Characterizing pathogenic effector T cells in adult atopic dermatitis

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ENGLISH ABSTRACT

Background: Atopic dermatitis is a chronic inflammatory skin disorder that follows a relapsing-remitting course. While historically considered a disease limited to childhood, recent studies demonstrate lifelong persistence in a large proportion of patients. Chronic inflammation in AD is mediated by both local and systemic T_H2-associated cytokines, of which IL-13 plays a central role. Dupilumab specifically targets IL-13/IL-4 signaling to control disease. While discontinuation of traditional treatments leads to relapse, the specific immune populations and mechanisms responsible for disease reactivation remain unknown. *S.aureus* and its secreted enterotoxins are key environmental factors in AD. The impact of host–pathogen interactions in chronic T helper 2 (T_H2)-dominated inflammation in AD remains poorly understood. The long-term effects of dupilumab on the persistence of pathological IL-13^+T cells remains to be clarified. We hypothesize that cutaneous host-pathogen factors may contribute to the persistence of pathogenic type 2 T cells following treatment with dupilumab in adult atopic dermatitis patients. In addition, we aimed to establish an *in vitro* model based on skin-derived factors relevant to AD that can replicate pathogenic type 2 T-cell and IL-13 cytokine functions in disease.

Methods: **A)** We performed a cross-sectional study with adults with moderate-to-severe AD, AD patients treated with dupilumab under disease remission, and age-matched healthy subjects. Cellular immunophenotyping was performed on PBMCs to identify *ex vivo* helper T cell subsets and intracellular cytokine production using multiparametric flow cytometry. **B)** PBMCs from healthy and AD patients were cultured *in vitro* for 7 days in the presence of *S. aureus*-derived superantigen (SEB) +/- thymic stromal lymphopoietin (TSLP) - conditions found in the skin of patients and known to expand IL-13^+ TH2 cells, with the aim of determining whether we can detect TH2 associated co-stimulatory factors (ICOS, OX40), canonical transcription factors (pSTAT6, GATA3), as well as memory/activated markers (CD45RO, HLA-DR) .

Results: **A.** As expected *ex vivo*, a profoundly decreased T_H1/T_H2 ratio was observed in AD patients relative to healthy subjects; notably, this persisted in a cohort of patients in remission with dupilumab-treatment. Intracellular cytokine detection demonstrated a greater proportion of CLA^+ IL-13 and IL-4 producing cells in AD patients compared to healthy subjects. We demonstrated that patients treated with dupilumab have (persistent elevated) activated memory TH2 cells, specifically skin homing CLA^+, compared to healthy subjects, despite minimal disease activity. A higher proportion of Treg cells were also detected in dupilumab-treated AD patients compared to healthy subjects. Notably, the level of IL-13 production (MFI) in dupilumab treated patients remains comparable to untreated AD patients, and is significantly higher compared to healthy subjects, despite decreased frequency of $CD4^+CLA^+IL\text{-}13^+$ T cells. Using skin-mimicking inflammatory conditions, we demonstrated that TSLP and SEB are sufficient to drive IL-13 secretion and pathogenic effector type 2 T cell responses from healthy PBMCs *in vitro*, replicating AD-like cytokine and T cell functions found in patients *ex vivo*.

Conclusions: Our findings demonstrate that despite long-term treatment of AD patients with dupilumab, a persistent level of IL-13 production from skin-homing T cells is detected in patients, despite clinical remission. Here, we propose that residual level of IL-13 production may serve as a potential source of disease relapse in patients. In addition, we show that skin-associated factors such as TSLP and SEB can promote type 2 responses to induce IL-13 production *in vitro,* suggesting that these factors may play a role upstream in the maintenance of residual disease activity following treatment. Together, this data provides insight on pathophysiology and potential mechanisms responsible for relapse, ultimately highlighting novel potential targets for therapeutic avenues.

FRENCH ABSTRACT

Contexte: La dermatite atopique (DA) est une maladie inflammatoire chronique de la peau qui évolue par poussées et rémissions. Historiquement considérée comme une maladie limitée à l'enfance, de récentes études démontrent qu'elle persiste tout au long de la vie pour une portion considérable de patients. L'inflammation chronique dans la DA est médiée par des cytokines locales et systémiques associées aux lymphocytes T_H2, parmi lesquels l'IL-13 joue un rôle central. Le dupilumab est un médicament qui cible spécifiquement la signalisation par les cytokines IL-13 et IL-4 pour contrôler la maladie de façon efficace. Bien que la suspension des traitements traditionnels entraîne une rechute, les cellules et les mécanismes immunitaires responsables de la réactivation de la maladie restent inconnus. Le *Staphylococcus aureus (S. aureus)* et ses entérotoxines sécrétées sont des facteurs environnementaux clés de DA. L'impact des interactions hôte-pathogène dans l'inflammation chronique dominée par les lymphocytes T_H2 dans la DA reste mal compris. Les effets à long terme du dupilumab sur la persistance des lymphocytes T pathologiques IL-13^+ restent à clarifier. Nous émettons l'hypothèse que les facteurs cutanés hôte-pathogène peuvent contribuer à la persistance des cellules T de type 2 pathogéniques après un traitement avec dupilumab chez les patients adultes atteints de DA. Pour ce faire, nous avons tenté d'établir un modèle *in vitro* basé sur des facteurs cutanés pertinents pour la DA, afin de reproduire et d'étudier les fonctions pathogéniques des lymphocytes T de type 2 et des cytokines IL-13 dans la maladie.

Méthodes: **A)** Nous avons réalisé une étude transversale auprès d'adultes atteints de DA modérée à sévère, de patients atteints de DA en rémission après traitement avec dupilumab, et de sujets sains appariés selon l'âge. Un immunophénotypage cellulaire a été performé sur des PBMC afin d'identifier *ex vivo* les sous-ensembles de cellules T auxiliaires et la production intracellulaire

de cytokines à l'aide de la cytométrie de flux multiparamétrique. **B)** Les PBMCs de patients sains et de patients atteints de DA ont été cultivés *in vitro* pendant 7 jours en présence de S. aureus (SEB) +/- lymphopoïétine stromale thymique (TSLP) - conditions présentes dans la peau des patients et connues pour augmenter les cellules T_H2 IL-13^+, dans le but de déterminer si nous pouvons détecter des facteurs de co-stimulation (ICOS, OX40), de transcription canoniques (pSTAT6, GATA3), ainsi que des marqueurs de mémoire/activation (CD45RO, HLA-DR) associés à une réponse T_H2.

Résultats: **A.** Comme prévu, un rapport T_H1/T_H2 profondément réduit a été observé *ex vivo* chez les patients atteints de DA par rapport aux sujets sains; cela a notamment persisté dans une cohorte de patients en rémission suite au traitement avec dupilumab. La détection intracellulaire des cytokines a révélé une plus grande proportion de cellules productrices d'IL-13 et d'IL-4 parmi les cellules CLA^+ chez les patients atteints de DA que chez les sujets sains. Nous démontrons que les patients traités avec dupilumab ont des cellules T_H2 mémoires activées (élevées et persistantes), en particulier des cellules CLA^+ à orientation cutanée, par rapport aux sujets sains, en dépit d'une activité minime de la maladie. Une proportion plus élevée de cellules Treg a également été détectée chez les patients atteints de DA traités avec dupilumab par rapport aux sujets sains. Notamment, le niveau de production d'IL-13 (MFI) chez les patients traités avec dupilumab reste comparable à celui des patients atteints de DA non traités, et est significativement plus élevé que chez les sujets sains, malgré une diminution de la fréquence des lymphocytes T $CD4^+CLA^+$IL-13^+.

B. En utilisant des conditions inflammatoires simulant la peau, nous démontrons que la TSLP et la SEB sont suffisantes pour entraîner la sécrétion d'IL-13 et des réponses pathogènes de cellules T effectrices de type 2 à partir de PBMCs sains *in vitro*, reproduisant les fonctions de cytokines et de cellules T similaires à celles de la DA trouvées chez les patients *ex vivo*.

Conclusions: Nos résultats démontrent que malgré un traitement à long terme des patients atteints de DA avec dupilumab, un niveau persistant de production d'IL-13 par les lymphoctyes T à localisation cutanée est détecté chez les patients, malgré une rémission clinique. Nous révélons ici que ce niveau résiduel de production d'IL-13 peut potentiellement contribuer à rechute de la maladie chez les patients. En outre, nous démontrons que les facteurs associés à la peau, tels que la TSLP et la SEB, peuvent promouvoir les réponses de type 2 pour induire la production d'IL-13 *in vitro*, ce qui suggère que ces facteurs peuvent jouer un rôle en amont dans le maintien de l'activité résiduelle de la maladie après le traitement. L'ensemble de ces données permet de mieux comprendre la physiopathologie et les mécanismes potentiels responsables des rechutes, et de mettre en évidence de nouvelles cibles potentielles pour le traitement de la DA.

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CONTRIBUTION OF AUTHORS

Harshita Patel, MSc thesis student, wrote the entire thesis with editorial input from Dr. Carolyn Jack and Dr. Ciriaco Piccirillo. Céleste Pilon provided editorial inputs on the French abstract of this thesis. Harshita Patel, Dr. Carolyn Jack, Dr. Ciriaco Piccirillo, Tho-Alfakar Al-Aubodah, and Dr. Fernando Alvarez conceptualized the experiments. Harshita Patel performed all the experiments (PBMC isolation, cell culture, flow cytometry acquisition) and collected data. Harshita Patel performed all statistical analysis with input from Dr. Carolyn Jack, Dr. Fernando Alvarez, Tho-Alfakar Al-Aubodah, Mikhäel Attias and Franck P. Dupuy.

I, Harshita Patel, have read, understood and abided by all norms and regulations of academic integrity of McGill University.

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LIST OF ABBREVIATIONS

Abbreviation	Definition
AD	Atopic dermatitis
AMP	Anti-microbial peptides
APC	Antigen-presenting cell
BSA	Body surface area
CCL17	C-C chemokine ligand 17
CCL22	C-C chemokine ligand 22
CCL27/CCL28	C-C chemokine ligand 27/28
CCR10	C-C chemokine receptor type 10
CCR4	C-C chemokine receptor type 4
CLA	Cutaneous lymphocyte antigen
CRTH2	Prostaglandin D2 receptor 2
DC	Dendritic cell
DLQI	Daily life quality index
EASI	Eczema Area and Severity Index
FLG	Filaggrin
Foxp3	Fork-head binding protein 3
GATA3	GATA binding protein 3
IL-13	Interlukin-13
IL-2	Interlukin-2
IL-25	Interlukin-25
IL-31	Interlukin-31
IL-33	Interlukin-33
IL-4	Interlukin-4
IL-5	Interlukin-5
ILC2	Type 2 innate lymphoid cells
MFI	Mean fluorescence intensity
MHC	Major histocompatibility complex
OX40L	OX40 ligand
POEM	Patient-Oriented Eczema Measure
S. aureus	*Staphylococcus aureus*
SEB	superantigen enterotoxin B
STAT6	Signal transducer and activation of transcription 6
TCI	Topical calcineurin inhibitors
TCR	T cell receptor
TCS	Topical corticosteroids
T_H1	T helper 1 cell
T_H17	T helper 17 cell
T_H2	T helper 2 cell
T_{REG}	Regulatory T cells
T_{RM}	tissue-resident memory T
TSLP	Thymic stromal lymphopoietin
TSLPR	TSLP receptor
vIGA	Validated Investigator Global Assessment

INTRODUCTION

Atopic dermatitis (AD), known as eczema, is the most common inflammatory skin disorder around the world, with highest burden of disease measured by disability-adjusted life years[1-3]. The disease has an early onset of diagnosis for the vast majority of people, however, adult onset of AD is reported more frequently with a different clinical presentation[4-6]. Localization of disease changes from infancy to adulthood, however, similar clinical manifestations of AD are present including eczematous lesions with exudation, blistering, erythema (redness), lichenification (thickening of skin), and intense pruritis (itch)[7, 8]. Advanced population studies have debunked the long-term understanding that AD is a disease limited to childhood, as there is a significant subset of adult patients who do not undergo natural remission, and progress to have chronic adult AD[9-11]. Large challenges remain as many patients with moderate-to-severe AD are resistant to traditional therapies (topical, phototherapy, systemic), and therefore, experience debilitating comorbidities[12]. Therefore, the factors that contribute to refractory disease in adults, permitting the lack of natural remission must be clarified.

Clinical heterogeneity is a prominent feature of AD , which leads to many difficulties for accurate diagnoses, and requires additional research in diverse populations[13]. Pathophysiology mechanisms of AD involves an interplay of complex factors including genetics, epidermal barrier deficiencies, dysbiosis of the skin microbiome, and importantly, deviation in immune homeostasis[8]. The inflammatory state in AD is characterized by an overreactive T_H2 response, primarily driven by Interlukin-13 (IL-13), which responsible for the inflammation and clinical manifestations observed in disease[14]. Environmental factors such as the overabundant colonization of *Staphylococcus aureus (S. aureus)* and its associated-superantigens may directly activate or strengthen a type 2 response in chronic AD[15]. In 2017, Dupixent (dupilumab) was the first human monoclonal antibody (targets IL-4/IL-13) approved for the treatment of moderate-to-severe AD in

adults[16]. While dupilumab has demonstrated clinical efficacy in patients[17, 18], there is limited data regarding the long-term effects of treatment, disease remission and relapse after discontinuation of treatment. To gain insight on disease relapse triggering populations, the identification and characterization of persistent conventional and non-conventional T cell populations in AD patients after treatment is required. We hypothesize that cutaneous host-pathogen factors may contribute to the persistence of pathogenic type 2 T cells following treatment with dupilumab in adult atopic dermatitis patients.

Objective: We sought to understand the role of heterogeneous T cell populations, specifically polar T cells/type 2 cells, contributing to chronic, unremitting moderate-to-severe disease in adult AD patients. Furthermore, we sought to elucidate the impact of long-term dupilumab treatment on the persistence of type 2 T cells in AD patients compared to untreated patients and healthy subjects.

Using a Canadian cohort of adult AD patients, we aimed to characterize the distinct T_H2 and non-conventional T_H2-like IL-13^+ cells during severe active disease and after dupilumab-induced disease remission.

Aims:

1. To identify and compare conventional and non-conventional IL-13^+ T cell populations found in peripheral blood of adult AD patients <u>at baseline. vs healthy controls</u>
2. To determine if IL-13^+ T cells persist despite successful remission of disease following dupilumab treatment, relative to baseline (cross-sectional analysis of EASI-90 responders)
3. To determine the skin-associated molecular factors promoting IL-13^+ T_H2 expansion in adult atopic dermatitis patients using an *in vitro* model for Type 2 immunity.

LITERATURE REVIEW

Epidemiology of AD

Epidemiological studies of AD at a global scale are challenging to perform due limitations in uniform diagnostic criteria, validity of questionnaires or surveys, and cost constraints[19]. The prevalence of AD varies largely across different countries, as well as between pediatric and adult AD. AD has historically been considered a disease of childhood, which resulted in majority of epidemiological AD studies being on a pediatric population of patients[9-11, 19]. The International Study of Asthma and Allergies in Childhood (ISAAC) is the largest study to provide validated global prevalence of AD using a standardized method of diagnosis across all countries, however, this lacks the inclusion of adults[20]. The phase 3 ISAAC study revealed that prevalence of AD in adults is increasing in developed nations, and demonstrates how some ethnic groups are disproportionately affected[20]. Many population-based studies show that AD is more prevalent in African American (AA) and Asian children compared to European American (EA) children[21]. Currently, there are insufficient studies that have investigated prevalence of adult AD[2]. A collection of US-population based studies performed on adult AD patients suggest an overall AD prevalence rate of 7% in US adults[22]. Limited data is available on Canadian cohorts of AD patients, however, the 'Skin I am in" report from Eczema Quebec, revealed that 96% of their Canadian AD cohort were of adolescent-adult age, and nearly half the patients said AD symptoms affected their daily life based on the DLQI test. Overall, AD can affect up to 20% of children and up to 10% of adults in populations world-wide[2, 23]. As the prevalence and/or incidence of adult AD may be on the rise globally, future studies using validated and standardized disease measurement tools must be used to better understand the natural history of this complex heterogenic disease across the lifespan.

Natural remission vs. refractory disease

AD manifests early during childhood representing the first stage of the atopic march for most children[4]. Approximately 50% of individuals with AD develop clinical symptoms by the first year of life, while ~95% will experience disease onset by 5 years of age [5]. Historically, by the beginning of adolescence, 75% of children outgrow disease and enter spontaneous natural remission[3]. Individuals that do not undergo natural remission may have chronic AD throughout their life-time, with variability in the relapse-remitting course of disease[5]. Studies demonstrating persistence of AD into adulthood has challenged the dogma of natural remission in childhood-adolescence. The PEER study is an ongoing cross-sectional cohort that enrolled children with AD to evaluate the natural history and persistence of disease over a 10 year period[11]. From 7157 children enrolled, the study demonstrated that, at every age (2-26 years), more than 80% of participants had persistent AD symptoms, for which they were using medication to treat symptoms. In addition, 50% of patients experienced their first 6-month symptom and treatment-free period only after 20 years of age[11]. More recently, adult/adolescent-onset of AD has been described as symptoms presenting after 16 years of age, determined to be as frequent as in 1 in 4 AD patients[24]. As patient-reported adult-onset may in fact be remitted childhood AD, compounded by recall bias, specificity of adult-onset vs. recurrent AD remains controversial[10]. Diagnostic criteria also remain a challenge as studies reveal that the clinical phenotypes of adult-onset AD differ compared to those of childhood onset[6, 25, 26]. Patients with chronic recurrent AD often have inadequate disease control with potent topical corticosteroids or calcineurin inhibitors, and therefore, require long-term systemic medication to reduce disease burden[27]. Traditional immunosuppressive therapies are effective to control an acute flare of AD, however, safety considerations limit their use and discontinuation of treatment results in disease relapse[12]. Newer targeted therapies are in clinical trials or approved for the treatment of chronic AD, however, there are several barriers to its continuous use including

the lack of studies evaluating long-term safety and efficacy, conflicting evidence, small sample size of studies, adverse effects, and cost[28]. Currently, there is a gap in understanding as to why some patients undergo natural remission whereas others have life-long disease; similarly, the determinants of response to therapeutic remission, or lack thereof, are still being defined. Studies investigating the mechanisms of disease persistence, specifically host-pathogen molecular and cellular immune factors that promote disease activity, are needed for disease-modulating long-term management of AD.

Pathogenesis of AD disease

Genetic Susceptibility

Parental family history of atopic diseases is a strong risk factor for the development of AD. Twin studies demonstrate that heritability of AD is 75% in monozygotic twins[29]. Multiple genes have been associated with susceptibility of AD, including those encoding epidermal barrier structure proteins, IL-13 and associated immune/ inflammatory molecules[8]. Filaggrin (FLG) is a filament-aggregating protein that binds keratin fibers in epithelial cells promoting epidermal differentiation, and supporting the corneal barrier of the upper epidermis[30]. Loss of function mutations in FLG can impair this integrity resulting in increased trans epidermal water loss, increased pH, and greater permeability penetration to exogenous allergens/microbial factors, contributing to the atopic traits asthma, hay fever and microbial or allergen specific-IgE[8, 31]. In some populations ~ 50% of AD patients have FLG mutations; however, while numerous FLG mutations are associated with Caucasian AD, a different picture emerges which considering diverse ethnicities[32]. In addition, more than 50% of FLG carrying individuals do not develop atopic diseases[33]. This suggests that FLG mutations may increase susceptibility but not required nor sufficient for AD. Other skin-associated genes such as *SPINK5/LEKT1* have also been associated with AD[34]; these are beyond the scope of this report. Notably, IL-13, OVOL, and FLG are the main three genes associated with

susceptibility to AD, with several other mutations in the T_H2 cytokine locus and its respective receptors[8, 35]. Finally, as majority of AD heritability remains unexplained by GWAS studies (~80%)[36], additional work is required, to understand individual risk[32], and to explore environmental: epigenetic factors .

Epidermal Barrier Dysfunction

The maintenance of an intact epidermis is needed for a protective physical and chemical barrier to the outside world. In AD, decreased expression of the epidermal differentiation complex is associated with altered lipids, including protective ceramides, and reduced tight junctions[37]. Functional barrier deficits lower the threshold for triggering the itch-scratch cycle[8]. A plethora of skin-damaging noxious insults (microbes, trauma, allergens), may penetrate or damage the skin epithelia and be sensed by live keratinocytes, thus activating powerful innate immune response 'alarmins', such as IL-25, IL-33, and thymic stromal lymphopoietin (TSLP)[8]. Specifically, TSLP has been best described for its role in promoting type 2 responses in allergic diseases, however, recent literature recognizes its pleotropic role in other functions such as host defence against infection, cancer, fat metabolism, and chronic inflammatory diseases[38]. In human AD skin lesions, TSLP is a cytokine that is highly expressed, and is produced in response to skin injury or *S. aureus* burden[39-41]. While TSLP promotes naïve CD4 T cell differentiation through acting on dendritic cells[39], it can also directly bind to TSLP receptor (TSLPR) on TH2 cells to produce inflammatory cytokines[42]. TSLPR has been found to be elevated in AD patients, correlating with disease severity[42, 43]. Alarmin-mediated downstream signaling initiates a pro-inflammatory T_H2 response through cytokine production, that will contribute to inflammation, stimulate itch-sensory neurons to perpetuate barrier defects[44, 45]. Further, T_H2 cytokines have been shown to decrease expression of anti-microbial peptides (AMP) on skin, facilitating colonization of antibiotic-resistant bacteria

such as *Staphylococcus aureus (S. aureus)* on lesional AD skin and increasing the risk of skin infections[37, 46]. While these cellular, molecular, and microbial factors drive an ongoing cycle of itch-scratch in AD resulting in tissue injury, their role in the maintenance of chronic severe disease remains unclear.

