In-situ spatial characterization of pathogenic effector T cells in the skin of adult atopic dermatitis patients

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Abstract (English)

Atopic dermatitis (AD), also known as atopic eczema, is a chronic inflammatory skin disorder affecting 15–20% of children and up to 10% of adults. Characterized by a compromised skin barrier and an aberrant immune response, AD was once considered primarily a childhood-limited condition. However, recent studies have highlighted a significant prevalence of adult-onset AD and high rates of persistence into adulthood. Th2-associated genes and cytokines, particularly IL-13 and IL-4, are believed to play a critical role in AD pathogenesis, however, IL-4 protein has not been found to be significantly elevated in skin. IL-13 may be the central mediator of disease, but cellular sources remain to be clarified in AD. A subset of IL-13-high and IL-4-low Th2 cells, known as Th2A or pathogenic effector Th2 (peTh2) cells expressing chemokine receptor CRTH2, were recently detected in adult AD skin, along with novel type 2 $CD8^+$ T cells. Elevated levels of IL-13 also appear to persist in the skin of patients with clinically resolved AD, even after treatment with dupilumab, the first FDA-approved long-term systemic therapy. Tissue-resident memory cells (TRMs) are known to be critical in maintaining immune memory across various tissues, but little is known about TRM peTh2-type cells in skin. We hypothesize that TRM cells with pathogenic effector functions ($CRTH2^+$ / $IL\text{-}13^+$) are elevated in AD patients, impeding full remission.

Using a case-control design (N=11 adult AD patients, N=6 healthy subjects (HS)) we first examined the expression of Th2 cytokines, IL-13 and IL-4, at the protein level in skin biopsies. Our findings confirmed very significantly increased IL-13 in AD skin across all compartments. While IL-4 protein was more difficult to detect, we found protein concentration significantly increased when focused on the epidermal compartment alone. Immunofluorescence staining

against CD3 and CD8, coupled with IL-13 and IL-4, revealed a significant accumulation of IL-13-protein^{+} CD8^{+} T cells in the dermis of AD skin. In contrast, this was not found for canonic Th2 (IL-4^{+} or IL-13^{+}) cells, suggesting alternative pathogenic effectors in AD skin. Preliminary data may suggest increased CD69^{+} TRM expressing IL-13 protein, but this needs more investigation.

To validate protein-based findings and phenotype more deeply, unbiased analysis of spatial single cell single molecule FISH (RNAscope) was conducted to characterize pathogenic effector T cells in AD skin in a sub-cohort of these patients (N=5 AD and HS). Using spatial topo maps, we found CD8, not CD4, CRTH2^{hi} TRM peTH2-type T cells were unique to AD epidermis, with the signature OX-40^{hi}GATA3^{hi}IL-13^{hi}IL-4^{lo} cytokine profile.

These findings underscore the pivotal role of IL-13 and tissue-resident memory cells in the severe adult subtype of AD, offering insights into potential therapeutic targets to achieve complete remission.

Abstract (Français)

La dermatite atopique (DA), également connue sous le nom d'eczéma atopique, est un trouble inflammatoire chronique de la peau qui touche 15 à 20 % des enfants et jusqu'à 10 % des adultes. Caractérisée par une barrière cutanée compromise et une réponse immunitaire aberrante, la DA était autrefois considérée comme une condition principalement limitée à l'enfance. Cependant, des études récentes ont mis en évidence une prévalence significative de la DA à apparition à l'âge adulte et des taux élevés de persistance à l'âge adulte. Les gènes et cytokines associés aux cellules Th2, en particulier l'IL-13 et l'IL-4, sont censés jouer un rôle clé dans la pathogenèse de la DA, cependant, la protéine IL-4 n'a pas été trouvée significativement élevée dans la peau. L'IL-13 pourrait être le médiateur central de la maladie, mais les sources cellulaires restent à clarifier dans la DA. Un sous-ensemble de cellules Th2 riches en IL-13 et faibles en IL-4, connues sous le nom de Th2A ou cellules Th2 effectrices pathogènes (peTh2), exprimant le récepteur des chimiokines CRTH2, a récemment été détecté dans la peau de patients adultes atteints de DA, ainsi que de nouvelles cellules T $CD8^+$ de type 2. Des niveaux élevés d'IL-13 semblent également persister dans la peau de patients dont la DA est cliniquement résolue, même après un traitement par dupilumab, la première thérapie systémique approuvée par la FDA pour un traitement à long terme. Les cellules mémoire résidentes des tissus (TRM) sont connues pour être essentielles dans le maintien de la mémoire immunitaire à travers divers tissus, mais on sait peu de choses sur les cellules TRM de type peTh2 dans la peau. Nous émettons l'hypothèse que les cellules TRM avec des fonctions effectrices pathogènes ($CRTH2^+$ / $IL\text{-}13^+$) sont élevées chez les patients atteints de DA, empêchant une rémission complète.

À l'aide d'une conception cas-témoin (N=11 patients adultes atteints de DA, N=6 sujets sains (HS)), nous avons d'abord examiné l'expression des cytokines Th2, IL-13 et IL-4, au niveau

protéique dans des biopsies cutanées. Nos résultats ont confirmé une augmentation très significative de l'IL-13 dans la peau des patients atteints de DA, dans tous les compartiments. Bien que la protéine IL-4 soit plus difficile à détecter, nous avons trouvé une concentration protéique significativement augmentée lorsqu'on se concentrait uniquement sur le compartiment épidermique. La coloration en immunofluorescence contre CD3 et CD8, couplée à IL-13 et IL-4, a révélé une accumulation significative de cellules T $CD8^+$ $IL\text{-}13^+$ dans le derme de la peau atteinte de DA. En revanche, cela n'a pas été observé pour les cellules Th2 canoniques ($IL\text{-}4^+$ ou $IL\text{-}13^+$), ce qui suggère d'autres effecteurs pathogènes dans la peau atteinte de DA. Les données préliminaires peuvent suggérer une augmentation des TRM $CD69^+$ exprimant la protéine IL-13, mais cela nécessite davantage de recherches.

Pour valider les résultats basés sur les protéines et caractériser plus en profondeur le phénotype, une analyse non biaisée des cellules individuelles par FISH à molécule unique et à cellule unique (RNAscope) a été réalisée pour caractériser les cellules T effectrices pathogènes dans la peau des patients atteints de DA dans une sous-cohorte de ces patients (N=5 DA et HS). En utilisant des cartes topographiques spatiales, nous avons découvert que les cellules T $CD8^+$ $CRTH2^{hi}$ TRM de type peTh2, et non $CD4^+$, étaient uniques à l'épiderme de la DA, avec un profil de cytokines signature $OX\text{-}40^{hi}GATA3^{hi}IL\text{-}13^{hi}IL\text{-}4^{lo}$.

Ces découvertes soulignent le rôle crucial de l'IL-13 et des cellules mémoire résidentes des tissus dans le sous-type adulte sévère de la DA, offrant des perspectives sur des cibles thérapeutiques potentielles pour parvenir à une rémission complète.

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Thank you all.

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Contribution of Authors

Individual contributions to thesis are as follows:

Vimal Murugesan: Conducted all experiments, analyzed and interpreted the results. Primary to thesis preparation, performed the literature review, and was responsible for troubleshooting and optimizing both the methodology and data analysis.

Gaurav Isola: Assisted in the development of the project and methodology and contributed to the understanding and analysis of the data.

Souad Mubaid: Reviewed and revised sections of the thesis, provided feedback on edits.

Carolyn Jack: Supervised the study, facilitated skin sample collection, editorial help and contributed to data interpretation.

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List of Abbreviations

AD	**Atopic dermatitis**
AE	**Atopic eczema**
AMP	**Anti-microbial peptides**
BSA	**Body surface area**
CLA	**Cutaneous lymphocyte-associated antigen**
CRTH2	**Chemoattractant homologous receptor expressed by Th2 cells**
DCs	**Dendritic cells**
EASI	**Eczema area severity index**
FLG	**Filaggrin**
GWAS	**Genome wide association studies**
ILCs	**Innate lymphoid cells**
JAK/STAT	**Janus kinase / signal transducers and activator of transcription**
LCs	**Langerhans cells**
MFI	**Mean fluorescent intensity**
MHC	**Major histocompatibility complex**
NK cells	**Natural killer cells**
peTRM	**Pathogenic effector tissue resident memory cells**
SC	**Stratum Corneum**
STAT	**Signal transducer and activator of transcription**
TEWL	**Trans epidermal water loss**
Tfh	**T follicular helper cells**
TFs	**Transcription factors**
Th2	**T helper 2**
TLR	**Toll-like receptors**
Tregs	**Regulatory T cells**
TRMs	**Tissue resident memory cells**
TSLP	**Thymic stromal lymphopoietin**
vIGA	**Validated investigator global assessment**

1. Atopic Dermatitis

Atopic Dermatitis (AD, also known as Atopic Eczema) is a heterogeneous chronic inflammatory skin disorder [1], highly prevalent in developed nations and, over the past decades, increasingly prevalent in low-income countries [2]. AD has become a significant global public health concern due to its substantial healthcare costs on a worldwide scale [3, 4]. It is associated with significant morbidity; the disease burden of AD is comparable to that of other chronic conditions like epilepsy, diabetes mellitus, and cystic fibrosis [5]. Approximately 15 – 20 % of children develop AD [6], with limited data on persistence across the lifespan; up to 50% of children affected will have AD persistence into adulthood and adult-onset patients developing AD is up to 10% [7, 8]. Recently, a distinct subset of patients with adult-onset AD has been identified [4]. Comprehensive longitudinal studies on the natural progression of AD throughout adulthood remain limited [9].

Theories about the development of AD include "outside in" and "inside out" hypothesis [10, 11]. The former theory implies the primary initiating factor is the impairment of the skin barrier which allows the invasion of allergens and microbes further contributing to the disease progression. Conversely, the latter hypothesis proposes that the onset of AD pathogenesis primarily involves active cutaneous inflammation which results in the compromise of the skin barrier, playing a crucial role in the development of systemic immune dysregulation [10, 12].

Comprehensive longitudinal studies on the natural progression of atopic dermatitis (AD), particularly in adulthood, remain limited due to several factors. Historically, AD was considered primarily a pediatric disease, leading to a greater research focus on its progression in children.

However, adult-onset AD is now gaining more prevalence, and the disease's course in adulthood has not been as thoroughly explored, requiring further research.

The variable nature of AD, characterized by periods of remission and flare-ups, adds complexity to designing long-term studies that accurately capture its progression. Additionally, the presence of comorbidities, such as asthma or allergies, can further obscure the specific trajectory of AD in adults, complicating research efforts. As a result, further investigation is needed to fully understand the natural history of adult-onset AD. The etiology of AD is multifactorial, with an intricate interplay between genetic, immune, and environmental factors [13]. Due to the complexity of factors contributing to AD, and its variable presentation at different stages of life and in different areas of the body with fluctuating severity, large gaps in understanding this disease remain. In the following introductory chapters, I will describe the various factors known to be involved in this disease, with a focus on immune factors and gaps in knowledge.

1.1 Clinical presentation and manifestation of AD

AD is heterogeneous and variable across the different ages, from infancy to adulthood [14-16]. Patients with AD live with dry, intense itchiness (pruritus), cracked, oozing, crusting, thickened skin, and open wounds due to scratching [17]. AD significantly negatively impacts the quality of life of patients due to intense pruritus and sleep disturbances, sleep-related impairments such as daytime somnolence, impaired functioning in school/work, social isolation, disruptions in family dynamics, along with secondary psychological conditions. [18, 19]. The clinical diagnosis of AD is based on the Hanifin and Rajka criteria established in the 1980s [20], where patients presenting with 3 of 4 major features and at least 3 minor features are considered AD (Suppl. Table 1) [21].

Internationally validated tools to measure the severity of AD are the Eczema Area and Severity Index (EASI), Body Surface Area (BSA), and validated Investigator Global Assessment (vIGA) [22, 23]. The vIGA is one of the simplest metrics, scaling from 0 to 1 (clear to almost clear) up to 4 (severe disease) (Suppl. Table 2) [23, 24]. The EASI is the most robust tool, assessing disease intensity for four parameters (erythema, papulation/edema, excoriation, and lichenification) weighted across the total body surface regions; head and neck, trunk, upper limbs, and lower limbs (Suppl. Suppl. Table 3) [23]. The BSA measurement is used to evaluate the extent of the lesions in the affected AD patients [22].

Various sub-types of atopic dermatitis (AD) have been reported, with 'extrinsic' and 'intrinsic' AD being the most common clinically [25]. This classification is based on the presence (extrinsic AD) or absence (intrinsic AD) of elevated immunoglobulin E (IgE, total and allergen-specific). Intrinsic AD comprises ~ 20% of patients with the disease [26]. A total IgE level above 200 kU/L indicates extrinsic AD, however, atopic patients frequently have much higher IgE levels (>1,000 kU/L); extremely high levels may indicate associated monogenic or complex trait immunodeficiency syndromes [14].

Knowing that AD is a heterogeneous disease, and it presents variably across different age groups and subtypes, further research is important to explore the underlying mechanisms driving the heterogeneity of AD.

1.2 Genetic factors involved in AD

Genetic factors influence the predisposition and development of AD [7]. If one parent is affected with AD, the risk of the child also developing AD is 1.5-fold, whereas if both the parents are affected with AD, the risk escalates to approximately 3-5-fold [7, 27]. The most well-known genetic risk factor for AD involves mutations in *FLG*, the gene responsible for encoding the epidermal protein filaggrin, which serves as the major structural component of the stratum corneum, i.e. the top layer of the skin [28, 29]. *FLG* mutation in AD shows a semi-dominant inheritance pattern which leads to a reduction in filaggrin expression [30]. In addition to *FLG*, *OVOL1* and *IL-13* are significantly associated with AD [28]. OVOL1 and OVOL2 are upstream transcription factors that play a critical role in regulating *FLG* expression. They regulate keratinocyte differentiation and proliferation in the skin [31].

As discussed earlier regarding the two primary hypotheses of AD pathogenesis, the "inside-out" hypothesis can be further explained by two key mechanisms involved in the IL-4/IL-13-mediated downregulation of filaggrin. These mechanisms include the inactivation of OVOL1 and the periostin-IL-24 signaling axis. [32]. IL-13 promotes *POSTN* (encoding periostin protein) expression in keratinocytes through the activation of STAT6 [33]. Periostin, in turn, induces keratinocytes to produce IL-24, which downregulates *FLG* expression via STAT3 activation [28, 34].

In addition to *FLG*, various other genetic factors have been linked to atopic dermatitis (AD) through genome-wide association studies (GWAS). Notably, different ethnic groups exhibit distinct *FLG* genetic mutations associations with AD. For instance, *SPRR3* has been significantly associated with AD, along with multiple studies implicating *OVOL [35]*. *CARD14*, a regulator of

FLG, is also involved in AD pathogenesis. *KIF3A*, located on the same chromosomal region (5q31) as the T-helper (Th)2 cytokine cluster, including *IL13*, has been associated with AD as well [27, 35]. A study identified around 202 unique variants, across a smaller number of loci, that have been reported to be linked with AD, highlighting the genetic complexity of the disease [36].

Further research is needed to explore the specific mechanisms by which these genetic variants contribute to AD pathogenesis across different populations and to better understand on how these genes interact with environmental factors, potentially leading to new treatment strategies.

1.3 Current Therapies for AD

The primary goal in managing atopic dermatitis (AD) is to reduce skin inflammation and pruritus, and to maintain long-term disease control, thereby allowing patients to function optimally in their daily lives. The selection of therapeutic strategies is primarily guided by the severity of the disease, with additional considerations such as the patient's age, risk factors, and the presence of comorbid conditions.

The first line of treatment for managing AD is with the use of topical therapies (corticosteroids, calcineurin inhibitors). When the disease is not controlled by topical measures, phototherapy using narrow band ultra-violet B can be used to control skin lesions and inflammation. When both approaches prove ineffective in managing AD, cyclosporine, a systemic immunosuppressive agent, is used as a short-term treatment for AD [18, 37].

The development of biologics for the treatment of AD has focused on targeting the Th2 pathway, which is central to disease's pathogenesis. Dupilumab, the first systemic drug specifically approved for AD, is a monoclonal antibody that targets the IL-4R alpha receptor, thereby inhibiting the signaling of both IL-4 and IL-13 [38]. Simpson *et al*. looked at two randomized placebo-controlled phase 3 enrolled adults with moderate-to-severe AD and assigned dupilumab treatment for 16 weeks compared to placebo. From 671 and 708 patients enrolled in each trial,‒primary outcome of 0 to 1 score on the Investigators' Global Assessment was observed in 38% and 36% of patients who received dupilumab every other week, respectively compared with 8% of patients who received placebo. In addition, 75% improvement in disease severity measured by the EASI score (EASI-75) was achieved in a significant number of dupilumab-treated patients compared to placebo at the end of 16 weeks [39].

Advancements in biologics targeting IL-13 have proven effective in the treatment of AD. They not only help control the disease but also lead to clinically meaningful responses in affected patients [40]. Tralokinumab is a fully human IgG4 monoclonal antibody, designed to specifically target IL-13 by neutralizing its activity via blocking its binding with receptors, thereby easing symptoms associated with AD [41]. Simpson *et al*. found that patients from two phase III clinical trials of tralokinumab for moderate-to-severe AD patients treated with tralokinumab versus placebo experienced clinically meaningful improvements in signs, symptoms, and quality of life. Clinically, responses were defined as at least a 50% improvement in the EASI, a 3-point or greater improvement in itch severity, or a 4-point or greater improvement in quality of life. Tralokinumab treatment was associated with greater proportions of patients achieving these clinically meaningful responses such as EASI-75 or EASI-90 improvements in disease severity scores [42].

Lebrikizumab is another monoclonal antibody that specifically targets IL-13 by blocking its downstream effects through inhibition of the IL-13Ra1/IL-4Ra signaling complex formation. It has only very recently been approved for the treatment of AD [43]. Lebrikizumab treated patients achieved better EASI scores as early as week 2, with clinically meaningful responses as well as additional outcomes such as 75% or 90% improvements in disease severity scores and significant improvements in skin symptoms, including reductions in itching [44]. Although direct comparison is not possible, lebrikizumab appears to achieve equivalent efficacy, as measured by EASI-75, when compared to dupilumab [45].

In addition to targeting cytokine binding, novel drugs target downstream receptor signaling via the JAK/STAT pathway. Such drugs include Upadacitinib, Abrocitinib, and Baricitinib, which have been approved for the treatment of AD [46]. The most effective drug, Upadacitinib, is an ATP-competitive Janus kinase 1 (JAK) inhibitor that blocks nucleotide binding by competing with ATP, thereby inhibiting kinase activity and preventing the phosphorylation of downstream effectors. This drug primarily targets the JAK1 pathway, with comparatively lower efficacy against other JAK pathways [47]. In clinical studies, Upadacitinib demonstrated significant efficacy in treating atopic dermatitis (AD). The percentages of patients achieving EASI responses were as follows: EASI-75 at 91.43%, EASI-90 at 74.29%, and EASI-100 at 60% [48].

Significant development has been made in understanding the treatment and pathogenesis of AD, from managing symptoms to targeting the underlying immunology of the disease. However, the ability to achieve complete remission varies across treatments. Topical corticosteroids (TCS) are

commonly used to control flare-ups, while biologics have demonstrated greater efficacy in both reducing flare-ups and controlling disease progression, although they often require long-term use. Most treatment options rely on continuous or intermittent administration to prevent relapses, highlighting that the mechanisms preventing complete remission remain poorly understood. Therefore, further research is needed to identify the specific immune cells and factors involved in AD pathogenesis and to develop strategies for achieving sustained remission.

2. AD Immunology and Pathogenic effector cells in AD

2.1 Immune cells and the role of Cytokines in AD

AD is characterized by immune dysregulation, predominantly mediated by a Th2 response, and more recent findings implicate Th22 and Th17 as well. In skin, chronic atopic dermatitis may also be characterized by concomitant presence of Th1-, Th2-, and Th17 -related gene pathways in bulk RNA-sequencing [49, 50]. Immunological studies assessing gene expression in the skin of AD patients consistently observe upregulation of Th2-related genes [51-53]. Clinical studies assessing the impact of targeting of IL-13/IL-4 receptor by dupilumab demonstrated that AD is driven by a Th2 response. Hamilton *et al*. performed transcriptomic studies using both lesional and non-lesional skin from AD patients receiving dupilumab treatment (4 weeks) compared to placebo. They found decreased expression in genes related to hyperplasia, T cells, dendritic cells, and Th2-associated chemokines, which were all correlated with improved disease severity scores [54]. A study by Bakker *et al*. examined the early and long-term effects of dupilumab treatment revealing that, at 4 weeks, there was a reduction in the frequency of skin-homing T cells producing Th2 and Th22 cytokines. However, by 52 weeks, no preferential skewing towards a specific T helper cell subset was observed. [55].

Single-cell RNA sequencing studies have revealed that the frequencies of CD4 /CD8 type 2 (IL-13) and type 17 (IL-26) cells were significantly increased in lesional AD compared with both non-lesional AD and control samples [56, 57]. Type 2, type 22, or type 17 T cell proportions were higher, ranging from 2.98- to 3.87-fold, compared to type 1 cells in lesional AD skin [57].

Transcription factors and cytokines play a major role in priming T cells and with the expression of chemokines recruit T cells to the tissues. [58]. IL-13 and IL-4, are the two most prominent cytokines thought to be involved in the disease pathogenesis of AD. Although these cytokines share overlapping functions and a receptor subunit and originate from the same chromosomal region (5q31), they exhibit distinct expression patterns *in vivo* [59, 60]. This differential expression occurs in a variety of cells that regulate both innate and adaptive immunity, suggesting that IL-4 and IL-13 may have distinct roles in Th2 immunity [59]. IL-4 is known to drive T cell differentiation and may play a crucial role in the early stages of atopic dermatitis (AD) pathogenesis, whereas IL-13 predominantly affects peripheral tissue cells and influences the effector phase of the immune response [28, 61, 62]. IL-13 in AD skin lesions is well-established at the mRNA level [46, 63-65]. Specifically, biopsies of AD skin show an overexpression of IL-13 in both lesional and non-lesional areas compared to healthy controls [32]. Furthermore, the severity of AD is directly correlated with elevated IL-13 protein expression in the lesional AD skin [16, 66]. A reduction in IL-13 levels has been associated with improved clinical outcomes, suggesting IL-13 plays a crucial role in the disease activity [66]. IL-4 drives the differentiation of naïve CD4+ T cells into Th2 cells [67]. This results in a significantly higher number of Th2 cells infiltrating both lesional and non-lesional AD skin. Mouse models have underscored the centrality of IL-4 in AD pathogenesis, demonstrating its ability to induce all histopathological features of the disease [68]. In contrast, there is an inconsistency in the levels of IL-4 protein expression in skin biopsies of AD patients [69], while others show near undetectable levels of IL-4 in the skin of AD patients [63]. The dominance of IL-13 is poorly understood, and the role of IL-4 is unclear in human. Thus, further investigation is needed separately for the role of both IL-13 and IL-4 in AD.

