSION

Enumeration of ILC2, Eosinophil Progenitors, Basophils and CD4+ Cells by

Flow Cytometry

In this study, we enumerated the number of ILC2s, eosinophil progenitors, basophils and CD4+ cells in lesional skin and peripheral blood by flow cytometry at baseline. We found that there was a higher number of total ILC2s in the lesional skin compared to blood. This maybe due to 2 reasons. Firstly, there is a recruitment of ILC2s from the bone marrow and circulation to the lesional skin. Secondly, there is maturation and proliferation of tissue resident ILC2Ps and mature ILC2s. This confirmed previous studies where skin-extracted ILC2s numbers were higher compared to blood (0.04-2.94% vs 0-0.18% respectively) (Salimi, Barlow, Saunders, Xue, Gutowska-Owsiak, et al., 2013). Activated ILC2s release Type 2 cytokines IL-5 and IL-13. Here, we reported IL-5+ and IL-13+ producing ILC2s in lesional skin, as well as peripheral blood. IL-5 and IL-13 are involved in the recruitment, developing, maturation, survival and activation of EoPs, and mature eosinophils. We did not assess skin homing markers such as CCR10 and Cutaneous Leucocyte-Associated Antigen Receptor (CLA) to determine the proportion of ILC2s that were from peripheral blood due to technical limitations. This should be a topic of future studies.

We identified EoPs in both the lesional skin and blood. EoPs were identified in allergic diseases such as asthma (Smith et al., 2016). EoPs have not previously been identified in AD. Here we report for the first time, EoPs were identified in AD lesional skin, as well as in peripheral blood from patients with moderate-to-severe AD. EoPs, or eosinophil progenitors, or eosinophil-lineage committed progenitor cells are derived from the bone marrow.

We identified basophils in the skin by flow cytometry and by differential cell counts in the blood. Basophils are identified in diseases such as allergic diseases, infections, cancer and autoimmune diseases. In the context of allergic diseases, basophils require TSLP and IL-3 for development, as well as tissue basophilia (Salter et al., 2015). Activated basophils release Type 2 cytokines IL-4, IL-5, IL-13 from cytokines such as TSLP and IL-3, as well as IgE. In addition, basophils release these cytokines following exposure from Derp 1 protease from HDM in vivo as reported by Philips et al (Phillips, 2003). In the context of ILC2s, Motomura et al reported that basophil-derived IL-4 by binding to the IL-4R α on ILC2s in a mouse model in lung inflammation (Motomura et al., 2014). Kim et al identified basophils and ILC2s in AD skin lesions (Brian S Kim et al., 2014). In that same study, basophils and ILC2s were shown to be in close proximity in lesional skin in mice and that the basophil responses preceded ILC2s and that basophils release IL-4 to recruit IL-4R α -expressing ILC2s. Mashiko et al reported that basophils and ILC2s were elevated in AD lesional skin, but not in blood, when compared to psoriatic skin lesions and blood. While the author reported a difference between skin lesions and blood in AD compared to psoriasis a healthy control group was not included. In our study, we reported that basophils were found in the skin lesions and blood of AD patients. However, no statistics were performed as both sample types were enumerated by different methods. Mashiko et al reported that there was a positive correlation between skin ILC2s and skin basophils ($r=0.8$, $p=0.016$) and a negative correlation between blood ILC2s and skin basophils ($r=-0.77$, $p=0.025$) as a percentage of CD45+ cells (Mashiko, Mehta, Bissonnette, & Sarfati, 2017). When we performed a correlation with our skin ILC2s, blood ILC2s, and skin basophils, we found no correlation with either datasets. Although the author had a smaller sample size compared to our study, this may be due to the different gating strategies used to identify ILC2s and basophils. Mashiko used c-Kit, CD45, CRTH2, CD127 and

separate CD3 and FcεR1 antibodies to identify ILC2s. In addition, the author manually created a lineage cocktail, whereas in our study we used a commercial lineage cocktail that had a different composition. Furthermore, we did not use c-Kit in our gating strategy, and the commercial lineage cocktail already had FcεR1 and CD3. This may potentially lead to a different enumeration of ILC2s in both our studies. Therefore, this may account for the discrepancy in ILC2 and basophil correlation