Dysbiosis of the Skin Microbiome

The human skin is inhabited by various bacteria, fungi, and viruses known as the skin microbiota[47]. Common skin commensals include *Propionobacterium* species, which colonize sebaceous sites of the skin, whereas *Staphylococcus* and *Corynebacterium* species thrive in moist humid areas of the skin[47]. Skin microorganisms have an essential role in protection against pathogen invasion, to educate innate and adaptive immune responses, and to promote epidermal barrier repair[47, 48]. Whether a dysbiosis of the skin flora can directly lead to development of AD requires further elucidation.

Abnormalities in *S. aureus* populations have strongly been associated with AD. Studies demonstrate that up to 90% of AD skin is colonized with *S. aureus*, in contrast to healthy subjects having a prevalence of approximately 20%, mainly localized in moist areas (armpits, elbow/knee creases)[47, 49-51]. High burden *S. aureus* colonization has been shown to positively correlate with disease severity in AD patients[15, 52-56]. Within the same patients, colonization of *S. aureus* was 70% and 39% in lesional vs non-lesional AD skin[57]. In addition, microbial diversity in AD patients is reduced, meaning AD patients have fewer protective commensal bacteria such as *S. epidermidis* and *S. hominis,* which secreate antimicrobial peptides to maintain a balance of *S. aureus*[58]. This dysbiosis of the skin microbiome contributes to increasing disease severity of AD. One study investigated shifts in skin microbiome from AD children during disease flare and after treatment by performing 16S ribosomal RNA bacterial gene sequencing[59]. In AD children, they found that

S. aureus sequences were highly expressed during flares compared to baseline or post-treatment[59]. Following treatment, *Streptococcus, Cutibacterium* and *Corynebacterium* species were increased in AD patients, suggesting a reversion to normal bacterial flora in the skin correlating with improved symptoms[59]. *S. aureus* is armed with numerous virulence factors including exogenous proteases, pore-forming toxins, metabolic adaptations, biofilm producing capacity, and importantly for AD, superantigens[60]. Exogenous proteases can disrupt the epidermal barrier and also have the capacity to inactivate antimicrobial peptides, which are responsible to degrade host-evading *S. aureus* biofilms on skin[61]. Bacterial biofilms are extremely cohesive and surface adherent which are held together by a self-producing extracellular matrix, rendering them highly resistant to antibiotic treatment[62, 63]. The ability of *S. aureus* to penetrate into the dermis layer and induce the production of T_H2 cytokines[58, 64] suggests that this may be a mechanism of disease pathology and requires further investigations.

Superantigens are potent immunostimulatory molecules that activate T cells, leading to rapid inflammation, excessive production of inflammatory cytokines, and toxic shock[65]. Specifically, staphylococcal superantigens (Sags) are comprised of staphylococcus enterotoxins (SEs), staphylococcus enterotoxin- like (SEls) proteins, and toxic shock syndrome toxin-1(TSST1) [65]. Conventional T cell activation is mediated through interaction of a peptide antigen presented via major histocompatibility complex (MHC) class II expressed on antigen-presenting cells (APC), with specific T cell receptors (TCR)[66]. Antigen recognition through MHC-TCR will result in downstream signalling to activate naïve T cells, the first signal promoting differentiation into effector and memory T cells. As the TCR repertoire is extremely diverse, a very small proportion of naïve T cells respond to a specific antigen, resulting in a normal adaptive inflammatory response[65]. In contrast, superantigens bind the exterior of MHC and TCR, not in the peptide

groove, crosslink the receptors and initiate robust non-specific T cell activation (~ 30% of total T cell pool, vs 0.01%) [65, 67]. Superantigen activation depends on TCR β-chain variable domains (Vβ)-whereby each superantigen bind certain TCR Vβ regions[67]. Currently, there are 20 distinct SAgs known, however, superantigen enterotoxin B (SEB) has been well described to play a prominent role in AD, over other enterotoxins [15,68-75]. SEB can stimulate multiple T cells which bear TCRvβ3, 12, 14, 15, 17, or 20[76]. The presence of SEB-specific IgE antibodies has been positively correlated with ,disease severity in AD patients [75, 77], suggesting an active etiologic role for *S. aureus*. Lin et al. demonstrated that SEB- stimulated T cells produced T_H2 cytokines from AD patients, while T_H1 cytokines were detected in healthy subjects[68, 78]. In addition, SEB has been described to promote itch in AD patients through the upregulation of IL-31- an inflammatory cytokine, mainly produced by T_H2 cells, mast cells, monocytes and macrophages, that has a key role in pruritis in allergic skin disease[79]. High levels of IL-31 production was detected *in vitro* from human SEB-stimulated monocytes and monocyte-derived macrophages in peripheral blood[80], while increased IL-31 mRNA was detected from lesional AD skin following SEB exposure[81]. As such, *S. aureus* burden in AD has the capacity to worsen disease activity through activation of inflammatory responses, however, the debate persists on its bystander versus causal role in AD.

Immune Processes in AD

Development of Type 2 responses

Type 2 immune responses are critical for protective immunity against extracellular parasitic infections and mediate pathogenesis of allergic diseases (eg. Asthma, allergic rhinitis, atopic dermatitis)[82, 83]. Amongst the T helper subset, T_H2 cells are lymphocytes that mediate cellular type 2 adaptive responses, and promote allergic diseases through key effector cytokines including IL-4, IL-5, and IL-13[83, 84]. Differentiation of naïve T cells into specific T helper subsets (T_H1, T_H2,

T_H17) require key polarizing cytokine signals[83, 84]. An autocrine and paracrine manner of IL-4 signaling regulates T_H2 responses[83, 84]. IL-4 drives T_H2 cell differentiation through activation of signal transducer and activation of transcription 6 (STAT6) tyrosine phosphorylation[83, 84]. This phosphorylation upregulates gene expression of GATA3, the canonical transcription factor defining functional T_H2 cell programs[83, 84]. Finally, T_H2 cell cytokines promote B cell class switch to generate IgE antibodies, IL-5-induced eosinophilia, and innate cell activation[83], all of which are important features of AD[8].

T_H2-mediated inflammation in AD is modulated by various skin associated molecular factors[44]. In response to external insults to the epidermal barrier or allergens, epithelial cells secrete "alarmins" including TSLP, IL-25, IL-33[44]. Keratinocyte-derived alarmins can directly promote T_H2 responses by induction of T_H2 cytokine production, or polarization of dendritic cells[44]. TSLP is largely produced in lesional AD skin, in response to tissue injury. TSLP activates dendritic cells (DC), which prime naïve T cells to differentiate into T_H2 cells[85]. Specifically, TSLP signaling via TSLP receptor (TSLPR) induces the expression of OX40 ligand (OX40L) on dendritic cells (DC), which is required for interaction with OX40 on naïve T cells to promote T_H2 differentiation[86]. In addition, TSLP stimulates itch-sensory neurons which evokes itch in AD[87]. Production of IL-4, IL-5, and IL-13 is elevated from T_H2 cells as well as ILC2s following TSLP-induced signalling via TSLP receptor[88]. Studies in murine models of atopic dermatitis demonstrate TSLPR-/- mice are protected from eosinophilia[89], and have decreased expression of T_H2 cytokines, whereas skin allergen exposure in the presence of TSLP leads to allergic inflammation[90]. While TSLP is constitutively expressed in healthy skin, it has been found to be significantly increased in lesional skin and serum of AD patients (adults and children), but lacking in non-lesional skin, compared to healthy subjects[39, 91-93]. Thus, TSLP is an integral factor contributing to the development and

maintenance of type 2 responses, however, further investigation is needed to fully elucidate its role in AD.

IL-25 is a cytokine of the IL-17 family that favors T_H2 inflammation, which has been found to be overexpressed in lesional AD skin[82, 94]. In addition to DC activation, this cytokine directly stimulates type 2 innate lymphoid cells (ILC2s) leading to type 2 cytokine production[82]. Allergen-stimulated keratinocytes produce high levels of IL-25, which has been shown to decrease expression of FLG protein at the epidermal barrier[94]. Similarly, IL-33 is a member of the IL-1 cytokines produced by epithelial cells of the skin, fibroblasts, and endothelial cells, which has been implicated in promoting type 2 immune responses in allergic diseases[95, 96]. IL-33 signals through its ST2 receptor found on $T_H2$cells, ILC2 cells, eosinophils, basophils, mast cells, and macrophages to initiate the production of T_H2 cytokines[95, 96]. In AD lesional skin, IL-33 mRNA and protein has been found to be elevated compared to non-lesional and healthy skin[95, 97, 98]. One study demonstrated that IL-33 and TSLP reciprocally potentiates each other's protein and receptor expression (i.e ST2, TSLPR) in type 2 airway inflammation. This mechanism may also be activate in AD to worsen disease[99]. Together, epithelial-derived alarmins, in response to skin injury and inflammation in AD, promote T_H2 cell generation to perpetuate disease progression and severity.

Key Type 2 cells in AD

It is well described that type 2 inflammation mediates AD disease pathology, predominately by T helper $CD4^+$ T_H2 cells[14]. Activation of conventional T_H2 cells results in effector cytokine production including IL-4, IL-5 and IL-13[14]. Chemokine receptors including CCR4, CCR10, and CRTH2 are preferentially expressed on circulating T_H2 cells in allergic diseases, especially AD, and used to define subsets in human studies[100, 101]. CCL17 (TARC) and CCL22 (MDC), ligands of CCR4, and CCL27/CCL28, ligands of CCR10, are biomarkers of AD[101-103].

CRTH2 receptor, expressed primarily on mast cells, eosinophils, and T_H2 cells, binds to its target PDG2 to induce downstream signaling leading to the production of inflammatory cytokines[104]. More recently, Wambre et al. have described a new subset of T_H2 cells known as T_H2A cells associated with allergic disorders in humans, absent in nonatopic individuals[105], T_H2A cells, defined as $CD4^+CRTH2^+CD161^+CD45RO^+CD27^-CD45RB^{low}CD49d^+$ T cells, have phenotypic and functional differences compared to conventional T_H2 cells, and may constitute a therapeutic target to treat allergic disorders[105]. Recent studies demonstrate a decrease in frequency of T_H2A cells following food oral immunotherapy[106-108]. Of note, T_H2A cells were found to persist in AD skin following treatment, suggesting that this subset may be important to understand AD relapse[109]. Further, T cells expressing cutaneous lymphocyte antigen (CLA) are skin-homing T cells that migrate from the systemic circulation into the barrier site to mediate inflammatory functions[110]. CLA+T cells can also recirculate back into the blood through lymphatics, which allows researchers to investigate these skin-homing T cells to gain insight regarding the immune process that occur in the skin[110]. High frequencies of skin-homing T_H2-cytokine producing T cells are found in refractory AD patients, highlighting their potential role for maintaining disease[111, 112].

In addition, ILC2s have been recently identified as contributors to the initiation and maintenance of type 2 cytokine responses[88]. ILC2s strongly rely on epithelium derived alarmins IL-25, IL-33, and TSLP to sense tissue injury or pathogen invasion, given they do not express pattern-recognition receptors like DCs[88]. Once activated, ILC2s mainly produce IL-5, IL-13, but not IL-4, to amplify type 2 immune responses[88]. ILC2s perpetuate downstream inflammation after T_H2 pathology in AD, and may also have a protective role to repair lesions in AD[113]. ILC2s are a major source of IL-13 in murine models and are elevated in AD skin lesions[98], however, their role in human AD remains unclear. In normal human healthy skin, ILC2s comprise 25% of total ILC

populations. Aside from inflammatory functions, ILC2s promote tissue repair and immune homeostasis following inflammation, through secretion of amphiregulin- an epidermal growth factor for keratinocyte differentiation and proliferation[114]. As such, the dual role of ILC2s to promote type 2 inflammatory responses vs tissue repair in AD must be clarified.

While classical effector cell types have been the primary focus of research, increasing evidence suggests the presence of polyfunctional T cells with functional overlap with T_H2, T_H2A and ILC2 cells in allergic diseases [106, 115]. Novel IL-13-secreting cells, specifically CD8 T cells referred as Tc2, have been identified in AD patients[116]. Skin-homing $CD8^+IL\text{-}13^+$ T cells have been found in blood and skin to correlate with disease severity in some AD patients [111, 116, 117]. Furthermore, a subset of memory T cells of the skin are tissue-resident memory T (T_{RM}) cells. They can be divided into both CD4 and CD8 subsets, whereby $CD8^+$ T_{RM} co-expressing CD69 and CD103 are found in the epidermis where the disease manifests[118]. $CD4^+$ and $CD8^+$ T_{RM} may be increased in AD skin compared to healthy skin, yet limited reports have characterized $CD8^+$ T_{RM} cells in disease [117, 119, 120]. $CD8^+$ T_{RM} cells persist in the epidermal skin compartment, and therefore, their role in maintenance of AD inflammation and potential contribution to initiation of a flare must further be investigated[121].

Heterogeneity of T helper cell responses in AD

Recent advancements challenge T_H2-centric paradigm of AD to describe the heterogeneity of cellular players across the lifespan and stage of disease[122]. Higher rates of severity and treatment resistance are reported in adult cohorts; to address questions of selection bias, age-related immune and host-pathogen environmental factors must be clarified in controlled longitudinal human studies. Infants with eczema have greater frequencies of T_H2 polarized cells trafficking to skin, which remain consistently elevated into adulthood compared to age-matched healthy subjects[123-

[129]. In lesional skin of children (<6 months; pediatric AD), a predominant T_H2 upregulation was observed with some T_H17/T_H22 skew, however, children lacked T_H1 expression detected in adult AD patients[130]. In healthy development, T_H1 cell frequencies and T_H1/T_H2 ratios gradually increase with age; here, the healthy development of skin-homing T_H1 populations is lacking in AD children, while skin-homing T_H2 populations predominate[112, 131]. Severe disease in adult AD patients is characterized by an expansion T_H2/Tc2 and Th22/Tc22 cells, with defects in the T_H1/Tc1 T cell populations in blood[111]. However, this phenomenon appears to change with increasing age; elderly (>60 years) AD patients demonstrate downregulated T_H2 and T_H22 expression compared to younger patients, with an increase in T_H1 and T_H17 responses, in skin (mRNA expression, immunohistochemistry) and blood (serum) [132]. Therefore, additional studies must be performed in blood and skin of AD patients to clarify and account for changes in T cell populations associated with progressing age, allowing researchers to develop tailored treatments.

Moreover, differences in cellular players are also detected based in disease state (non-lesional, acute lesional, chronic lesional). Non-lesional skin may have some subtle immunohistological changes and T cell infiltration, which largely remains asymptomatic[133, 134]. Acute lesional skin is primarily dominated by a T_H2 signature, along with an upregulation in T_H22 cells[135]. Progression into the chronic lesional state continues to have a steady level of T_H2 expression, with an increase in T_H1 and T_H17 expression[136, 137]. Due to challenges in obtaining human skin biopsy samples, there is a gap in knowledge regarding T cell populations at various disease states (non-lesional, acute, chronic) in AD, which requires further elucidation.

Although studies are limited in patients with skin of color, it has become evident that clinical phenotypic differences exist across ethnic backgrounds[21]. European American AD patients present with strong upregulation in both T_H2 and T_H22 axes, with minimal activity in the T_H1 and

T_H17 axes compared to both Asian and African American patients[44]. In contrast, Asian patients appear to have the strongest T_H17 skew amongst all cohorts in addition to T_H2 activity[13, 44]. Finally African American patients demonstrate low T_H1/T_H17 activity with upregulated T_H2/T_H22 pathways, which may be still lower compared to European American patients[21, 44]. These findings have important implications for the development of targeted therapies in AD, as majority of AD research findings is based on European AD populations. Inclusive and diverse longitudinal studies are needed to clarify eczema immune deviations across various ethnic and age groups.

Regulatory T cell responses in AD

Regulatory T (T_{REG}) cells are potent suppressors of exaggerated immune responses and mediate tolerogenic responses to self-antigens[138]. T_{REG} cells, commonly defined as $CD25^{HIGH}CD127^{-}CD4^{+}$, comprise up to 20% of all T cells in human skin, whereas only ~5% are found in peripheral blood[139]. The lineage defining transcription factor of T_{REG} cells is fork-head binding protein 3 (Foxp3), which is responsible for its development and suppressive function[140]. In normal steady state skin, Langerhans cells promote the survival of T_{REG} cells, which produce anti-inflammatory cytokines such as IL-10, IL-35, and TFG-B to maintain tolerance[141]. However, the role of T_{REG} cells in AD remains unclear[142]. A strong evidence that T_{REG} cells are important for regulation of inflammation in AD arises from the symptoms observed in immune dysregulation, polyendocrinopathy, enteropathy, X-linked (IPEX) patients[143]. Mutations in Foxp3 cause Treg cell dysfunction, leading to IPEX syndrome, where patients develop autoimmune enteropathy, anemia, endocrinopathy, and specifically, eczematous dermatitis[143]. Human studies and mice models of AD demonstrates an important role of T_{REG} cells in AD, however, there is a lack of consensus on whether this response is impaired[144]. Numerous studies have demonstrated increased numbers of $CD4^{+}CD25^{+}FOXP3^{+}$ T_{REG} cells in AD patients from peripheral blood and skin, which has been

positively correlated with disease severity[145-147]. However, the suppressive capacity of T_{REG} cells in AD remains to be fully elucidated. Reefer et al. showed that *in vitro* stimulation of circulating $CD25^{high}Foxp3^{+}$ T_{REG} from AD patients produced T_H2-cytokines, implicating a dysfunctional regulatory response[148]. Immunohistochemistry revealed an absence of $CD25^{+}Foxp3^{+}$ T cells in lesional AD skin although IL-10 and TGF-b was detected in the skin[149]. $CD25^{+}Foxp3$ T_{REG} failed to inhibit effector T cell mediated keratinocyte cell death in an artificial co-culture model[149]. Impaired suppressive function of Treg cells may be mediated by repeated exposure to allergic antigens, such as *S. aureus*-derived enterotoxins in AD skin. Upon stimulation with SEB, $CD25^{+}FOXP3^{+}$ T_{REG} cells fail to suppress proliferation of effector T cells in AD patients, however, this suppressive capacity is maintained with anti-CD3 stimuation[146, 150]. Thus, the ability of T_{REG} cells to retain their suppressive capacity in response to AD-associated inflammation must be further investigated.

Role of IL-13 in AD

IL-13 is a critical mediator of allergic inflammation and required to sustain disease activity of AD[14, 151, 152]. T_H2 cells are the primary source of IL-13 in AD, however, eosinophils, basophils, mast cells, macrophages, and B cells are also producers of IL-13[151]. The homeostatic milieu of the skin and barrier surface are heavily impacted by downstream effects of IL-13 signalling in AD. IL-13 downregulates the expression of various structural barrier proteins such as filaggrin, keratin, and involucrin[14]. IL-13 mediated lipid abnormalities are observed in AD lesional skin, compared to healthy subjects[14, 153]. These changes in the epidermal barrier facilitates transmembrane water loss, worsening the skin[14]. Direct IL-13 signalling decreases keratinocyte-derived antimicrobial peptides, which allows increased colonization of *S. aureus* on the skin[14]. As previously discussed, AD patients have a high *S. aureus* burden that induces robust inflammatory response upon

production of superantigen enterotoxins. IL-13 activates keratinocytes to produce specific chemoattractant (CCL26, CCL22) to recruit inflammatory cells, further driving inflammation at the barrier site[154]. Fibrotic tissue remodeling is observed in AD through decreased expression of MMP-9 in keratinocytes driven by IL-13 signalling, and contributes to lichenification of chronic lesions[14, 155]. Finally, itch-sensory neurons can be directly activated by IL-13 driving the itch-scratch cycle[14, 156]. While the central role of IL-13 in AD has been established through numerous studies, it is unclear whether IL-13 producing T cell populations are the main drivers of disease relapse in patients following treatment.

Disease Measurement of AD

Objective Tools

Validated Investigator Global Assessment (vIGA)

An Investigator, or Physician, Global Assessment (IGA or PGA) is an objective measure to measure overall disease severity[157]. This assessment takes 4 clinical features of AD into account including erythema, edema, lichenification, and oozing/crusting.

Score range: 0= clear; 1= almost clear; 2= mild; 3=moderate; 4= severe[157].

Eczema Area and Severity Index (EASI)

An EASI score is a validated tool used to precisely measure the extent and severity of AD. This tool incorporates 4 objective AD features (erythema, edema, excoriations, lichenification), as well as the body surface area affected[157]. A change in EASI score from baseline is used to monitor therapeutic response such as 50% (EASI50) or 75% (EASI75) improved[157].

Score range: 0 = clear; 0.1–1.0 = almost clear; 1.1–7.0 = mild; 7.1–21.0 = moderate; 21.1–50.0 = severe; 50.1–72.0 = very severe.

SCORAD

SCORAD is a tool used to assess the severity, extent, and intensity of AD. This test incorporates both objective features (erythema, edema, excoriations, lichenification, oozing/crust, xerosis), as well as subjective features (daily itch and sleeplessness) [157].

Score range: mild= <25; moderate= ≥25<50; severe= ≥50

Body Surface Area (BSA)

BSA assesses the body surface area involved in AD[157]. However, modifications have been made to now use vIGAxBSA, which was found to correlate better with EASI[157].