IL-13 is an immunoregulatory cytokine primarily produced by Th2 cells, as well as mast cells, basophils, and innate lymphoid cells (ILCs) [70]. In the skin of murine models, dermal ILC2s secrete IL-13 independently of allergen exposure and without the involvement of alarmins such as IL-2, TSLP, and IL-33 [59]. This IL-13 secretion drives the differentiation of dendritic cell precursors, which primes Th2 cells [59]. The signaling of IL-13 is mediated through a complex receptor system. When IL-13 binds to the functional heterodimeric IL-4Rα/IL-13Rα1 receptor, it triggers the activation of downstream pathways, including tyrosine kinase 2 (TYK2) and Janus kinase 1 (JAK1), leading to the activation of signal transducer and activator of transcription (STAT) proteins, specifically STAT3, STAT1, and STAT6 [61, 65, 70]. The activation of the JAK-STAT pathway results in the increased secretion of chemokines for eosinophils, cytokines, angiogenic factors, subsequently leading to an elevated production of IgE by B cells [71]. The binding of IgE to mast cell receptors leads them to release inflammatory mediators, including histamine, cytokines, and proteases, contributing to the worsening of the inflammatory process in AD [72, 73].

Other than T cells, mast cells, eosinophils, DCs, ILCs, NK T cells play a role in AD pathogenesis [74]. In humans, dendritic cells (DCs) are categorized into two major subsets: CD11c+ myeloid dendritic cells (mDCs) and CD123+ plasmacytoid dendritic cells (pDCs). The function of mDCs is crucial in the etiology of allergic diseases, as they drive Th2 immune responses to inhaled allergens[75]. In AD, myeloid DCs, including inflammatory dendritic epidermal cells, are the most prominently expanded immune cell population in lesions [76, 77]. Studies looking at both humans and murine models, indicate that there is an increased number of mast cells found in AD, and they

contribute to skin inflammation [78]. Mast cells in response to IgE stimulus release various mediators, which include cytokines such as IL-4 and TNF as well as itch mediators such as histamines [79]. Itching and scratching promote keratinocyte activation which releases TSLP and IL-33, resulting in the activation of a range of myeloid cells [80, 81]. The involvement of mast cells also includes regulation and recruitment of chemotaxis in other inflammatory cells, which may amplify disease progression [82].

Since 2020, multiple studies have used single-cell RNA sequencing technology to compare the gene expression of the different types of cells in AD patients compared to healthy subjects [57, 83-92]. While these studies confirm the presence of a type 2 immune response, their findings have revealed that there are poorly described immune cell populations in skin that may be associated with AD, creating an urgent need to characterize specific immune cells and to assess their role in driving the pathogenesis.

2.2 T cell activation and co-stimulatory molecules in AD

The activation of T cells involves three key signals: antigen presentation by MHC, co-stimulatory molecules, and polarizing cytokines. These signals collectively activate T cells. OX40L, a co-stimulatory molecule expressed on the surface of both $CD4^+$ and $CD8^+$ T cells, plays a key role in enhancing their effector functions, and thought to play a role in AD progression [93].

OX40/OX40L involvement has also been observed in other atopic and autoimmune diseases, such as asthma, multiple sclerosis (MS), Crohn's disease, irritable bowel syndrome (IBS), and rheumatoid arthritis. [94]. Targeting OX40 has emerged as a promising therapeutic strategy to

inhibit Th2 cells in AD, leading to the development of various antibodies against OX40, including Rocatinlimab, Telazorlimab, and Amlitelimab [94, 95] Upon antigen stimulation, OX40 is rapidly and transiently expressed on both memory and effector T cells. Its ligand, OX40L, is a co-stimulatory molecule expressed on B cells, Langerhans cells (LCs), and dendritic cells (DCs), as well as on other cell types, including ILC2s, fibroblasts, and mast cells [96, 97]. Studies have shown that lesional skin of AD patients exhibits high OX40 and OX40L expression as well as increased numbers of OX40L-expressing cells, such as monocytes, mast cells, dendritic cells and ILCs [94]. In addition, co-localization of OX40 (expressed by T cells) and OX40 ligand (OX40L) (expressed by monocytes) in the skin of AD patients have been noted [98, 99]. A study by Ilves *et al*. investigated the expression of OX40-OX40L in patients with atopic dermatitis (AD). Their findings revealed a significant upregulation of OX40-OX40L expression in lesional skin compared to non-lesional skin in AD patients. Additionally, the study identified that keratinocytes in the epidermis of AD skin also express OX40L [100].

OX40 is critical in T-cell activation and may contribute to the progression of AD; however, the mechanisms underlying OX40-OX40L interactions, particularly between these molecules and keratinocytes, remain incompletely understood and require further investigation.

2.3 Pathogenic role of effector cells in AD

A subset of Th2 cells, termed by various groups as pathogenic effector Th2 (peTh2) cells (or Th2A cells), have been described in allergic diseases [101]. peTh2 cells have been shown in individuals with allergic diseases such as food allergy (FA), allergic rhinitis (AR), asthma, and eosinophilic esophagitis (EoE) [101, 102]. PeTh2 cells exhibit enhanced function in allergic diseases, with elevated expression of IL-5 and IL-13 and were therefore termed pathogenic effector peTh2 cells.

These cells are $CD4^+$ T cells characterized by the expression of chemoattractant receptor–homologous molecule expressed on Th2 cells (CRTH2) [103]. Prostaglandin D2 (PGD2), which binds to the CRTH2 receptor, is produced by various immune cells, including Th2 helper T cells, dendritic cells (DCs), skin Langerhans cells, and keratinocytes [104, 105]. PGD2 mediates its effects including chemotaxis through the receptors DP1 and CRTH2 [101]. CD161 is a C type lectin marker known to be expressed on ILC2, Natural killer (NK) cells, Th17 cells, and memory T cells [106]. Importantly, Bangert *et al*., revealed a persistent population of cells in AD patients undergoing a year-long treatment with dupilumab, which had an increased expression of $CRTH2^+$ $CD161^+$, consistent with the characteristics of peTh2 cells [107].

Further research is needed to understand the specific mechanisms driving the persistence of peTh2 cells in AD, particularly in patients undergoing treatments. This could reveal therapeutic strategies to target these cells more effectively and improve long-term disease management.

2.4 Role of Innate Lymphoid cells in AD

Innate lymphoid cells (ILCs) originate from common lymphoid progenitor cells (CLPs). ILCs are categorized into five subtypes, including NK cells, group 1 ILCs, group 2 ILCs, group 3 ILCs, and lymphoid tissue-inducing (LTi) cells [108]. ILCs, especially group 2 ILCs (ILC2s) can produce type 2 cytokines[105]. Alkon *et al*. used 14 enzymatically digested skin samples from atopic dermatitis (AD) patients and 51 healthy controls for explant cultures *in vitro*. The study revealed an increased number of innate lymphoid cells (ILCs) in patients with atopic dermatitis (AD), with these ILCs exhibiting CRTH2 expression. Their localization in the epidermis and upper dermis was determined through immunofluorescence using multiple markers and validated by flow cytometry. Given the challenges in precisely characterizing ILCs, the researchers employed

CD161+ and CD94+, which are primarily NK cell markers but can also be expressed by other cell types. Although these markers do not definitively identify ILCs, they are known to be expressed on ILC populations. The identified ILCs were found to express type 2 inflammatory cytokine genes, contributing to the inflammatory response in AD [84]. Expanding the investigation to a larger cohort of AD skin biopsy samples would be highly valuable to confirm if cellular and molecular signals driving ILCs are important in comparison to T cells.

Epidermal alarmins, including TSLP, IL-33, and IL-25, are released by epithelial cells and play a key role in the activation of a distinct subset of Th2 cells, known as Th2A or peTh2 cells. Unlike conventional Th2 cells, Th2A cells are directly activated by these alarmins in response to inflammation, bypassing the traditional activation pathway [101, 102]. IL-33 binds to its receptor ST2 (IL1RL1) and, together with the IL-1 receptor accessory protein (IL-1RAcP), activates intracellular signaling pathways that drive type 2 immune responses. *In vitro* studies using murine and human ILC2s have shown that IL-33 signaling induces the expansion of ILC2s and stimulates the production of type 2 cytokines [109]. In the skin, IL-33, IL-25, and thymic stromal lymphopoietin (TSLP) have been shown to play crucial roles in inducing ILC2 responses and inflammation in a mouse model of AD [110]. The complex mechanisms of AD involve critical interactions between ILC2s and other innate immune cells, such as mast cells and basophils.[111, 112]. Studies using mouse models and *in vitro* systems have demonstrated the involvement of innate lymphoid cells (ILCs) in atopic dermatitis (AD)-like phenotypes. Additionally, single-cell RNA (scRNA) studies have identified the presence of ILCs in human AD skin. However, further research is needed to fully elucidate the role of ILCs in AD pathogenesis in human subjects.

ILCs play a crucial role in amplifying the immune response in AD, bridging innate immunity and the activation of pathogenic effector cells, while keratinocytes contribute to this process by releasing signals that further recruit and activate these immune cells, creating a cycle of inflammation.

2.5 Interaction between keratinocytes and pathogenic effector cells

Keratinocytes act as the first line of defense against the pathogens invading the skin [113]. Disruption of the epidermal barrier keratinocytes leads to the release of damage-associated molecular patterns (DAMPs), commonly DNA, cytosolic peptides, antimicrobial peptides (AMPs) such as defensins and alarmins such as IL-25, IL-33, and TSLP [114, 115]. Alarmins, which are endogenous danger signals, play a key role in cutaneous immune surveillance. Alarmins initiate defensive immune responses by tissue cells as innate cytokines, and may contribute to tailoring adaptive immune responses in the skin [116]. Defensins are anti-microbial peptides that disrupt microbial membranes as endogenous antibiotics [117] Overall, DAMPs bind innate pattern-recognition receptors such as toll-like receptors, while alarmin can also promote T cell polarization such as Th2 (T-helper 2) responses, further contributing to inflammation [118] [119] Chronic secretion of TSLP by damaged keratinocytes in AD skin lesions may drive Th2 inflammation and disease severity [119-123].

IL-33, a member of the IL-1 family, is another alarmin shown to induce Th2 cytokine production [124]. Keratinocytes serve as the primary source of IL-33, which binds the ST2 receptor . Increased levels of IL-33 can stimulate ILC2s, and increased Th2-type cytokines. [122]. Studies from human plasma and mouse AD models has shown that IL-33 also activates antigen-specific regulatory T cells (Tregs), which help mitigate the increased skin inflammation. The IL-33/ST2 signaling

pathway additionally activates sensory neurons, contributing to itch and pain responses in AD [122, 123]. To date, clinical trials are ongoing that aim to block alarmins such as TSLP and IL-133 in AD patients; however, responses appear limited and very little tissue-based data is available for study. Despite the insights on the various factors that are involved in AD, the factors that drive persistent, skin-specific pathogenic cells in AD skin across the lifespan remain unclear, and the role of other immune players such as ILC2 in AD requires further investigation.

2.6 Role of microbes in AD

Staphylococcus aureus is a predominant colonizer of atopic skin and is known to secrete a diverse range of endotoxins and exotoxins [125]. Exposure to *S. aureus* in humans has been shown to increase the production of TSLP and interleukin-33 (IL-33) in keratinocytes, contributing to immune dysregulation [126, 127]. The recognition of *S. aureus* surface lipoproteins is mediated by the Toll-like receptor 2 (TLR2) and Toll-like receptor 6 (TLR6) heterodimer, which promotes Th2-mediated inflammation through TSLP production. Furthermore, *S. aureus* produces pathogen-associated molecular patterns (PAMPs), which, when detected by TLR2, stimulate the epidermis to generate a Th2-dependent inflammatory response [128].

In addition to these mechanisms, *S. aureus* secretes various virulence factors, including exogenous proteases, biofilm, and superantigens [116]. Superantigens, which include staphylococcal enterotoxins (SEs), staphylococcal enterotoxin-like (SEls) proteins, and toxic shock syndrome toxin-1 (TSST1), are potent immunostimulatory molecules that activate T cells. This activation

can lead to rapid inflammation, excessive cytokine production, and potentially severe outcomes, such as toxic shock syndrome [116].

2.7 Barrier dysfunction in AD

Skin barrier dysfunction in AD is driven by a combination of intrinsic genetic and extrinsic environmental factors [129]. The epidermal differentiation complex (EDC) is a gene cluster necessary for normal epidermal terminal differentiation and the formation of the stratum corneum-the outermost layer of the skin that provides its primary barrier function. EDC genes include families such as S100 calcium-binding proteins, small proline-rich proteins, and late cornified envelope essential barrier proteins, which include filaggrin (*FLG*), loricrin (*LOR*), involucrin (*IVL*) [130].

FLG encodes is a keratin aggregating protein, and *FLG* mutations have long been recognized as the strongest genetic risk factor in European and some Asian populations [131]. Null mutations of FLG may be carried by approximately half of all moderate-to-severe AD patients. [131] While heterozygous carriers can demonstrate an eight-fold increased risk for AD, this increases closer to 90% in patients with biallelic mutations [132]. The breakdown products of FLG, including histidine, play important roles in maintaining epidermal hydration and regulating lamellar body secretion and lipid organization critical for barrier function [133]. Epidermal proteases and their inhibitors, including kallikreins, cathepsins, cystatin A, transcription factors such as OVOL1, which controls FLG expression, and corneodesmosomal genes, including desmoglein and desmocollin are also involved [134]. Several additional genes that play a role in the pathogenesis of atopic dermatitis are listed in Table 1. In AD, two opposing pathways regulate the skin barrier: the AHR axis and the IL-13/IL-4-JAK-STAT6/STAT3 axis. The aryl hydrocarbon receptor is an

important transcription factor that increases the expression of EDC genes. The latter suppresses the expression of the key structural proteins FLG, LOR, and IVL, thus impairing terminal differentiation of keratinocytes and weakening skin barrier integrity [138]. Itch-induced scratching may lead to sufficient damage to activate type 2 responses in the right context, where the secretion of IL-13 and subsequent downregulation of *FLG* and barrier protein expression can further compromise the skin barrier, thus driving AD pathogenesis.

Table 1: Genes involved in Atopic Dermatitis [140]		
Genes Encoding Epidermal Proteins	**FLG**	Filaggrin (loss-of-function variants; essential for skin barrier function)
	FLG2	Filaggrin family member 2
	SPINK5	Serine protease inhibitor LETKI, regulates protease activity
	KLK5/SCTE	Kallikrein-related peptidase 5 (stratum corneum tryptic enzyme)
	KLK7/SCCE	Kallikrein-related peptidase 7 (stratum corneum chymotryptic enzyme)
	CLDN1	Claudin-1, critical for maintaining tight junctions in the epidermis
	SPRR3	Small proline-rich protein 3
	TMEM79	Transmembrane protein 79 (mattrin), associated with epidermal barrier integrity
Genes Encoding Immune Proteins	**FCER1A**	Fc fragment of high-affinity IgE receptor I, α chain
	TLR2, 4, 6, 9	Toll-like receptors 2, 4, 6, and 9, involved in pathogen recognition
	IRF2	Interferon regulatory factor 2
	IL4, 5, 12B, 13, 18, 31	Interleukins associated with immune regulation and inflammation
	IL4RA, IL5RA, IL13RA	Interleukin-4, -5, and -13 receptors, α subunits
	GM-CSF	Granulocyte–macrophage colony-stimulating factor
	CD14	Monocyte differentiation antigen CD14
	DEFB1	β-defensin 1, involved in antimicrobial defense
	GSTP1	Glutathione S-transferase P1, involved in detoxification pathways
	CMA1	Mast cell chymase

	CCL5/RANTES	Chemokine (C-C motif) ligand 5, also known as RANTES, contributes to inflammation
	TSLP	Thymic stromal lymphopoietin, regulates immune cell activation
	MIF	Macrophage migration inhibitory factor
	VDR	Vitamin D receptor, involved in immune modulation and skin health

3. Tissue Resident Memory cells in AD

Tissue Resident Memory T cells (TRMs) occupy specific anatomical niches serving as the frontline defense against pathogens upon re-exposure to antigens [141]. TRMs are long-lived cells that, once established in peripheral tissues such as the skin, remain localized for extended periods without recirculating back to the lymph nodes [142]. Even in the absence of detectable pathogens, these cells persist in the skin for years and may play significant role in the chronic nature of AD.

TRMs may contribute to local inflammation characteristic of AD, even in the absence of ongoing exposure to allergens or irritants [143]. Their ability to rapidly respond to environmental triggers may lead to recurrent flare-ups of the disease [144]. Upon re-exposure to specific antigens, TRMs can swiftly reactivate, producing pro-inflammatory cytokines such as IL-13, IL-4, and IL-17 [145, 146]. These cytokines are key drivers of the inflammatory response in AD, with IL-17 playing a particularly crucial role.

The distribution and composition of different TRM cell clusters within the skin may provide insights into the cellular landscape of AD, offering potential targets for therapeutic intervention aimed at achieving disease remission.

3.1 Characterization of Tissue resident memory cells

TRMs are characterized by distinguished surface markers and transcriptional profiles from circulating memory T cells [147]. In healthy human skin, the majority of TRMs are dermal $CD4^+$ T cells, while $CD8^+$ TRMs localize mainly in the epidermis [148]. TRMs underpin the immune surveillance as cytotoxic killer cells and rapidly express proinflammatory cytokines and chemokines after reinfection [149]. They express CD69 (C-type lectin molecule), CD103 (the E-cadherin receptor), and CD49a ($CD8^+$ TRM cell marker) depending on the TRM subset and tissue location [150-153].

In humans and mice, CD69 is expressed on both $CD4^+$ and $CD8^+$ TRMs in the skin. The prolonged expression of CD69 differentiates resident T memory cells from circulating T memory cells [142, 147, 152]. CD69 participates by the downregulating the cell surface expression and function of S1PR1 [150, 154]. S1P protein, creates a gradient, with higher concentrations in the blood and lower in tissues. This gradient guides T cells to exit lymphoid organs and migrate back into circulation [154]. By downregulating S1PR1, TRMs become less responsive to S1P and are retained in the tissues instead of following the S1P gradient back into circulation, taking up residency in skin [155].

CD103, α-chain component of the integrin αEβ7, is a commonly known marker for TRMs in the skin [156]. Enhanced expression of CD103, observed in inflammatory disorders, especially those characterized by T cell infiltration into epithelial tissues like AD, signifies its crucial role in disease progression [157].

The role of TRMs, particularly those expressing CD69 and CD103, in skin immunity and inflammation is important, and the specific mechanisms that regulate their retention, activation, and contribution to disease progression in AD, —especially in relation to their interactions with other immune cell subsets, are not fully understood and require further investigation.

3.2 Role of Tissue Resident Memory T cells in pathogenesis of AD

TRMs are heterogeneous, and their characteristics are influenced by the microenvironment of different tissues and diseases. Upon reactivation of inflammation, pathogen-specific TRMs proliferate and perform effector functions. In the skin, TRMs patrol for pathogens by migrating within the confined epidermal compartment and navigating between keratinocytes through dynamic dendritic projections. This mechanism facilitates rapid and effective protection against subsequent exposures to the same pathogen [158, 159]. Numerous studies have highlighted the essential roles of TRMs in defending against microbial infections, including herpes simplex virus 2, *Leishmania*, bacterial pathogens, and various viruses, particularly in mucosal sites such as the vagina and lungs. Both $CD8^+$ and $CD4^+$ TRMs have been shown to play pivotal roles in these protective immune responses [160, 161].

A study from He *et al*., showed that single-cell RNA sequencing (5 AD patients and 7 healthy controls) demonstrated increased TRMs in lesional AD skin compared to non-lesional and healthy controls skin. Notably, the TRMs predominantly belonged to the $CD8^+$ subset and were primarily localized in the epidermis of lesional AD skin [57].

In AD skin, approximately half of TRMs in the dermis express the immune checkpoint inhibitor, programmed cell death protein 1 (PD-1) [158]. PD-1, and its ligands have been found to play major

roles in maintaining the T cell homeostasis in cancer and allergic diseases [162]. Using immunofluorescence experiments, an expansion of $CD69^{+}CD103^{+}$ cells in lesional AD skin has been observed compared to non-lesional AD skin and healthy controls [163].

A study by Bangert *et al*. used single-cell RNA sequencing on blister fluid samples obtained from patients treated with dupilumab for over a year versus healthy controls. They identified a large cluster of T cells expressing both CD69 and CD103, comprising cells from both CD4 and CD8 lineages, along with other clusters containing Tregs expressing FOXP3 and a small population of mast cells [107]. In this study, TRMs were found sustained in dupilumab-treated AD lesions, characterized by CD69 and CD103 expression, in addition to expressing CD3D. Among these TRMs, a small subset of cells also exhibited markers indicative of central memory T cells, including CCR7 and CD62L [85]. These findings from this study strongly suggest the presence of TRMs in the AD skin in patients who have clinically resolved skin.

Studies from other inflammatory skin diseases such as psoriasis have shown that the TRMs are retained in skin lesional sites even after months of clinical remission with the levels of inflammatory infiltrates reduced in skin [164]. Bangert *et al*. identified a large cluster of TRMs in AD, with $CD8^{+}$ T cells being the largest subset. They also observed sustained TRMs in patients treated with dupilumab [85]. Similarly, He *et al*. found $CD8^{+}$ TRMs localized in the epidermis of AD skin [57]. Liu *et al*. further identified multiple clusters of TRM cells expressing both CD4 and CD8 markers [86]. Rindler *et al*. reported T cell clusters containing $CD69^{+}$ cells [165], and several studies have demonstrated the persistence of CD69-expressing $CD8^{+}$ T cells in resolved AD skin,

indicating the formation of immune memory by these cells, as shown through single-cell RNA sequencing (scRNA-seq).

4. Hypothesis and Aims

Hypothesis of this study:

"**Tissue resident memory T cells (TRMs) with pathogenic effector functions (CRTH2$^+$ and/or IL-13$^+$) are increased in atopic dermatitis skin and may impair remission"**.

Objective:

To characterize the pathogenic effector T cell populations in adult human AD skin

Specific Aims:

Objective I: To test skin for IL-13$^+$ / IL-4$^+$ cells (protein) by immunofluorescence in healthy subjects versus atopic dermatitis patients

Objective II: To apply HIPLEX RNAscope (multiplex fluorescence in-situ hybridization) to characterize pathogenic effector Th2-like cells in skin (mRNA)

5. Methods

5.1 Study design

This is a case: control study of skin samples from patients from the Dermatitis Biobank (DBB) 11 patients met criteria for inclusion in the multi-center Canadian Atopic Dermatitis Cohort for Translational Immunology and Imaging (CACTI) study (REB: 2022-8004); namely, adult AD patients with moderate-severe disease meeting Hanifin & Rajka criteria per the validated Investigator Global Assessment for AD scale, with an EASI score at or above the moderate disease range (≥7.1) as per published severity strata.

All patients and healthy subjects provided written informed consent to participate in the study. 5mm punch biopsy was collected from the patients and healthy subjects after informed consent. The patients were screened for any medications and were enrolled into the study if they are baseline, indicating no prior treatment or medication. A washout phase of 7 days was required for patients taking any TCS. Physician reported clinical parameters (EASI, vIGA, BSA) and patient reported subjective measures were collected from participants. Clinical laboratory test results were used to obtain IgE levels from AD patients (normal, <200 kU/L).

5.2 Processing of skin samples

After obtaining the informed consent, a 5-millimeter skin punch biopsy is performed preferably antecubital fossa. The skin biopsies are subjected to fixation and permeabilization using 1x Fix solution from True-Nuclear™ Transcription Factor Buffer Set (Biolegend, 424401), and this mixture was agitated with a thermomixer maintained at a temperature of 37 ºC for a duration of 45 minutes.