We identified CD4⁺ cells in both AD lesional skin, and blood. Although there was a significantly higher number of total CD4⁺ cells in the blood compared to the skin, there was a variable median number of activated CD4⁺ cells in the blood and skin. We asked ourselves why CD4⁺ cells were lower than ILC2s in the skin compared to blood. From our literature search, we found that ILC2s were the main producers of IL-5 and IL-13, compared to CD4⁺ cells (Zhou et al., 2016). Zhou et al reported that there was a higher number of IL-5 and IL-13 producing ILC2s compared to CD4⁺ cells. However, other studies have shown that IL-5 and IL-13 producing CD4⁺ cells are higher than IL-5 and IL-13 producing ILC2s. But having said that, no comparison was done to compare cytokine levels between CD4⁺ cells and ILC2s in AD. A suitable experiment would be to isolate ILC2s and CD4⁺ cells separately and measure cytokine levels with, and without stimulation and quantify with ELISA. This way we can elucidate if ILC2s are the main drivers for eosinophilic inflammation in AD, instead of CD4⁺ cells, by producing higher amounts of cytokines, or if they work in tandem with CD4⁺ cells.

In a study reported by Drake et al, CD4⁺ cells interact with lung ILC2s by the OX40-OX40L interaction in a mouse model to synergistically promote airway inflammation (Drake, Iijima, Bartemes, & Kita, 2016). Mirchandani et al reported that CD4⁺ T cell-derived IL-2 was involved in ILC2 cytokine production in lung tissue in a mouse model. Furthermore, ILC2s express

MHC-II that has the capacity to perform as an APC. In addition, ILC2s enhanced CD4+ cell responses, which mirrored the synergistic effects reported by Drake (Mirchandani et al., 2014). In a study by Smith et al, CD4+ cells and ILC2s were identified in both blood and sputum samples in both mild and severe asthmatic patients (Smith et al., 2016). However, to our knowledge, CD4+ cells were not reported together with ILC2s in patients with AD, except as part of a lineage cocktail. Here, we report for the first time, CD4+ cells in blood and skin of AD patients.

The limitation of this flow cytometry study is the lack of a healthy, or a non-lesional control group, or a mild AD group to compare with the moderate-to-severe AD cohort. However, it is due to technical limitations. During our optimization, we had very little single cells extracted from non-lesional skin and mild AD lesional skin, and it was not possible to stain for flow cytometry with our current panel. This is especially true for rare cell populations like ILC2s where a high starting number of cells is required to detect it. Extracting single cells from our moderate-to-severe AD cohort gave us a varied number of cells, from a maximum of 12 million cells to a minimum 70,000 cells. Therefore, a suitable workaround to determine the number of ILC2s in non-lesional skin is by histopathology, specifically by immunofluorescence staining.

Sex differences in ILC2s in Atopic Dermatitis

There have been studies that reported that there is a sex difference in the number of ILC2s in asthma, in both humans and mouse models (Laffont et al., 2017)(Cephus et al., 2017)(Warren et al., 2017). What these research groups found was that females had a higher number of ILC2s, compared to males, in both humans and mouse models. This is possible due to the expression of the androgen-receptor on ILC2Ps which prevents the maturation and development to mature ILC2s upon exposure to an androgen hormone. Currently, there are no studies that reported the sex

differences of ILC2s in patients with moderate-to-severe AD. Here, we report for the first time, that total ILC2s were significantly higher in female blood, compared to males in patients with moderate-to-severe AD. In addition, there was also a higher number of skin ILC2s in females, compared to males, but was not statistically significant. As for activated ILC2s, there was a higher number of IL-5+, IL-13+, and IL-5+IL-13+-producing ILC2s in females, compared to males, in both blood and skin, but not statistically significant. This is possible due to our sample size (n=16). Having a larger sample size may increase the power of this comparison. Our study confirmed previous studies that ILC2s were higher in blood from females, compared to males. However, our study did not specifically look at levels of sex hormones such as testosterone, free testosterone, and 5-DHT. Therefore, we cannot say that the different levels of ILC2s in males and females may be attributed to these sex hormones. Furthermore, as we did not show significant sex differences in the skin of AD subjects, it is difficult to extrapolate any disease related role that sex-hormones may be playing via effects on ILC2.