SUBJECTIVE

Patient-Oriented Eczema Measure (POEM)

Patient-Oriented Eczema Measure (POEM) is a validated tool measuring the frequency of 7 subjective symptoms (1)itch frequency 2) bleeding 3) oozing/weeping/scabs 4) dryness/scaling 5) flaking 6) cracking 7) sleep loss) of AD over the past week [157]. The frequency of each is scored on the following scale: 0 = no days, 1 = 1 to 2 days, 2 =3 to 4 days, 3= 5 to 6 days, and 4 = every day, with a maximum score of 28[157].

Dermatology Life Quality Index (DLQI).

The DLQI is a 10 question tool including symptoms and feelings, daily activities, leisure activities, work/school, personal relationships, and treatments received, to assess the health-related quality of life of patients[157].

Current Treatments for AD

Topical treatment, Phototherapy

Management of atopic dermatitis begins with preventative therapies including skin moisturization and the use of emollients to replenish hydration of the epidermis, reducing dry skin and lessening the urge to scratch[5, 158]. Non-specific irritants such as allergens (pollen, dust mites, animal dander, food allergen), specific soaps and harsh clothing's are also avoided by patients[5, 158]. Disease exacerbation requires pharmacological treatments to control and reduce the degree of flare[5, 158].

Topical corticosteroids (TCS) are anti-inflammatory drugs used as the first-measure of treatment for moderate-severe AD[5, 158]. They are grouped into seven classes of potency (Class 1 highest, Class 7 lowest) based on the vasoconstrictor assay[5, 158]. Usage of TCS ranges from 1-2 weeks to achieve control of disease, depending on the potency of the drug, area of body to treat, and age of the patient[5, 158]. Side effects of local application include epidermal thinning, atrophy, striae, purpura, and therefore, as not recommended for long-term use[159]. Topical calcineurin inhibitors (TCI), including pimecrolimus cream and tacrolimus ointment, are used as a second-line of treatment for disease flare, and long-term preventative measures[5, 158]. Calcineurin inhibitors provide broad immunosuppression without the epidermal side-effects seen in topical corticosteroids[5, 158]. Both TCIs are approved for use in children and adults[158]. Phototherapy is another treatment for AD which employs narrowband UVB and UVA1 to mediate immunosuppression[160]. Specifically, it inhibits keratinocyte proliferation, alters cytokine production, reduces the antigen-presentation capacity of Langerhans cells, and changes the microbial diversity of the skin[27, 160]. While clinical efficacy is observed in some patients after therapy, the willingness to use phototherapy is low due to its inconvenient treatment course[160].

While clinical efficacy is observed in some patients after therapy, the willingness to use phototherapy is low due to its inconvenient treatment course[160]. Topical treatments and

phototherapies are effective pillars of AD treatment, however, adult AD patients with persistent disease fail to respond to traditional therapies, and require more targeted systemic treatments.

Systemic Treatment: Cyclosporine

Cyclosporine, an oral calcineurin inhibitor, is approved in Europe for AD and used as a first line of systemic medication for adults with moderate-to-severe AD[161]. However, it is used off-label in North America for the treatment of AD[162]. Binding of cyclosporine to cyclophilin forms a complex that inhibits calcineurin, blocking downstream signalling responsible for synthesis of interlukin-2 (IL-2)[163]. IL-2 is essential for T cell differentiation and activation[164], thus, cyclosporine provides broad immunosuppression by preventing T cell function[163]. Frequently, it is used as a rescue drug for rapid disease control during an flare. A meta-analysis of 15 controlled and uncontrolled studies assessing cyclosporine treatment outcomes demonstrated 55% relative effectiveness after 6-8 weeks of treatment[165]; unfortunately, cessation of treatment leads to disease relapse in ~50% of patients within 2-3 months[166]. Exposure to cyclosporine has important safety considerations, and long term use is associated with risks including nephrotoxicity, hypertension as well as infectious and neoplastic complications[27]. Few studies have evaluated the immune modulatory effects of cyclosporine treatment in AD patients. Khattri et al. demonstrated significant decreases in gene expression pathways of T_H2, T_H17, and T_H22 molecules in lesional and non-lesional skin 2 and 12 weeks after cyclosporine treatment in AD patients[167].

Biologic: Dupilumab

Dupilumab is the first targeted biologic therapy approved for the long-term treatment of refractory moderate-severe AD[168]. It is a fully human monoclonal antibody that targets IL-4Ra on type I and II receptors, which inhibits IL-4 and IL-13 signalling[168]. While dupilumab is well tolerated, common adverse effects include injection site allergic reaction, conjunctivitis, and headache[169].

Two randomized, placebo-controlled phase 3 enrolled adults with moderate-to-severe AD and assigned dupilumab treatment for 16 weeks compared to placebo[17]. From 671 and 708 patients enrolled in each trial, primary outcome of 0 to 1 score on the Investigator's Global Assessment was observed in 38% and 36% of patients who received dupilumab every other week, respectively[17]. In addition, EASI-75 was significantly achieved in dupilumab treated patients compared to placebo at the end of 16 weeks. Dupilumab has been found to be clinically safe, with few adverse effects in patients[17]. Hamilton et al. first performed transcriptomic analyses from lesional and non-lesional skin of AD patients on dupilumab treatment (4 weeks), compared to placebo[170]. They detected decreased expression in genes related to hyperplasia, T cells, dendritic cells, and T_H2-associated chemokines, which were all correlated to improvements in clinical scores[170]. Although dupilumab is approved for severe disease management, the effects of long-term dupilumab treatment on immune populations remains to be clarified. Bakker et al. first investigated the early and long-term effects of dupilumab treatment (4 weeks vs 52 weeks) on T cell cytokine production[171]. At 4 weeks, T_H2 and T_H22 cytokine producing skin-homing T cell frequencies were decreased, however, no skew towards a specific T helper cell was observed at 52 weeks [171]. Similarly, a gradual decrease in memory T_H2 cells (CXCR3-CCR6-), and T_H1 cells was detected after dupilumab treatment in AD patients with moderate disease[171]. Thorough characterization of T helper subsets, and cytokine production from AD patients after long-term dupilumab treatment are required to gain insight on target cell populations, and potential residual disease activity.

Since the approval of dupilumab, other biologics (tralokinumab and lebrikizumab) targeting the IL-13 pathway at different stages have been in clinical trials[168]. Large long-term

studies will be required for each medication individually and in comparison to dupilumab, to assess clinical efficacy and modulation in the immune landscape.

Persistent T cell populations following treatment in AD

Adult AD patients with uncontrolled moderate-to-severe disease are frequently unresponsive to traditional treatments, and require targeted therapies to induce disease remission[12]. Discontinuation of treatment results in disease relapse, for both dupilumab and cyclosporine, which is quicker for the latter[166, 172]. Although T_H2-mediated IL-13 responses are critical to maintain disease activity, large gaps exist in understanding treatment responses and residual disease activity at the molecular and cellular level in adult AD patients. Bangert et al. used a multi-omics approach to study the persistence of specific immune cells in healed AD skin following 1 year of dupilumab treatment[109]. Single-cell RNAseq (scRNA) performed on skin blister biopsies from AD patients showed increased expression $CD4^+IL13^+$ (T_H2) and $CD8A^+IL13^+$ (Tc2) cells after 1 year of dupilumab treatment, which were higher compared to healthy subjects[109]. Overall IL-13 mRNA, as well as IL-13 protein detected from skin blister fluid, remained elevated despite dupilumab treatment[109]. Given that dupilumab blocks both IL-13 and IL-4 receptor binding, an accumulation of protein is expected, however, IL-4 protein was not found in skin blister fluid following dupilumab treatment. CD8 T cells and CD4 "T_H2A" remained elevated in dupilumab treated skin, however, it was absent in healthy skin[109]. In a cohort of moderate AD patients, elevated frequencies of skin-homing $CCR4^+CLA^+IL\text{-}13^+$ were detected, and shown to persist 52 weeks after treatment[171]. Information regarding the level of IL-13 production after dupilumab treatment is lacking. Similarly, after cyclosporine treatment, non-responder patients lacked improvement in genes expressing T_H2/IL-13 cytokines, whereas, responder patients had reductions in this pathway[167, 173]. Some studies demonstrate that cyclosporine may differentially impact T_H2

cells, specifically having reduced ability to inhibit IL-13 production[174, 175]. Together, these findings emphasize the residual disease activity observed in AD patients after treatment, in clinically resolved disease.

METHODS

Study Design

This cross-sectional study enrolled patients from the Canadian Atopic Dermatitis Cohort for Translational Immunology and Imaging (CACTI) study (REB: 2022-8004). Adult AD patients with moderate-severe disease (no systemic treatment, median EASI: 23.6) meeting Hanifin & Rajka criteria per the validated Investigator Global Assessment for AD scale, and an Eczema Area and Severity Index (EASI) score at or above the moderate disease range (≥7.1) as per published severity strata were enrolled in the study. Patients treated with dupilumab (median treatment length: 2.28 years; median EASI: 3.9) according to routine clinical care were also recruited. Age-matched healthy subjects were enrolled. All patients and healthy subjects provided written informed consent to participate in the study, and filled out a patient-reported questionnaire. Blood samples were collected from patients and healthy subjects. Physician reported clinical parameters (EASI, vIGA, BSA) and patient reported subjective measures (POEM, DLQI, ITCH PP-NRS,) were collected from participants. Clinical laboratory test results was used to obtained IgE levels from AD patients (normal, <200 kU/L). For *in vitro* T_H2 expansion, we performed a case-control study with an additional cohort of AD patients with moderate-severe disease meeting Hanifin & Rajka criteria were recruited along with healthy controls.

Isolation, freezing, and thawing of peripheral blood mononuclear cells

Peripheral blood mononuclear cells (PBMC) were isolated from whole blood using Ficoll-Paque Plus (Cytiva Life Sciences) gradient centrifugation. Undiluted whole blood was laid on top of Ficoll prior to centrifugation. After centrifugation, plasma (top layer) was first collected and

aliquoted. PBMCs were collected from the white "buffy" coat at the plasma-ficoll interface, and washed with PBS (Wisent). Red blood cell lysis was performed with eBioscience 1X RBC Lysis Buffer (Invitrogen). Cells were washed with PBS and resuspended in appropriate solutions for subsequent processes. To freeze PBMC following isolation, cells were resuspended in heat-inactivated fetal bovine serum (FBS) (Wisent). A cell count was performed with Trypan blue using a hemocytometer. A maximum of 10 million cells/ml were stored per vial. PBMCs were frozen in 10% dimethyl sulfoxide (DMSO) (Sigma-Aldrich) and 90% FBS (Wisent) in cryogenic vials. Vials were immediately placed in Styrofoam containers and placed in a -80 °C freezer. PBMCs were thawed in a 37°C water bath and washed with pre-warmed complete RPMI 1640 (cRPMI) with L-glutamine (Gibco) supplemented with 10% FBS (Wisent), 1% sodium pyruvate (Wisent), 1% hepes (Wisent), 1% penicillin/streptomycin (Wisent), and 1% non-essential amino acids (Wisent). Cells were resuspended in cRPMI. DNase1 (Sigma) (final concentration: 0.25mg/ml) was added to the cell suspension and incubated at room temperature for 10 minutes. Cells were washed with cRPMI and used for *ex vivo* multiparametric flow cytometry staining.

In vitro T_H2 expansion

Freshly isolated PBMCs were resuspended in complete RPMI 1640 (Gibco) supplemented with 10% autologous human plasma, 1 mM sodium pyruvate (Hyclone), 25 mM hepes, 100 ug/ml penicillin/streptomycin (HyClone), 2mM L-glutamine, and 1X non-essential amino acids. In a 24-well plate coated with collagen I from rat tail (Corning), 2.5 x 10^6 cells/well in 450ul were seeded in triplicates and rested overnight at 37°C in a 5% CO_2 incubator. To replicate the inflammatory milieu of AD skin, PBMCs were stimulated with 2ng/mL of staphylococcal enterotoxin B (SEB) (Toxin Technologies) and 120ng/mL of recombinant human thymic stromal lymphopoietin (TSLP) (Biolegend) in the following conditions: Untreated, TSLP, SEB, TSLP+SEB (Day 0). Every 2 days, 500ul of complete RPMI was added to each well. On day 7, the cells were harvested

and a cell count for each condition (one well) was performed. In 96-well V bottom plate, 2.5 x 10^5 cells/well were plated for subsequent flow cytometry experiments. The volume required to obtain 2.5 x 10^5 cells was determined from the TSLP+SEB condition, and the same volume was taken from each condition to maintain the culture conditions.

Extracellular and Intracellular staining

For *ex vivo* immunophenotyping, PBMC were thawed and rested for 2h at 37°C. For intracellular cytokine detection, cells were stimulated with 20ng/ml of phorbol 12-myristate 13 acetate (PMA) (Sigma-Aldrich) and 750ng/ml of ionomycin (Sigma-Aldrich) in the presence of Monensin based Golgi Stop (1:1000 dilution) for 3 hours at 37°C in 5% CO_2 incubator prior to staining. Single cell suspensions were stained with Fixable Viability Dye eFluor 780 (eBioscience, 1:1000) and Human TruStain FcX blocking solution (Biolegend, 1:100) in PBS (Wisent) for 15 mins at 4°C. Extracellular surface markers were stained for 30 mins at 4°C with the following fluorochrome-conjugated monoclonal antibodies raised against human proteins prepared in PBS (according to experiment): anti-CD4 FITC (RPA-T4, BDBiosciences), anti-CD4 APC (RPA-T4, Biolegend), anti-CD8α PerCP-Cy 5.5 (RPA-T8, BDBiosciences), anti-CD8α V500 (RPA-T8, BDBiosciences), anti-CD3 BV785 (OKT3, Biolegend), anti-CD45RA AF700 (HI100, Biolegend), anti-CLA FITC (HECA-452, Biolegend), anti-CRTH2 PE-CF594 (BM16, BDBiosciences), anti-CXCR3 BV421 (1C6/CXCR3, BDBiosciences), anti-CCR6 PE-Cy7 (GO34E3, Biolegend), anti-CD25 APC (2A3, BDBiosciences), anti-CD127 PE-eFluor-610 (eBioRDR5, eBioscience), anti-HLA-DR V500 (G46-46, BDBiosciences). Subsequently, cells were fixed and permeabilized using the Foxp3 Transcription Factor Fixation/Permeabilization buffer set (eBioscience) (*ex vivo*) or True-Nuclear Transcription Factor Fixation/Permeabilization Buffer set (Biolegend) (*in vitro* culture) according to manufacturer's instructions. Intracellular staining was performed with fluorochrome-conjugated monoclonal antibodies raised against intracellular human proteins

prepared in permeabilization buffer: anti-GATA3 BUV395 (L50-823, BDBiosciences), anti-FOXP3 PE (236A/E7, Invitrogen), anti-Helios Pacific Blue (22F6, Biolegend), anti-IL-13 PE (JES10-5A2, BDBiosciences), anti-IL4 APC (8D4-8, BDBiosciences), anti-IFN-γ PE-Cy7 (4S.B3, BDBiosciences), anti-IL17a V450 (N49-653, BDBiosciences), anti-IL-2 PerCP-Cy 5.5 (MQ1-17H12, BDBiosciences). Cells stimulated with PMA and ionomycin were stained intracellularly for CD4 and CD8α. Samples were acquired on a BD LSRFortessa X-20 flow cytometer (BD Bioscience) and analyzed using FlowJo version 10 software.

Phosphoflow Staining

For intranuclear phospho-protein staining, cells were re-stimulated with human anti-CD3/CD28/CD2 (StemCell, 1:100) for 24 hours at 37°C in 5% CO_2 incubator prior to staining. Single cell suspensions were stained with Fixable Viability dye Zombie UV (Biolegend, 1:1000) in PBS (Wisent) for 15 mins at 4°C. Cells were fixed and permeabilizated using the True-Nuclear Transcription Factor Fixation/Permeabalization set (Biolegend) for 30 mins at 37°C in 5% CO_2 incubator. Nuclear permeabilization was performed with BD Phosflow™ Perm Buffer III (BDBiosciences) on ice for 30 mins. Cells were then washed twice with PBS. Extracellular and intranuclear markers were stained at room temperature for 30 mins with the following monoclonal antibodies raised against human proteins prepared in permeabilization buffer, Human TruStain FcX blocking solution (Biolegend), and Brilliant Stain buffer (BD Biosciences): anti-CD4 APC (RPA-T4, Biolegend), anti-CD8α BV605 (SK1, Biolegend), anti-CD3 V500 (UCHT1, BDBiosciences), anti-CD45RO APC/Fire 750 (UCHT1, Biolegend), anti-GATA3 PE (L50-823, BDBiosciences), anti-pSTAT6 Alexa Fluor 488 (A15137E, Biolegend), anti-OX40 BV711 (Ber-ACT35, Biolegend), anti-ICOS AF700 (C398.4A, Biolegend), anti-HLA-DR BV395 (G46-6, BDBiosciences). Cells were washed and resuspended in PBS for acquisition. Samples were

acquired on a BD LSRFortessa X-20 flow cytometer (BD Bioscience) and analyzed using FlowJo version 10 software.

Statistical Analysis

Multiple comparisons between three groups were tested using a unpaired, non-parametric Kruskal-Wallis test followed by a Dunn's multiple comparisons test (*ex vivo*). Multiple comparisons in the *in vitro* culture (to control: untreated) were tested using a paired, non-parametric Friedman Test followed by a Dunn's multiple comparisons test, between AD and healthy subjects separately for each condition. Data are displayed as mean $\pm$ standard deviation. P value of ≤ 0.05 was considered significant. P values were indicated on graphs as $* \ p \leq 0.05$, $** \ p \leq 0.01$, $*** \ p \leq 0.001$, $**** \ p \leq 0.0001$. All statistical analyses were performed using GraphPad Prism, version 7 (GraphPad Software).

RESULTS

Aim 1-2:

In order to understand the role of heterogeneous T cell populations contributing to chronic AD, we performed a cross-sectional study with adult AD patients meeting the Hanifin and Rajka diagnostic criteria (n=21, no systemic treatment), and age-matched healthy subjects (n=10), in addition to a cohort of AD patients in near clinical remission (dupilumab-treated, n=13). Untreated adult AD patients had severe disease as measured by Eczema Area Severity Index (EASI) score (median EASI: 23.1; range: 11.6-46.8). In the Dupilumab treatment cohort, disease was controlled and below the baseline target treatment goal of EASI 7[176]. AD patients treated with dupilumab had mild disease (median EASI: 3.1; range: 0.6-8.3) (Table 1). Prior to dupilumab treatment, this cohort of AD patients also had severe disease (median EASI: 35.65; range: 16-51; IgE) comparable to the untreated AD cohort (no significant differences), minimizing the impact of cohort specific differences on the data (Table 2). The median treatment length: 1.93 years and range of dupilumab treatment length was 0.51-2.29 years. Objective clinician-reported severity assessment using the vIGA, %BSA, as well as the subjective patient-reported (POEM), and itch PP-NRS demonstrate that AD patients had a significantly higher disease severity compared to dupilumab treated patients (Table 1.) Comorbidities within the cohorts included asthma (AD: 31.2%; dupilumab AD: 83.3%), and allergic rhinitis (AD: 56.2%; dupilumab AD: 75%).

Table 1. Laboratory and clinical information for cross sectional study: ex vivo T cell immunophenotyping

	Healthy (n=10)	AD (n=16)	AD + Dupilumab (n=12)	*ρ*-value
Age (yrs)	27 (21-60)	32.7 (21.9-60.7)	37(25-70.9)	ns
Sex (F/M)	5/4 (1 N/A)	11/5	3/9	
Ethnicity				
European	4	9	6	
Asian	2	7	3	
Middle Eastern	3	N/A	2	
Latin American	N/A	N/A	1	
Treatment length (yrs)	N/A	N/A	1.93 (0.51-2.29)	
*IgE (kU/L)	N/A	2203.71 (99-62235)	1442.62 (53-8459)	ns
**LDH (U/L)	N/A	221.5 (151-482)	177 (125-211)	ns
EASI Score	N/A	23.1 (11.6-46.8)	3.1 (0.6-8.3)	<0.0001
vIGA	N/A	3 (2-4)	1 (1-3)	<0.0001
BSA%	N/A	42 (10-90)	2 (1-20)	<0.0001
POEM	N/A	23 (9-28)	4 (3-17)	<0.0001
DLQI	N/A	12 (7-22)	1 (0-19)	<0.001
ITCH PP-NRS	N/A	7 (3-10)	2 (0-5)	<0.0001
Asthma	N/A	5 (31.2%)	10 (83.3%)	
Allergic Rhinitis	N/A	9 (56.2%)	9 (75%)	
Childhood AD onset	N/A	13 (81.2%)	11 (91.6%)	
Family Hx AD	N/A	8 (n=15; 53.5%)	6 (50%)	
Family Hx Asthma	N/A	3 (n=15; 20%)	6 (50%)	
Family Hx Allergic Rhinitis	N/A	12 (n=15; 80%)	9 (75)	
Dust Allergies	N/A	11 (n=15; 73.3%)	10 (83.3%)	
Animal Allergies	N/A	8 (n=15; 53.3%)	9 (75%)	

Data are presented as median values with range unless otherwise specified.
*Reference range: 0 to 200 kU/L for adults
**Reference range: 140 to 280 U/L for adults

Objective measures:
EASI: Eczema Area and Severity Index
VigA: Validated Investigator Global Assessment scale for AD
BSA%: Body Surface Area affected

Subjective measures:
POEM: Patient Oriented Eczema Measure
DLQI: Dermatology Life Quality Index
ITCH PP-NRS: Peak Pruritus Numerical Rating Scale

Table 2. Laboratory and clinical information for AD patients' treated with dupilumab.