After the fixation and permeabilization process, the skin biopsy was kept in a 30% sucrose solution and incubated overnight at 4 ºC before being frozen using Optimal Cutting Temperature (OCT) medium (Fisher Scientific, 23-730-571). Following the incubation, the skin biopsy was carefully dried and fixed to a biopsy cryomold using OCT, and stored at -80º C.

Further, the frozen skin sections were made into 10-micron-thin slices using a cryostat, and the slides are examined for any folds or damage before storing in -20º C

5.3 Immunofluorescence staining of fixed-frozen skin samples

Immunofluorescence staining was performed on 10-µm sections of fixed frozen skin samples. The slides are washed twice with 1x TBS (Wisent, (811-030) and air-dried. A hydrophobic barrier was created using a pap pen. To identify the T cell population in AD, the sections were treated with monoclonal antibodies against CD3 (Invitrogen, CD3-12), CD8 (Biolegend, RPA-T8), IL-4 (Santa Cruz, sc-12723) and IL-13 (Peprotech, 500-P13) at the concentration of 1:50. Additionally, cytokine expression was analyzed using polyclonal antibodies targeting human IL-13 (company, cat. no.) and IL-4 (company, cat. no.), also following the same concentration. The primary antibody cocktail also had DAPI (nuclear stain), 10% NHS (Non homologous serum) was used to prevent binding to non-target reactive sites. The slides were then incubated overnight at 4 ºC.

After the overnight incubation, the slides were washed with 1x TBS and subsequently re-treated with secondary antibodies against the primary host species: anti-mouse Alexa fluor 488 (Thermofisher Scientific, A28175), anti-rat Alexa fluor 555 (Thermofisher Scientific, A21147), and anti-rabbit Alexa fluor 647 (Thermofisher Scientific, A31573) at room temperature for 1 hour.

For negative control, the primary cocktail did not contain any target antibodies and was treated in the same conditions to that of the real stain. Following this incubation, the slides were washed twice with 1x TBS and air-dried.

Finally, the dried slides were mounted using aqueous fluorescent mounting medium (Sigma Aldrich, F6182) and carefully mounted without any bubbles for imaging. Confocal imaging was performed using Zeiss Microscope LSM 880, using the 20x optical lens and the ZEN software.

5.4 RNA – *in situ* hybridization (RNAscope HiPlex assay)

The RNAscope HiPlex assay is a sequential procedure designed to accommodate up to 12 distinct RNA probes. Sample pre-treatment was done by dehydrating tissue sections through a sequential immersion in varying concentrations of alcohol, followed by air drying. Antigen retrieval was then performed by placing slides within a steamer maintained with antigen retrieval solution (part of RNAscope Multiplex Fluorescent Reagent Kit v2, Cat. No. 323100) at 99°C for 5 minutes, succeeded by washing in distilled water, immersing in ethanol for 3 minutes, and air drying. A hydrophobic barrier is established on the desiccated slides using a pap-pen, thereby preparing the slides for further treatment.

Subsequently, protease treatment was carried out by incubating the slides in the HybEZ oven with protease for 30 minutes at 40°C, followed by a distilled water wash. Simultaneously, probe cocktails and amplification reagents are prepared, with probes being prewarmed at 40°C for 10 minutes and a probe cocktail prepared by combining 40 µL of each probe stock with 1520 µL of RNAscope HiPlex probe diluent to form a 2 mL solution encompassing all 12 probes. For our panel, we selected 12 probes (Table 2).

Table 2: 12-plex RNAscope panel		
Sr. No	**Marker**	**Marker function**
1	IL-13	Th2 cytokine
2	CD8	T cell lineage marker
3	CD45	Leucocyte marker
4	CD4	T helper cell lineage marker
5	IL-4	Key differentiation cytokine Th2
6	OX40	Co-stimulatory marker
7	CRTH2	Pathogenic effector marker – inflammatory mediator
8	CD103	Integrin (TRMs marker)
9	CD69	C-type lectin, early activation and tissue-residency marker
10	TSLPR	Alarmin receptor
11	TCR	T cell marker
12	GATA3	Transcription factor key for Th2 cells

The slides were incubated for 2hours with the probe cocktail at 40°C, followed by two washes with 1x wash buffer. The subsequent stages encompassed three amplification steps, each employing a specific amplification solution (AMP1, AMP2, and AMP3), with each step involving a 30-minute incubation at 40°C and an intervening wash with 1x wash buffer. Following amplification, the day 1 fluorophores (T1-T3) are applied for a 15-minute incubation at 40°C, followed by two 2-minute washes in 1x wash buffer. The slides were then air-dried, treated with

approximately 3-4 drops of DAPI for no more than 30 seconds, mounted using an Antifade mounting medium, and stored at 2-8°C until the imaging process.

Following the initial imaging session on day 1, the glass slides were subjected to a defluorination process using a 4x saline-sodium citrate (SSC) solution for 30 minutes at room temperature. This treatment facilitated the gentle detachment of coverslips, preparing the slides for the subsequent day 2 procedure. After the 4x SSC treatment, the slides underwent a dual application of a cleaving solution stock, as supplied by the RNAscope kit, with each instance lasting 15 minutes at room temperature. The slides were then thoroughly washed with fresh phosphate-buffered saline with Tween-20 (PBST, 0.5%) and subsequently dried. Following these preparatory steps, the slides were subjected to day 2 fluorophores T4-T6, mirroring the procedures employed on day 1.

These procedures are subsequently repeated for days 3, and 4, employing the respective fluorophores (T7-T9, and T10-T12).

The acquisition of RNAscope images paralleled the procedure outlined previously for immunofluorescence on day 1. However, for subsequent days (days 2, 3, and 4), a critical requirement entailed the precise alignment of image dimensions. This alignment was necessary to facilitate the recognition by the HiPlex IRS software, which subsequently overlaid the images onto the day 1 image and amalgamated them into a single composite image.

5.5 Obtaining images using confocal microscopy

For the immunofluorescence imaging, the slides were positioned on the microscope stage, and the 20x objective lens was selected based on the experimental requirements. ZEN software was used to acquire images, allowing for precise control over the laser excitation wavelengths, which were chosen according to the fluorescent markers used in the panel of interest. After optimizing the laser

settings, the specimen was scanned point-by-point, and the emitted fluorescent light was captured through a pinhole to minimize out-of-focus background signals. The slides were then adjusted for Z-stacking, enabling detailed imaging of the skin segment in multiple planes.

Once the parameters for the slides were established and fine-tuned, a manual assessment of each fluorescence channel was conducted to ensure optimal image quality and signal intensity, including thresholding for each channel. After verifying all the settings, the slides were subjected to tile scanning, depending on the length of the sample, and the imaging process was executed to capture the desired composite image.

5.6 Quantification of immunofluorescence images

The images were exported to ImageJ software, and post-processing steps were performed. The images were subjected to removal of artifacts and clearing the blank spaces on the images were removed manually. For cytokine markers IL-13/ IL-4, we consistently removed about 1.5 rete ridges of the skin sample; to eliminate the edge effect and the images were subjected to further analysis.

5.7 Quantification of RNAscope images

Image quantification was performed using ImageJ software. The post-processing of RNAscope images involves a detailed series of steps. Initially, the images were checked for quality and manually the empty area around the sample tissue was removed, followed by background subtraction was performed using negative controls for comparison. Thresholds were set and validated for the images. Subsequently, images were separated according to individual probes and renamed appropriately according to meet HiPlex Image Registration software requirements. These

images were then imported into the software for merging and superimposition. Afterward, the images were exported for analysis.

Next, the images underwent primary analysis using ImageJ software, including thresholding and sequential operations such as watershed, dilation, and hole filling. Following these steps, the images were mapped to identify regions of interest. Each probe was manually checked and adjusted to measure its signal quality. Finally, quantification for each probe was performed using the "Find Maxima" function to capture "punctum," where each punctum represents one mRNA transcript. The results were then exported to an Excel spreadsheet for further data refinement.

5.8 Gating of $CD45^+$ immune cells using Excel

The output from RNAscope experiments was obtained as a heatmap using Excel, displaying expression levels for the 12 AD-markers. Post-processing, the data were cleaned and prepared for analysis. Immune cells were identified by gating for the CD45 gene, a marker specific for immune cells. Cells were classified as $CD45^+$ if they exhibited more than one punctum (expression / mRNA transcript), indicating significant CD45 expression. This gating was performed by filtering cells with non-zero CD45 expression values using the sort of function in Excel.

Within the $CD45^+$ population, T cells were further identified by sub-gating based on TCR gene expression. A custom sort of function was applied to isolate TCR^+ and TCR- cells within the $CD45^+$ group. The absolute counts of $CD45^+$ and $CD45^+$ TCR^+/- cells were then used to generate plots using GraphPad PRISM 10.

5.9 Statistical analysis

Multiple comparisons between three groups were tested using an unpaired, non-parametric Kruskal-Wallis test followed by a Dunn's multiple comparisons test. Multiple comparisons were tested between lesional, non-lesional and healthy samples using one-way ANOVA, and paired tests were carried out using Wilcoxon test.

Data are displayed as mean $^{+}$ standard deviation. P value of ≤ 0.05 was considered significant. All statistical analyses were performed using GraphPad Prism, version 10 (GraphPad Software).

6. Results

Study design: case-control of skin biopsies from severe AD patients compared to healthy subjects.

Skin biopsies from 11 patients diagnosed with AD and 6 healthy subjects were included in this study, with their demographic and disease information presented in Table 3. The mean age for the AD patients in this group was 37.8 years, slightly higher than the mean age of healthy subjects at 31.6 years. The cohort had an average Eczema Area and Severity Index (EASI) score of 33.1, indicative of severe disease, with Investigator's Global Assessment (vIGA) score of 3.18 and averaged 53.8% body surface area affected by lesions. The clinical laboratory findings note mean Immunoglobulin E (IgE) level of 29904.76 µg/L and a mean Lactate Dehydrogenase (LDH) level of 265.76 U/L. Table 4 summarizes the sub-cohort of 5 AD patients and 5 healthy subjects with skin samples further analyzed by RNAscope analysis.

Table 3. Clinical data for whole cohort of AD patients; immunofluorescence (protein)			
		AD skin (n= 11) (Lesional and non-lesional)	**Healthy Subjects (n=6)**
Age		37.81 ± 12.53	31.6 ± 16.1
Sex			
	Female	6	6
	Male	5	-
Ethnicity			
	Southeast Asian	1	-
	White	4	5
	Indigenous	1	-
	South Asian	2	-
	East Asian	2	1
	Other	1	-
Clinical scores			
	EASI	33.1 ± 14.30	N/A
	vIGA score	3.18 ± 0.38	N/A
	BSA%	53.8 ± 29.24	N/A
	IgE (ug/L)	29904.76 ± 45544.64	N/A
	LDH (U/L)	265.76 ± 103.31	N/A

Table 4. Clinical data for sub-cohort of AD patients; RNAscope (FISH)			
		AD skin (n= 5)	**Healthy Subjects (n=5)**
Age		41.50 ± 14.51	33.2 ± 14.33
Sex			
	Female	2	4
	Male	3	1
Ethnicity			
	Southeast Asian	1	-
	White	2	4
	Indigenous	1	-
	South Asian	-	1
	Other	1	-
Clinical scores			
	EASI	31.08 ± 13.51	N/A
	vIGA score	3	N/A
	BSA%	42 ± 27.76	N/A
	IgE (ug/L)	13720.5 ± 17186.51	N/A
	LDH (U/L)	210 ± 54.70	N/A

Variable expression of IL-13 protein observed in human AD skin.

We aimed to assess the expression of Th2 cytokines, IL-13 and IL-4, in the skin of AD patients' and matched healthy subject skin. To this end, we performed immunofluorescence analysis of these cytokines using 5mm punch biopsies from 11 AD patients, compared to their respective isotype controls (Figs. 1 and 2).

Our results revealed readily detectable (high) expression of IL-13 in the lesional skin of AD patients, albeit variable across subjects (Fig. 1, left panels) compared to healthy skin (Fig. 1, right panels). On the other hand, although IL-4 expression was detectable (low expression), it was not as significant as the expression of IL-13 in the lesional skin of AD patients (Fig. 2, left panels) compared to healthy skin (Fig. 2, right panels).

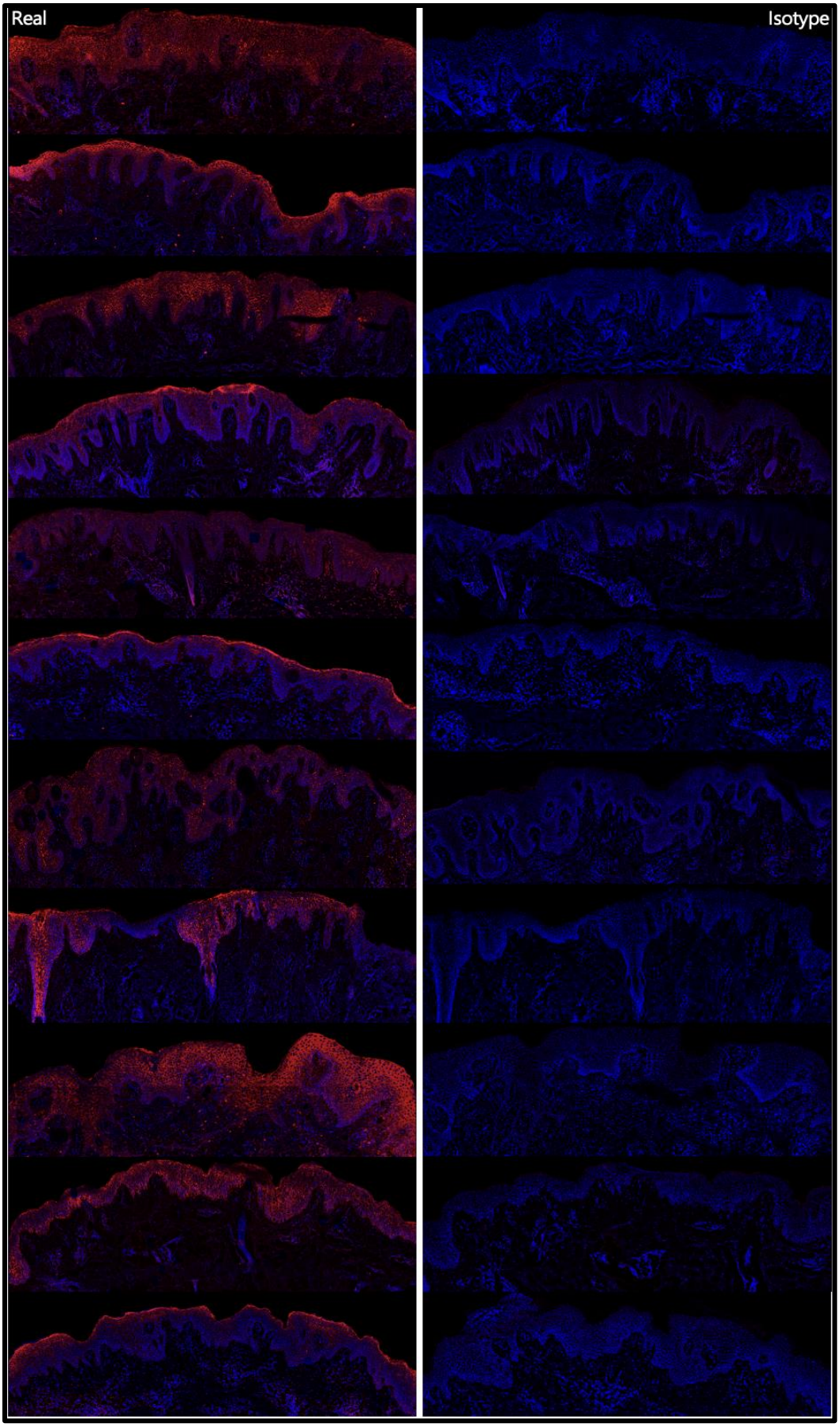

Real
Isotype

Figure 1: IL-13 protein is readily detected in lesional skin at variable levels in adult AD patients.

Immunofluorescence images of IL-13 protein in 5mm punch biopsies by tile scan of AD patient skin. Anti-IL-13 antibody (red fluorescence), DAPI (blue fluorescence) staining of cell nuclei. Each row corresponds to a different patient, with the left panel showing the IL-13 staining and the right panel showing the corresponding isotype control for each patient. N=11 AD patient.

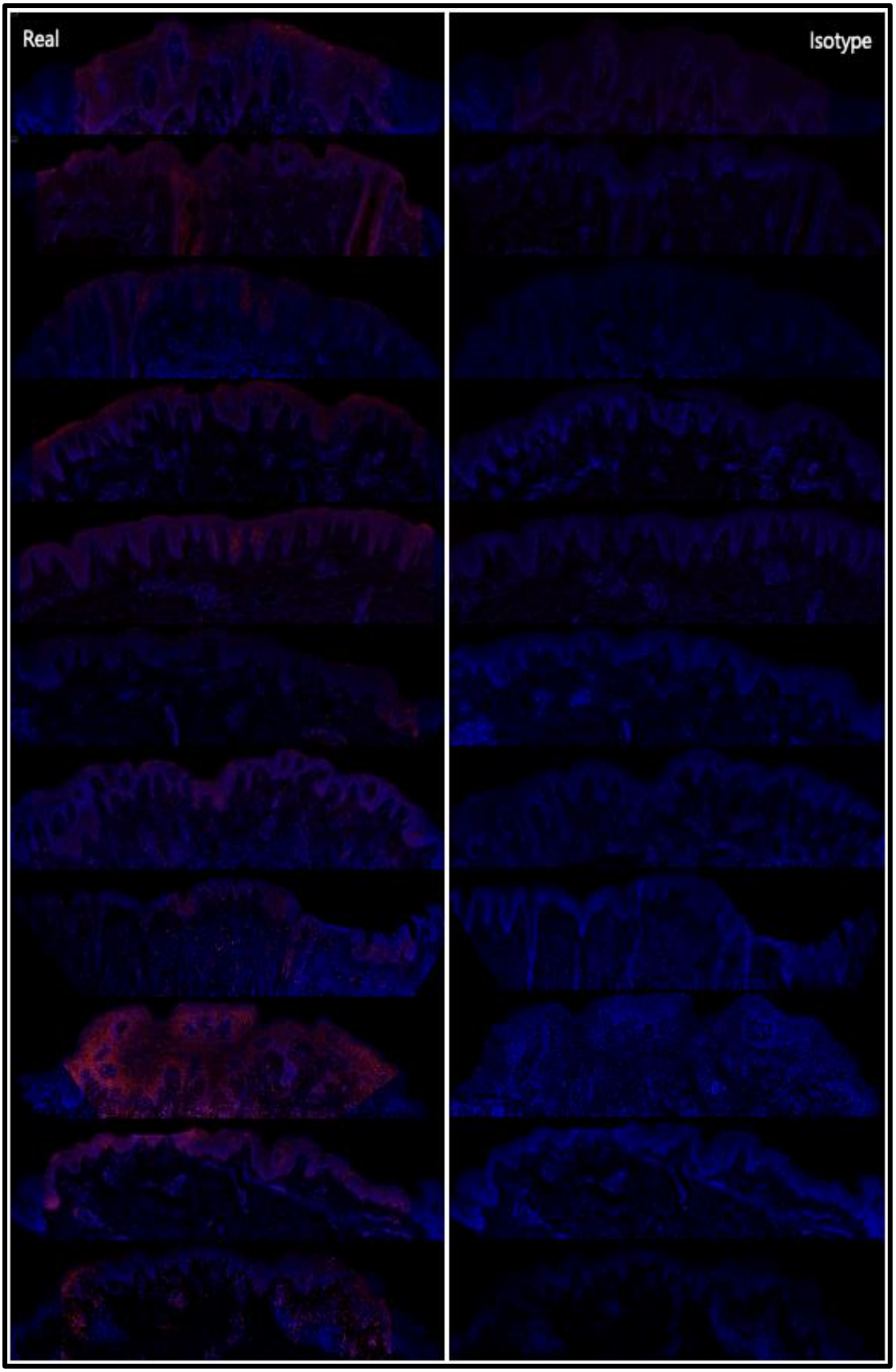

Real
Isotype

Figure 2: IL-4 protein is low and variably detectable in the lesional skin of adult AD patients.

Immunofluorescence images of IL-4 protein in 5mm punch biopsies by tile scan of AD patient skin. Anti-IL-4 antibody, (red fluorescence). DAPI (blue fluorescence) staining of cell nuclei. Each row corresponds to a different patient, with the left panel showing the IL-4 staining and the right panel showing the corresponding isotype control for each patient. N=11 AD patient.

Weighted IL-13 protein is significantly elevated in both lesional and non-lesional AD skin, in contrast to IL-4.

To our observation, we quantified the mean fluorescent intensity (MFI) of the signal obtained from the IL-13 and IL-4 immunofluorescence images (Fig. 1 and 2) using ImageJ software. To remove non-specific signal, we subtracted the MFI value of the negative staining control not incubated with the primary antibodies (isotype – right panels) from the real stain (Fig. 1 and 2), generating the delta MFI values. Estimation of total protein in full thickness 5mm skin biopsies of AD skin must also address expanded weight/volume of affected skin lesions, with thickening (acanthosis) of the epidermis more than the dermis. Delta MFI weighted to account for total skin area across the tile scan for IL-13 staining is shown in Fig. 3a-c, and for IL-4 staining in Fig. 3d-f. Our data show a significant increase in IL-13 protein in lesional, as well as non-lesional, AD skin compared to healthy. IL-13 levels are also significantly higher in lesional than non-lesional AD skin (Fig. 3a). When considering skin compartments alone, we observed a significant increase in both the dermis and the epidermis of lesional skin only (Fig. 3b and c). In contrast, the expression of IL-4 in total skin was not significantly elevated in comparison with healthy skin (Fig. 3d). However, there was a trend for significant increase in IL-4 protein, localized to the epidermis of lesional AD skin (Fig. 3e, f).