Identification and Quantification of ILC2s and CD3+ Cells by Immunofluorescence Staining

Flow cytometry is still considered the gold standard to identify and enumerate multiple cell types. One of the challenges of flow cytometry is acquiring sufficient number of single cells after extraction for a staining panel. Typically, in a staining tube, 2 million cells are required. When extracting from peripheral blood, an abundant number of single cells can be extracted for our 4-staining tube panel (i.e.: 8 million cells). However, when digesting lesional skin, we found that our single cell counts varied between patients, from a maximum of 12 million cells to a minimum 70,000 cells. Therefore, we had to make a decision each time to determine if we were to do a full 4-tubes panel, or to just put all our cells in our specific antibody tube, without any isotype controls. If we were to extract single cells from non-lesional skin, there wouldn't be any cells for the specific

tube. In studies by Kim et al, and Salimi et al (Salimi, Barlow, Saunders, Xue, Gutowska-Owsiak, et al., 2013)(Brian S Kim et al., 2014), what these authors did to counter this challenge was to acquire skin from routine abdominoplasty surgeries for healthy controls. However, the authors of these studies did not specify the amount of skin used for digestion. Whether they used the entire abdominoplasty skin, or a specific dimension. These authors compared ILC2s in skin from a healthy control group, and another group that has AD. Furthermore, they did not specify the severity of disease of the patients recruited in their studies (i.e.: mild or moderate or severe AD).

Currently to our knowledge, no studies have been done to look at the ILC2 landscape in lesional-skin, non-lesional skin, allergen-challenged-skin, and diluent-challenged skin from the same patient. Here, we report for the first time, the ILC2 landscape in various skin biopsies. Our IF staining panel is based on the staining panel by Brügger et al (Brügger et al., 2016). However, the limitation with the author's study is firstly, the antibodies used was optimized for flow cytometry, and not for immunofluorescence or immunohistochemistry. Secondly, the author used frozen sections for her IF staining. The limitation with frozen sections is that the tissue and cellular morphology might not be detailed and accurate and that staining might not end up very well. In addition, frozen tissues cannot be stored for long-term use for future sectionings and stainings. Therefore, formalin-fixed paraffin-embedded (FFPE) is a suitable platform for immunohistochemistry and immunofluorescence. Kim et al also identified ILC2s by IF staining on mouse and human FFPE tissues (Brian S Kim et al., 2014). The author used CD3-IL-33R+ to identify ILC2s. While ILC2s are CD3-, IL-33R is not exclusively expressed by ILC2s and CD3+ cells. IL-33R is also expressed by airway smooth muscles, basophils, bronchial epithelial cells, CD34+ progenitor cells, goblet cells, fibroblasts, and mast cells (R Sehmi et al., 2016)(Kaur et al., 2015)(Salter et al., 2016)(Zimmermann et al., 2008)(Bianchetti et al., 2012)(Guo et al.,

2014)(Tanabe, Shimokawaji, Kanoh, & Rubin, 2014)(Han et al., 2017)(H. Y. Kim et al., 2012)(Bartemes, Kephart, Fox, & Kita, 2014). This will artificially inflate the number of ILC2s due to the false positive counts. Therefore, having a lineage cocktail to exclude cells that express these lineage markers to identify ILC2s is paramount. As stated earlier, we based our staining panel on Brügger's panel (Brügger et al., 2016). For this thesis, we improved an immunofluorescence protocol to detect ILC2s by using a comprehensive panel consisting of a lineage cocktail as reported by Brügger. However, in our staining panel, we also included an antibody that was specific for Fc Epsilon Receptor 1 (FcεR1) in our lineage cocktail, by excluding FcεR1+ cells, such as basophils, eosinophils, and mast cells. In addition, we stained the tissue with a lineage marker cocktail, CRTH2 and GATA3. From this, we identified ILC2s by Lin-CRTH2+GATA3+. Also, we used antibodies that were optimized for immunofluorescence staining, and not for flow cytometry. Finally, we used FFPE tissues, instead of frozen tissues as we can store at room temperature for long-term storage and for any repeated stainings or to investigate any other markers.