	Pre-Dupilumab (n=12)	**Post-Dupilumab (n=12)**	***ρ*-value**
Treatment length (yrs)	N/A	1.93 (0.51-2.29)	
*IgE (kU/L)	10272.28 (1682-33650)	1442.62 (53-8459)	<0.0001
**LDH (U/L)	295.5 (195-471)	177 (125-211)	ns
EASI Score	**35.65 (16-51)**	**3.1 (0.6-8.3)**	**<0.0001**
vIGA	3.5 (3-4)	1 (1-3)	<0.0001
BSA%	70 (20-90)	2 (1-20)	<0.001

Data are presented as median values with range unless otherwise specified.

*Reference range: 0 to 200 kU/L for adults
**Reference range: 140 to 280 U/L for adults

Objective measures:
EASI: Eczema Area and Severity Index
VigA: Validated Investigator Global Assessment scale for AD
BSA%: Body Surface Area affected

Circulating activated T cells and Th2 cells persist in AD patients despite achieving clinical remission on Dupilumab

Dupilumab inhibits the IL-4Ra-mediated signaling involved in the differentiation, function, and survival of T_H2 T cells[84]. To investigate whether type 2 T cells persist in AD patients following long-term treatment with dupilumab, we first focused on identifying and characterizing T cell populations from peripheral blood *(ex vivo*) of AD patients, AD patients treated with dupixent and age-matched healthy subjects.

Cell surface markers were used to identify subsets of T_H2 ($CRTH2^+CD4^+$), T_H1 ($CXCR3^+CD4^+$), T_H17 ($CCR6^+CD4^+$), activated ($HLA\text{-}DR^+$ $CD4^+$) T cells, and Treg cells ($CD127^+Foxp3^+Helios^+$) by flow cytometry. Gating strategy for subsequent analyses is demonstrated in Supplemental Figure 1. Prior studies have demonstrated that memory T cells are elevated in peripheral blood and skin of pediatric AD patients compared to healthy subjects[167, 177], although there are conflicting reports on which specific memory T helper subsets (T_H1, T_H2, and

T_H17) contribute to disease activity in adult AD patients. Memory T cells may reflect cutaneous immune responses, given their migratory properties to inflamed non-lymphoid tissues. We sought to characterize subsets of antigen-experienced memory T cells in the systemic compartment of our cohorts to identify their potential contribution to immune deviation in the skin.

We first demonstrated that activated T cells (HLA-DR$^+$), T_H1 (CXCR3$^+$), T_H2 (CRTH2$^+$), and T_H17 (CCR6$^+$) cells from total CD4 T cells can be detected from peripheral blood of adult AD patients, AD patients treated with dupilumab, and healthy subjects. **(Suppl. Figure 2).** Elevated proportions of activated HLA-DR$^+$ T cells were observed in AD patients compared to healthy subjects, however, no significant differences were observed in T_H1 (CXCR3$^+$), T_H2 (CRTH2$^+$), and T_H17 (CCR6$^+$) cells **(Suppl. Figure 2)**. Within total CD8 T cells, we detected HLA-DR$^+$, CRTH2$^+$, CXCR3$^+$, and CCR6$^+$ T cells, although no differences were observed between the three cohorts (Suppl. Figure 3). Here, we established that our cohort of AD patients are representative of T lymphocytes populations observed in AD patients in the current body of literature.

Further, we investigated memory T cells to clarify their role in maintaining chronic AD disease and to identify how dupilumab treatment impacts this population. Memory T cells are defined as CD45RA$^-$ CD4 T cells (Figure 1a). Similar proportions of naïve and memory CD4 and CD8 T cells were detected from all three groups (Suppl. Figure 4). AD patients were found to have a significantly higher frequency of activated memory CD4 T cells (HLA-DR$^+$), and increased HLA-DR expression-level (MFI) in AD patients compared to healthy subjects (1b). Furthermore, we found elevated proportions of activated memory CD4 T cells persisted in dupixent-treated patients despite disease control, as compared to healthy subjects (Figure 1b). The subset of memory CRTH2$^+$ T_H2, but not CXCR3$^+$ T_H1 nor CCR6$^+$ T_H17 cells, was found to be significantly elevated in untreated AD patients, compared to healthy subjects (Figure 1c). Notably, in dupixent-

treated patients, this subset of highly pathogenic Th2 cells is reduced and not significantly elevated above healthy subjects. In contrast, CRTH2 expression (gMFI) per cell remains significantly increased relative to healthy subjects and comparable between untreated AD and dupilumab-treated patients (Figure 1C), suggesting that cell trafficking to the skin may be facilitated by higher levels of this chemokine receptor. No significant differences were observed in the proportion of $CXCR3^+$ T_H1 and $CCR6^+$ $CD4^+$ T_H17-like cells across the cohorts, however, dupixent-treated patients expressed lower CCR6 expression levels compared to both AD patients and healthy subjects (Figure 1 D and E). Amongst memory CD8 T cells, a unique population of $CRTH2^+CD8$ T cells was identified in AD patients, which was significantly elevated compared to healthy subjects (Suppl. Figure 5). In dupilumab-treated patients, the frequency of $CRTH2^+CD8^+$ T cells appears to decrease. Similar expression of HLA-DR, CXCR3, and CCR6 was observed on CD8 T cells between all three cohorts.

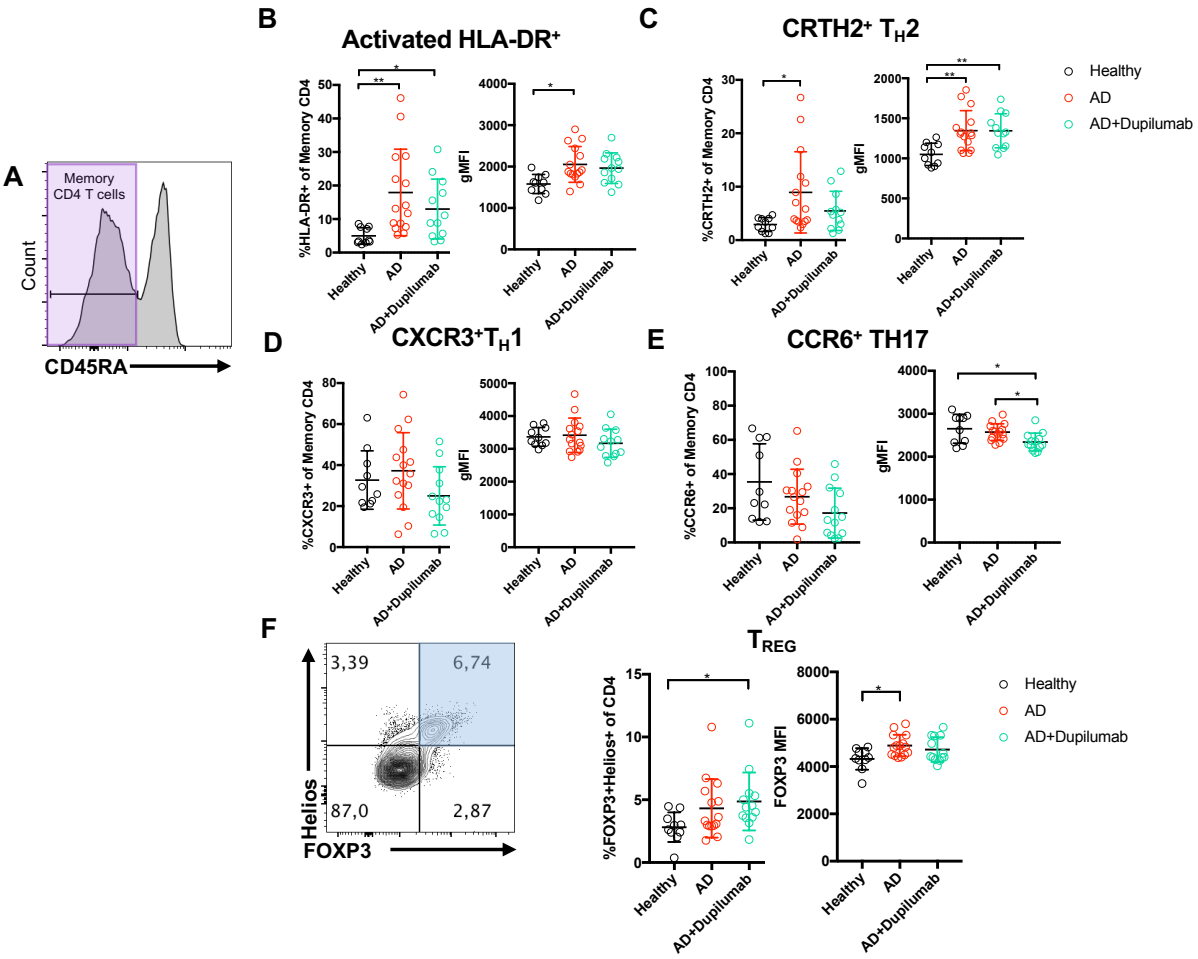


Figure 1. Elevated levels of activated memory TH2 cells are found in atopic dermatitis patients, despite a modest increase in Treg cells. PBMCs were thawed in cRPMI and rested for 2 hours prior to *ex vivo* staining for extracellular and intracellular markers. Multiparametric flow cytometry was used to measure the expression of each marker. Cells were gated for doublet exclusion, followed by live cells, and CD3+ T cells according to gating strategy. **(a)** Representative flow cytometry plot gated on memory (CD45RA-) CD4 T cells for subsequent analyses. Histograms from memory CD4 T cells depicting frequency of cells and level of expression (gMFI) for **(b)** activated T cells **(c)** TH2 cells **(d)** TH1 cells **(e)** TH17 cells. **(f)** Representative flow cytometry plot and histogram from total CD4 T cells depicting the proportion of Foxp3+Helios+ Tregs and level of Foxp3 expression (gMFI). Statistical significance was determined by a Kruskal-Wallis test (non-parametric). *$P \leq 0.05$, **$P \leq 0.01$, ***$P \leq 0.001$, and **** $P \leq 0.0001$.

Regulatory T (T_{REG}) cells are potent suppressors of hyperinflammatory responses and maintain immune homeostasis and self-tolerance[138]. Currently, there are gaps and conflicting evidence regarding the immunoregulatory role of T_{REG} cells in AD. To determine whether the proportion of T_{REG} cells is altered , we quantified T_{REG} cells, defined as $CD4^+Foxp3^+Helios^+$ T cells, as well as conventionally described as $CD4^+Foxp3^+CD25^{HIGH}CD127^{LOW}$ T cells by flow cytometry. The proportion of $CD4^+Foxp3^+Helios^+$ Treg cells was lowest in healthy subjects, with slight increased numbers detected in AD patients. Notably, this population was significantly increased in dupilumab-treated patients in remission, but only relative to healthy controls (Figure 1F). Higher level Foxp3 expression was observed in AD patients compared to healthy subjects. Comparably, the proportion of $CD4^+Foxp3^+CD25^{HIGH}CD127^{LOW}$ Tregs in dupilumab treated patients was similar to $Foxp3^+Helios^+$ T cells.

Overall, we demonstrate that Treg cells are expanded in AD patients, regardless of treatment, compared to healthy subjects. Together, our findings demonstrate that adult AD patients have elevated levels of activated and memory T_H2 cells, compared to healthy subjects, clarifying previous findings. Specifically, we demonstrate that memory T_H2 cells are reduced following dupilumab treatment, however, expression of CRTH2 on the cell surface remains unchanged after treatment.

<u>Selective modulation of $CRTH2^+$ T_H2 cells in dupixent-treated patients is specific to the skin-homing CLA^+ compartment</u>

Skin-homing memory T cells express the cutaneous lymphocyte-associated antigen (CLA) known to mediate T cell migration to the skin, promoting inflammation in the context of AD[110]. Several studies have demonstrated that specific CLA^+ T cell subsets, defined by IL-13, IFN-γ, IL-22 production, are crucial to drive disease activity in AD[111, 178]. Despite their clear role in AD, the impact dupilumab treatment on skin-homing T cells has yet to be clarified. In addition, limited

studies have described CRTH2$^+$ T cells in AD patients; Bakker et al., 2021 detected a minute frequency (~1%) of CLA$^+$CRTH2$^+$ T cells in untreated AD patients, which remained unchanged after dupilumab treatment[171]. Thus, in our long-term treatment cohort, we sought to identify and determine the persistence of these specific T cell subsets. We sought to characterize CLA$^+$ (skin-homing) and CLA$^-$ (non-skin homing) T cells in our cohorts (Figure 2a).

Although similar proportions of total CLA$^+$ CD4 were detected between all three cohorts, we observed a significantly higher frequency of CLA$^+$CD8 T cells in healthy subjects compared to AD patients (Figure 2b). Specifically, a significantly higher percentage of activated T cells and T_H2 cells were identified from the skin-homing CLA$^+$ group, but not CLA$^-$, in AD patients compared to healthy subjects (Figure 2 C and D). The overall frequency of activated T cells and T_H2 cells was higher in CLA$^+$ T cells compared to prior memory T cell group. In addition, increased cell surface expression of CRTH2 was observed in AD patients regardless of treatment in comparison to healthy subjects, while increased HLA-DR expression was only detected in AD patients compared to healthy subjects and lost in patients in remission, similar to findings in memory T cells (Figure 2c). In line with memory T cell populations, no differences were observed in CXCR3$^+$ and CCR6$^+$ from both CLA$^+$ and CLA$^-$ populations.

Amongst skin-homing CD8 T cells, we detected CRTH2-expressing CD8 T cells that were significantly increased in AD patients compared to healthy subjects, but appear to be lower in dupilumab treated patients (Figure 3A). Activated HLA-DR$^+$ T cells were also significantly increased in untreated AD, which then proceeded to decrease after treatment (Figure 3A). Interestingly, these findings were not observed in the non-skin homing compartment, nor were any differences observed in CXCR3$^+$ and CCR6$^+$ populations from both CLA$^+$ and CLA$^-$ groups in

CD8 T cells. Collectively, this data indicates that a selective modulation in the skin-homing $CD4^+$ and $CD8^+$ T cell population is observed in AD patients.

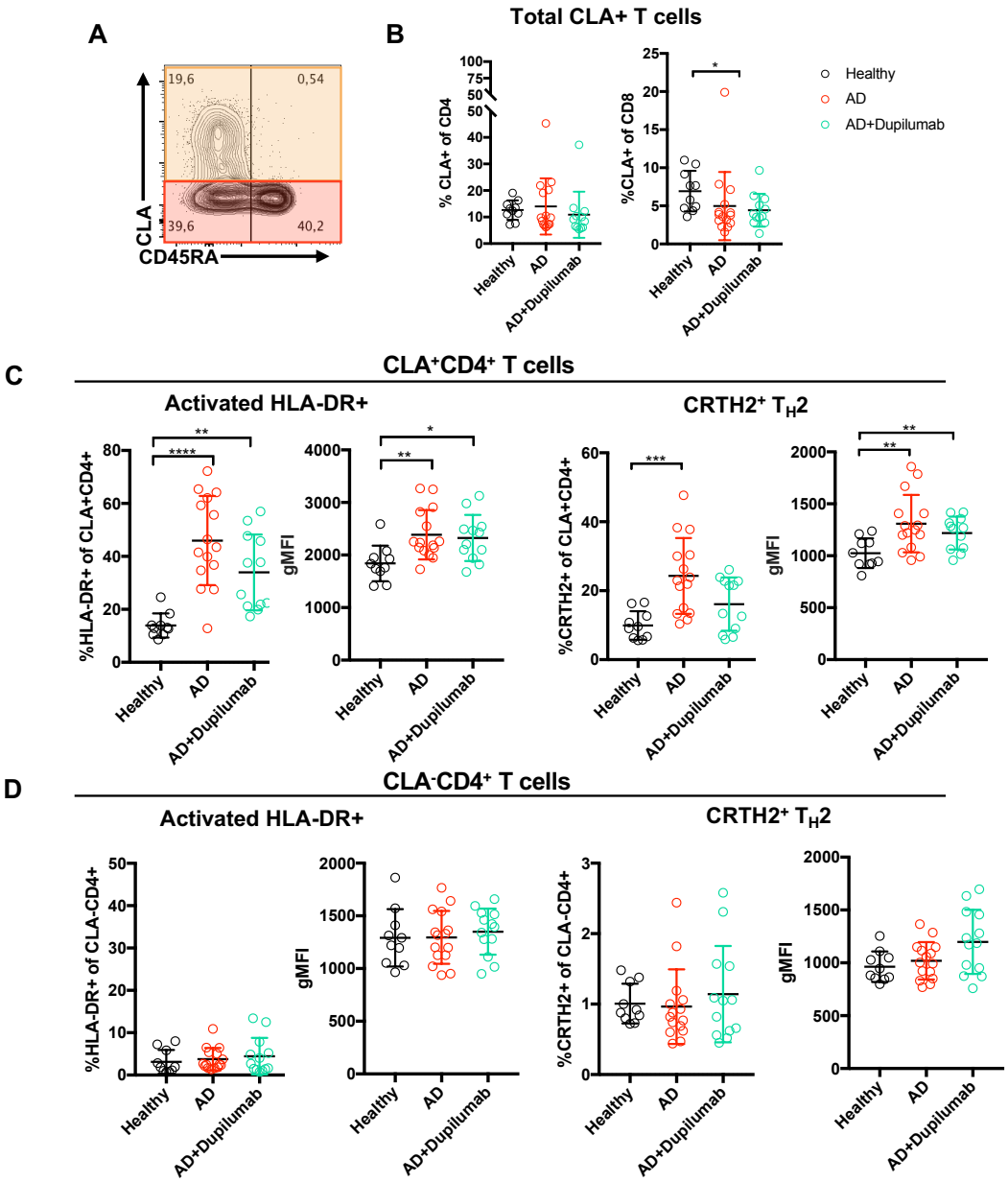


Figure 2. Skin-homing (CLA$^+$) TH2 cells remain elevated in dupilumab-treated atopic dermatitis patients. PBMCs were thawed in cRPMI and rested for 2 hours prior to *ex vivo* staining for extracellular and intracellular markers. Multiparametric flow cytometry was used to measure the expression of each marker. **(a)** Representative flow cytometry plot displaying CLA+ (top, beige) and CLA- (bottom, red) CD4 T cells for subsequent analyses. **(b)** Histogram showing proportion of CLA+ T cells from CD4 and CD8 T cells. **(c)** Histograms displaying TH2 and activated T cells from CLA+CD4+ T cells. **(d)** Histograms displaying TH2 and activated T cells from CLA-CD4+ T cells. Statistical significance was determined by a Kruskal-Wallis test (non-parametric). *$P \leq 0.05$, **$P \leq 0.01$, ***$P \leq 0.001$, and **** $P \leq 0.0001$.

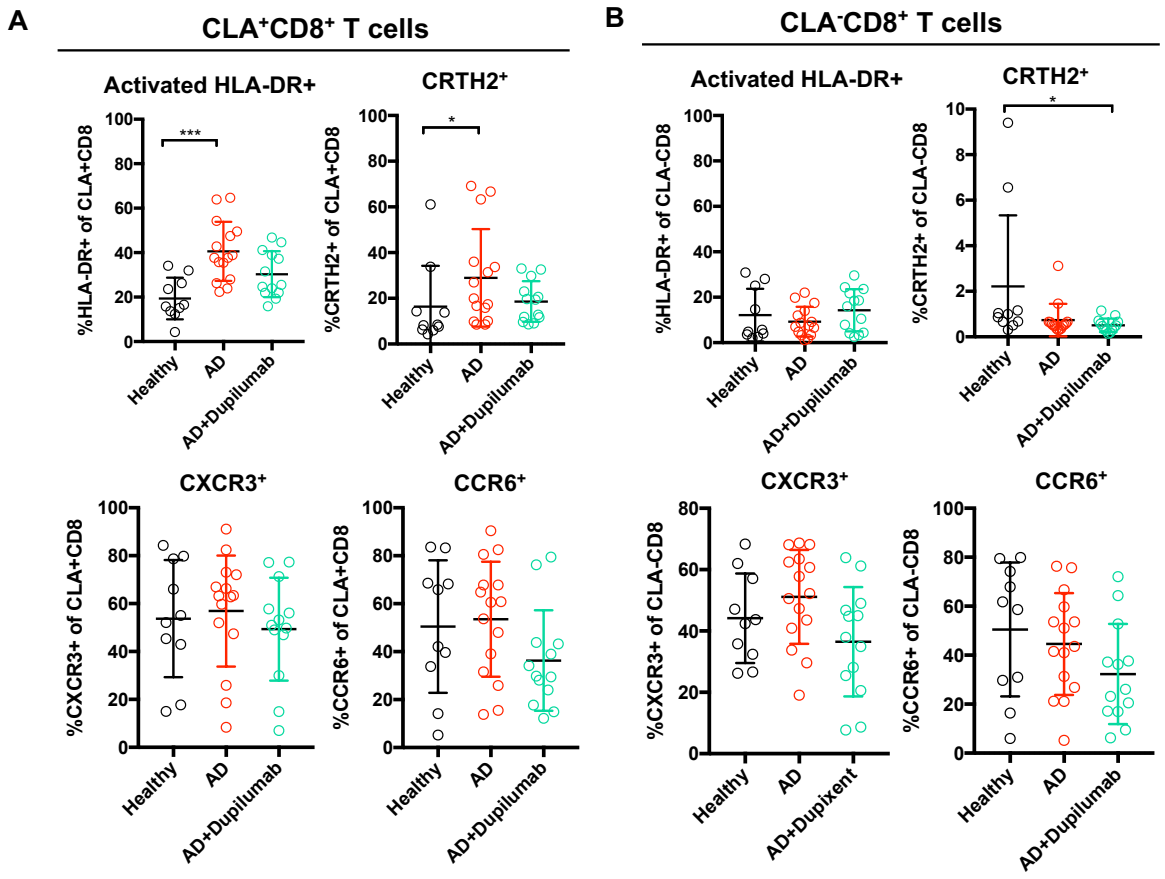


Figure 3. Skin-homing CRTH2+CD8+ T cells are detected in atopic dermatitis patients. PBMCs were thawed in cRPMI and rested for 2 hours prior to *ex vivo* staining for extracellular and intracellular markers. Multiparametric flow cytometry was used to measure the expression of each marker. **(a)** Histograms showing the proportion of activated HLA-DR+, CRTH2+, CXCR3+ and CCR6+ from CLA+ CD8+ T cells. **(b)** Histograms showing the proportion of activated HLA-DR+, CRTH2+, CXCR3+ and CCR6+ from CLA- CD8+ T cells. Statistical significance was determined by a Kruskal-Wallis test (non-parametric). *$P \leq 0.05$, **$P \leq 0.01$, ***$P \leq 0.001$, and **** $P \leq 0.0001$.