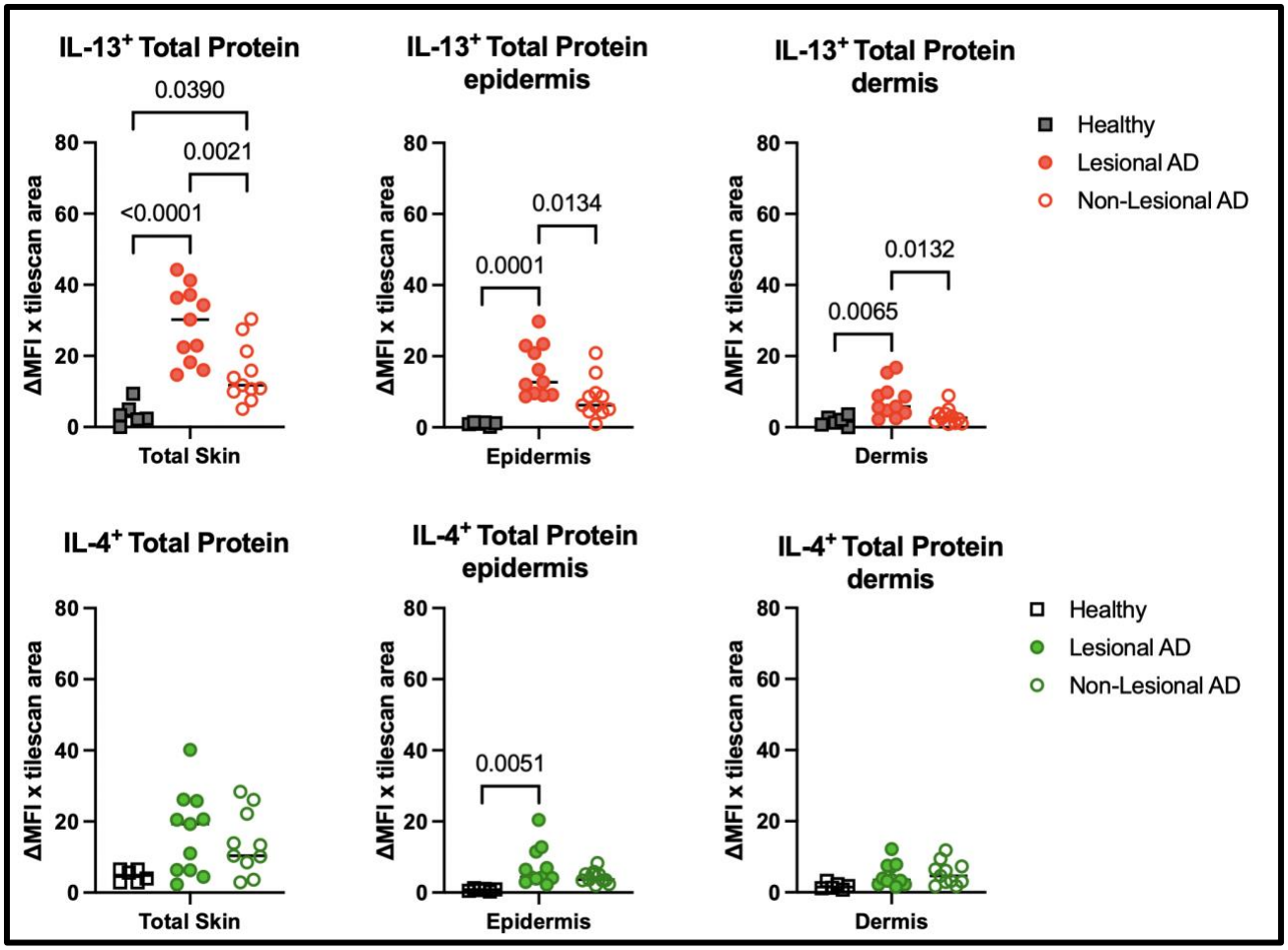


Figure 3: IL-13 total protein is significantly increased in both lesional and non-lesional AD skin, in contrast to IL-4.
Quantification of weighted total IL-13 (red) or IL-4 (green) protein in full 5mm punch biopsies by tile scan of skin from AD patients versus healthy. Weighted to account for different skin thickness between groups (delta MFI x area of skin), lesional (L) and non-lesional (NL); Left panels: full thickness skin (dermis and epidermis); middle panels, epidermis; right, dermis. N=11 AD patients, N=6 healthy controls. Statistical significance was obtained using one-way ANOVA (Tukey's multiple comparison test).

To measure and visualize the fold-increase over normal skin, this data is represented in bar graphs in Figure 4. When considering a full-thickness biopsy (epidermis and dermis combined), IL-13 protein was 8-fold higher than healthy skin; in non-lesional skin, a 4-fold increase. In contrast, IL-4 protein was non-significant at 2.5-fold in lesional skin. When skin compartments are considered independently, the data reveals subtle differences. The epidermis of lesional skin has a striking

weighted 15-fold increase in IL-13 protein compared to healthy skin. A significant 5-fold increase in IL-4 was also found in the epidermis. In line with fewer receptor-expressing cells, protein levels are more modest in the dermis, where lesional IL-13 protein was significantly elevated over healthy skin, but only 4-fold.

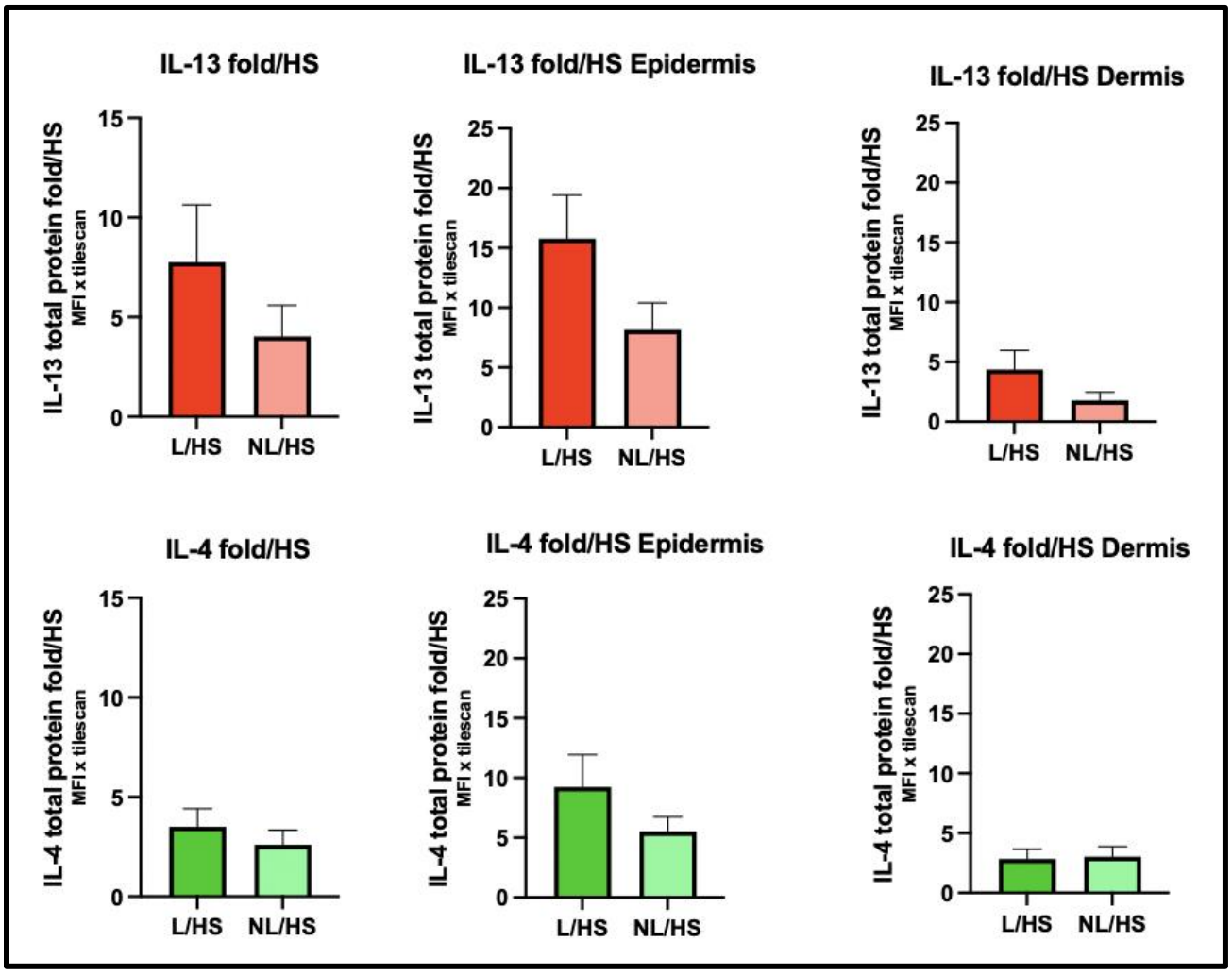


Figure 4: IL-13 total protein is elevated in both epidermal and dermal compartments in AD skin, in contrast to IL-4.

Fold-change for weighted total IL-13 (red) or IL-4 (green) protein in AD over healthy skin, full 5mm punch biopsies. Weighted to account for different skin thickness between groups (delta MFI x area of skin), lesional (L) and non-lesional (NL); Left panels: full thickness skin (dermis and epidermis); middle panels, epidermis; right, dermis. N=11 AD patients, N=6 healthy controls. Data

presented as mean fold change ± standard error of the mean (SEM). Statistical significance was obtained using one-way ANOVA (Tukey's multiple comparison test).

In the epidermis, increased concentration of both IL-13 and IL-4 protein is detected.

To assess concentration (signal intensity) of cytokine without consideration of differences to volume expansion in diseased skin, delta MFI alone is also presented. (Fig. 5 and 6). In full-thickness skin, we found parallel results for both IL-13 and IL-4. Interestingly, when considering intensity of protein signal alone, both IL-13 and IL-4 are clearly significantly increased in both lesional, as well as non-lesional, epidermis. When comparing to Figures 3 and 4, the distinction between lesional and non-lesional skin is less evident, particularly for the low intensity IL-4 protein.

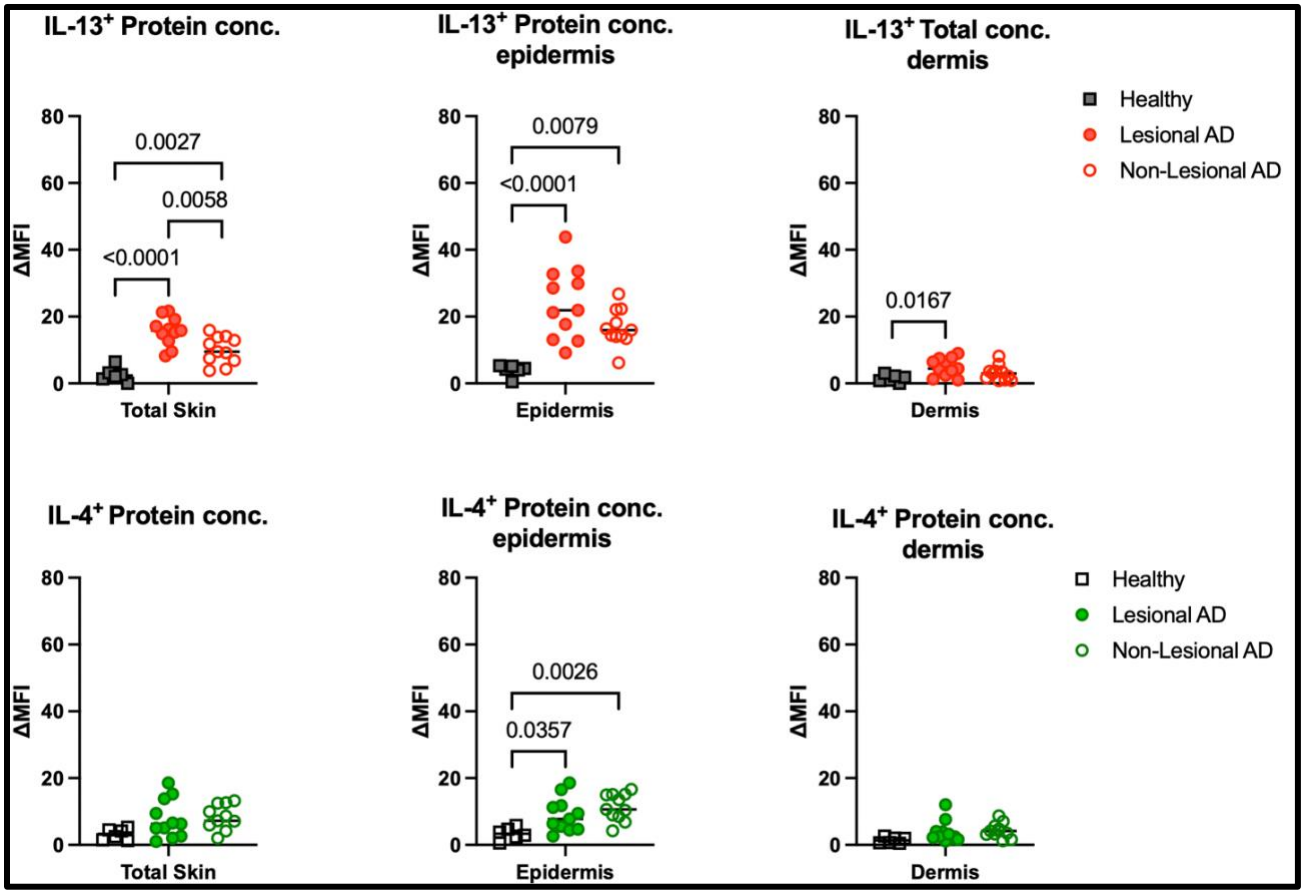


Figure 5: The concentration of both IL-13 and IL-4 protein is significantly increased in AD epidermis, both in lesional and non-lesional skin.

Concentration (intensity alone, delta MFI) of IL-13 (red) or IL-4 (green) in 5mm punch biopsies, tile scan of AD patient skin versus healthy subjects; Left panels: total skin (dermis and epidermis); middle panels, epidermis; right panels, dermis. The delta MFI or concentration shown here is not weighted to account for thickness of skin. N=11 AD patients, N=6 healthy controls. Statistical significance by one-way ANOVA (Tukey's multiple comparison test).

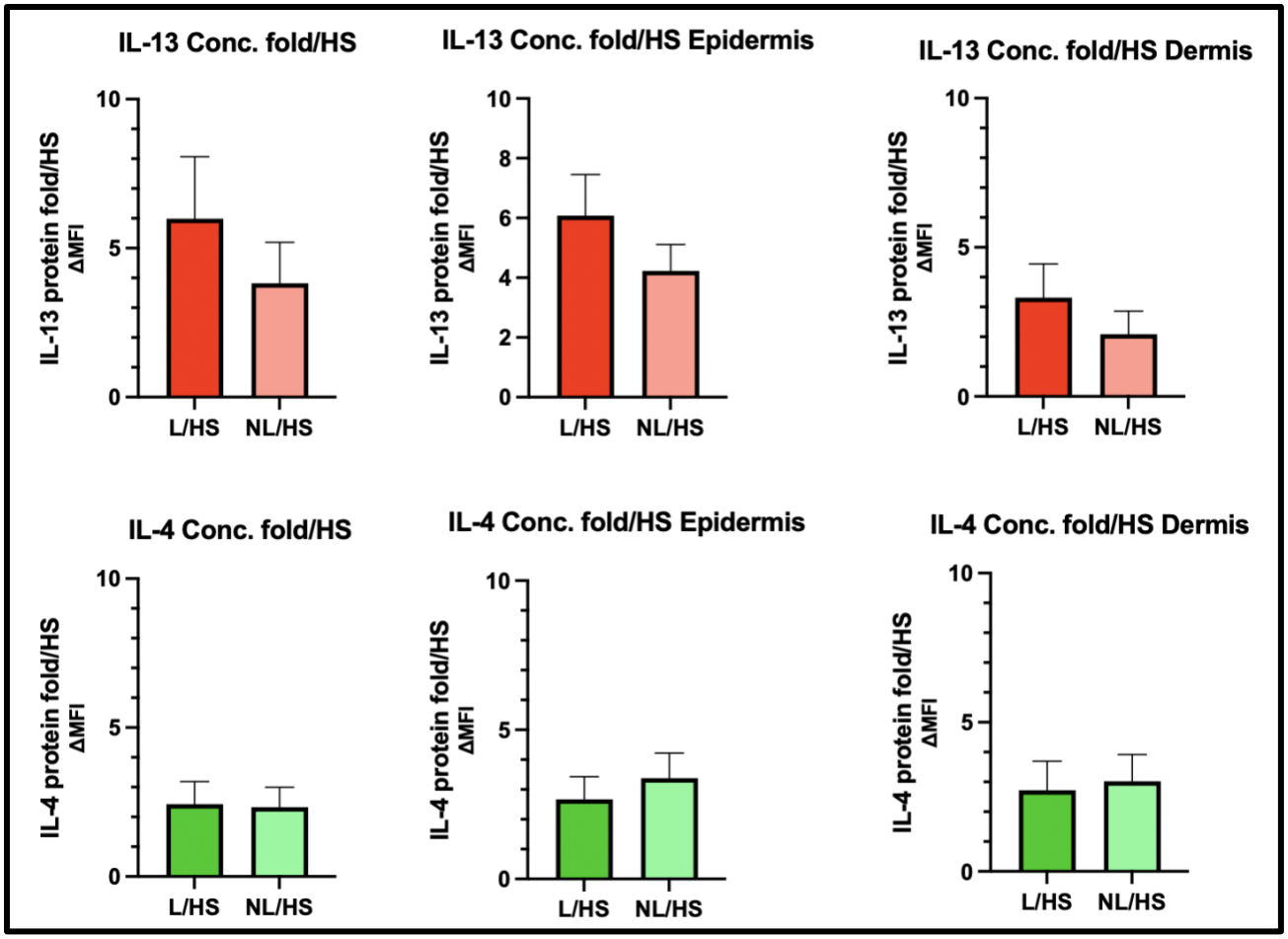


Figure 6: The concentration of IL-4 protein in AD skin lesions is equivalent to non-lesional skin.

Fold-change in AD over healthy skin for IL-13 (red) or IL-4 (green) protein concentration (intensity alone, delta MFI) of IL-13 (red) or IL-4 (green) in 5mm punch biopsies, tile scan of AD patient skin versus healthy subjects; Left panels: full thickness skin (dermis and epidermis); middle panels, epidermis; right, dermis. The delta MFI or concentration shown here is not weighted to account for thickness of skin. Data are presented as mean fold change ± standard error of the mean (SEM), which are baseline corrected. N=11 AD patients, N=6 healthy controls. Statistical significance was obtained using one-way ANOVA (Tukey's multiple comparison test).

In summary, weighted IL-13 protein is most strikingly elevated in the epidermis of lesional skin, where IL-13 receptor is expressed by keratinocytes. IL-4 protein is more difficult to detect in the skin of AD patients, yet analysis of the epidermis alone appears to show *in situ* evidence of this key Th2 cytokine elevated, despite being far out weighted by IL-13.

Overall, our results demonstrate a significantly increased expression of IL-13 protein in the full lesional, and non-lesional AD skin compared to healthy skin (Fig. 3a), which becomes only significant in lesional AD epidermis and dermis when the compartments are assessed individually (Fig. 3b and c).

IL-13 protein co-localizes with T cells in the dermis of AD patient skin.

Next, we were interested in assessing the number of T cells in each skin section expressing IL-13 and IL-4 in AD skin compared to healthy skin. To this end, we performed immunofluorescence experiments assessing the number of cells co-expressing CD3 and IL-13, versus IL-4, using lesional and non-lesional AD skin, as well as healthy skin (Fig. 7). A significantly higher number of IL-13^+ $CD3^+$ T cells were found in lesional full-thickness AD skin compared to healthy skin ($p = 0.0412$) (Fig. 7, top panel), indicating increased T cell infiltration. In contrast, IL-4^+ $CD3^+$ co-expression was not significantly increased (Fig. 7, top panel). When analyzing the epidermal and dermal compartments individually, we found significantly increased IL-13^+ $CD3^+$ T cells ($p = 0.0022$) localized to the dermal compartment, while no difference in IL-13^+ $CD3^+$ T cells nor IL-4^+ $CD3^+$ T cells was noted between the other groups (Fig. 7, lower panels). Overall, $CD3^+$ T cells expressing IL-13 were increased in the lesional skin of AD patients compared to the non-lesional AD and healthy skin, and this increase was most evident in the dermal compartment, while $CD3^+$ T cells expressing IL-4 were not significantly different in AD skin compared to healthy skin, despite small increases.

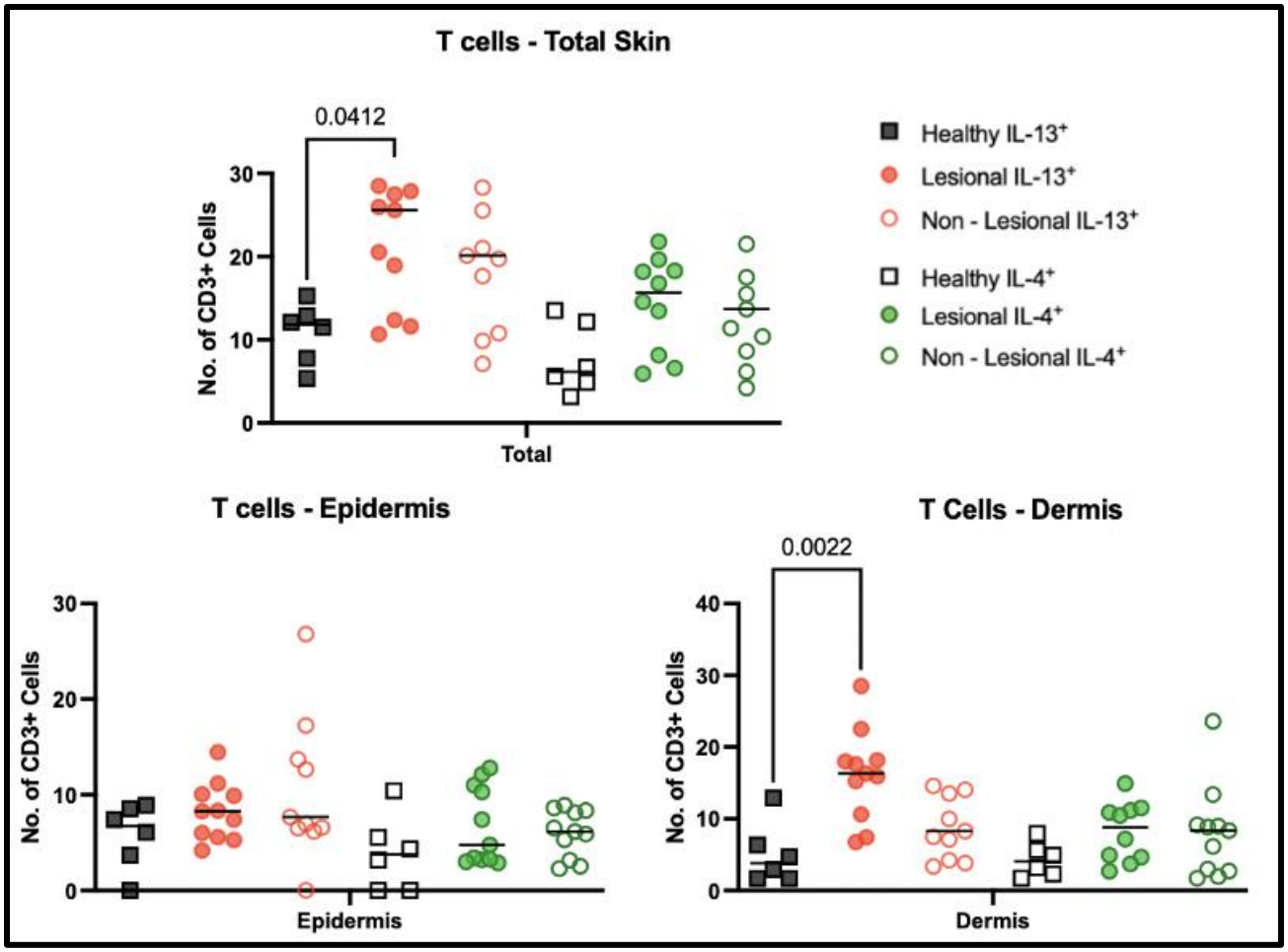


Figure 7: IL-13-protein co-localizes with T cells in the dermis of AD patient skin.