We identified ILC2s in lesional skin, non-lesional skin, allergen-challenged skin, and diluent-challenged skin. While in Brügger's study where the author reported no ILC2s in normal human skin, we did not look for ILC2s in normal skin in a healthy control group (Brügger et al., 2016). However, we did identify ILC2s in non-lesional skin. This may be due to their analysis as they used a program (StrataQuest software) to detect ILC2s and therefore, may have false negatives. In our study, we manually counted ILC2s and looked at individual channels and excluded CD3+GATA3+CRTH2+ cells. However, a possible limitation is the introduction of false positives. When manually counting cells, there is a possibility of cells that have background staining and that might be deemed to be a positive staining. A workaround for this is to introduce

a secondary observer to confirm the counts. When identifying ILC2s, we excluded CD3⁺ cells and looked for Lin-GATA3⁺CRTH2⁺. Cells that were Lin⁺GATA3⁺CRTH2⁺ were thought to be CD4⁺ Th2 cells. Th2 cells express GATA3 to produce Type 2 cytokines IL-5 and IL-13. In our raw absolute counts, we found a higher number of ILC2s in the allergen-challenged skin, followed by the lesional skin, and similar counts seen non-lesional skin and diluent-challenged skin. This may be due to 2 reasons: 1) Recruitment of ILC2s from the bone marrow and circulation to the site of the allergen challenge, and/or 2) Maturation and proliferation of tissue resident ILC2Ps to ILC2s. There was no to minimal numbers of ILC2s in the non-lesional skin and diluent-challenged skin which showed that ILC2s are only activated and recruited during sensitization to an allergen. The challenge to detecting ILC2s by IF staining was that, when comparing to flow cytometry where we digested a 3 x 1 cm tissue excision biopsy, whereas for IF staining, we took a 4 mm punch biopsy and then a 5 µm section from that punch biopsy. This may not be an accurate representation as cells do not move in a 2-dimensional manner like in a 5µm section, but rather in a 3-dimensional manner. Also, confirming Brüggen's study, ILC2s were mainly localized in the dermal-epidermal junction (Brüggen et al., 2016).

We also counted the number of CD3⁺ cells in our skin biopsies and found no trend in any of the skin biopsies in either the raw absolute counts or normalized to cells per mm². This may be due to technical limitations. Our current software was unable to count CD3⁺ stainings together with DAPI stainings to get an accurate count. This was why we decided to count for ILC2s manually as they are a rare cell population, and as the program was unable to detect cells with multiple stainings. In the interest of time and technical issues, we were unable to count CD3⁺ cells as in the biopsies, the CD3⁺ cells tend to form huge clusters. Therefore, making manual counting near impossible to do so. Furthermore, the StrataQuest software Brüggen used was very expensive

for our research group to purchase. A workaround is perhaps the use of bioinformatics to create a series of codes to count cells using software such as MATLAB. The main challenge to learning coding for this study is time which this author did not have; as such manual counting had to be done.

One of the questions we wanted to ask was whether ILC2s promoted tissue eosinophilia. Using our ILC2 IF counts and the skin eosinophil counts from another study from our research group (Price E et al, 2018), we found no correlation between these 2 cell types. This may be due to 2 reasons: 1) Small sample size and may be increasing the sample size might be able to form a trend and increase significance and/or 2) The mature eosinophils degranulated and could not be detected by H&E staining that picks up whole cells and not degranulated cells. A workaround this challenge would be to stain for the granule proteins such as MBP, ECP, EDN and EPX as detailed by Leiferman et al (Kristin M. Leiferman et al., 1985). These experiments are underway. We also investigated whether ILC2s and CD4+ cells correlated with EoPs. Separately, there was no correlation with ILC2s, CD4+ cells and EoPs. However, when we combined our ILC2 and CD4+ cell counts, we found a significant correlation with EoPs.

We investigated if whether alarmin cytokines such as TSLP, IL-33 and IL-25 are responsible for ILC2 activation. we correlated with our ILC2 IF counts and found no correlation. This may be due to 2 reasons: 1) Sample size and/or 2) Other factors that may activate and recruit ILC2s such as Prostaglandin D2, IL-4, IL-2, IL-7 and Leukotriene B4/C4.