Elevated TH2 and diminished TH1 responses are consistently observed in AD patients and patients after dupixent treatment.

Chronic inflammation in AD is mediated by both local and systemic type 2-associated cytokines, of which IL-13 plays a central role. Recent reports suggest the dominant role of IL-13 and its modulation in contrast to IL-4 in AD, therefore, we sought to investigate this dynamic in comparison to other T helper cells (TH1, TH17, Tregs) following long-term dupilumab treatment. To investigate functional cytokine profile, *ex vivo* PBMCs were stimulated with PMA/ionomycin/Golgi stop. Intracellular cytokine staining was performed for T_H2 (IL-4, IL-13), T_H1 (IFN-γ), T_H17 (IL-17) cytokines along with IL-2.

In atopic patients, a significantly greater proportion of IL-4^+CD4^+T cells, and significantly decreased proportion of IFN-γ^+CD4^+ T cells were found, compared to healthy subjects (Supplemental 7 A,B). Dupilumab treated patients had a similar proportion of IL-4^+CD4^+ T cells compared to AD patients (P value:0.7080), although this was not significantly different compared to healthy subjects (P value: 0.6258) (Supplemental 7 A,B). However, a consistent significant decrease in the proportion of IFN-γ^+CD4 T cells were observed in dupilumab treated patients compared to healthy subjects (Supplemental 7 A,B). Amongst the total proportion of IL-13^+CD4^+ T cells, no significant differences were observed in AD or dupilumab-treated patients, however significantly higher IL-13 production (MFI) was observed in both AD and dupilumab-treated patients, compared to healthy subjects (Supplemental 7 A). No differences were detected in the frequency of IL-17 or IL-2-producing T cells or MFI (Supplemental 7C,D). A decreased T_H1/T_H2 ratio (IFN-γ IL-13 producing T cells) is observed in AD patients compared to healthy subjects (Supplemental 7E). From CD8 T cells, fewer T_H2 cytokines (IL-4, IL-13) , yet greater IFN-γ and IL-2-producing $CD8^+$ T cells were detected from AD patients, with no significant differences

observed. Overall, this data demonstrates a persistent T_H2 skew in the systemic compartment of AD patients, that is not normalized following dupixent treatment, despite clinical improvement.

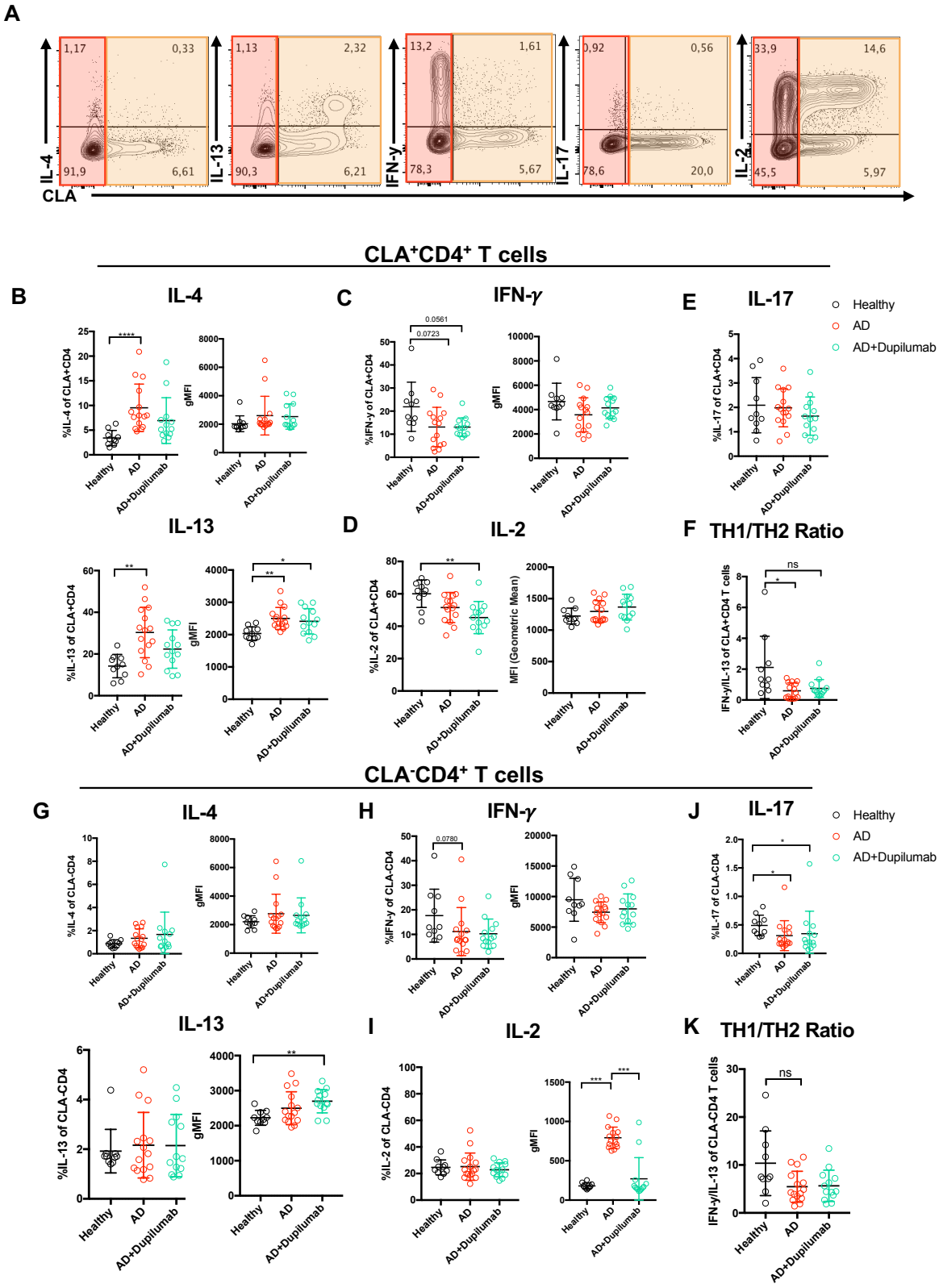
Skin-homing T cells have persistent levels of IL-13 production from AD patients after dupilumab treatment

To further investigate polar T cell subset function, we stratified CLA^+ (skin-homing) and CLA^- (non-skin homing) T cells (Figure 4A). Similar to total $CD4^+$ cells (Supp Fig. 7), the proportion of IL-4^+ CLA^+CD4 T cells was significantly increased in AD patients compared to healthy subjects, with a trend towards an increase in dupilumab treated patients (P value= 0.0607). However, the level of IL-4 production was comparable between all groups (P value AD vs healthy= 0.0780; AD+dupilumab = 0.2356) (Figure 4B). Amongst non-skin homing T cells, the proportion of IL-4 producing cells and the level of cytokine production (MFI) remained unchanged between the three cohorts (Figure 4G). CLA^+IL-13^+CD4^+ T cells are significantly expanded in AD patients compared to healthy subjects; this was not observed dupilumab-treated group , compared to untreated AD patients (*P* value= 0.3694) (Figure 4B). Despite this trend, significantly higher IL-13 production per CLA^+CD4^+ T cell (gMFI) is found in both patient cohorts, relative to healthy subjects (Figure 4B). While proportions of IL-13^+ $CD4^+$ T cells were very low and comparable across groups in the non-skin homing (CLA^-) compartment, higher IL-13 (gMFI) per cell was noted in the treated cohort (Figure 4G). Lower proportion of skin-homing Th1 IFN-γ producing T cells are found in AD patients (P=value 0.0723) and in the treatment cohort (P value=0.0561)(Figure 4 C and D), which is consistent with a decreased trend in $CXCR3^+$ T_H1 expression on memory CD4 T cells (Figure 1).

In CLA^-CD4 T cells, a slight trend to defective T_H1 responses persists relative to healthy subjects (Figure 4H). Interestingly, AD patients produce slightly less IFN-γ (gMFI) in contrast to

healthy subjects, however, in the dupixent cohort, IFN-γ production is comparable with healthy subject levels. In contrast, CLA^+IL-2 producing T cells were significantly higher in healthy subjects compared to dupilumab treated patients, while the level of cytokine production was similar between cohorts. Interestingly, no differences were observed in the frequency of CLA-IL-2 T cells, however, IL-2 production was strikingly higher in AD patients compared to both healthy subjects and dupilumab treated patients. Overall, a decreased T_H1/T_H2 ratio was observed in AD patients compared to healthy subjects (Figure 4F), and this skew is clearly notable in the CLA^- compartment (Figure 4K). Further, CD8 T cells were stratified between CLA^+ and CLA^- to investigate cytokine production. In CLA^+, but not CLA^-, CD8 T cells, we detected elevated proportions of IL-13 producing cells (non-significant), yet significantly lower IFN-γ producing T cells from AD patients compared to healthy subjects (Figure. 5B and C). Dupilumab treated patients appear to have lower proportions of $CD8^+$IL-13^+ T cells compared to untreated patients (non-significant, P value>0.9999) (Figure 5B). Overall, no differences were detected from CLA^+ and CLA^- IL-2^+ T cells between the three groups (Figure 5 D and G).

Thus, our data strongly indicates that IL-13 production from skin-homing CLA^+ T cells persists following long-term treatment with dupilumab in AD patients, despite clinical remission, which may be one avenue to trigger disease relapse.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Healthy
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AD+Dupilumab
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IFN-γ/IL-13 of CLA-CD4 T cells
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Figure 4. IL-13 and IL-4 producing skin-homing T cells remain elevated in dupilumab treated atopic dermatitis patients. PBMCs were thawed in cRPMI, rested for 2 hours, and stimulated with PMA/Ionomycin/Golgi Stop (3 hrs) for cytokine production. Intracellular staining for IL-4, IL-13, IFN-γ, IL-17 and IL-2 was performed *ex vivo* and measured by flow cytometry. Cytokine production from CLA+ and CLA- CD4 T cells was assessed. **(a)** Representative flow cytometry plot displaying CLA+ (beige) and CLA- (red) CD4 T cells for subsequent analyses. Histograms depicting frequency of cells and level of expression (gMFI) of **(b)** IL-4 and IL-13 **(c)** IFN-y **(d)** IL-17 and **(e)** IL-2 , and **(f)** TH1/TH2 ratio from CLA+CD4+ T cells. Histograms depicting frequency of cells and level of expression (gMFI) of **(g)** IL-4 and IL-13 **(h)** IFN-y **(i)** IL-17 and **(j)** IL-2 , and **(k)** TH1/TH2 ratio from CLA-CD4+ T cells. Statistical significance was determined by a Kruskal-Wallis test (non-parametric). *$*P \leq 0.05$, **$P \leq 0.01$, ***$P \leq 0.001$, and **** $P \leq 0.0001$.

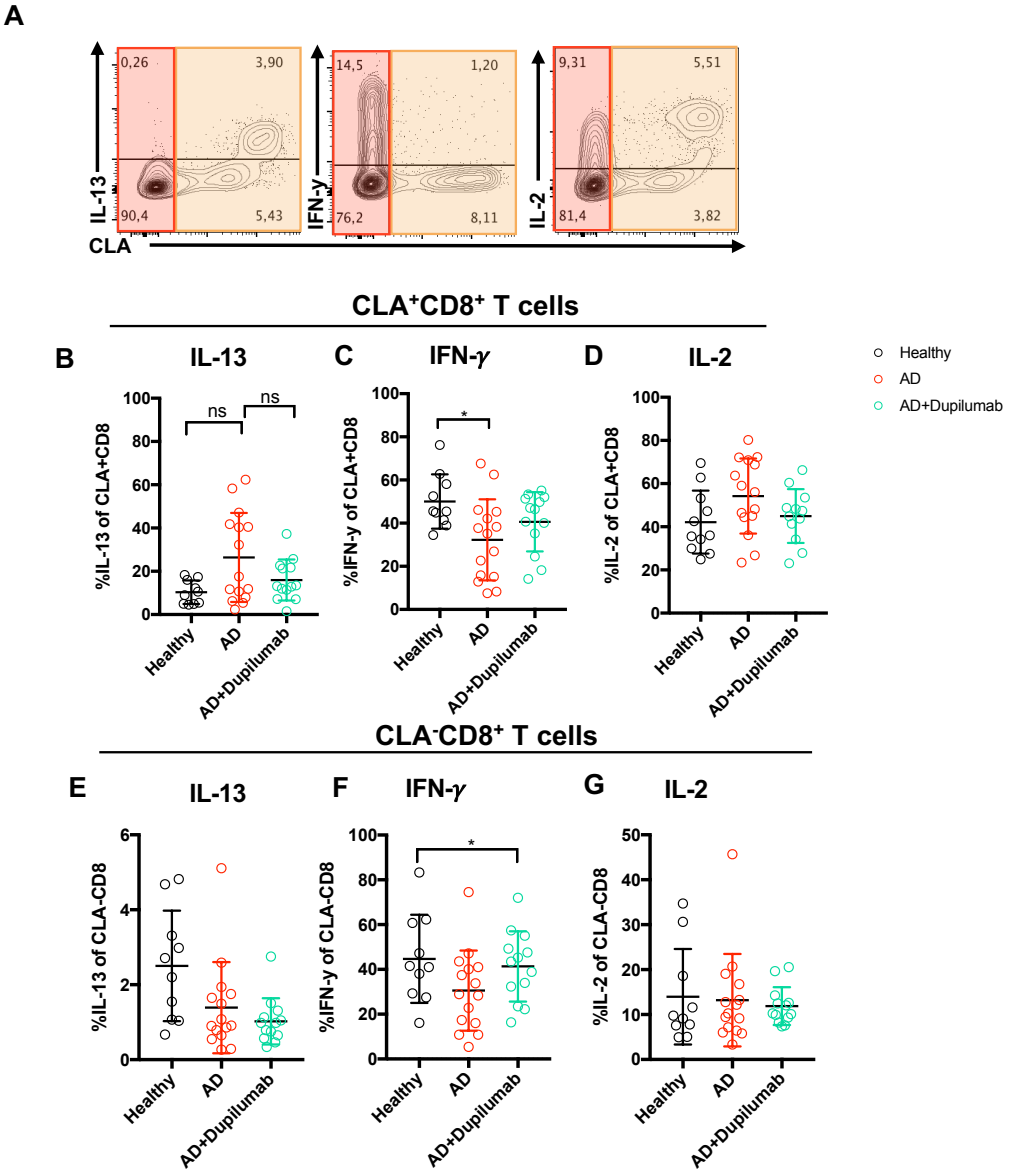


Figure 5. Skin-homing CD8+ IL-13 producing T cells are detected in atopic dermatitis patients. PBMCs were thawed in cRPMI, rested for 2 hours, and stimulated with PMA/Ionomycin/Golgi Stop (3 hrs) for cytokine production. Intracellular staining for IL-4, IL-13, IFN-γ, IL-17 and IL-2 was performed *ex vivo* and measured by flow cytometry. Cytokine production from CLA+ and CLA- CD4 T cells was assessed. **(a)** Representative flow cytometry plot displaying CLA+ (beige) and CLA- (red) CD4 T cells for subsequent analyses. Histograms depicting frequency of cells **(b)** IL-13 **(c)** IFN-y **(d)** IL-2 from CLA+CD4+ T cells. Histograms depicting frequency of cells **(e)** IL-13 **(f)** IFN-y **(g)** IL-2 CLA-CD4+ T cells. Statistical significance was determined by a Kruskal-Wallis test (non-parametric). **P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001, and **** P ≤ 0.0001.*

Aim 3:

TSLP+SEB is sufficient to promote the expansion of IL-13 producing CD4 T cells and non-conventional $CD8^+IL\text{-}13^+$ T cells

In our *ex vivo* study, elevated levels of circulating $IL\text{-}13^+T_H2$ cells were found in AD patients, however, patients under remission with dupilumab presented with lower levels of these pathogenic cells. However, upstream molecular factors that contribute to the development of these pathogenic T cells in AD remain to be clarified. To understand the mechanisms leading to type 2 T cell development, we sought to test molecular factors promoting IL-13 type 2 T cell expansion in an inflammatory milieu recapitulating cutaneous environmental AD disease factors. Typically, *in vitro* generation of T_H2 cells is induced by IL-4 and anti- IFN-γ antibodies. Our lab's novel *in vitro* model expands human type 2 T cells in PBMC by mimicking key skin factors known to play pathogenic role in AD at the end-organ level.

We assessed the functional capacity of expanded T cells to produce IL-13 by intracellular cytokine detection. We used a case-control study of healthy and AD patients PMBCs, freshly cultured for 7 days in the presence of *S. aureus*-derived superantigen (SEB) +/- TSLP (Table 2). Following a 7-day culture, cells were restimulated and Intracellular cytokine staining was performed to detect IL-4, IL-13, and IFN-γ production. We found that TSLP+SEB significantly increases the proportion of IL-13 producing $CD4^+$ T compared to control (untreated) (Figure 7B). Of note, TLSP+SEB is sufficient to increase $IL\text{-}13^+CD4^+$ T cells in PBMCs from both AD patients and healthy subjects. The level of IL-13 production (MFI) was also significantly higher in the TSLP+SEB condition compared to untreated (Figure 7B). Notably, in healthy subjects we also found $IL\text{-}13^+CD8^+$ T cells were also significantly increased in TSLP+SEB compared to untreated, (Figure 7C). The frequency of $CD4^+$ IFN γ^+ T cells was lower in AD patients compared to healthy subjects, regardless of culture condition (Untreated vs TSLP vs SEB vs TSLP+SEB). Discrete

populations of $CD4^+$ IL-13^+ IFN-γ^- and $CD4^+$IL-13^- IFN γ^+ cells were detected, as were smaller proportions of polyfunctional polyclonal-like $CD4^+$ IL-13^+ IFN-γ^+ T cells primarily in the SEB and TSLP+SEB condition. Thus, the robust increase of circulating IL-13^+ T_H2 cells as well as CD8 T cells (Tc2) from AD patient blood following TSLP+SEB demonstrates the ability of these skin-associated molecular factors to promote expansion of pathogenic inflammatory T cell populations and contribute to disease persistence. This platform allows to further investigate whether persisting IL-13^+T_H2 following treatment are capable of expanding and producing cytokines to the same capacity as baseline AD T cells.

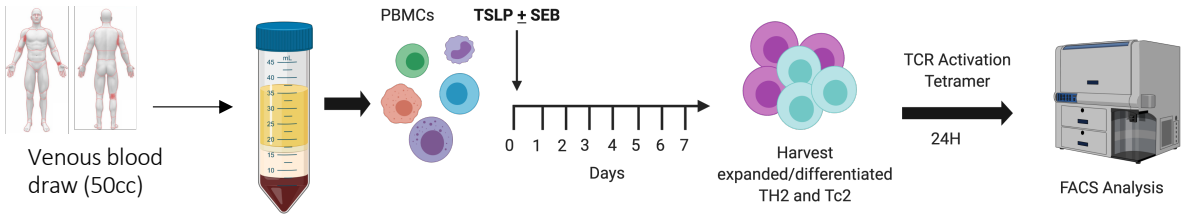


Figure 6. ***In vitro*** **TH2 expansion model.**

Table 3. Laboratory and clinical data of AD patients and healthy subjects for *in vitro* TH2 expansion

	Patients with AD (n=10)	**Healthy Subjects (n=8)**
Age (yrs)	39 (21-60)	24 (21-27)
Sex (F/M)	7/1	6/2
Ethnicity		
European	4	3
Asian	4	1
Middle Eastern	N/A	3
IgE* (kU/L)	2044.33 (200-7327)	N/A
LDH (U/L)	215 (145-277)	N/A
EASI Score	16 (8.6-40.1)	NA
VigA	2.5 (2-4)	NA
BSA%	22.5 (8-70)	NA

Data are presented as means ± SD unless otherwise specified.