Cell counts of $CD3^+$ T cells co-expressing IL-13 or IL-4 in 5mm punch biopsies, tile scan of AD patient skin versus healthy skin. The number of $CD3^+$ cells is compared among healthy (black squares), IL-13^+ lesional and non-lesional (red circles), and IL-4^+ lesional and non-lesional (green circles) skin samples. Top panel: total skin (dermis and epidermis); lower left panel, epidermis; lower right panel, dermis. N=11 AD patients and N=6 healthy controls. Statistical significance is indicated by one-way ANOVA (Kruskal-Wallis test).

$CD8^+$ IL-13^+ T cytotoxic-type cells are significantly elevated in AD compared to healthy skin.

To determine the lineage of these $CD3^+$ T cells expressing IL-13 and IL-4, we aimed to determine whether they were T helper cells or T cytotoxic cells. Colocalization of CD3 and CD8 was used to identify T cytotoxic type, versus CD8 negative cells, considered to be primarily T helper cells.

Notably, the analysis of T cytotoxic and T helper cell expression in AD revealed significant differences in T cytotoxic cells between AD (lesional and non-lesional) and healthy skin. Results indicated a marked increase in the number of IL-13$^+$ T cytotoxic cell counts in the full biopsy of both lesional ($p = 0.412$) and non-lesional ($p = 0.0038$) skin compared to healthy biopsies (Fig. 9, top panel). This significant increase within the epidermal layer ($p = 0.0100$ and $p = 0.0490$), was seen in lesional and non-lesional skin, respectively (Fig. 9, lower left panel). However, within the dermal layer, only lesional IL-13$^+$ T cytotoxic cells (CD3$^+$ CD8$^+$ IL13$^+$) were significantly increased (not in non-lesional) ($p = 0.0120$) (Fig. 9, lower right panel). In contrast, IL-4 expressing T cytotoxic cell counts did not differ significantly between the different groups in the total skin nor the separate compartments (Fig. 9). Unexpectedly, IL-13$^+$ or IL-4$^+$ T helper cells did not show any difference in counts between full AD skin and healthy skin, nor in separate compartments (Fig. 8).

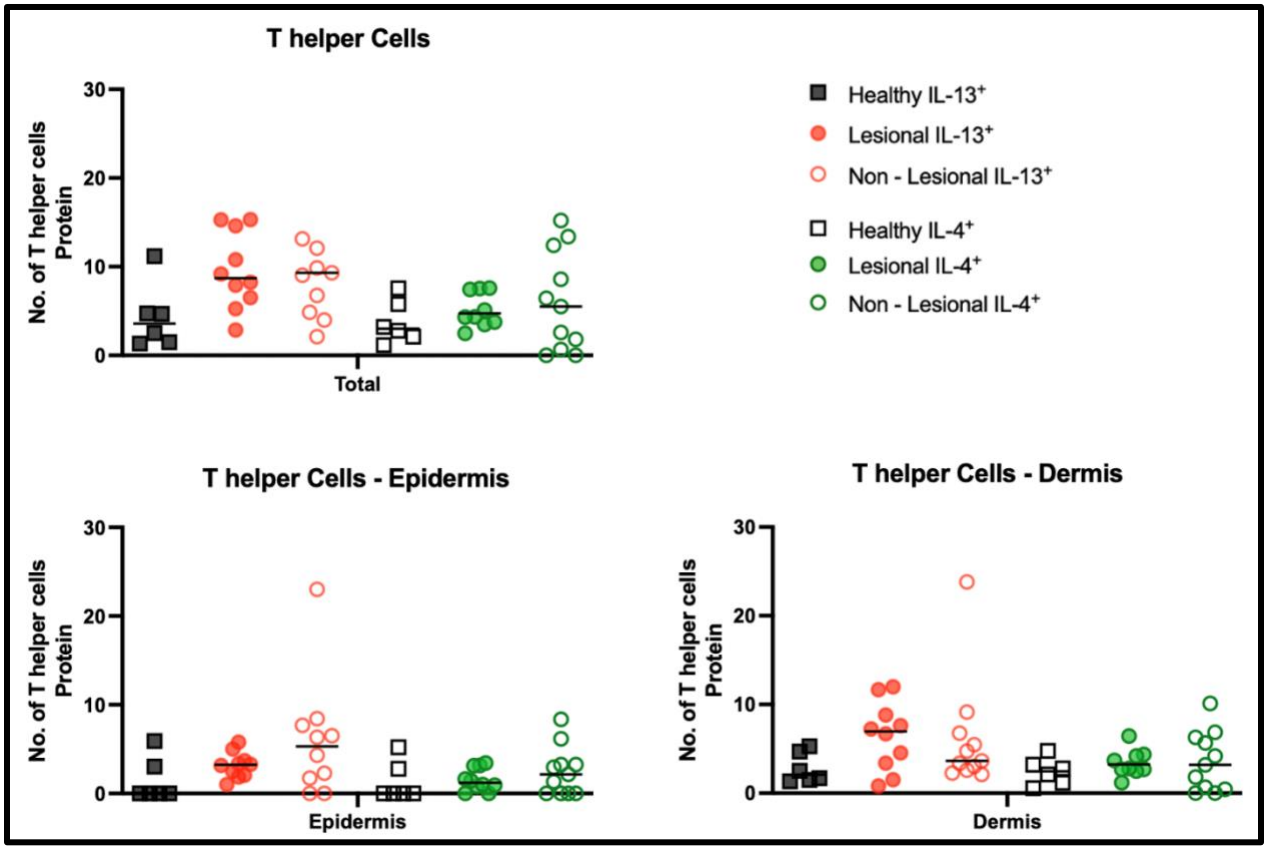


Figure 8: IL-13$^+$ T helper cells are not significantly increased in AD skin.
Cell counts of helper T cells expressing IL-13 or IL-4 in full 5mm punch biopsies, by tile scan of skin from AD patients versus healthy. Immunofluorescence staining was conducted with monoclonal antibodies against CD3, CD8, IL-13, and IL-4. DAPI was used to stain nuclei. The number of CD3$^+$ cells is compared among healthy (black squares), IL-13$^+$ lesional and non-lesional (red circles), and IL-4$^+$ lesional and non-lesional (green circles) skin samples. Top panel: total skin (dermis and epidermis); lower left panel, epidermis; lower right panel, dermis. N=11 AD patients, N=6 healthy controls. Statistical significance was obtained using one-way ANOVA (Kruskal-Wallis test).

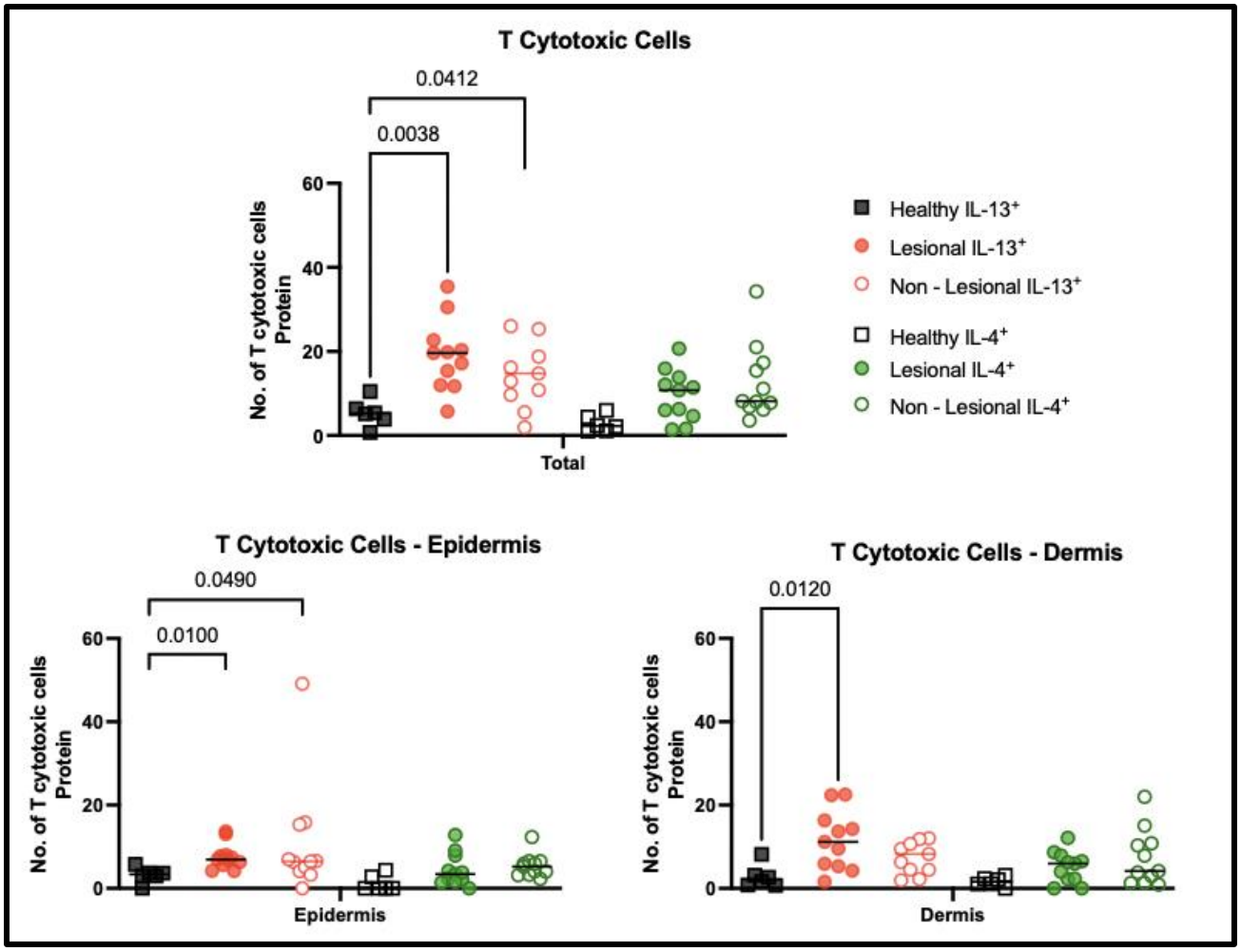


Figure 9: IL-13$^+$ T cytotoxic cells are significantly increased in AD skin in contrast to IL-4.

Cell counts of cytotoxic T cells expressing IL-13 or IL-4 in full 5mm punch biopsies, by tile scan of skin from AD patients versus healthy. Immunofluorescence staining was conducted with monoclonal antibodies against CD3, CD8, IL-13, and IL-4. DAPI was used to stain nuclei. The number of CD3$^+$ cells is compared among healthy (black squares), IL-13$^+$ lesional and non-lesional (red circles), and IL-4$^+$ lesional and non-lesional (green circles) skin samples. Top panel: total skin (dermis and epidermis); lower left panel, epidermis; lower right panel, dermis. N=11 AD patients, N=6 healthy controls. Statistical significance was performed by one-way ANOVA (Kruskal-Wallis test).

We conducted preliminary analyses using immunofluorescence staining to examine TRMs at the protein level, focusing on CD69$^+$ T cells across lesional, non-lesional, and healthy skin. These preliminary results seem to confirm that lesional skin has a higher number of CD69$^+$ T cells (Suppl. Fig 2 & 3) compared to both non-lesional and healthy skin, with non-lesional skin also showing a

moderate increase. Future studies are needed to expand an immunophenotyping panel at the protein level for spatial multiplex.

Overall, these findings demonstrate the significant presence of T cytotoxic cells expressing IL-13 in the inflammatory environment of adult AD patient skin. Only $CD8^+$ $IL\text{-}13^+$ type 2 T "Tc2" cells are found in significant numbers in both the dermis and epidermis of severe AD skin lesions in this study. These IL-13-Tc2 (type 2 cytotoxic T) cells are not increased in the dermis of patients' non-lesional skin. Canonical T helper cells with IL-13 or IL-4 co-localization were not significantly increased. When considering the pattern of type 2 cytokines in AD skin at the protein-level globally, (Fig. 3-6), IL-13-producing CD8 T cells appear to correlate best with cytokine accumulation. Further investigation is needed to clarify these non-canonical effectors and immune pathways in the pathogenesis of AD.

Spatial mapping of $IL\text{-}13^+$ and $IL\text{-}4\ mRNA^+$ cellular distribution in AD Skin.

Having established that $CD8^+$ $IL\text{-}13^+$ T cytotoxic-like cells are increased at the protein level in AD skin, we next sought to determine if mRNA expression levels of IL-13 and IL-4 in T cells is also found within these cells. In addition, we aimed to characterize the sub-populations of lymphocytes, focusing on of tissue-residency markers (CD103, CD69), the type 2 transcription factor GATA3, the Th2-associated co-stimulatory receptor IL13, IL4, OX40, the innate cell alarmin receptor TSLPR), and the atopy-associated prostaglandin receptor CRTH2, considered a pathogenic effector marker when expressed on T helper (peTh2) cells (Table 5) (see section 2.3 of intro).

To this end, we used an advanced form of single-cell single-molecule fluorescent *in situ* hybridization, multiplexing 12 probes. We used ImageJ software to automate the signal quantification for each target mRNA, then collaborated with C3G to adapt a single-cell automated multiplex pipeline for RNA (SCAMPR)-developed by neuronal imaging investigators [166]. This methodology allows quantification and spatial localization using classification of T cell types based on the combination of mRNAs expressed by each cell across the entire 5mm punch biopsy. We analyzed the skin of 5 AD patients and 3 healthy subjects (see Table 4), generating spatial topology maps of the full tile scan skin sections with sub-populations colour-coded, as shown in representative epidermal regions of one patient's lesional skin (Fig. 10 and 11). Dots represent the spatial distribution of IL-13^+ (Fig. 10) and IL-4^+ (Fig. 11) cells co-localizing with specified lineage markers within the total biopsy.

As illustrated, we found both IL-4 and IL-13-producing $CD8^+$ tissue resident memory T cells (peTRM Tc) cells, expressing the pathogenic effector marker CRTH2 in AD skin, predominantly located in the epidermis. When comparing the representative areas for IL-13 and IL-4, we observed distinct populations of anticipated as well as novel cells (Fig. 10 and 11, left zoomed images). Importantly, it was not rare to see peTRM cells that were double-positive for CD4 and CD8 and expressing IL-13 and/or IL-4.

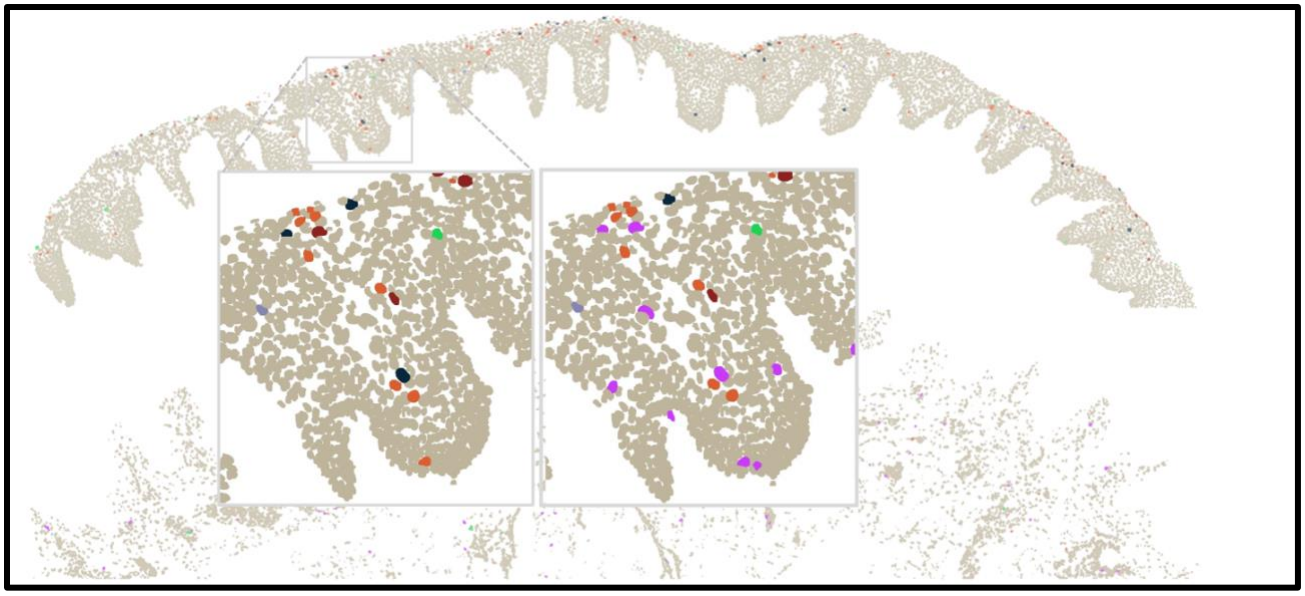


Figure 10: Spatial Topo map of IL-13^{+} mRNA cellular distribution and marker expression in AD skin.

The spatial topo map visualizes the distribution of annotated IL-13^{+} cell types in AD skin, lineage overlaid with nuclei. The zoom (LEFT panel) highlights the clustering of effector TRM populations based on the expression of specific markers: pathogenic effector (pe)TRM-Th cells (TCR^{+}CD4^{+}CRTH2^{+}CD69^{+}) lavender dots; peTRM-Tc cells (TCR^{+}CD8^{+}CRTH2^{+}CD69^{+}) burgundy-red dots; peTRM-dp cells (TCR^{+}CD4^{+}CD8^{+}CRTH2^{+}CD69^{+}) black dots. TRM T-cells (TCR^{+}CD4^{+}CD8^{+}CD69^{+}) and TRM non-T cells (CD4^{-}CD8^{-}CD69^{+}) without CRTH2 are shown as brown and green dots, respectively; on the RIGHT: IL-13^{+} cells are overlaid on the populations as purple dots; cells without IL-13^{+} retain lineage colors. N= 5 AD patients, N=5 healthy controls.

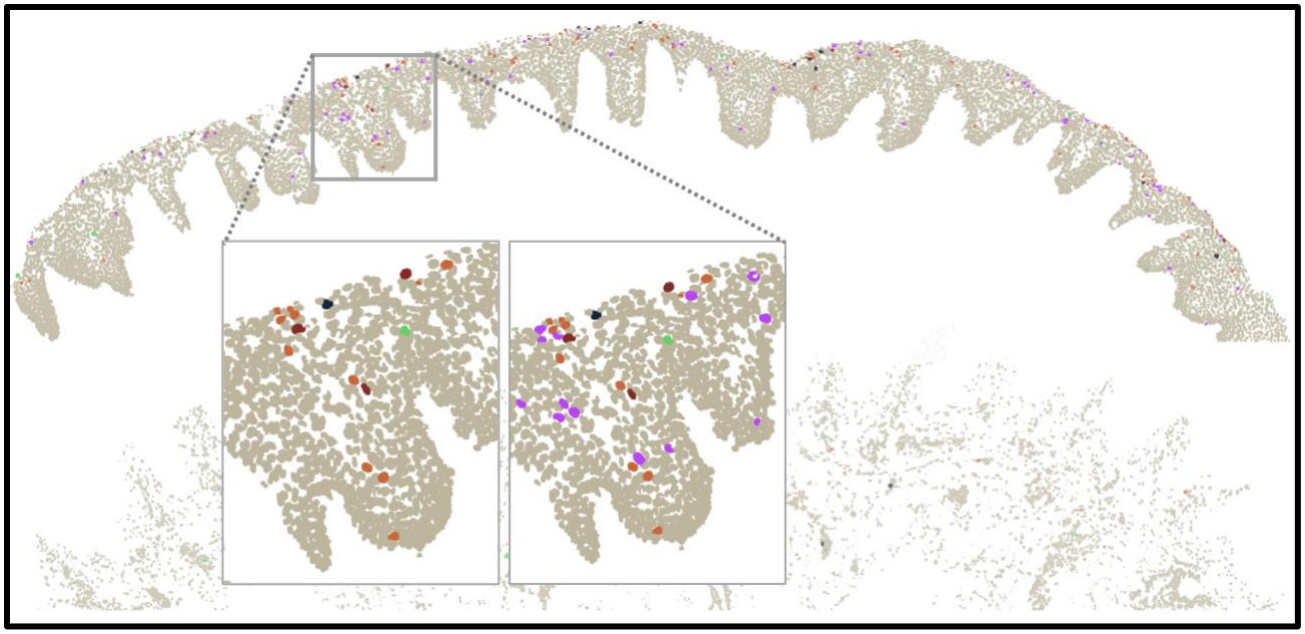


Figure 11: Spatial Topo map of IL-4^{+} mRNA cellular distribution and marker expression in AD skin

The spatial topo map visualizes the distribution of annotated IL-4^{+} cell types in AD skin. The zoom (left panel) highlights the clustering of effector TRM populations based on the expression of specific markers: pathogenic effector (pe)TRM-Th cells (TCR^{+}CD4^{+}CRTH2^{+}CD69^{+}) lavender dots; peTRM-Tc cells (TCR^{+}CD8^{+}CRTH2^{+}CD69^{+}) burgundy-red dots; peTRM-dp cells (TCR^{+}CD4^{+}CD8^{+}CRTH2^{+}CD69^{+}) black dots. TRM T-cells (TCR^{+}CD4^{+}CD8^{+}CD69^{+}) and TRM non-T cells (CD4^{-}CD8^{-}CD69^{+}) without CRTH2 are shown as brown and green dots, respectively; on the RIGHT: IL-4^{+} cells are overlaid on the populations as purple dots; cells without IL-4^{+} retain lineage colors. N= 5 AD patients, N=5 healthy controls.

Table 5. RNAscope panel genes and their function		
Sr. No	**Gene used**	**Function**
T1	IL-13	Th2 cytokine, known to be the dominant cytokine in the disease progression
T2	CD8	T cell marker for cytotoxic T cells, which play a role in the immune response.
T3	CD45	Leukocyte common antigen involved in signaling and activation of immune cells.
T4	CD4	T cell marker for helper T cells, involved in immune response and T cell differentiation.
T5	IL-4	Th2 cytokine which promotes Th2 differentiation and is known to be involved in allergic inflammation.
T6	OX40	Costimulatory molecule that enhances T cell survival and function, implicated in Type 2 inflammation
T7	CRTH2	Receptor involved in Th2 cell recruitment and activation in allergic diseases.
T8	CD103	Integrin involved in T cell localization to epithelial tissues and skin homing.
T9	CD69	An early activation marker of T cells involved in immune response regulation and Tissue residency.
T10	TSLPR	Receptor for thymic stromal lymphopoietin, which is involved in allergic inflammation and atopic dermatitis.
T11	TCR a/b	T cell receptor, which is crucial for antigen recognition by T cells.
T12	GATA3	Transcription factor that regulates Th2 cell differentiation and cytokine production.

Table 6: Annotation of cell types identified in RNAscope experiments using skin tissue from healthy and AD patients.		
Cell cluster	**Cell marker cluster**	**Representative color**
TRM T-cells	TCR^+ ($CD4^+$/$CD8^+$) $CD69^+$	Brown dots
TRM non-T-cells	TCR^- $CD4^-$ $CD8^-$ $CD69^+CD103^{+/-}$	Green dots
Pathogenic effector (peTRM) – Th cells	TCR^+ $CD4^+$ $CRTH2^+$ $CD69^+$ $CD103^{+/-}$	Lavender dots
Pathogenic effector (peTRM) – Tc cells	TCR^+ $CD8^+$ $CRTH2^+$ $CD69^+CD103^{+/-}$	Red dots
Pathogenic effector (peTRM) – T dp cells	TCR^+ $CD4^+$ $CD8^+$ $CRTH2^+$ $CD69^+CD103^{+/-}$	Black dots
Th2 cytokines	IL-4^+ / IL-13^+	Purple dots

Unbiased analysis of single-cell single-molecule FISH: expression levels of AD related markers.