We also did a Spearman Correlations with clinical data such as AD patient surveys and clinician assessments and found a statistically significant correlation with total skin ILC2s, IL-13+ ILC2s and IL-5+ IL-13+ ILC2s, with the POEM score which is an AD patient survey to determine

the duration of pruritus, sleep loss, bleeding and oozing. This is interesting as IL-13 is involved in pruritus. In a study by Oh et al, a mouse model was used to identify IL-13 binding to the transient receptor potential ankyrin 1 (TRPA1) which was required for histamine-independent pruritus. Inhibition of TRPA1 attenuated pruritus in mice. In addition, IL-13 contributed to an increased expression of TRPA1 in dermal sensory nerve fibers and mast cells. TRPA1 was expressed in lesional biopsies from AD patients (Oh et al., 2013). From our statistically correlation of activated ILC2 (IL-13+ ILC2s and IL-5+ IL-13+ ILC2s) and disease severity are comparable with Smith et al showing an increase in airways levels of ILC2 was associated with uncontrolled airway eosinophilia (Smith et al., 2016). Beck et al, reported that there was a significant improvement in the 5-D pruritus scale score and the pruritus numerical-rating scale score, which is an indication for itching after administration of Dupilumab. This shows that Dupilumab may target ILC2-derived IL-13 and thus have disease modifying effects. Although we did not find a correlation with our skin ILC2s and eosinophils, staining for activated eosinophil or eosinophil-derived granule proteins may provide a correlation with ILC2 levels. These studies are underway currently.

We also performed a Spearman correlation on our ILC2s and CD4+ cells with our EoP counts and found no correlations. Interestingly, when we combined our ILC2s and CD4+ cells and performed a Spearman correlation on our EoPs, there was a significant correlation ($r=0.58$, $p=0.03$) (Figure 17). This mirrored a study by Liu et al that there was a collaborative effect of ILC2s and CD4+ cells in BALB/c mice in an asthma model. ILC2s and CD4+ cells combined promoted airway eosinophilia and goblet cell hyperplasia by release of cytokines (Liu, Lee, Chen, Hershey, & Wang, 2015).

4.1: Conclusions

Using flow cytometry, we identified ILC2s, EoPs, CD4+ cells, and basophils in peripheral blood and lesional skin in patients with moderate-to-severe Atopic Dermatitis at baseline. Total ILC2s and EoPs were found to be elevated in the skin, compared to blood. Activated ILC2s and EoPs were defined as cells that produce Type 2 cytokines IL-5 and IL-13, which were also higher in the skin, compared to blood. Conversely, we found that total CD4+ cells were higher in the blood, compared to skin. We found an equal activation of CD4+ cells in the blood and skin. The challenge with flow cytometry is acquiring an adequate number of cells. A suitable workaround is the use of immunofluorescence staining to look for ILC2s, which is a rare cell population.

We improved a previously staining protocol to identify ILC2s by immunofluorescence on formalin-fixed paraffin-embedded skin biopsy tissues. Our ILC2 IF study is novel as we stained for ILC2s in lesional-skin, non-lesional skin, allergen-challenged skin, and diluent-challenged skin. We reported detecting ILC2s in these skin biopsies. ILC2s were higher in allergen-challenged skin, followed by lesional skin, and then an equal number of ILC2s in the controls. This novel protocol is a suitable workaround to counter the challenge faced with flow cytometry.

We proposed that the presence of ILC2s promoted the activation, migration, survival and development of EoPs and mature eosinophils, thereby creating tissue eosinophilia. From our flow cytometry data, we detected IL-5 and IL-13, which are key cytokines for eosinophils. Currently, with our data, we could not determine if that is the case as we did not perform any mechanistic studies.

Currently, the gold standard of treatment for moderate-to-severe Atopic Dermatitis is the use of systemic treatments such corticosteroids and cyclosporine. The problem with the long-term

use of these drugs is the range of side effects such as, but not limited to, high blood pressure, edema, slow wound healing, glaucoma, depression and osteoporosis. There has been a paradigm shift on the use of biologics such as monoclonal antibodies. Currently, there are several clinical trials with the use of monoclonal antibodies. For example, our research group is investigating the use of Benralizumab in asthmatic patients, which targets IL-5R α (Roma Sehmi et al., 2018). There are also antibodies that target IL-5 such as Mepolizumab and Reslizumab (Mukherjee et al., 2018) (R. Sehmi et al., 2016). There is Dupilumab, which is approved for AD, targets the IL-4R α , which IL-4 and IL-13 binds to (Beck et al., 2014). Future experiments are needed to define the mechanistic role of ILC2s in the context of EoP and mature eosinophil activation, recruitment, development and survival.