*Reference range: 0 to 200 kU/L for adults

Objective measures:
EASI: Eczema Area and Severity Index
VigA: Validated Investigator Global Assessment scale for AD
BSA%: Body Surface Area affected

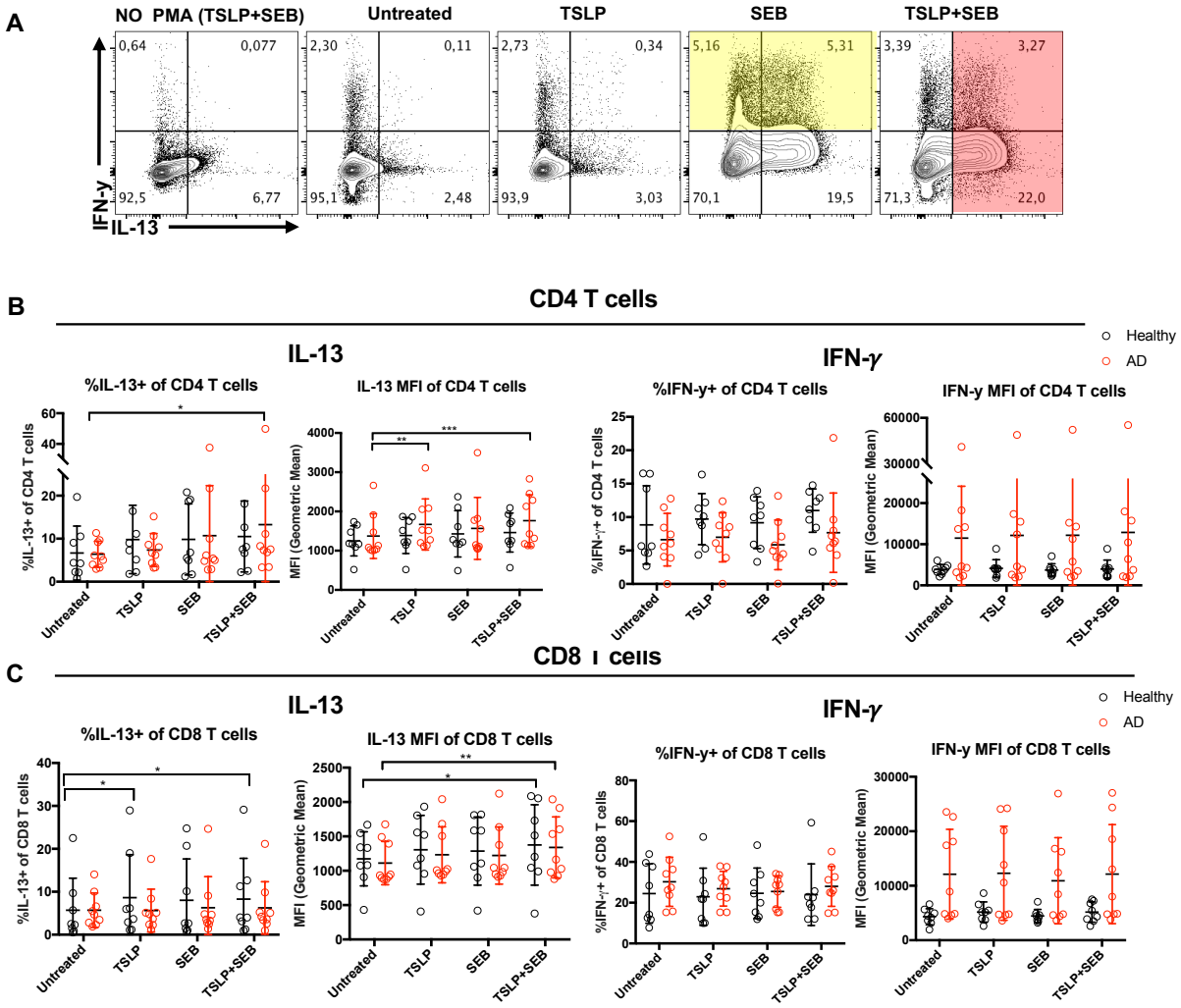


Figure 7. TSLP+SEB is sufficient to promote the expansion of IL-13 producing CD4 T cells and non-conventional CD8 IL-13+ T cells. PBMCs cultured with TSLP +/- SEB from AD patients and healthy subject were re-stimulated with PMA/Ionomycin/Golgi Stop for cytokine production. Intracellular staining for IL-13, and IFN-γ was performed and measured by flow cytometry. **(a)** Representative flow cytometry plots demonstrating proportion of IL-13 and IFN-γ-producing cells. The proportion of cells expressing **B)** IL-13 and **C)** IFN-γ, and level of cytokine production (MFI) is displayed through the histograms. Statistical significance was determined by a paired two-way ANOVA, followed by a Dunnett's test for multiple comparisons. Experiments performed in duplicates or triplicates (N=10 AD; N=8 HS). *$P \leq 0.05$, **$P \leq 0.01$, ***$P \leq 0.001$, and **** $P \leq 0.0001$.

Skin-mimicking inflammatory conditions enriches TH2 cells from adult patients with refractory disease.

We sought to further characterize *in vitro* enriched T_H2 cells. Cellular immunophenotyping of (HLA-DR), memory (CD45RO), T_H2 co-stimulatory (OX40, ICOS) and transcription factors (pSTAT6, GATA3) was performed by flow cytometry. A similar proportion of memory CD4 and $CD8^+$ T cells were observed across all conditions between AD patients and healthy subjects (Figure 8B and C). The frequency of activated HLA-DR^+CD4^+ T cells from AD patients was significantly increased following TSLP+SEB compared to control, of note, TSLP and SEB alone was also sufficient to expand activated T cells from AD patients but not healthy subjects (Figure 8B). Co-stimulatory molecules including ICOS and OX40 are important in promoting T_H2 cell differentiation, therefore, we investigated the impact of TSLP+SEB on these factors. From $CD4^+$ T cells, the proportion of both ICOS and OX40 expressing T cells were increased in the presence of TSLP+SEB compared to the untreated condition (Figure 9B), however, this pattern was only observed with OX40 on CD8 T cells. Finally, we quantified $CD4^+$ and $CD8^+$ T cells expressing T_H2-associated transcriptions factors (pSTAT6, GATA3) in AD patients and healthy subjects (Figure 10). As expected, a high level of pSTAT6 expression was observed in both CD4 and CD8 T cells due to re-stimulation with anti-CD3/CD28/CD2. Interestingly, the proportion of $GATA3^+$ CD4 T cells from AD patients, but not healthy subjects, expanded in the presence of TSLP+SEB compared to control. In summary, we demonstrate that AD skin-associated factors TSLP and SEB synergistically have the capacity to increase type 2 T cells from AD patients, and can be a potential target for new therapies.

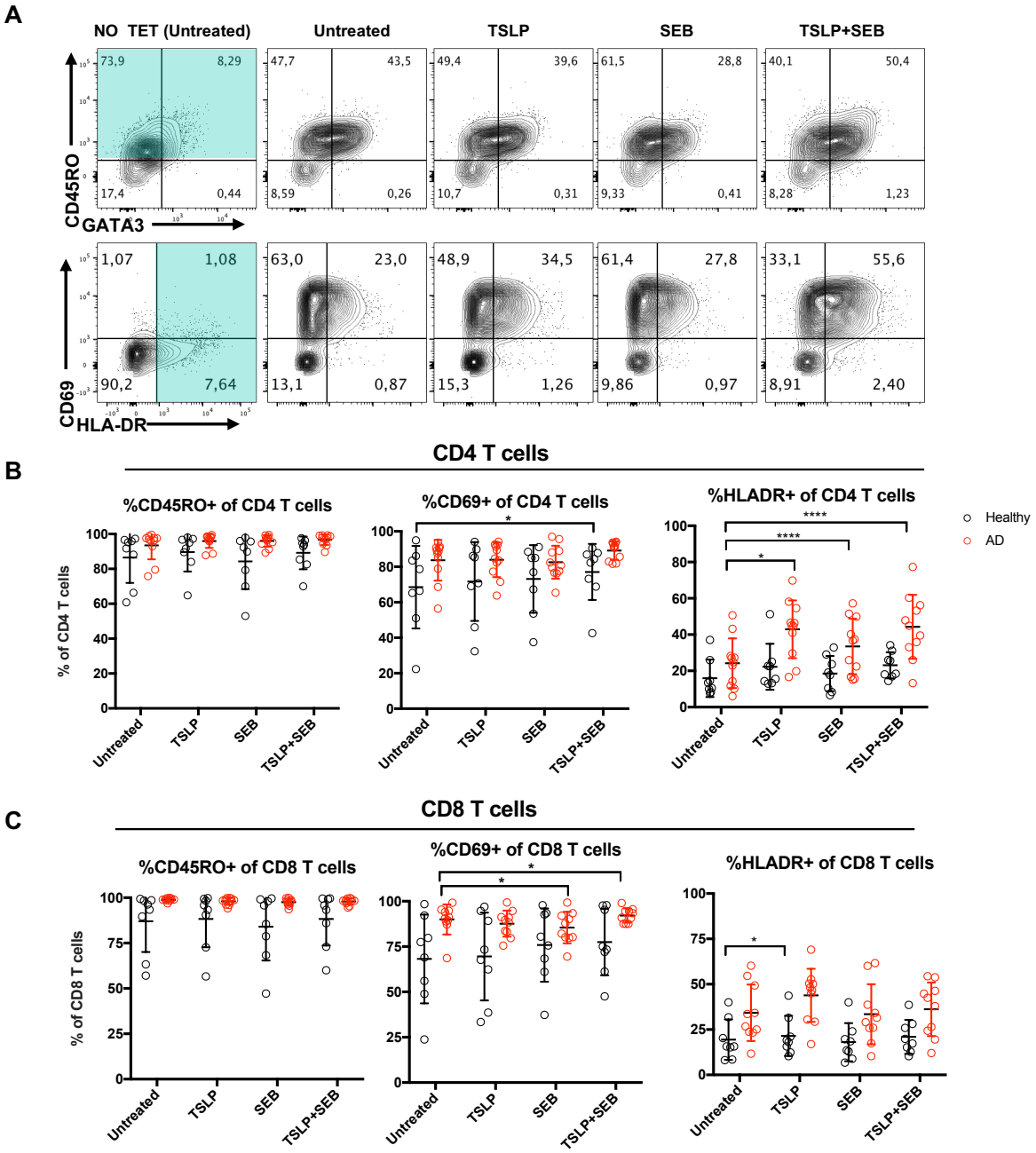


Figure 8. Expansion of activated and memory CD4 T cells from AD patients following TSLP+SEB stimulation. PBMCs cultured with TSLP +/- SEB for 7-days from AD patients and healthy subject were re-stimulated with anti-CD3/CD8/CD2 for 24h prior to staining for cellular markers. Expression levels of each marker was measured by flow cytometry. **(a)** Representative flow cytometry plots depicting proportions of CD45RO, CD69, and HLA-DR+ CD4 T cells from each condition. Histograms representing the proportion of **(b)** CD4 T cells and **(c)** CD8 T cells expressing CD45RO+, CD69, and HLA-DR+ cells. Statistical significance was determined by a paired two-way ANOVA, followed by a Dunnett's test for multiple comparisons. Experiments performed in duplicates or triplicates (N=10 AD; N=8 HS). *$P \leq 0.05$, **$P \leq 0.01$, ***$P \leq 0.001$, and **** $P \leq 0.0001$.

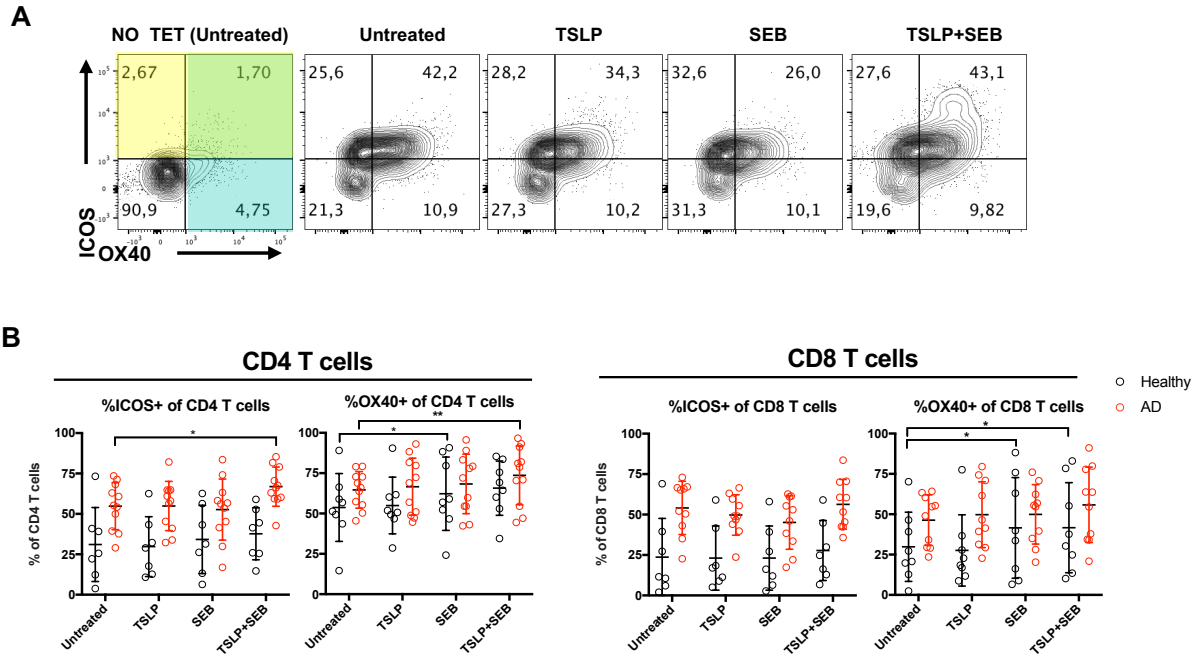


Figure 9. Expression TH2-associated costimulatory markers from CD4 and CD8 T cells in adult AD patients with refractory disease relative to healthy control. PBMCs cultured with TSLP +/- SEB for 7-days from AD patients and healthy subject were re-stimulated with anti-CD3/CD8/CD2 for 24h prior to staining for cellular markers. Expression levels of each marker was measured by flow cytometry. **(a)** Representative flow cytometry plots depicting proportions of ICOS and OX40+ CD4 T cells from each condition. **(b)** Histograms representing the proportion of ICOS+ and OX40+ expressing CD4 and CD8 T cells. Statistical significance was determined by a paired two-way ANOVA, followed by a Dunnett's test for multiple comparisons. Experiments performed in duplicates or triplicates (N=10 AD; N=8 HS). *$P \leq 0.05$, **$P \leq 0.01$, ***$P \leq 0.001$, and **** $P \leq 0.0001$.

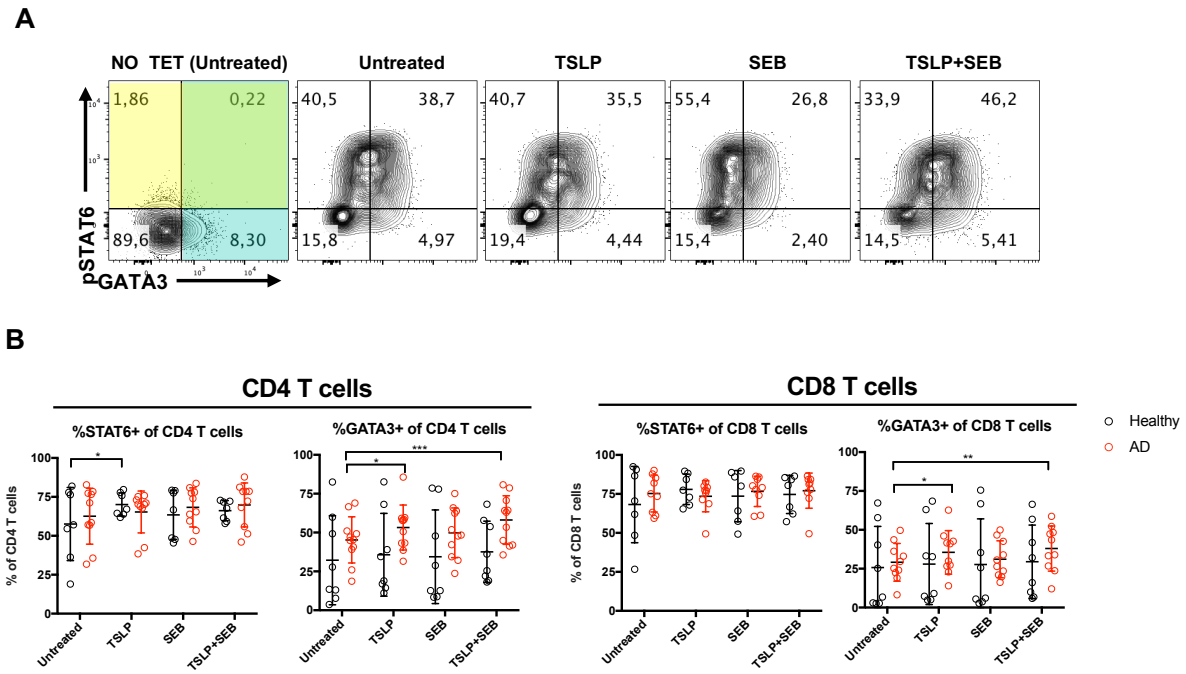
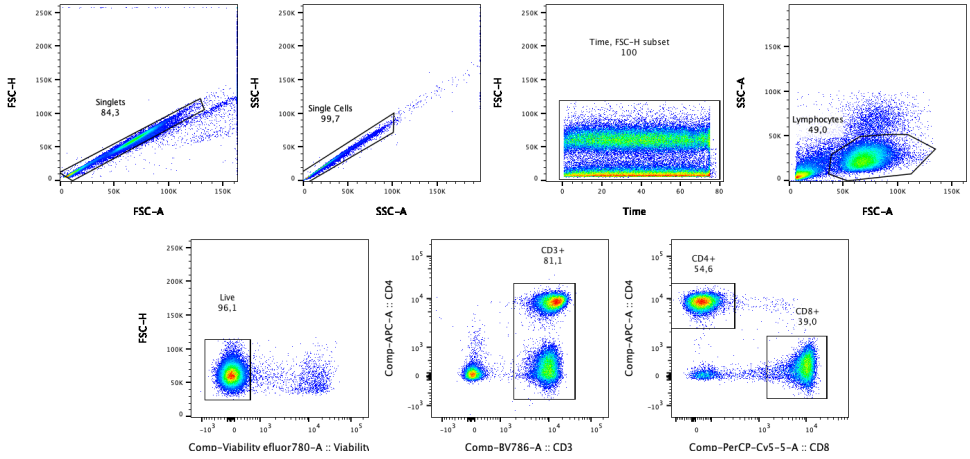
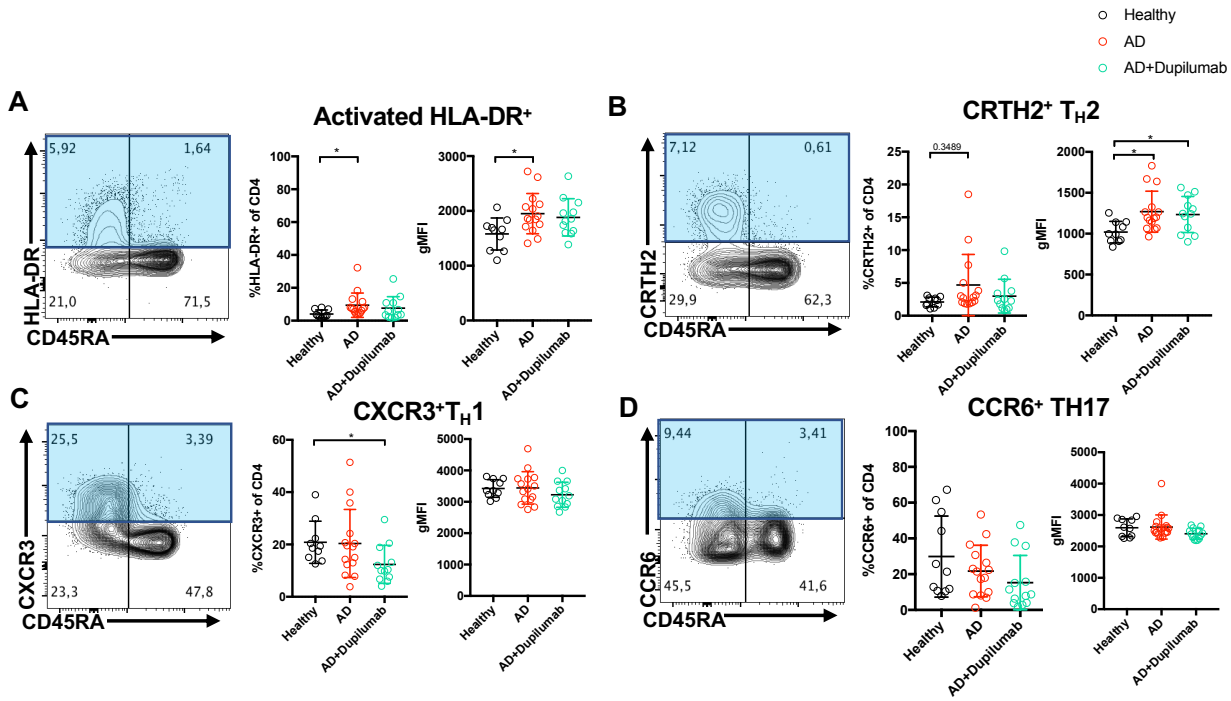


Figure 10. Skin-mimicking inflammatory conditions expand TH2 cells from adult patients with refractory disease compared to healthy subjects. PBMCs cultured with TSLP +/- SEB for 7-days from AD patients and healthy subject were re-stimulated with anti-CD3/CD8/CD2 for 24h prior to staining for cellular markers. Expression levels of each marker was measured by flow cytometry. **(a)** Representative flow cytometry plots depicting proportions of pSTAT6 and GATA3+ CD4 T cells from each condition. **(b)** Histograms representing the proportion of pSTAT6+ and GATA3+ expressing CD4 and CD8 T cells without stimulation. Statistical significance was determined by a paired two-way ANOVA, followed by a Dunnett's test for multiple comparisons. Experiments performed in duplicates or triplicates (N=10 AD; N=8 HS). *$P \leq 0.05$, **$P \leq 0.01$, ***$P \leq 0.001$, and **** $P \leq 0.0001$.