To further delineate the cell types identified, this single cell RNAscope data from AD patients lesional skin and healthy controls (HC) was analyzed and visualized with the help of Canadian Centre for Computational Genomics (C3G) using our manual annotation and Flow SOM which was used for clustering in an approach with parallels to the unbiased analysis of flow cytometry data. Dot plots were generated to quantify lineage and pathogenic marker expression levels across different cell types across the full 5mm skin section and sub-divided into the separate skin layers (epidermis, dermis). (Fig.13 and 14). The size of the dots represents the proportion of cells expressing the marker (Nonzero Count), while the color intensity indicates the expression level.

This unbiased analysis demonstrates the elevation of pathogenic marker expression and cell counts most evident in epidermal skin of AD patients compared to healthy subjects, which is consistent for $CD8^+$ Tc, $CD4^+$ Th, as well as the novel populations (Fig. 13 and 14). Our data demonstrate higher expression and cell count of CD69 and GATA3 in all four cell types in AD patients' skin compared to healthy (Fig. 13 and 14), indicative of a type 2-skewed immune response. In addition, Th, Tc, and Tdp cells exhibit higher OX40 expression in the epidermis of AD skin compared to healthy epidermis, indicative of the activation and potential survival of these cells (Fig. 12 and 13) [95].

Looking at individual cell types, Th cells from AD patients' epidermis seem to express higher levels of IL-4 and TSLPR compared to healthy epidermis and compared to Tc cells from AD epidermis as well (Fig. 12). In contrast, Tc cells from the epidermis of AD patients exhibit significantly elevated mRNA expression levels of key pathogenic effectors, such as IL-13, CRTH2, and CD103, compared to both healthy epidermal Tc cells and Th cells from the epidermis of AD patients (Fig. 12). The combination of CD69 and CD103 is well known to identify skin TRMs. As such, it seems like the TRMs in AD are more CD8 cytotoxic than T helper. Unexpectedly, our data revealed a novel population of double positive T (Tdp) cells ($CD4^+CD8^+$) with pathogenic effector functions in AD skin. Notably these cells are highly activated and express all the target markers within the epidermis of our AD patients, but not in our healthy skin epidermis (Fig. 13). These cells show highly elevated levels of CD69 and GATA3, like the Th and Tc cells, as well as CD103, OX40, CRTH2, TSLPR, and both IL-13 and IL-4. This indicates that this population is active, exhibits tissue residency memory as well as innate-like pathogenic properties due to the expression of CRTH2 and TSLPR (Fig. 13). Moreover, these cells express IL-13 and IL-4.

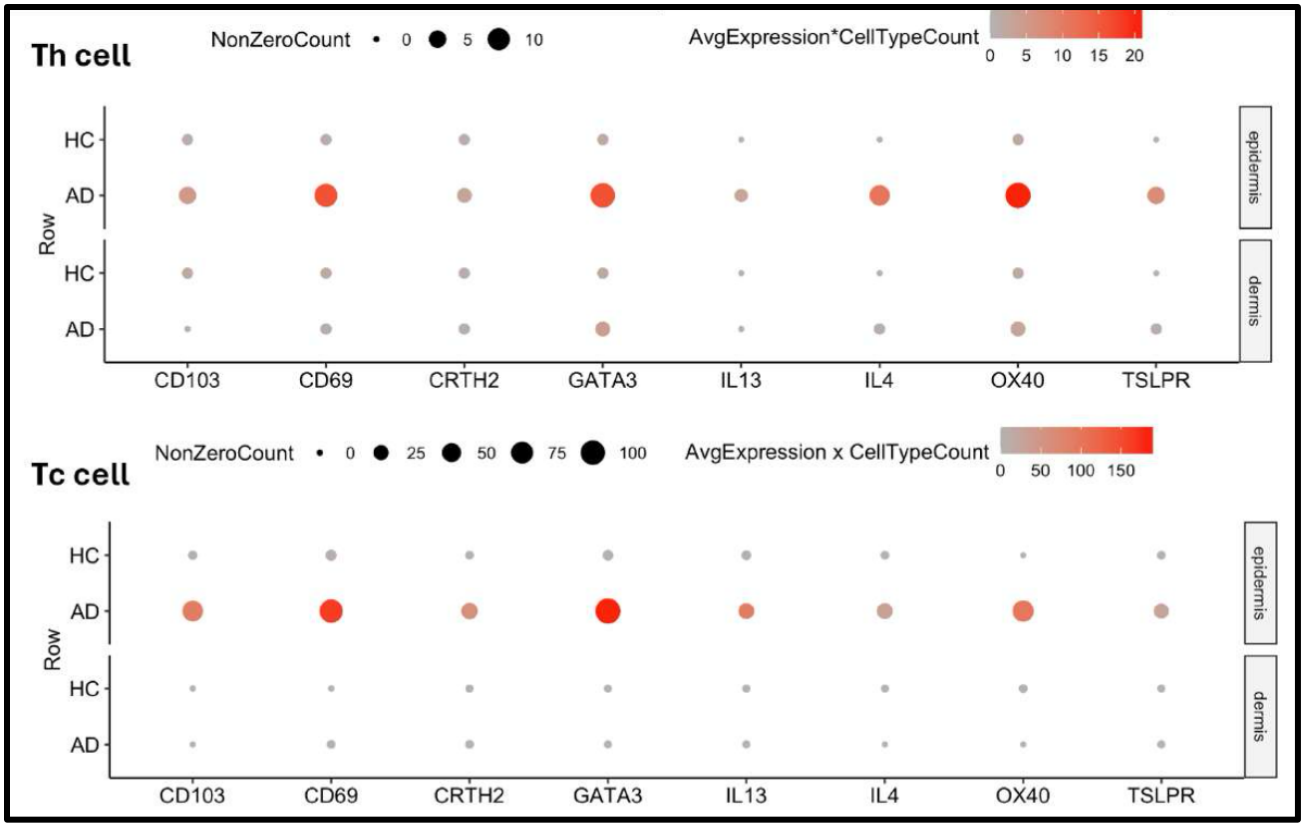


Figure 12: Expression of pathogenic effector cells in AD patients vs healthy controls

Expression of AD-related markers (CD103, CD69, CRTH2, GATA3, IL13, IL4, OX40, TSLPR) in T helper cells (top panel) and T cytotoxic cells (bottom panel) in 5mm punch biopsies, tile scan of AD patient skin versus healthy subjects. The x-axis represents the markers, and the y-axis categorizes the sample sources (HC and AD) and skin layers (epidermis and dermis). The variations in color intensity shows the differences in marker expression levels (grey, red) and the dot size the proportion of cells expressing these markers. N= 5 AD patients, N=5 healthy controls.

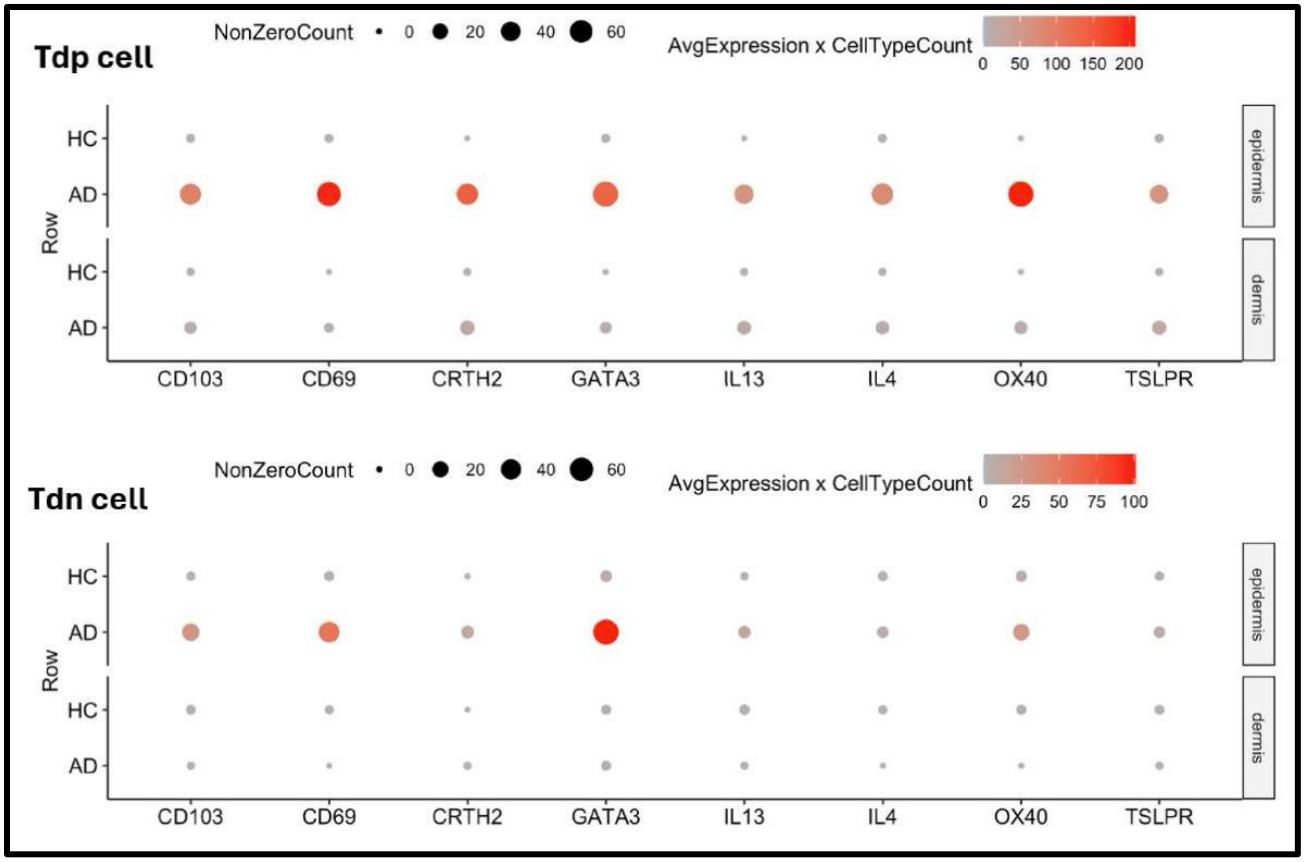


Figure 13: Expression of novel pathogenic effector markers in double-positive T cells in AD patients vs healthy controls

Expression of different AD-related markers (CD103, CD69, CRTH2, GATA3, IL13, IL4, OX40, TSLPR) in double positive T (Tdp) cells (top panel) and double negative T cells (Tdn) (bottom panel) in 5mm punch biopsies, tile scan of AD patient skin versus healthy subjects. The x-axis represents the markers, and the y-axis categorizes the sample sources (HC and AD) and skin layers (epidermis and dermis). The variations in dot size and color intensity shows the differences in marker expression levels and the number of cells expressing these markers. N= 5 AD patients, N=5 healthy controls.

7. Discussion

Atopic dermatitis is a chronic inflammatory skin disorder, primarily characterized by an impaired skin barrier and type 2-mediated inflammation, with Th2 cytokines being the key drivers of the disease. [167]. Recent studies have established IL-13 as the dominant cytokine associated with the disease, showing a strong correlation with disease severity [32, 63]. In contrast, IL-4 has not been consistently detected significantly elevated at the protein level in the skin of AD patients, despite its key role in Th2 differentiation and type 2 immune responses [32, 63]. There is a gap in understanding this lack of IL-4 as well as the pathogenic cells and pathways active in the local skin environment of chronic adult AD skin. Our working hypothesis is that skin tissue resident memory T cells (TRMs) with pathogenic effector functions are increased in atopic dermatitis and may impair remission. Pathogenic markers have been proposed in AD, including the expression of the innate-cell chemokine receptor CRTH2 on a subset of atopy-specific T cells, in addition to type 2 cytokine profiles biased to IL-13. In this study, we aimed to characterize these cells in adult patient skin *in situ,* by combining immunofluorescence for IL-13 versus IL-4-producing T cells at the protein level, combined with single cell single molecule FISH for deeper phenotyping of tissue-specific populations.

This was a case-control study of skin biopsies from severe adult AD patients compared to healthy subjects. The full cohort (skin biopsies from 11 patients and 6 healthy subjects) was included in our first aim focused on detection and quantification of protein levels of type 2 cytokines. Our confocal imaging across full thickness 5mm biopsies analyzed weighted protein, accounting for the expanded volume, or thickness, of diseased skin. We found that total IL-13 protein was significantly higher in lesional as well as non-lesional AD skin compared to healthy skin. The highest significance was observed in the epidermis of lesional skin, with a 15-fold increase in IL-

13 protein levels. In line with expanded epidermal thickness, differences were also noted between lesional and non-lesional skin. This emphasizes the role of IL-13 as a dominant cytokine in atopic dermatitis and demonstrates that the unaffected non-lesional skin of AD skin is also subjected to high IL-13 levels. While total weighted IL-4 protein was not significantly elevated across the lesional and non-lesional total skin compared with healthy, a trend toward significance was observed in the epidermis of lesional skin, with approximately a 5-fold increase in IL-4 protein levels. Interestingly, when data was analyzed for concentration (intensity) of protein without weighting the amount of skin present, both IL-4 and IL-13 were significantly elevated in the epidermis of AD lesions, and the distinction between lesional and non-lesional skin was harder to appreciate.

We tested for IL-13 protein positive, versus IL-4 protein positive T cells *in situ* in adult severe AD, including both helper and cytotoxic subsets. Our results showed a significant increase in IL-13^+ T cells in full thickness skin, localized primarily within the dermis. We examined the proportions of type 2 T helper and CD8+ T cytotoxic type cells in AD skin. While no significant increase was observed in IL-13^+ or IL-4^+ T helper cells in severe adult skin, IL-13^+ CD8 cytotoxic-type T cells were significantly elevated in both lesional and non-lesional skin of patients, localized to both compartments (epidermis versus dermis). Our preliminary data (see Supplemental Figures) appears to show higher number of IL-13^+ $CD69^+$ T cells. Preliminary findings may suggest very low increased numbers of $CRTH2^+$ cells expressing IL-13 (Suppl. Fig 4A), TCR (Suppl. Fig 4B), and CD69 (Suppl. Fig 4C) at the protein level in lesional skin compared to healthy skin, particularly in the dermis, but this data has not been validated within the cohort. Further studies with well-developed multiplexing panels for spatial proteomics are needed to expand

immunophenotyping at the protein level to characterize pathogenic effector or tissue-residency markers *in situ* in lesional skin.

Overall, our study suggests that $CD8^+$ $IL\text{-}13^+$ cytotoxic T cells may play a more active role in the pathophysiology of severe adult atopic dermatitis than conventional Th2 cells. This finding also suggests that there is a functional alteration to canonical cell-mediated cytotoxicity in AD skin. Like NK cells, $CD8^+$ T cells play an important immunoregulatory role and can limit excessive activation of cells like macrophages. These altered dynamics may contribute to the chronic inflammation in AD with elevated levels of *Staphylococcus aureus*. Given the fact that IL-13 and IL-4 are on the same locus, transcription and epigenetic factors involved in regulating these genes differentially also requires further study. [168] A better understanding of environmental, genetic and epigenetic factors could shed light on persistent and adult-onset AD and provide new avenues for therapeutic intervention. Access to multi-omics platforms to test AD skin tissue will help to investigate the pathways driving IL-13 high IL-4 low super-polarized responses in this disease.

We phenotyped IL-13 and IL-4-expressing cells at the mRNA level *in situ* to characterize sub-populations of T lymphocytes. Using single-molecule single-cell fluorescent *in situ* hybridization (RNAscope), multiplexing 12 probes for quantification and spatial localization across the entire 5mm punch biopsy, we analyzed the skin of 5 AD patients and 3 healthy subjects in a sub-cohort. We found patient-specific cell populations expressing 12 AD-related markers. Within the T helper cell population, we identified a small number of IL-4 Th2 cells expressing CD69, GATA3, and OX40, with low CD103 and TSLPR, suggesting these cells are activated T cells with some tissue-residency functions. In contrast, we found large numbers of IL-13-high IL-4-low, CD8 cytotoxic-like tissue-resident memory T cell expressing OX40 in the epidermis with low CRTH2 and very

high GATA3, consistent with novel pathogenic effectors in AD. $CD8^+$ TRMs in AD and other allergic diseases are still being studied. [169-171].

Notably, we also identified a double-positive $CD4^+$ and $CD8^+$ TRM T cell population using unbiased analysis, with the most striking expression of pathogenic functional markers, GATA3, OX40, TSLPR, as well as high CRTH2, IL-13 and IL-4. The existence of these cells may be explained by the fact that T cell populations in chronic inflammation may resemble thymocyte-like cells; Bang *et al*. previously identified $CD4^+$ $CD8^+$ T cells in the blood and skin of AD patients, although this data was from skin cell lines [172]. These thymocyte-like cells are also identified in conditions such as myasthenia gravis and leprosy and have been described to express various cytokine profiles (including IL-13, IL-4, and IL-5) in cancer studies [173], however, studies characterizing them, and their function are limited. We note that publicly available single-cell RNA sequencing data of AD skin show the presence of these cells, supporting the hypothesis of their potential role in AD pathogenesis [57].

We also identified a population of double negative T cells with high GATA3 and lower TRM markers, a subset that may be similar to mature T lymphocytes found in tissues and linked to autoimmune and inflammatory conditions, which also remain to be studied in AD. In addition, we note $CD45^+$ TCR^- cells were found, which may be innate immune cells such as NK cells or ILC2, or even mast cells. While the scope of our study is focused on T cell populations numerous innate immune cell types contribute to AD pathogenesis. More recent data implicating innate lymphoid cells may be strongest for another cytotoxic-type cell, NK cells, and their role in AD disease progression and severity together with CD8 T cells remains to be clarified [112, 174]. The detection of CD45- TCR+ cells may suggest the presence of a novel cell type, but it also raises

concerns about potential technical limitations when using FISH, highlighting the need to validate the findings and assess more comprehensive testing panels. Although the RNAscope assay that we use allows the simultaneous assessment of 12 probes, which is an improvement of the original fluorescent *in-situ* hybridization (FISH) assay, it is not sufficient to reveal the full transcriptomics of cells. Therefore, analyzing our results within known single-cell RNA sequencing datasets would provide a better insight into the immune cells involved in the pathogenesis of AD. Such studies will also allow for the comparison with the increasingly diverse T cell populations that simultaneously express type 2, type 17, and type 22 cytokines in the lesional skin of AD patients [107].

8. Conclusion

This study highlights the important role of IL-13 in the pathogenesis of severe adult AD, with significant variability in its expression among patients. IL-13 is predominantly expressed in lesional skin, where it is strongly associated with increased T cell infiltration, as shown by both protein and mRNA assays, highlighting its importance as a therapeutic target. Although IL-4 can be found increased at the protein level in lesional epidermis, IL-13 remains a far more prominent cytokine capable of driving the cellular inflammatory process specific to skin. A comparison between lesional and non-lesional skin revealed that non-lesional skin harbors significant levels of IL-13 in severe disease, which requires further study. Our RNAscope analyses demonstrated the localization of peTRMs alongside IL-13 expression within the epidermis, supporting the proposed hypothesis. Furthermore, our findings revealed that both mRNA and protein exhibit comparable expression levels in AD skin. With the help of C3G, we also investigated T cell lineages in AD, focusing on AD-related markers. Interestingly, this revealed the presence of double-positive T cells in AD, an area that has been relatively understudied in AD.

Future research should focus on further elucidating the dominant role of IL-13 as related to tissue-resident pathogenic effector cell functions to aid in developing more effective treatments aimed at complete remission.

APPENDIX: Supplementary tables and figures

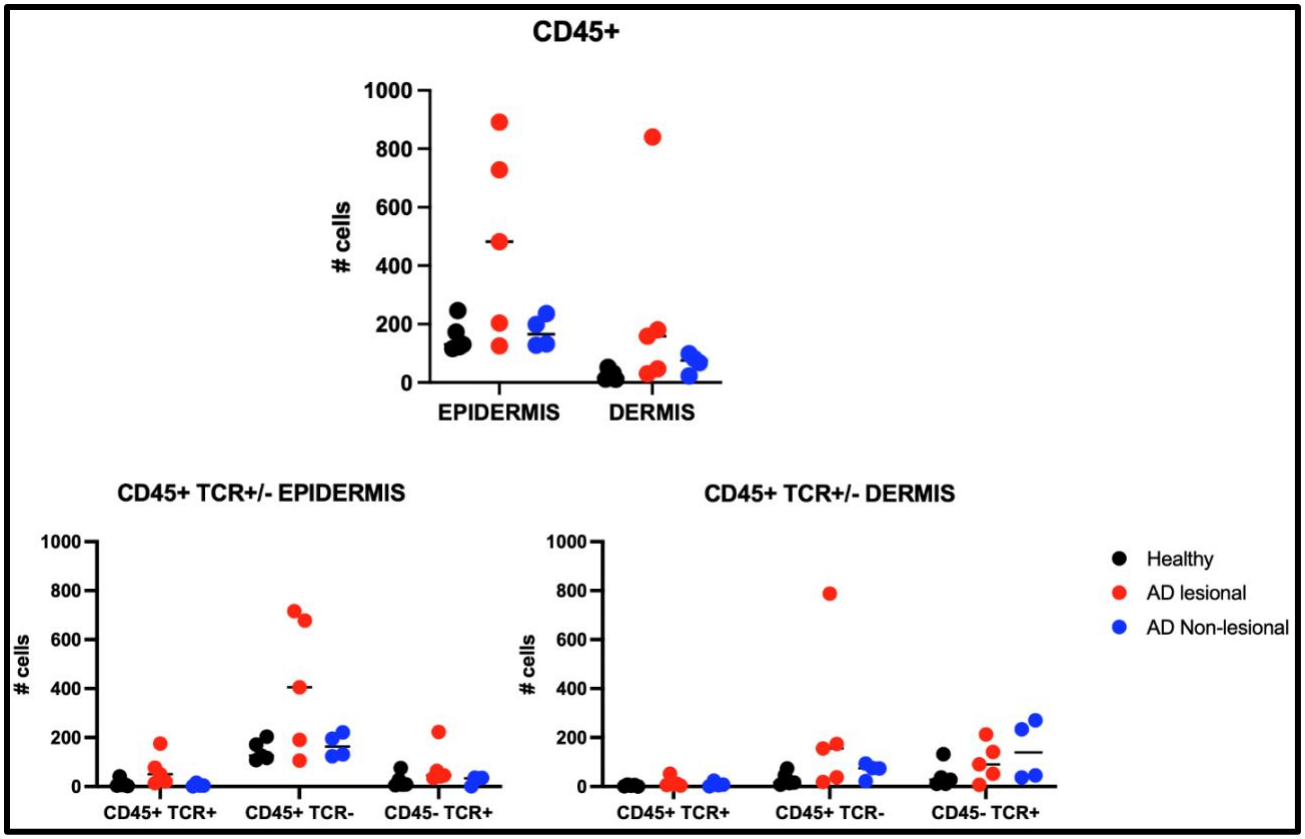
Suppl Table 1: Hanifin & Rajka criteria for AD [7, 21, 175]
Must have 3 or more basic features
- Pruritus - Typical morphology and distribution - Chronic or relapsing dermatitis - Personal or family history of atopy
At least 3 or more minor features
- Xerosis - Ichthyosis/palmer hyper linearity/keratosis pilaris - Immediate skin test reactivity - Elevated Serum IgE levels - Age of onset - Tendency towards cutaneous infection (staph infection) - Non-specific dermatitis - Nipple eczema - Cheilitis - Dennie-Morgan infraorbital fold - Orbital darkening - Pityriasis alba - Anterior neck folds - Anterior subcapsular cataracts - Intolerance to wool and lipid solvents - Perifollicular accentuation - Food intolerance - Facial erythema - Recurrent conjunctivitis - Keratoconus - Itch when sweating - Course influenced by environmental / emotional factors / white demographism / delayed blanch