4.2: Future Directions

This thesis aimed to look at the levels of ILC2s, EoPs, CD4+, and basophils in lesional skin and peripheral blood in patients with moderate-to-severe AD baseline. This thesis is a small part of an overarching study which investigates the effects of oral corticosteroids on ILC2s, EoPs, CD4+, and basophils in a double-blind, placebo-controlled study. Our main hypothesis is that ILC2s are increased in lesional and allergen-challenged skin and oral corticosteroids reduces the number of ILC2s.

The reason why we are unable to report the post-treatment data is that the study is still ongoing where we are currently recruiting 20 patients and have processed 16 patients so far. As the study is still ongoing, we are currently blinded to which group the patients are randomized to. This is a 2-week study with 4 visits per patient. Patients that are on oral cyclosporine and calcineurin inhibitors are to go for a 30-day washout prior to commencement of the study, while

patients on oral corticosteroids to washout for 7 days prior. Two weeks prior to the start of the study, patients undergo an 8-day steroid run-in at a concentration of 0.25mg/kg body weight, after which there is an 8-day washout, as previously reported in Materials and Methods chapter.

During the first visit, typically on a Monday, patients will undergo a skin prick test to confirm the RAST result. After selecting a suitable allergen, a titration is performed to find a suitable dilution for the allergen intradermal challenge. Twenty-four hours later, the patient returned for a second visit and a series of punch and excision biopsies as well as a draw of 20mL of peripheral blood in sodium heparin tubes. The patient is then randomized to the steroid (prednisolone) group, or the placebo group at a concentration of 0.75mg/kg body weight for the rest of the week. The patient will then return for the third visit the following Monday for another round of allergen and diluent challenge. The patient will again return 24 hours later for the fourth visit for another round of biopsies and blood draw. The patient will then be given a lower dose of prednisolone for the next 3 days (0.50mg/kg body weight), followed by a lower dose of prednisolone for the last 4 days (0.25mg/kg body weight). The reason for this tapering is to prevent steroid withdrawal in patients. If patients were to immediately stop the prednisolone without any tapering, it will cause steroid withdrawal, which may create problems as the adrenal glands are not given enough time to return to normal levels of hormone secretion. Side effects of steroid withdrawal include, fatigue, weight loss, nausea, diarrhea, dizziness and depression.

Like the methodology reported in this thesis, the same methods will be used on the post-treatment samples. After completing 20 patients, the code will be broken to determine which groups the patients are randomized, and the proper statistics will be employed.

During the course of this study, as reported in the Materials and Methods chapter, we are also collecting the supernatants from allergen-challenged and lesional skin punch biopsies. We will be sending the supernatants to a third-party laboratory (Eve Technologies) to identify and quantify cytokines. The supernatants are currently stored at -80°C in our laboratory. The panel is being determined to find out which cytokines are of interest. But we will be looking for alarmin cytokines, TSLP, IL-25, and IL-33. We are also interested in looking at γ -chain cytokines IL-2, IL-4 and IL-7, and lipid mediators Prostaglandin D2, and Leukotriene B4/C4, and Type 2 cytokines, IL-4, IL-5, IL-9, and IL-13. We aim to publish our findings after collecting all our data. Upon completion of this oral steroid study, our research group will commence on a similar study, using Benralizumab which targets IL-5R α , again with patients with moderate-to-severe AD.

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APPENDIX**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.