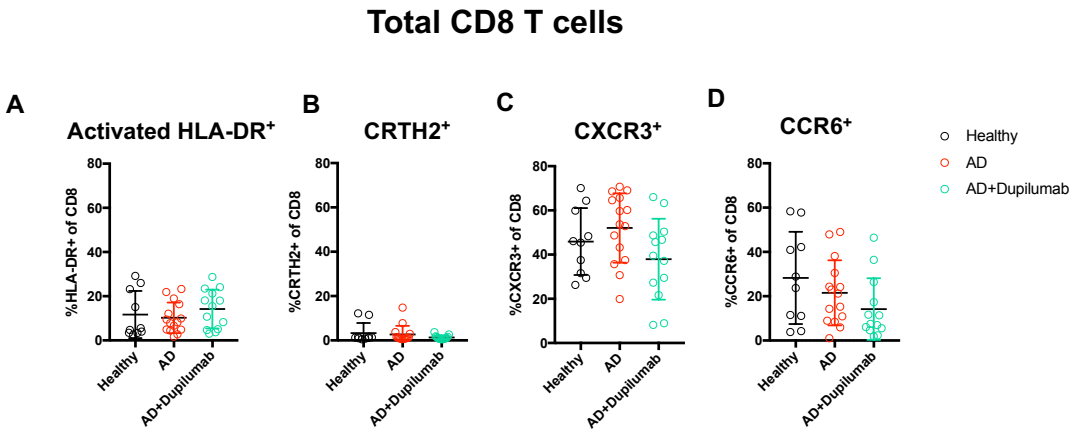
SUPPLEMENTARY FIGURES



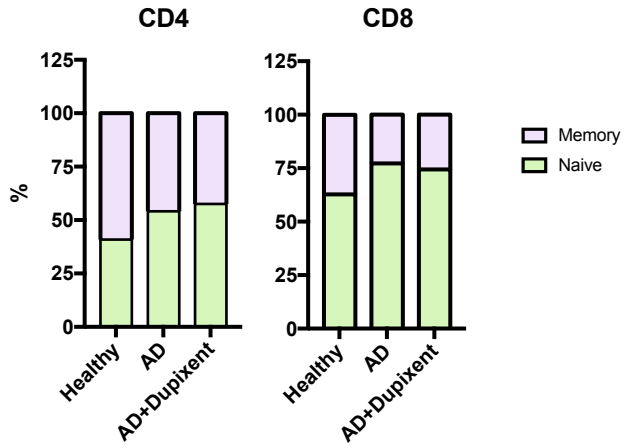
Suppl. Figure 1. Gating strategy for phenotypic/cytokine analyses. Acquired cells were gated for single cells, lymphocytes, and live cells . CD4+ and CD8+ T cells were gated from CD3+ T cells for subsequent analyses.



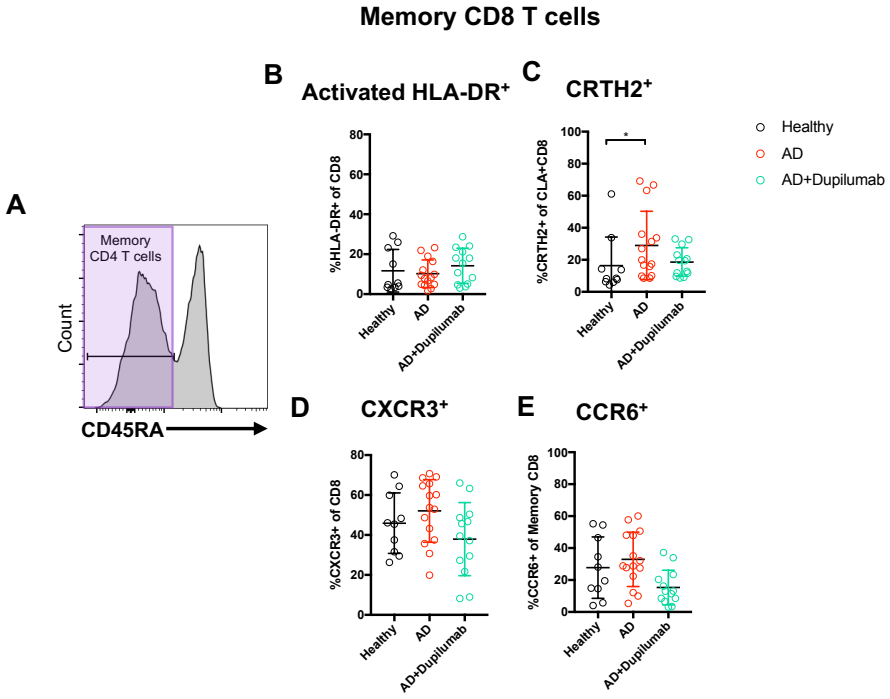
Suppl. Figure 2. Detection of TH1, TH2, and TH17 cells from peripheral blood of adult atopic dermatitis patients. Whole blood was collected from healthy subjects (n=10), AD patients (n=16), and AD patients on dupixent treatment (n=12), and PBCMs were isolated using Ficoll gradient centrifugation and frozen. Cells were thawed in cRPMI and rested for 2 hours prior to *ex vivo* staining for extracellular and intracellular markers. Multiparametric flow cytometry was used to measure the expression of each marker. Cells were gated for doublet exclusion, followed by live cells, and CD3+ T cells according to gating strategy. Representative flow cytometry plots and histograms from total CD4 T cells depicting frequency of cells and level of expression (gMFI) for **(a)** activated T cells **(b)** TH2 cells **(c)** TH1 cells **(d)** TH17 cells. Statistical significance was determined by a Kruskal-Wallis test (non-parametric). *$P \leq 0.05$, **$P \leq 0.01$, ***$P \leq 0.001$, and **** $P \leq 0.0001$.



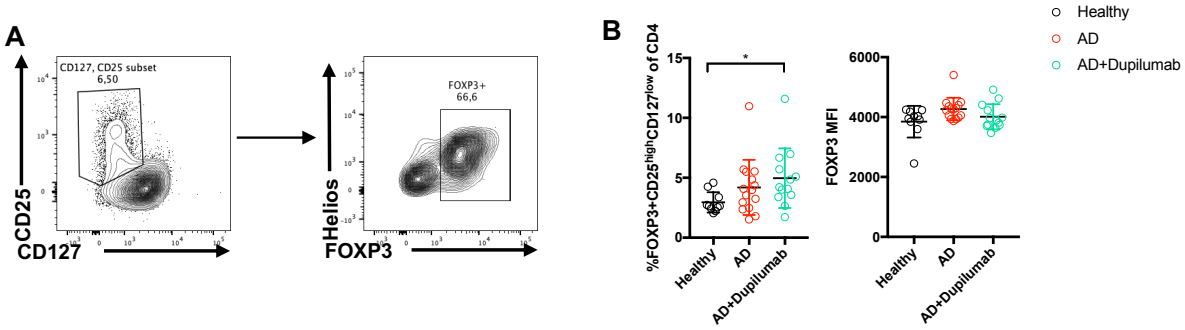
Suppl. Figure 3. Detection of CD8 T cells from peripheral blood of adult atopic dermatitis patients. Whole blood was collected from healthy subjects (n=10), AD patients (n=16), and AD patients on dupixent treatment (n=12), and PBCMs were isolated using Ficoll gradient centrifugation and frozen. Cells were thawed in cRPMI and rested for 2 hours prior to *ex vivo* staining for extracellular and intracellular markers. Multiparametric flow cytometry was used to measure the expression of each marker. Cells were gated for doublet exclusion, followed by live cells, and CD3+ T cells according to gating strategy. Histograms from total CD8 T cells depicting frequency of cells (**a)** HLA-DR+**(b)** CRTH2+ **(c)** CXCR3+ **(d)** CCR6+. Statistical significance was determined by a Kruskal-Wallis test (non-parametric). *$*P \leq 0.05$, **$**P \leq 0.01$, ***$***P \leq 0.001$, and ****$**** P \leq 0.0001$.



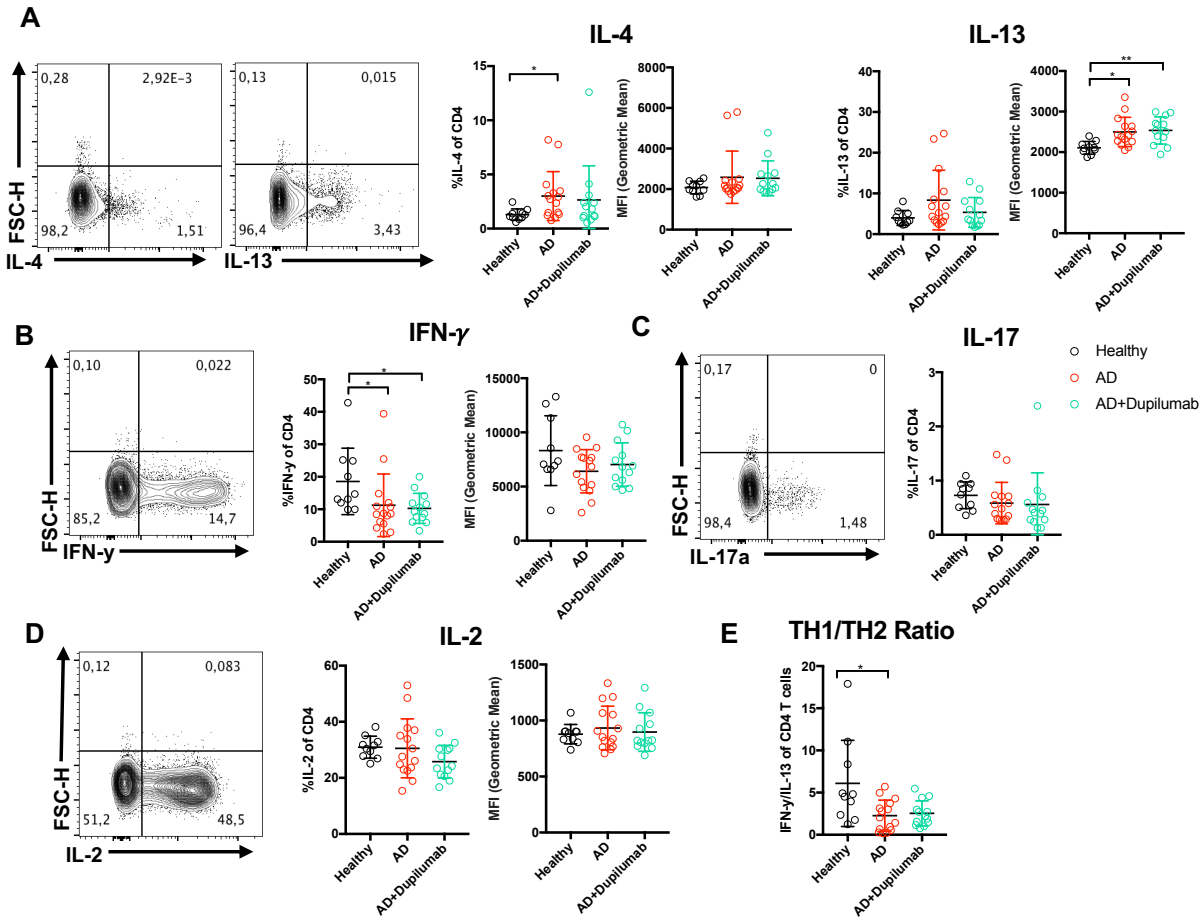
Suppl. Figure 4. Naïve and memory T cells from peripheral blood of adult atopic dermatitis patients. Cells were thawed in cRPMI and rested for 2 hours prior to *ex vivo* staining for extracellular and intracellular markers. Multiparametric flow cytometry was used to measure the expression of each marker. Cells were gated for doublet exclusion, followed by live cells, and CD3+ T cells according to gating strategy. Representative histograms from total CD4 and CD8 T cells depicting naïve (CD45RA+) and memory (CD45RA-) T cells.



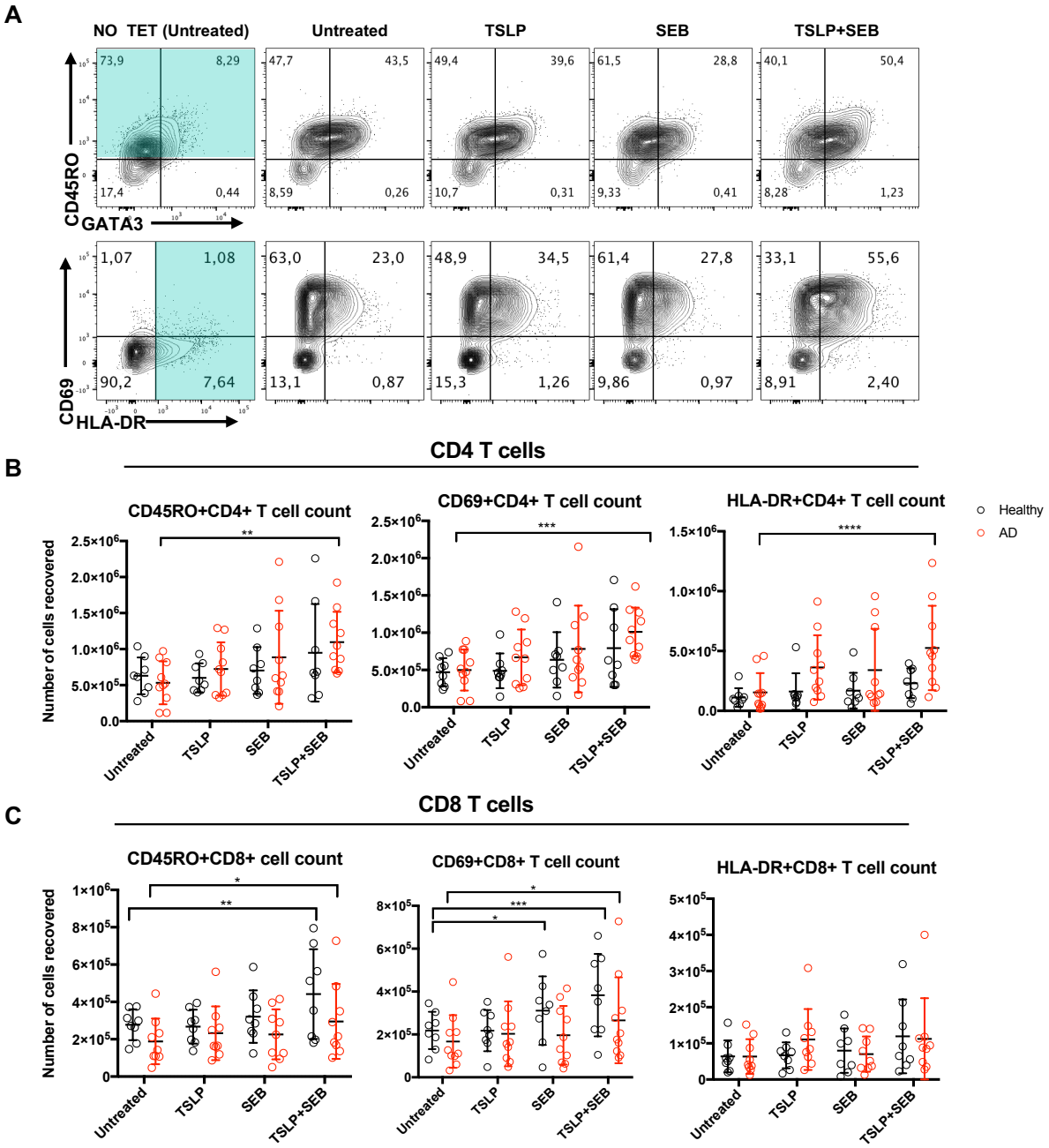
Suppl. Figure 5. CRTH2+ expressing CD8 T cells are elevated in AD patients. PBMCs were thawed in cRPMI and rested for 2 hours prior to *ex vivo* staining for extracellular and intracellular markers. Multiparametric flow cytometry was used to measure the expression of each marker. Cells were gated for doublet exclusion, followed by live cells, and CD3+ T cells according to gating strategy. **(a)** Representative flow cytometry plot gated on memory (CD45RA-) CD4 T cells for subsequent analyses. Histograms from memory CD8 T cells depicting frequency of (**b)** HLA-DR+ **(c)** CRTH2+ **(d)** CXCR3+ **(e)** CCR6+. Statistical significance was determined by a Kruskal-Wallis test (non-parametric). *$P \leq 0.05$, **$P \leq 0.01$, ***$P \leq 0.001$, and **** $P \leq 0.0001$.



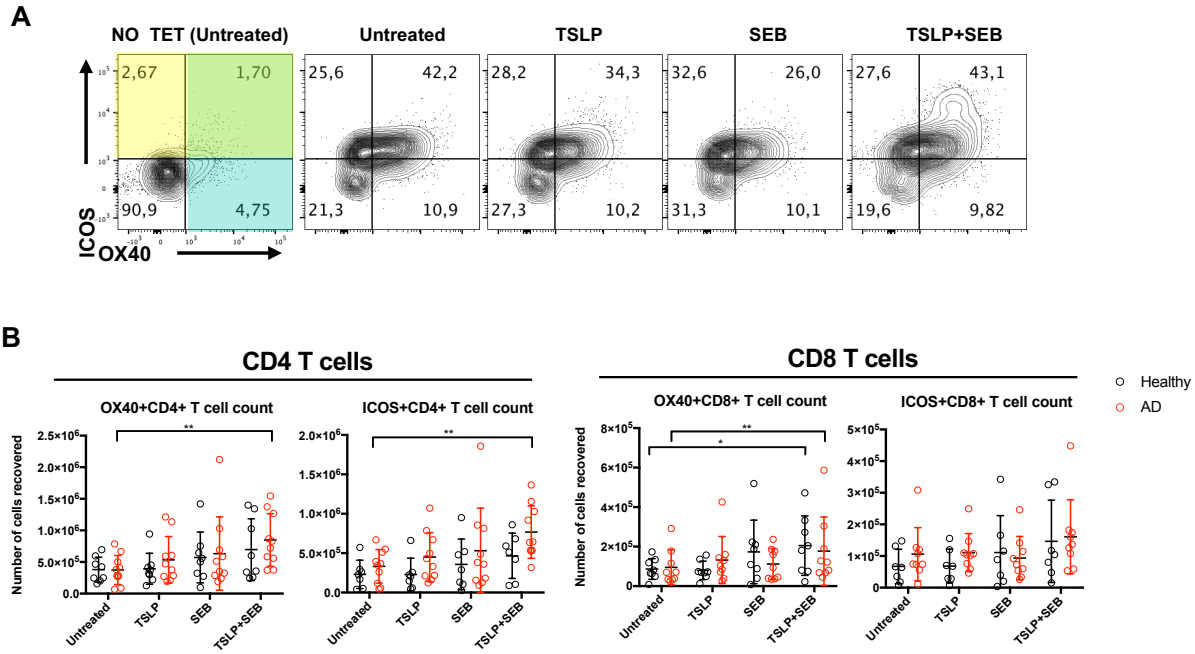
Suppl. Figure 6. Higher proportion of Treg cells detected in dupixent-treated AD patients compared to healthy subjects. Whole blood was collected from healthy subjects (n=10), AD patients (n=16), and AD patients on dupixent treatment (n=12), and PBCMs were isolated using Ficoll gradient centrifugation and frozen. Cells were thawed in cRPMI and rested for 2 hours prior to *ex vivo* staining for extracellular and intracellular markers. Multiparametric flow cytometry was used to measure the expression of each marker. **(a)** Representative flow cytometry plots and histograms from total CD4 T cells depicting frequency of CD25highCD127low T cells and level of Foxp3 expression (gMFI). **(b)** Histograms representing %Treg cells. Statistical significance was determined by one-way ANOVA (Kruskal-Wallis test). *$P \leq 0.05$, **$P \leq 0.01$, ***$P \leq 0.001$, and **** $P \leq 0.0001$.