Suppl Table 2: EASI calculation for adult atopic dermatitis							
Area score [23]							
% Involvement	**0**	**1-9%**	**10-29%**	**30-49%**	**50-69%**	**70-89%**	**90-100%**
Area score	**0**	**1**	**2**	**3**	**4**	**5**	**6**

EASI calculator (adults) [23]							
Body region	**Erythema (0-3)**	**Edema (0-3)**	**Excoriation (0-3)**	**Lichenification (0-3)**	**Area score (0-6)**	**Multiplier**	**Score**
Head/ Neck					x	x 0.1	
Trunk					x	x 0.3	
Upper extremities					x	x 0.2	
Lower extremities					x	x 0.4	
						The final EASI score (0-72)	

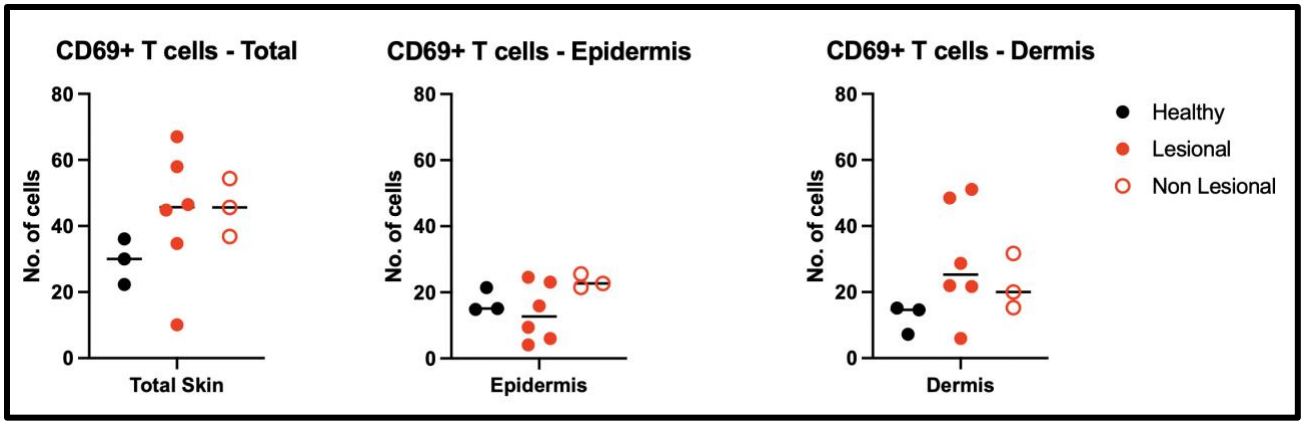
EASI score scale [23]					
Clear	**Almost clear**	**Mild**	**Moderate**	**Severe**	**Very severe**
0	0.1 – 1.0	1.1 – 7.0	7.1 – 21.0	21.1 – 50.0	50.1 – 72.0

Suppl Table 3: vIGA scale for AD [176]	
vIGA score	Clinical description
0 – Clear	No inflammatory signs of atopic dermatitis, such as erythema, induration or papulation, lichenification, or oozing and crusting. However, post-inflammatory changes, including hyperpigmentation and/or hypopigmentation, may be present.
1 – Almost clear	Mild erythema, slight induration or papulation, and minimal lichenification are present, with no evidence of oozing or crusting.
2 – Mild	There is slight but noticeable erythema (pink in color), mild induration or papulation, and/or subtle lichenification, with no signs of oozing or crusting.
3 – Moderate	There is clearly perceptible erythema (dull red in color), noticeable induration or papulation, and/or distinct lichenification. Oozing and crusting may also be present.
4 - Severe	There is marked erythema (deep or bright red), significant induration or papulation, and/or pronounced lichenification. The disease is widespread, with possible oozing or crusting.



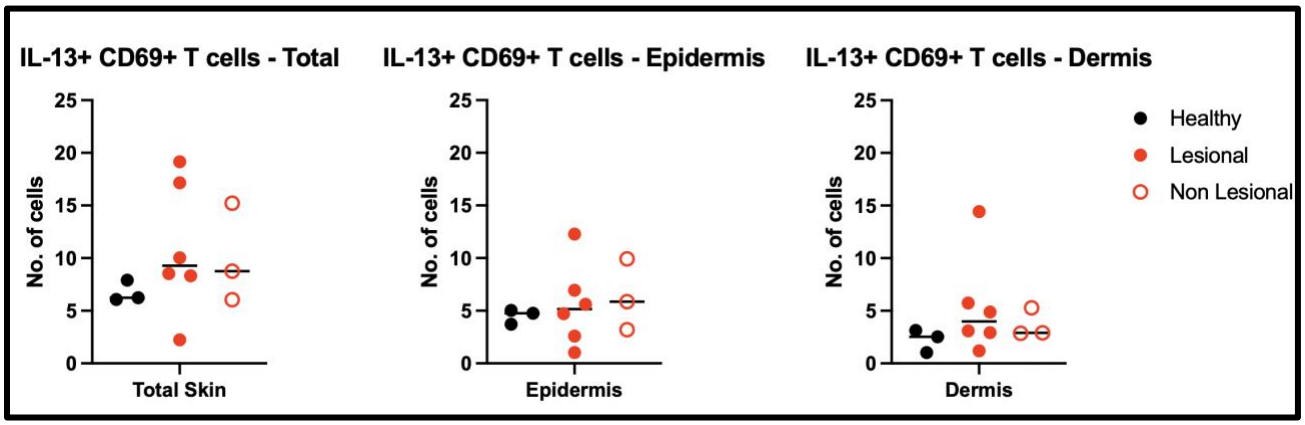
Suppl Figure 1: Proportion of CD45$^+$ cells are increased in AD skin

Cell counts of CD45$^+$ cells (mRNA) detected by RNAscope experiments done on 5mm punch biopsies, tile scan of AD patient skin versus healthy subjects. Top panel, Dot plot comparing the cell count of CD45$^+$ cells in the epidermis and dermis in AD lesional (red circles), AD non-lesional (blue circles), and healthy (black circles) skin: Lower left panel, Dot plot comparing the cell count of CD45$^+$ TCR$^+$, CD45$^+$ TCR$^-$ and CD45$^-$ TCR$^+$ cells in the epidermis among the three groups; Lower right panel, Dot plot comparing the cell count of CD45$^+$ TCR$^+$, CD45$^+$ TCR$^-$ and CD45$^-$ TCR$^+$ cells in the dermis among the three groups. N= 5 lesional AD, N=4 non-lesional AD, N=4 healthy skin.



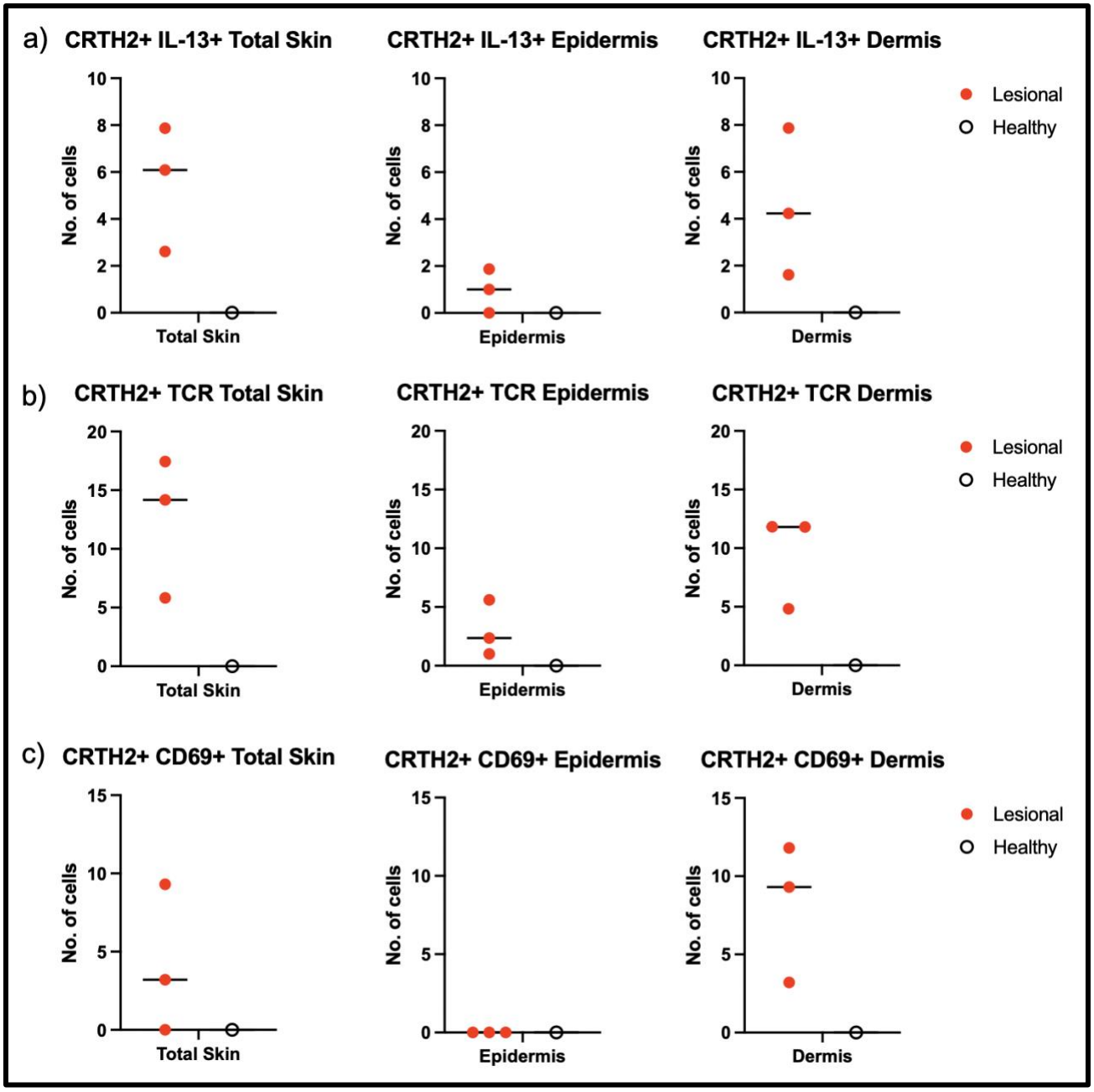
Suppl. Figure 2: Increased number of TRMs ($CD69^+$) in AD skin compared to healthy skin, protein level.

Cell counts of $CD3^+$ $CD69^+$ cells in 5mm punch biopsies, tile scan of AD patient skin versus healthy subjects using immunofluorescence staining. Quantifications were plotted for lesional AD skin as filled red circles, non-lesional as red empty circles, and healthy as black filled circles. Left panel, total skin (dermis and epidermis); middle panel, epidermis; right panel, dermis. N=6 lesional AD, N=3 non-lesional AD, N=6 healthy control. Statistical significance was performed by one-way ANOVA (Kruskal – Wallis test).



Suppl. Figure 3: Increased number of $IL\text{-}13^+$ TRMs in AD skin compared to healthy skin, protein level.

Cell counts of $CD3^+$ $CD69^+$ $IL\text{-}13^+$ cells in 5mm punch biopsies, tile scan of AD patient skin versus healthy subjects. using immunofluorescence staining. Quantifications were plotted for lesional AD skin as filled red circles, non-lesional as red empty circles, and healthy as black filled circles. Left panel: total skin (dermis and epidermis); middle panel, epidermis; right panel, dermis. N=6 lesional AD, N=3 non-lesional AD, N=6 healthy controls. Statistical significance was performed by one-way ANOVA (Kruskal – Wallis test).



Suppl. Figure 4: Preliminary plots for CRTH2$^+$ cells in AD skin, protein level.
Cell counts of CRTH2$^+$ cells in 5mm punch biopsies, tile scan of AD patient skin versus healthy subjects. using immunofluorescence staining. A) CRTH2$^+$ IL-13$^+$ cells B) CRTH2$^+$ TCR$^+$ C) CRTH2$^+$ CD69$^+$ cells. Quantifications were plotted for lesional AD skin as filled red circles, and healthy as black outline circles. Left panel: total skin (dermis and epidermis); middle panel, epidermis; right panel, dermis. N=3 lesional AD, N=1 healthy controls.

References

1. Bieber, T., et al., *Atopic dermatitis: pathomechanisms and lessons learned from novel systemic therapeutic options.* J Eur Acad Dermatol Venereol, 2022. **36**(9): p. 1432-1449.
2. Hadi, H.A., et al., *The Epidemiology and Global Burden of Atopic Dermatitis: A Narrative Review.* Life (Basel), 2021. **11**(9).
3. Adamson, A.S., *The Economics Burden of Atopic Dermatitis.* Adv Exp Med Biol, 2017. **1027**: p. 79-92.
4. Manjelievskaia, J., et al., *The direct and indirect costs of adult atopic dermatitis.* J Manag Care Spec Pharm, 2021. **27**(10): p. 1416-1425.
5. Ali, F., J. Vyas, and A.Y. Finlay, *Counting the Burden: Atopic Dermatitis and Health-related Quality of Life.* Acta Derm Venereol, 2020. **100**(12): p. adv00161.
6. Napolitano, M., et al., *Children atopic dermatitis: Diagnosis, mimics, overlaps, and therapeutic implication.* Dermatol Ther, 2022. **35**(12): p. e15901.
7. Weidinger, S., et al., *Atopic dermatitis.* Nat Rev Dis Primers, 2018. **4**(1): p. 1.
8. Chovatiya, R. and J.I. Silverberg, *Evaluating the Longitudinal Course of Atopic Dermatitis: Implications for Clinical Practice.* Am J Clin Dermatol, 2022. **23**(4): p. 459-468.
9. Boguniewicz, M., *Biologic Therapy for Atopic Dermatitis: Moving Beyond the Practice Parameter and Guidelines.* J Allergy Clin Immunol Pract, 2017. **5**(6): p. 1477-1487.
10. Sroka-Tomaszewska, J. and M. Trzeciak, *Molecular Mechanisms of Atopic Dermatitis Pathogenesis.* Int J Mol Sci, 2021. **22**(8).
11. Leung, D.Y. and E. Guttman-Yassky, *Deciphering the complexities of atopic dermatitis: shifting paradigms in treatment approaches.* J Allergy Clin Immunol, 2014. **134**(4): p. 769-79.
12. Silverberg, N.B. and J.I. Silverberg, *Inside out or outside in: does atopic dermatitis disrupt barrier function or does disruption of barrier function trigger atopic dermatitis?* Cutis, 2015. **96**(6): p. 359-61.
13. Sullivan, M. and N.B. Silverberg, *Current and emerging concepts in atopic dermatitis pathogenesis.* Clin Dermatol, 2017. **35**(4): p. 349-353.
14. Tokura, Y. and S. Hayano, *Subtypes of atopic dermatitis: From phenotype to endotype.* Allergol Int, 2022. **71**(1): p. 14-24.
15. Werfel, T., et al., *Cellular and molecular immunologic mechanisms in patients with atopic dermatitis.* J Allergy Clin Immunol, 2016. **138**(2): p. 336-49.
16. Czarnowicki, T., et al., *Atopic dermatitis endotypes and implications for targeted therapeutics.* J Allergy Clin Immunol, 2019. **143**(1): p. 1-11.
17. Lugovic-Mihic, L., et al., *Atopic Dermatitis: Disease Features, Therapeutic Options, and a Multidisciplinary Approach.* Life (Basel), 2023. **13**(6).
18. Torres, T., et al., *Update on Atopic Dermatitis.* Acta Med Port, 2019. **32**(9): p. 606-613.
19. Bosma, A.L., et al., *Work ability and quality of working life in atopic dermatitis patients treated with dupilumab.* J Dermatol, 2021. **48**(9): p. 1305-1314.
20. Rothe, M.J. and J.M. Grant-Kels, *Diagnostic criteria for atopic dermatitis.* Lancet, 1996. **348**(9030): p. 769-70.
21. Hanifin, J.M. and G. Rajka, *Diagnostic Features of Atopic Dermatitis.* Acta Dermato-Venereologica, 1980. **60**(92): p. 44-47.

22. Maintz, L., et al., *Measuring Atopic Dermatitis Disease Severity: The Potential for Electronic Tools to Benefit Clinical Care.* J Allergy Clin Immunol Pract, 2021. **9**(4): p. 1473-1486 e2.
23. Hanifin, J.M., et al., *The Eczema Area and Severity Index-A Practical Guide.* Dermatitis, 2022. **33**(3): p. 187-192.
24. Simpson, E.L., et al., *The Validated Investigator Global Assessment for Atopic Dermatitis (vIGA-AD): a clinical outcome measure for the severity of atopic dermatitis.* Br J Dermatol, 2022. **187**(4): p. 531-538.
25. Tokura, Y., *Extrinsic and intrinsic types of atopic dermatitis.* J Dermatol Sci, 2010. **58**(1): p. 1-7.
26. Wollenberg, A., et al., *Targeting immunoglobulin E in atopic dermatitis: A review of the existing evidence.* World Allergy Organ J, 2021. **14**(3): p. 100519.
27. Paternoster, L., *Genetic landscape of atopic dermatitis.* Curr Opin Allergy Clin Immunol, 2024.
28. Furue, K., et al., *The IL-13-OVOL1-FLG axis in atopic dermatitis.* Immunology, 2019. **158**(4): p. 281-286.
29. Akhtar, S., et al., *Epigenetic control of inflammation in Atopic Dermatitis.* Semin Cell Dev Biol, 2024. **154**(Pt C): p. 199-207.
30. Cetinarslan, T., L. Kumper, and R. Folster-Holst, *The immunological and structural epidermal barrier dysfunction and skin microbiome in atopic dermatitis-an update.* Front Mol Biosci, 2023. **10**: p. 1159404.
31. Paternoster, L., et al., *Meta-analysis of genome-wide association studies identifies three new risk loci for atopic dermatitis.* Nat Genet, 2011. **44**(2): p. 187-92.
32. Napolitano, M., et al., *The hidden sentinel of the skin: An overview on the role of interleukin-13 in atopic dermatitis.* Frontiers in Medicine, 2023. **10**.
33. Mitamura, Y., et al., *Hierarchical control of interleukin 13 (IL-13) signals in lung fibroblasts by STAT6 and SOX11.* J Biol Chem, 2018. **293**(38): p. 14646-14658.
34. Hirota, T., et al., *Genome-wide association study identifies eight new susceptibility loci for atopic dermatitis in the Japanese population.* Nat Genet, 2012. **44**(11): p. 1222-6.
35. Schuler, C.F.t., et al., *Genetic and Immunological Pathogenesis of Atopic Dermatitis.* J Invest Dermatol, 2023.
36. Budu-Aggrey, A., et al., *European and multi-ancestry genome-wide association meta-analysis of atopic dermatitis highlights importance of systemic immune regulation.* Nat Commun, 2023. **14**(1): p. 6172.
37. Ross, G., *Treatments for atopic dermatitis.* Aust Prescr, 2023. **46**(1): p. 9-12.
38. Seegräber, M., et al., *Dupilumab for treatment of atopic dermatitis.* Expert Rev Clin Pharmacol, 2018. **11**(5): p. 467-474.
39. Simpson, E.L., et al., *Two Phase 3 Trials of Dupilumab versus Placebo in Atopic Dermatitis.* N Engl J Med, 2016. **375**(24): p. 2335-2348.
40. Guttman-Yassky, E., et al., *Targeting IL-13 with tralokinumab normalizes type 2 inflammation in atopic dermatitis both early and at 2 years.* Allergy, 2024. **79**(6): p. 1560-1572.
41. Wollenberg, A., et al., *Tralokinumab for moderate-to-severe atopic dermatitis: results from two 52-week, randomized, double-blind, multicentre, placebo-controlled phase III trials (ECZTRA 1 and ECZTRA 2).* Br J Dermatol, 2021. **184**(3): p. 437-449.

42. Simpson, E.L., et al., *Tralokinumab Provides Clinically Meaningful Responses at Week 16 in Adults with Moderate-to-Severe Atopic Dermatitis Who Do Not Achieve IGA 0/1.* Am J Clin Dermatol, 2024. **25**(1): p. 139-148.
43. Silverberg, J.I., et al., *Patients with Moderate-to-Severe Atopic Dermatitis Maintain Stable Response with No or Minimal Fluctuations with 1 Year of Lebrikizumab Treatment.* Dermatol Ther (Heidelb), 2024.
44. Silverberg, J.I., et al., *Two Phase 3 Trials of Lebrikizumab for Moderate-to-Severe Atopic Dermatitis.* N Engl J Med, 2023. **388**(12): p. 1080-1091.
45. Rand, K., et al., *Matching-Adjusted Indirect Comparison of the Long-Term Efficacy Maintenance and Adverse Event Rates of Lebrikizumab versus Dupilumab in Moderate-to-Severe Atopic Dermatitis.* Dermatol Ther (Heidelb), 2024. **14**(1): p. 169-182.
46. Muller, S., L. Maintz, and T. Bieber, *Treatment of atopic dermatitis: Recently approved drugs and advanced clinical development programs.* Allergy, 2024.
47. Mohamed, M.F., et al., *Upadacitinib: Mechanism of action, clinical, and translational science.* Clin Transl Sci, 2024. **17**(1): p. e13688.
48. Guttman-Yassky, E., et al., *Once-daily upadacitinib versus placebo in adolescents and adults with moderate-to-severe atopic dermatitis (Measure Up 1 and Measure Up 2): results from two replicate double-blind, randomised controlled phase 3 trials.* Lancet, 2021. **397**(10290): p. 2151-2168.
49. Brandt, E.B. and U. Sivaprasad, *Th2 Cytokines and Atopic Dermatitis.* J Clin Cell Immunol, 2011. **2**(3).
50. Fania, L., et al., *Multiple Roles for Cytokines in Atopic Dermatitis: From Pathogenic Mediators to Endotype-Specific Biomarkers to Therapeutic Targets.* Int J Mol Sci, 2022. **23**(5).
51. Furue, M., *T helper type 2 signatures in atopic dermatitis.* Journal of Cutaneous Immunology and Allergy, 2018. **1**(3): p. 93-99.
52. Wynn, T.A., *Type 2 cytokines: mechanisms and therapeutic strategies.* Nat Rev Immunol, 2015. **15**(5): p. 271-82.
53. Lugovic, L., J. Lipozenocic, and J. Jakic-Razumovic, *Atopic dermatitis: immunophenotyping of inflammatory cells in skin lesions.* Int J Dermatol, 2001. **40**(8): p. 489-94.
54. Hamilton, J.D., et al., *Dupilumab improves the molecular signature in skin of patients with moderate-to-severe atopic dermatitis.* J Allergy Clin Immunol, 2014. **134**(6): p. 1293-1300.
55. Bakker, D.S., et al., *Early and Long-Term Effects of Dupilumab Treatment on Circulating T-Cell Functions in Patients with Moderate-to-Severe Atopic Dermatitis.* J Invest Dermatol, 2021. **141**(8): p. 1943-1953 e13.
56. He, H., et al., *Tape-Strip Proteomic Profiling of Atopic Dermatitis on Dupilumab Identifies Minimally Invasive Biomarkers.* Front Immunol, 2020. **11**: p. 1768.
57. He, H., et al., *Single-cell transcriptome analysis of human skin identifies novel fibroblast subpopulation and enrichment of immune subsets in atopic dermatitis.* J Allergy Clin Immunol, 2020. **145**(6): p. 1615-1628.
58. Kortekaas Krohn, I., et al., *T-cell subsets in the skin and their role in inflammatory skin disorders.* Allergy, 2022. **77**(3): p. 827-842.