(Reprinted) *Arch Dermatol.* 2004;140(12):1513-1519
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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	4	50-69% of the respective body region
1	< 10% of the respective body region	5	70-89% of the respective body region
2	10-29% of the respective body region	6	90-100% of the respective body region
3	30-49% 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

INVESTIGATORS GLOBAL ASSESEMENT

Score: _____

IGA Scoring Table (Eichenfield et al)

0	Clear (no inflammatory signs of AD)
1	Almost clear (just perceptible erythema, and just perceptible papulation/infiltration)
2	Mild disease (mild erythema, and mild papulation/infiltration)
3	Moderate disease (moderate erythema, and moderate papulation/infiltration)
4	Severe disease (severe erythema, and severe papulation/infiltration)
5	Very severe disease (severe erythema, and severe papulation/infiltration with oozing/crusting)

Scoring completed by_____
Date

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	<input type="checkbox"/> <input type="checkbox"/> <input type="checkbox"/> <input type="checkbox"/>	
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	<input type="checkbox"/> <input type="checkbox"/> <input type="checkbox"/> <input type="checkbox"/>	
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	<input type="checkbox"/> <input type="checkbox"/> <input type="checkbox"/> <input type="checkbox"/>	Not relevant <input type="checkbox"/>
4.	Over the last week, how much has your skin influenced the clothes you wear?	Very much A lot A little Not at all	<input type="checkbox"/> <input type="checkbox"/> <input type="checkbox"/> <input type="checkbox"/>	Not relevant <input type="checkbox"/>
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	<input type="checkbox"/> <input type="checkbox"/> <input type="checkbox"/> <input type="checkbox"/>	Not relevant <input type="checkbox"/>
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	<input type="checkbox"/> <input type="checkbox"/> <input type="checkbox"/> <input type="checkbox"/>	Not relevant <input type="checkbox"/>
7.	Over the last week, has your skin prevented you from working or studying?	Yes No	<input type="checkbox"/> <input type="checkbox"/>	Not relevant <input type="checkbox"/>
	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	<input type="checkbox"/> <input type="checkbox"/> <input type="checkbox"/>	
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	<input type="checkbox"/> <input type="checkbox"/> <input type="checkbox"/> <input type="checkbox"/>	Not relevant <input type="checkbox"/>
9.	Over the last week, how much has your skin caused any sexual difficulties?	Very much A lot A little Not at all	<input type="checkbox"/> <input type="checkbox"/> <input type="checkbox"/> <input type="checkbox"/>	Not relevant <input type="checkbox"/>
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	<input type="checkbox"/> <input type="checkbox"/> <input type="checkbox"/> <input type="checkbox"/>	Not relevant <input type="checkbox"/>

SCORAD INDEX

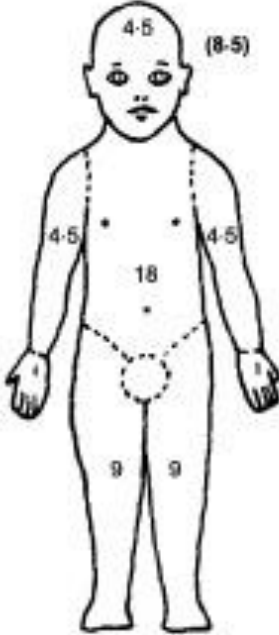
EUROPEAN TASK FORCE ON ATOPIC DERMATITIS

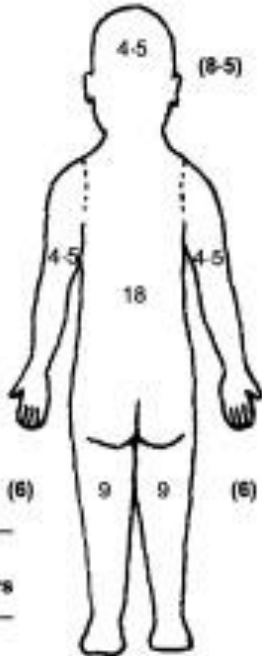
Last Name

First Name

Date of Birth: DD/MM/YY

Date of Visit:





Figures in parenthesis for children under two years

A: EXTENT Please indicate the area involved

B: INTENSITY

C: SUBJECTIVE SYMPTOMS
PRURITUS + SLEEP LOSS

A/5 + 7B/2 + C

CRITERIA	INTENSITY
Erythema	
Oedema/Papulation	
Oozing/crust	
Excoriation	
Lichenification	
Dryness*	

* Dryness is evaluated on uninvolved areas

MEANS OF CALCULATION
INTENSITY ITEMS (average representative area)
0 = absence
1 = mild
2 = moderate
3 = severe

Visual analog scale (average for the last 3 days or nights)

PRURITUS (0 to 10) **0** **10**

SLEEP LOSS (0 to 10)