59. Lamiable, O., M. Brewerton, and F. Ronchese, *IL-13 in dermal type-2 dendritic cell specialization: From function to therapeutic targeting.* European Journal of Immunology, 2022. **52**(7): p. 1047-1057.
60. Bao, K. and R.L. Reinhardt, *The differential expression of IL-4 and IL-13 and its impact on type-2 immunity.* Cytokine, 2015. **75**(1): p. 25-37.
61. Gandhi, N.A., et al., *Targeting key proximal drivers of type 2 inflammation in disease.* Nat Rev Drug Discov, 2016. **15**(1): p. 35-50.
62. Ong, P.Y., *Atopic dermatitis: Is innate or adaptive immunity in control? A clinical perspective.* Front Immunol, 2022. **13**: p. 943640.
63. Tsoi, L.C., et al., *Atopic Dermatitis Is an IL-13-Dominant Disease with Greater Molecular Heterogeneity Compared to Psoriasis.* J Invest Dermatol, 2019. **139**(7): p. 1480-1489.
64. Mayer, J.U., et al., *Homeostatic IL-13 in healthy skin directs dendritic cell differentiation to promote T(H)2 and inhibit T(H)17 cell polarization.* Nat Immunol, 2021. **22**(12): p. 1538-1550.
65. Tubau, C. and L. Puig, *Therapeutic targeting of the IL-13 pathway in skin inflammation.* Expert Rev Clin Immunol, 2021. **17**(1): p. 15-25.
66. Bieber, T., *Interleukin-13: Targeting an underestimated cytokine in atopic dermatitis.* Allergy, 2020. **75**(1): p. 54-62.
67. Chiricozzi, A., et al., *Targeting IL-4 for the Treatment of Atopic Dermatitis.* ImmunoTargets and Therapy, 2020. **Volume 9**: p. 151-156.
68. Sehra, S., et al., *IL-4 regulates skin homeostasis and the predisposition toward allergic skin inflammation.* J Immunol, 2010. **184**(6): p. 3186-90.
69. Gittler, J.K., et al., *Progressive activation of T(H)2/T(H)22 cytokines and selective epidermal proteins characterizes acute and chronic atopic dermatitis.* J Allergy Clin Immunol, 2012. **130**(6): p. 1344-54.
70. Moyle, M., et al., *Understanding the immune landscape in atopic dermatitis: The era of biologics and emerging therapeutic approaches.* Exp Dermatol, 2019. **28**(7): p. 756-768.
71. Leung, D.Y. and T. Bieber, *Atopic dermatitis.* Lancet, 2003. **361**(9352): p. 151-60.
72. Szalus, K., M. Trzeciak, and R.J. Nowicki, *JAK-STAT Inhibitors in Atopic Dermatitis from Pathogenesis to Clinical Trials Results.* Microorganisms, 2020. **8**(11).
73. Kawakami, T., et al., *Mast cells in atopic dermatitis.* Curr Opin Immunol, 2009. **21**(6): p. 666-78.
74. Szymanski, U., et al., *Cytokines and apoptosis in atopic dermatitis.* Postepy Dermatol Alergol, 2021. **38**(2): p. 1-13.
75. Peng, Y.Q., et al., *Effects of myeloid and plasmacytoid dendritic cells on ILC2s in patients with allergic rhinitis.* J Allergy Clin Immunol, 2020. **145**(3): p. 855-867 e8.
76. Wollenberg, A., et al., *Immunomorphological and ultrastructural characterization of Langerhans cells and a novel, inflammatory dendritic epidermal cell (IDEC) population in lesional skin of atopic eczema.* J Invest Dermatol, 1996. **106**(3): p. 446-53.
77. Chu, C.C., P. Di Meglio, and F.O. Nestle, *Harnessing dendritic cells in inflammatory skin diseases.* Semin Immunol, 2011. **23**(1): p. 28-41.
78. Otsuka, A. and K. Kabashima, *Mast cells and basophils in cutaneous immune responses.* Allergy, 2015. **70**(2): p. 131-40.
79. Tominaga, M. and K. Takamori, *Peripheral itch sensitization in atopic dermatitis.* Allergol Int, 2022. **71**(3): p. 265-277.

80. Voss, M., et al., *Mast Cells in the Skin: Defenders of Integrity or Offenders in Inflammation?* Int J Mol Sci, 2021. **22**(9).
81. Kuhn, H., et al., *Mas-related G protein-coupled receptor X2 and its activators in dermatologic allergies.* J Allergy Clin Immunol, 2021. **147**(2): p. 456-469.
82. Cho, B.O., et al., *Diospyros lotus leaf and grapefruit stem extract synergistically ameliorate atopic dermatitis-like skin lesion in mice by suppressing infiltration of mast cells in skin lesions.* Biomed Pharmacother, 2017. **89**: p. 819-826.
83. Alkon, N., et al., *Single-cell RNA sequencing defines disease-specific differences between chronic nodular prurigo and atopic dermatitis.* J Allergy Clin Immunol, 2023. **152**(2): p. 420-435.
84. Alkon, N., et al., *Single-cell analysis reveals innate lymphoid cell lineage infidelity in atopic dermatitis.* J Allergy Clin Immunol, 2022. **149**(2): p. 624-639.
85. Bangert, C., et al., *Persistence of mature dendritic cells, T(H)2A, and Tc2 cells characterize clinically resolved atopic dermatitis under IL-4Rα blockade.* Sci Immunol, 2021. **6**(55).
86. Liu, Y., et al., *Classification of human chronic inflammatory skin disease based on single-cell immune profiling.* Sci Immunol, 2022. **7**(70): p. eabl9165.
87. Mitamura, Y., et al., *Spatial transcriptomics combined with single-cell RNA-sequencing unravels the complex inflammatory cell network in atopic dermatitis.* Allergy, 2023. **78**(8): p. 2215-2231.
88. Zhang, B., et al., *Single-cell profiles reveal distinctive immune response in atopic dermatitis in contrast to psoriasis.* Allergy, 2023. **78**(2): p. 439-453.
89. Zhou, J., et al., *Single-cell RNA-seq reveals abnormal differentiation of keratinocytes and increased inflammatory differentiated keratinocytes in atopic dermatitis.* J Eur Acad Dermatol Venereol, 2023. **37**(11): p. 2336-2348.
90. Reynolds, G., et al., *Developmental cell programs are co-opted in inflammatory skin disease.* Science, 2021. **371**(6527).
91. Rojahn, T.B., et al., *Single-cell transcriptomics combined with interstitial fluid proteomics defines cell type-specific immune regulation in atopic dermatitis.* J Allergy Clin Immunol, 2020. **146**(5): p. 1056-1069.
92. Schabitz, A., et al., *Spatial transcriptomics landscape of lesions from non-communicable inflammatory skin diseases.* Nat Commun, 2022. **13**(1): p. 7729.
93. Furue, M. and M. Furue, *OX40L-OX40 Signaling in Atopic Dermatitis.* J Clin Med, 2021. **10**(12).
94. Guttman-Yassky, E., et al., *The role of OX40L/OX40 axis signalling in atopic dermatitis.* Br J Dermatol, 2024.
95. Croft, M., et al., *OX40 in the Pathogenesis of Atopic Dermatitis-A New Therapeutic Target.* Am J Clin Dermatol, 2024. **25**(3): p. 447-461.
96. Croft, M., *Control of immunity by the TNFR-related molecule OX40 (CD134).* Annu Rev Immunol, 2010. **28**: p. 57-78.
97. Halim, T.Y.F., et al., *Tissue-Restricted Adaptive Type 2 Immunity Is Orchestrated by Expression of the Costimulatory Molecule OX40L on Group 2 Innate Lymphoid Cells.* Immunity, 2018. **48**(6): p. 1195-1207 e6.
98. Esaki, H., et al., *Early-onset pediatric atopic dermatitis is T(H)2 but also T(H)17 polarized in skin.* J Allergy Clin Immunol, 2016. **138**(6): p. 1639-1651.

99. Elsner, J.S., et al., *The OX40 Axis is Associated with Both Systemic and Local Involvement in Atopic Dermatitis.* Acta Derm Venereol, 2020. **100**(6): p. adv00099.
100. Ilves, T. and I.T. Harvima, *OX40 ligand and OX40 are increased in atopic dermatitis lesions but do not correlate with clinical severity.* J Eur Acad Dermatol Venereol, 2013. **27**(2): p. e197-205.
101. Huang, Z., et al., *Th2A cells: The pathogenic players in allergic diseases.* Front Immunol, 2022. **13**: p. 916778.
102. Wambre, E.R.G., et al., *TH2A Cells As a Unique TH2 Cell Subset in Allergic Individuals: Steps Toward a T Cell Biomarker For Allergy.* Journal of Allergy and Clinical Immunology, 2012. **129**(2).
103. Mitson-Salazar, A., et al., *Hematopoietic prostaglandin D synthase defines a proeosinophilic pathogenic effector human T(H)2 cell subpopulation with enhanced function.* J Allergy Clin Immunol, 2016. **137**(3): p. 907-18.e9.
104. He, R., et al., *The prostaglandin D(2) receptor CRTH2 is important for allergic skin inflammation after epicutaneous antigen challenge.* J Allergy Clin Immunol, 2010. **126**(4): p. 784-90.
105. Honda, T. and K. Kabashima, *Prostanoids in allergy.* Allergol Int, 2015. **64**(1): p. 11-6.
106. Cella, M., H. Miller, and C. Song, *Beyond NK cells: the expanding universe of innate lymphoid cells.* Front Immunol, 2014. **5**: p. 282.
107. Bangert, C., et al., *Persistence of mature dendritic cells, T(H)2A, and Tc2 cells characterize clinically resolved atopic dermatitis under IL-4Ralpha blockade.* Sci Immunol, 2021. **6**(55).
108. Kim, B.S., *Innate lymphoid cells in the skin.* J Invest Dermatol, 2015. **135**(3): p. 673-678.
109. Barlow, J.L., et al., *Innate IL-13-producing nuocytes arise during allergic lung inflammation and contribute to airways hyperreactivity.* J Allergy Clin Immunol, 2012. **129**(1): p. 191-8 e1-4.
110. Kim, B.S., et al., *TSLP Elicits IL-33–Independent Innate Lymphoid Cell Responses to Promote Skin Inflammation.* Science Translational Medicine, 2013. **5**(170).
111. Bruggen, M.C., et al., *In Situ Mapping of Innate Lymphoid Cells in Human Skin: Evidence for Remarkable Differences between Normal and Inflamed Skin.* J Invest Dermatol, 2016. **136**(12): p. 2396-2405.
112. Jia, H., H. Wan, and D. Zhang, *Innate lymphoid cells: a new key player in atopic dermatitis.* Front Immunol, 2023. **14**: p. 1277120.
113. Chieosilapatham, P., et al., *Keratinocytes: innate immune cells in atopic dermatitis.* Clin Exp Immunol, 2021. **204**(3): p. 296-309.
114. Pan, Y., et al., *The Roles of Innate Immune Cells in Atopic Dermatitis.* J Innate Immun, 2024. **16**(1): p. 385-396.
115. Suwanchote, S., et al., *Role of antimicrobial peptides in atopic dermatitis.* Int J Dermatol, 2022. **61**(5): p. 532-540.
116. Rider, P., et al., *Alarmins: Feel the Stress.* J Immunol, 2017. **198**(4): p. 1395-1402.
117. Schauber, J. and R.L. Gallo, *Antimicrobial peptides and the skin immune defense system.* J Allergy Clin Immunol, 2008. **122**(2): p. 261-6.
118. El-Naccache, D.W., G. Hasko, and W.C. Gause, *Early Events Triggering the Initiation of a Type 2 Immune Response.* Trends Immunol, 2021. **42**(2): p. 151-164.
119. Soumelis, V., et al., *Human epithelial cells trigger dendritic cell mediated allergic inflammation by producing TSLP.* Nat Immunol, 2002. **3**(7): p. 673-80.

120. Zhang, Y. and B. Zhou, *Functions of thymic stromal lymphopoietin in immunity and disease.* Immunol Res, 2012. **52**(3): p. 211-23.
121. Yamaguchi, H.L., Y. Yamaguchi, and E. Peeva, *Role of Innate Immunity in Allergic Contact Dermatitis: An Update.* Int J Mol Sci, 2023. **24**(16).
122. Trier, A.M., et al., *IL-33 signaling in sensory neurons promotes dry skin itch.* J Allergy Clin Immunol, 2022. **149**(4): p. 1473-1480 e6.
123. Yamamura, Y., C. Nakashima, and A. Otsuka, *Interplay of cytokines in the pathophysiology of atopic dermatitis: insights from Murin models and human.* Front Med (Lausanne), 2024. **11**: p. 1342176.
124. Lloyd, C.M. and E.M. Hessel, *Functions of T cells in asthma: more than just T(H)2 cells.* Nat Rev Immunol, 2010. **10**(12): p. 838-48.
125. Chung, E.J., et al., *Immunomodulatory Role of Staphylococcus aureus in Atopic Dermatitis.* Pathogens, 2022. **11**(4).
126. Tian, X., et al., *A review of the mechanisms of keratinocytes damage caused by Staphylococcus aureus infection in patients with atopic dermatitis.* J Leukoc Biol, 2021. **110**(6): p. 1163-1169.
127. Schlievert, P.M., et al., *Staphylococcal TSST-1 Association with Eczema Herpeticum in Humans.* mSphere, 2021. **6**(4): p. e0060821.
128. Chen, H., et al., *Exploring the Role of Staphylococcus aureus in Inflammatory Diseases.* Toxins (Basel), 2022. **14**(7).
129. Yang, G., et al., *Skin Barrier Abnormalities and Immune Dysfunction in Atopic Dermatitis.* Int J Mol Sci, 2020. **21**(8).
130. Stawczyk-Macieja, M., et al., *Genetic background of skin barrier dysfunction in the pathogenesis of psoriasis vulgaris.* Postepy Dermatol Alergol, 2015. **32**(2): p. 123-6.
131. Irvine, A.D., Mclean, W.H.I., Leung, D.Y.M., *Filaggrin Mutations Associated with Skin and Allergic Diseases.* New England Journal of Medicine, 2011. **365**: p. 1315–1327.
132. Gupta, J. and D.J. Margolis, *Filaggrin gene mutations with special reference to atopic dermatitis.* Curr Treat Options Allergy, 2020. **7**(3): p. 403-413.
133. Moosbrugger-Martinz, V., et al., *Revisiting the Roles of Filaggrin in Atopic Dermatitis.* Int J Mol Sci, 2022. **23**(10).
134. Nedoszytko, B., et al., *Genetic and Epigenetic Aspects of Atopic Dermatitis.* Int J Mol Sci, 2020. **21**(18).
135. Maintz, L., et al., *From Skin Barrier Dysfunction to Systemic Impact of Atopic Dermatitis: Implications for a Precision Approach in Dermocosmetics and Medicine.* J Pers Med, 2022. **12**(6).
136. Quan, V.L., et al., *Atopic Dermatitis Across Shades of Skin.* Am J Clin Dermatol, 2023. **24**(5): p. 731-751.
137. Yoshida, T., L.A. Beck, and A. De Benedetto, *Skin barrier defects in atopic dermatitis: From old idea to new opportunity.* Allergol Int, 2022. **71**(1): p. 3-13.
138. Lu, H.F., et al., *Involvement and repair of epithelial barrier dysfunction in allergic diseases.* Front Immunol, 2024. **15**: p. 1348272.
139. Katsarou, S., et al., *The Role of Tight Junctions in Atopic Dermatitis: A Systematic Review.* J Clin Med, 2023. **12**(4).
140. Maeve A. McAleer, G.M.O.R.a.A.D.I., *Atopic Dermatitis*, in *Dermatology*. 2018, Elsevier.

141. Shin, H. and A. Iwasaki, *Tissue-resident memory T cells.* Immunol Rev, 2013. **255**(1): p. 165-81.
142. Long, B., et al., *Tissue-Resident Memory T Cells in Allergy.* Clin Rev Allergy Immunol, 2024. **66**(1): p. 64-75.
143. Samat, A.A.K., et al., *Tissue-Resident Memory T Cells in Chronic Inflammation-Local Cells with Systemic Effects?* Cells, 2021. **10**(2).
144. Zheng, C., et al., *Neutrophil recruitment by CD4 tissue-resident memory T cells induces chronic recurrent inflammation in atopic dermatitis.* Clin Immunol, 2023. **256**: p. 109805.
145. Amezcua Vesely, M.C., et al., *Effector T(H)17 Cells Give Rise to Long-Lived T(RM) Cells that Are Essential for an Immediate Response against Bacterial Infection.* Cell, 2019. **178**(5): p. 1176-1188 e15.
146. Migayron, L., et al., *Resident memory T cells in nonlesional skin and healed lesions of patients with chronic inflammatory diseases: Appearances can be deceptive.* J Allergy Clin Immunol, 2023.
147. Szabo, P.A., M. Miron, and D.L. Farber, *Location, location, location: Tissue resident memory T cells in mice and humans.* Sci Immunol, 2019. **4**(34).
148. Kabashima, K., et al., *The immunological anatomy of the skin.* Nat Rev Immunol, 2019. **19**(1): p. 19-30.
149. Yenyuwadee, S., et al., *The evolving role of tissue-resident memory T cells in infections and cancer.* Science Advances, 2022. **8**(33).
150. Mackay, L.K., et al., *Cutting edge: CD69 interference with sphingosine-1-phosphate receptor function regulates peripheral T cell retention.* J Immunol, 2015. **194**(5): p. 2059-63.
151. Mueller, S.N., A. Zaid, and F.R. Carbone, *Tissue-resident T cells: dynamic players in skin immunity.* Front Immunol, 2014. **5**: p. 332.
152. Pasparakis, M., I. Haase, and F.O. Nestle, *Mechanisms regulating skin immunity and inflammation.* Nature Reviews Immunology, 2014. **14**(5): p. 289-301.
153. Cheuk, S., et al., *CD49a Expression Defines Tissue-Resident CD8(+) T Cells Poised for Cytotoxic Function in Human Skin.* Immunity, 2017. **46**(2): p. 287-300.
154. Skon, C.N., et al., *Transcriptional downregulation of S1pr1 is required for the establishment of resident memory CD8+ T cells.* Nat Immunol, 2013. **14**(12): p. 1285-93.
155. Baeyens, A., et al., *Exit Strategies: S1P Signaling and T Cell Migration.* Trends Immunol, 2015. **36**(12): p. 778-787.
156. Steinert, E.M., et al., *Quantifying Memory CD8 T Cells Reveals Regionalization of Immunosurveillance.* Cell, 2015. **161**(4): p. 737-49.
157. Tokura, Y., et al., *Pathophysiology of Skin Resident Memory T Cells.* Front Immunol, 2020. **11**: p. 618897.
158. Emmanuel, T., et al., *Tissue-Resident Memory T Cells in Skin Diseases: A Systematic Review.* Int J Mol Sci, 2021. **22**(16).
159. Khalil, S., et al., *Tissue-resident memory T cells in the skin.* Inflamm Res, 2020. **69**(3): p. 245-254.
160. Clark, R.A., *Resident memory T cells in human health and disease.* Sci Transl Med, 2015. **7**(269): p. 269rv1.
161. Murakami, M., *Tissue-resident memory T cells: decoding intra-organ diversity with a gut perspective.* Inflamm Regen, 2024. **44**(1): p. 19.

162. Galvan Morales, M.A., et al., *New Insights into the Role of PD-1 and Its Ligands in Allergic Disease.* Int J Mol Sci, 2021. **22**(21).
163. Chen, L. and Z. Shen, *Tissue-resident memory T cells and their biological characteristics in the recurrence of inflammatory skin disorders.* Cell Mol Immunol, 2020. **17**(1): p. 64-75.
164. Kasprowicz-Furmanczyk, M., et al., *Assessment of the Tissue Resident Memory Cells in Lesional Skin of Patients with Psoriasis and in Healthy Skin of Healthy Volunteers.* Int J Environ Res Public Health, 2021. **18**(21).
165. Rindler, K., et al., *Spontaneously Resolved Atopic Dermatitis Shows Melanocyte and Immune Cell Activation Distinct From Healthy Control Skin.* Front Immunol, 2021. **12**: p. 630892.
166. Ali Marandi Ghoddousi, R., et al., *SCAMPR, a single-cell automated multiplex pipeline for RNA quantification and spatial mapping.* Cell Rep Methods, 2022. **2**(10): p. 100316.
167. Wasserer, S., et al., *Characterization of High and Low IFNG-Expressing Subgroups in Atopic Dermatitis.* Int J Mol Sci, 2024. **25**(11).
168. Kim, J.E., et al., *Molecular Mechanisms of Cutaneous Inflammatory Disorder: Atopic Dermatitis.* Int J Mol Sci, 2016. **17**(8).
169. Gadsboll, A.O., et al., *Pathogenic CD8(+) Epidermis-Resident Memory T Cells Displace Dendritic Epidermal T Cells in Allergic Dermatitis.* J Invest Dermatol, 2020. **140**(4): p. 806-815 e5.
170. Leijten, E.F., et al., *Tissue-Resident Memory CD8+ T Cells From Skin Differentiate Psoriatic Arthritis From Psoriasis.* Arthritis Rheumatol, 2021. **73**(7): p. 1220-1232.
171. Hennino, A., et al., *Skin-infiltrating CD8+ T cells initiate atopic dermatitis lesions.* J Immunol, 2007. **178**(9): p. 5571-7.
172. Bang, K. and et al., *CD4 CD8 (thymocyte-like) T lymphocytes present in blood and skin from patients with atopic dermatitis suggest immune dysregulation.* British Journal of Dermatology, 2001. **144**(6).
173. Overgaard, N.H., et al., *CD4+/CD8+ double-positive T cells: more than just a developmental stage?* J Leukoc Biol, 2015. **97**(1): p. 31-8.
174. Yamanaka, K., et al., *The Interplay of Type 1, Type 2, and Type 3 Lymphocytes and Cytokines in Atopic Dermatitis.* Int J Mol Sci, 2023. **24**(4).
175. Kulthanan, K., et al., *Clinical features of the extrinsic and intrinsic types of adult-onset atopic dermatitis.* Asia Pac Allergy, 2011. **1**(2): p. 80-6.
176. Simpson, E., et al., *The Validated Investigator Global Assessment for Atopic Dermatitis (vIGA-AD): The development and reliability testing of a novel clinical outcome measurement instrument for the severity of atopic dermatitis.* J Am Acad Dermatol, 2020. **83**(3): p. 839-846.