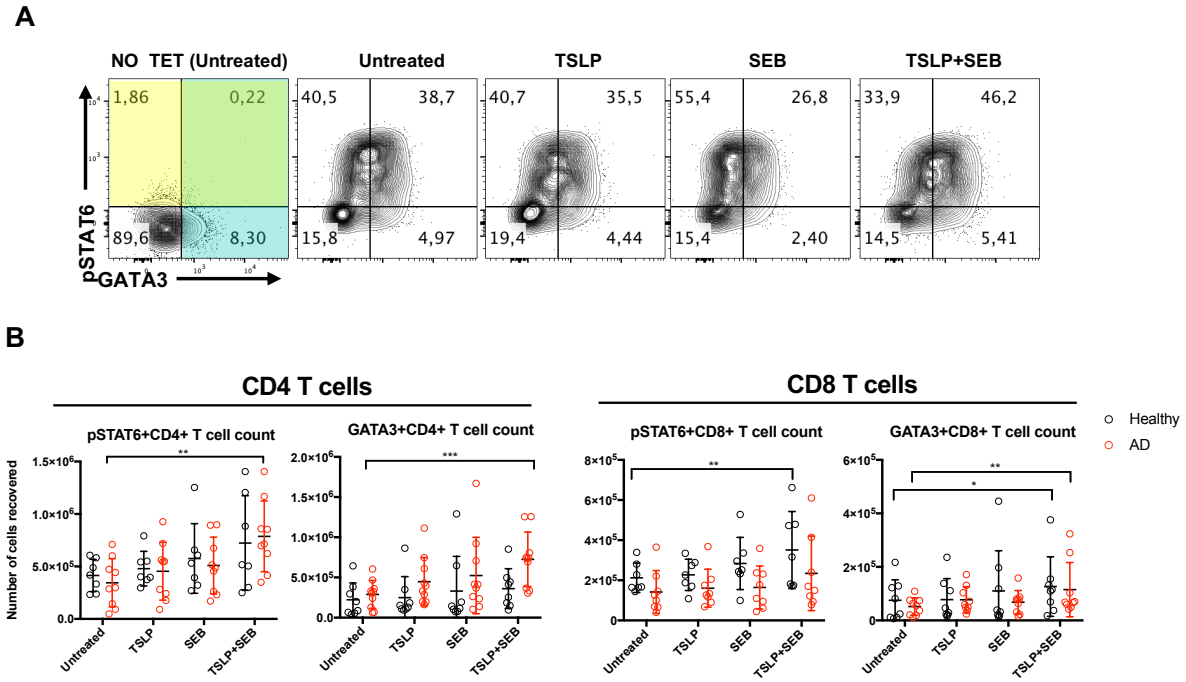
Suppl. Figure 7. Elevated TH2 and diminished TH1 responses are consistently observed in AD patients. PBMCs were thawed in cRPMI, rested for 2 hours, and stimulated with PMA/Ionomycin/Golgi Stop (3 hrs) for cytokine production. Intracellular staining for IL-4, IL-13, IFN-γ, IL-17 and IL-2 was performed *ex vivo* and measured by flow cytometry. Cytokine production from total CD4 T cells was assessed. Representative flow cytometry plot and histograms from total CD4 T cells depicting frequency of cells and level of expression (gMFI) of **(a)** IL-4 and IL-13 **(b)** IFN-γ **(c)** IL-17 and **(d)** IL-2. **(e)** Histogram displaying TH1/TH2 ratio. Statistical significance was determined by a Kruskal-Wallis test (non-parametric). **P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001, and **** P ≤ 0.0001*.



Suppl. Figure 8. Expansion of activated and memory CD4 T cells from AD patients following TSLP+SEB stimulation. PBMCs cultured with TSLP +/- SEB for 7-days from AD patients and healthy subject were re-stimulated with anti-CD3/CD8/CD2 for 24h prior to staining for cellular markers. Expression levels of each marker was measured by flow cytometry. **(a)** Representative flow cytometry plots depicting proportions of CD45RO and HLA-DR+ CD4 T cells from each condition. **(b)** Histograms representing the number of CD45RO+, HLA-DR+, and CD69 expressing CD4 T cells. **(c)** Histograms representing the number of CD45RO+, HLA-DR+, and CD69 expressing CD8 T cells. Statistical significance was determined by a paired two-way ANOVA, followed by a Dunnett's test for multiple comparisons. Experiments performed in duplicates or triplicates (N=10 AD; N=8 HS). **P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001, and **** P ≤ 0.0001.*



Suppl. Figure 9. Expression TH2-associated costimulatory markers from CD4 and CD8 T cells in adult AD patients with refractory disease relative to healthy control. PBMCs cultured with TSLP +/- SEB for 7-days from AD patients and healthy subject were re-stimulated with anti-CD3/CD8/CD2 for 24h prior to staining for cellular markers. Expression levels of each marker was measured by flow cytometry. **(a)** Representative flow cytometry plots depicting proportions of ICOS and OX40+ CD4 T cells from each condition. **(b)** Histograms representing the number of ICOS+ and OX40+ expressing CD4 and CD8 T cells. Statistical significance was determined by a paired two-way ANOVA, followed by a Dunnett's test for multiple comparisons. Experiments performed in duplicates or triplicates (N=10 AD; N=8 HS). *$P \leq 0.05$, **$P \leq 0.01$, ***$P \leq 0.001$, and **** $P \leq 0.0001$.



Suppl. Figure 10. Skin-mimicking inflammatory conditions expand TH2 cells from adult patients with refractory disease compared to healthy subjects. PBMCs cultured with TSLP +/- SEB for 7-days from AD patients and healthy subject were re-stimulated with anti-CD3/CD8/CD2 for 24h prior to staining for cellular markers. Expression levels of each marker was measured by flow cytometry. **(a)** Representative flow cytometry plots depicting proportions of pSTAT6 and GATA3+ CD4 T cells from each condition. **(b)** Histograms representing the number of pSTAT6+ and GATA3+ expressing CD4 and CD8 T cells. Statistical significance was determined by a paired two-way ANOVA, followed by a Dunnett's test for multiple comparisons. Experiments performed in duplicates or triplicates (N=10 AD; N=8 HS). *$P \leq 0.05$, **$P \leq 0.01$, ***$P \leq 0.001$, and **** $P \leq 0.0001$.

Table 4. *Ex vivo* Flow Cytometry Panels

Marker	Fluorochrome
Viability	Efluor780
CD4	APC
CD8	PerCP-Cy5.5
CD3	BV786
CD45RA	Alexa700
CLA	FITC
GATA3	BUV395
CRTH2	PE-CF594
CXCR3	BV421
CCR6	PE-Cy7
HLA-DR	V500

Marker	Fluorochrome
Viability	Efluor780
CD4	Alexa700
CD8	V500
CD3	BV786
CLA	FITC
IL-13	PE
IL-4	APC
IFN-y	PE-Cy7
IL-17a	V450
IL-2	Per-CP

Marker	Fluorochrome
Viability	Efluor780
CD4	FITC
CD8	V500
CD3	BV786
CD45RA	Alexa700
CRTH2	BV711
FOXP3	PE
CD25	APC
CD127	Texas Red
Helios	Pacific Blue
CTLA-4	Per-CP-Cy 5.5

Table 5. Antibody catalogue

Antibody	Fluorophore	Clone
Anti-CD8	PerCP-Cy5.5	RPA-T8
Anti-GATA3	BUV395	L50-823
Anti-CRTH2	PE-CF594	BM16
Anti-CXCR3	BV421	562558
Anti-CD8	V500	RPA-T8
Anti-IL-13	PE	JES10-5A2
Anti- IL-4	APC	8D4-8
Anti- IFN-y	PE-Cy7	4S.B3
Anti-IL-17a	V450	N49-653
Anti- IL-2	PerCP-Cy5.5	MQ1-17H12
Anti-CD4	FITC	RPA-T4
Anti-FOXP3	PE	236A/E7
Anti-CD25	APC	2A3
Anti-CD4	APC	RPA-T4
Anti-CD3	BV786	OKT3
Anti-CD45RA	Alexa700	HI100
Anti-CLA	FITC	HECA-452
Anti-CCR6	PE-Cy7	G034E3
Anti-Helios	Pacific Blue	22F6
Anti-CD4	AF700	RPA-T4
Anti-CD127	Texas Red	eBioRDR5
Anti-HLA-DR	V500	G46-6
Viability Dye	Eflour780	

DISCUSSION

While several studies have demonstrated the clinical efficacy of dupilumab to significantly ameliorate AD disease severity and patient-reported outcomes[179-183], cessation of dupilumab treatment has been associated with disease reactivation in patients[184]. Limited research has been performed to assess the long-term effects of dupilumab in AD patients, specifically to see whether subsets of IL-13$^+$ T cells persist following long-term treatment. Our cross-sectional study successfully characterized heterogeneous T cell populations, specifically polar T cells/type 2 cells, contributing to chronic, unremitting moderate-to-severe disease in adult AD patients. Our findings demonstrate that circulating activated T cells and T_H2 cells persist in AD patients despite achieving clinical remission on dupilumab. Specifically, we show that severe AD patients have significantly higher levels of memory CRTH2$^+$$T_H2$ cells compared to healthy subjects, found exclusive to skin-homing (CLA$^+$) T cells. Following dupilumab treatment, a persistent level of IL-13 production (MFI) in skin-homing T cells was observed in the systemic compartment of AD patients, which was not normalized following dupilumab treatment, despite clinical improvement. This may be a potential avenue to trigger disease relapse. Using our *in vitro* model composed of TSLP+SEB, we demonstrate that the combination of TSLP+SEB is sufficient to induce IL-13 production, Th2 transcription factors, as well as co-stimulatory receptor expression from CD4 and CD8 T cells of AD patients and healthy subjects.

To our knowledge, this is the first study to have investigated the long-term effects (>52 weeks) of dupilumab treatment on residual disease activity in AD patients. In-depth characterization with chemokine receptors (CRTH2, CXCR3, CCR6) was used to detect T helper subsets (T_H1, T_H2, T_H17, T_{REG}), while intracellular cytokine production revealed their functional capacity to produce cytokines (IL-4, IL-13, IFN-γ , IL-17, IL-2). Consistent with previous studies, our disease cohort of AD patients, treated or untreated, had deviations in the T_H2 axis, specifically

increased IL-13 production and elevated CRTH2 receptor expression (MFI), with decreased T_H1 cell activity demonstrated by lower IFN-γ production, and stable Tregs levels, suggesting that the latter subsets may play a role in disease maintenance or improvement.

Prostaglandin D2 (PGD_2) is a lipid mediator derived from arachidonic acid, primarily produced by IgE-activated mast cells, as well as dendritic cells, macrophages, eosinophils, T helper type 2 (Th2) cells, and endothelial cells during allergic inflammation[185]. PGD_2 exerts its effects through two receptors: D- prostanoid (DP1) and CRTH2. PGD_2-DP1 signaling leads to downstream effects of vasodilation, smooth muscle relaxation, and inhibition of platelet aggregation and cell migration[104]. In contrast, binding of PGD2 to CRTH2, an inhibitory G-protein coupled receptor, inhibits cAMP production, which initiates a cascade of signaling events which lead to an increase of $Ca2^+$ influx, resulting in migration and activation of mast cells, eosinophils, and T_H2 cells, and increased production of inflammatory cytokines (IL-4, IL-5, IL-13) [104]. While many studies have investigated the pathological role of CRTH2 in asthma, conflicting findings are available on the contributory role of CRTH2 in AD[64]. It has been reported that CRTH2-deficient mice exhibited lower infiltration of TH2 cells following epicutaneous challenge in an OVA-induced AD murine model[61]. In addition, CRTH2 is necessary for the chemotaxis of ILC2s, which may be important players of inflammation in AD skin, however, a recent clinical trial of a CRTH2 antagonist did not demonstrate any clinical efficacy in AD[186]. In humans, CRTH2 is described as the most reliable marker to detect T_H2 cells [187], therefore, we employed this marker to identify T_H2 cells in our cohort of AD patients.

We demonstrate that severe AD patients have significantly higher levels skin- homing memory $CRTH2^+T_H2$ cells compared to healthy subjects. In our study, patients treated with dupilumab appear to have decreased proportions of skin homing $CRTH2^+$ T_H2 cells relative to

untreated AD patients. Contrary to our findings, Bakker et al. detected no differences in CLA^+CRTH2^+ T cells amongst healthy subjects, baseline AD patients, and dupilumab treated patients (4, 16, 52 weeks) [171]. This difference may be explained by varying disease severity between cohorts, as their cohort had moderate AD (EASI: 16.8) compared to our AD patients with severe disease (EASI 23.6). Nevertheless, this suggests that CRTH2 may be used as a biomarker to identify severe AD. In addition, our study demonstrated that CRTH2 expression levels (MFI) in patients treated with dupilumab remains elevated and equal to levels found in untreated AD patients, compared to healthy subjects. A higher abundance of CRTH2 receptor on T_H2 cells would facilitate binding of PGD2 secreate from leukocytes, inducing a stronger downstream signal to secreate greater levels of IL-13, IL-4, and IL-5, and promote migration of T_H2 to inflamed skin[188, 189]. IL-4Ra blockade by dupilumab can directly inhibit the differentiation of naïve T cells into T_H2 cells [82], however, its impact on existing T_H2 cells function must further be determined.

Recently, Bangert et al. investigated whether tissue-resident immune memory persists in the resolved skin of patients (mean reduction in EASI from baseline of 72.0% and 88.8% by 16 weeks and 1 year treated with dupilumab) after short-term and long-term dupilumab treatment (16 weeks and 1 year)[109]. Through sc-RNAseq analysis of skin suction blisters (i.e. epidermis) , they demonstrated active PTGDR2 gene expression (i.e. CRTH2) in skin of clinical resolved AD patients after one year of dupilumab treatment, which was absent in healthy subjects[109]. Immunofluorescence also identified the persistence of $CD3^+CRTH2^+$ cells, defined as "Th2A", in the epidermis of dupilumab treated patients[109]. Our results from peripheral blood demonstrate that a proportion of $CLA^+CRTH2^+TH2^+$ cells, with unchanged CRTH2 cell surface expression persist in dupilumab treated patients compared to healthy subjects, which are in line with these findings. This suggests that these cells may migrate to the skin for long-term residence, however, additional

work is required to identify these cells with tissue-resident markers including CD69 and CD103. Contrary to the strict dogma that tissue-resident cells are solely compartmentalized in peripheral tissues, Klicznik et al. have demonstrated that healthy human $CD4^+CLA^+$ $CD69^+CD103^+$ T_{RM} cells in skin have the ability to downregulate CD69, to exit from the skin into the systemic circulation[190]. In addition, they detected that a population of $CD4^+CLA^+CD103^+$ T cells from blood is clonally similar to that found skin amongst the same donors, and can recirculate back to the skin. Skin-specific host antigens released following tissue damage may be a source of antigen for circulating T cells in chronic AD[191]. As previously described, serum IgE levels are elevated in the majority of AD patients, with variable specificity to antigens, including aeroallergens in sensitized individuals and, frequently, *S. aureus*-derived superantigens[192]. Bystander activation of T cells through superantigens, especially *S. aureus* SEB, has the potential to act to drive chronic inflammation in AD[191, 193].IgE autoantibodies found in AD patients have also been found to react to human keratinocyte proteins including Hom S 1-5, as well as human manganese superoxide dismutase (MnSOD), RP1, eukaryotic translation initiation factor 6 (eIF6), dense fine speckles (DFS), and human thioredoxins (hTrx)[191]. This provides an exciting avenue to investigate skin-resident cells in AD through the systemic circulation, overcoming the barrier of skin-tissue samples needed from patients.

In a murine allergic contact inflammation model of human AD, inhibition of CRTH2 prevented edema formation, reduced inflammatory cell infiltration and skin pathology, and repressed inflammatory cytokine gene expression in experimental mice[194], suggesting its role in mediating allergic-inflammation. CRTH2 inhibitors, fevipiprant (Clinicaltrials.gov Identifier: NCT01785602) and temapiprant (Clinicaltrials.gov Identifier: NCT02002208), were clinically tested in phase 2 trials for improvement of moderate to severe AD in adults for 12 and 16 weeks

respectively. Patients received oral treatment (fevipiprant or temapiprant) vs placebo; primary outcome was a significant change in EASI score from baseline between intervention vs placebo. Each trial failed to reach primary endpoint [195]. However, these inhibitors demonstrate clinical efficacy to improve asthma or eosinophilic diseases[196], suggesting that patients with intrinsic AD, characterized by high IgE levels and eosinophilia, may experience therapeutic benefits with a CRTH2 inhibitor.

Major histocompability complex (MHC) class II antigen, HLA-DR, is a marker of chronic T cell activation that is known to increase in autoimmune diseases as well as with age in healthy adults[197, 198]. In patients with AD, infants had similar levels of HLA-DR expression compared to healthy subjects, with an increase in AD children and adolescents with age. However, adult AD patients have been found to have significant higher levels of HLA-DR expressing CLA^+/CLA^- CD4 and CD8 T cells[177]. Consistent with previous findings, our data demonstrates that chronic AD patients have greater frequencies of $CLA^+HLA\text{-}DR^+$ CD4 and CD8 T cells relative to healthy subjects, however, this is absent in CLA^- populations[112]. Of note, we show that, despite clinical improvement, dupilumab treatment does not fully normalize the proportion of activated T cells within CD4 T cells, as the levels remain higher than healthy subjects. In treated patients, a decrease in HLA-DR expression was observed in skin-homing CD4 T cells compared to untreated patients. As age was not a confounding factor between the three cohorts (no significant differences observed), this observation is in line with changes associated with chronic inflammation, and not simply increase in $HLA\text{-}DR^+$ T cells due to aging[199]. Upon recognition of a foreign or skin-derived self-peptide such as S. aureus derived SEB , chronically activated $HLA\text{-}DR^+$ skin-homing T cells will rapidly mount an inflammatory response that may become uncontrolled following discontinuation of treatment.

T_{REG} cells are critical regulators of hyperinflammatory responses and self-tolerance, however, their ability to control inflammation in human AD remains unclear. Numerous studies demonstrate the expansion of Treg cells in lesional skin or peripheral blood in AD patients compared to healthy subjects [200, 201], however, there is conflicting evidence to suggest reduced capacity of Treg cells to produce suppressive cytokines and control proliferation of effector T cells[148, 202] versus $CD25^{high}$ Treg cells to have a "T_H2 cell" phenotype. Bakker et al. recently found that $CD25^+FOXP3^+$ $CD4^+$ Treg cells increase from baseline to 52 weeks of dupilumab treatment[171]. Our results are in line with these findings and show following long-term (2.28 years) treatment with dupilumab, patients have increased proportions of $Foxp3^+Helios^+CD4^+$ Treg cells, compared to healthy subjects and this is comparable to untreated AD patients. In addition, the level of FOXP3 expression per Treg cell is greater in AD patients. Expanded Treg cells may be opposing the persistent IL-13 effector T cell response to maintain clinical remission in dupilumab treated patients, as viewed by improved disease severity measures. Treg cells in AD patients may also be contributing to maintenance of remission in the presence of chronically activated $HLA\text{-}DR^+$T cells. Additional work must be performed to assess the suppressive capacities and cytokine profile of Treg cells in dupilumab treated patients.

Amongst other T_H2 cytokines, IL-13 has been well described to have a preferential role to mediate disease activity in AD[14, 151, 152]. Previous literature has demonstrated that skin-homing IL-13 producing T cells are increased in AD patients compared to healthy subjects [111, 203]**.** Limited literature is available with regards to modulation of the immune system following any systemic or biologic therapy for AD. Following oral cyclosporine treatment of 2 and 12 weeks, patients experienced clinical improvement (SCORAD), as well as significant downregulation in IL-13 mRNA within lesional skin[167]. However, persistent $IL\text{-}13^+$ T cells, especially in the systemic

compartment, following dupilumab has not been well characterized. In line with previously published research, our cohort of untreated AD patients have increased levels of both skin-homing IL-4 and IL-13^{+}CD4^{+} T cells compared to healthy subjects. In comparison, no significant differences were observed in CLA^{+}CD8 T cells between untreated AD patients, treated AD patients, and healthy subjects. In addition, we observed that patients under dupilumab treatment in clinical remission appear to have lower frequencies of skin-homing IL-13 producing CD4 and CD8 T cells. We sought to further determine the level of IL-13 production (MFI) by T cells, as this will impact the degree of inflammatory response during relapse and to compare whether differences are observed following dupilumab treatment. Persistent levels of IL-13 production from skin-homing T cells was detected after dupilumab treatment. Surprisingly, this was also observed from non-skin homing T cells, whereas differences for other cytokines were not observed in CLA^{-} T cells.

In line with our findings, Bangert et al. recently reported that the frequency of IL-13^{+}CD4^{+} T cells (scRNA-seq) in AD skin and IL-13 protein in skin blister fluid was significantly elevated after a year of dupilumab treatment compared to healthy subjects[14, 109]. Maintenance of elevated skin IL-13 levels in patients under remission remains to be elucidated.. Given that skin injury triggers a release of alarmins, which can promote IL-13 production, it is possible that sub-clinical inflammation in lesional skin may result in the secretion of these upstream regulators[44]. As high IL-13 levels are evidenced in patients, therapy with Tralokinumab, or a newly labelled Lebrikizumab, both IL-13 neutralizing antibodies[204], is a promising therapeutic avenue. While combination therapies are currently beyond the capacity of health systems, it may be ideal to target both receptor and cytokine in parallel. In addition to persistent IL-13, Bangert et al. found that levels of IL-22 and IL-26 remained high compared to healthy subjects, following dupilumab

treatment[109]. Both IL-22 and IL-26 are a part of the IL-10 cytokine family and are involved in tissue homeostasis during infection, upregulation of innate and adaptive immune, and tissue repair[205]. IL-26 is a newly discovered cytokine that induces inflammatory cytokine production, but also serves as a carrier for extracellular DNA and exhibits antimicrobial properties[206]. Whether additional factors, such as IL-22 and IL-26, are involved in disease persistence requires further research in order to develop effective combination therapies for a heterogeneous disease like AD.

Thus, persistent levels of IL-13 production, even in the systemic circulation, following long-term treatment with dupilumab provides evidence of residual disease activity, and highlights a potential mechanism of relapse in patients if medication is discontinued. To thoroughly address residual disease, we would need to investigate a cohort of adult AD patients before and after therapy in remission, and perform analyses on blood and skin to identify changes in immune signature. Amongst skin-homing CD4 T cells, no significant differences were observed in the proportion of IL-17 producing cells between healthy subjects, and untreated/treated AD patients, which correlated with our findings of CCR6 expression in memory CD4 T cells. The frequency $CLA^{+}IL\text{-}17^{+}$ CD4 T cells found in our cohort AD patients are similar to that described in the literature. T_H2 and T_H17 cells have been previously characterized to have reciprocal regulation of inflammatory pathways, which supports the low frequency of T_H17 cells yet high T_H2 cells in our findings[207-209].

Furthermore, we demonstrate that AD patients and dupilumab treated patients consistently have lower proportions of skin-homing IFN-γ and IL-2-producing cells, compared to healthy subjects. IL-2 is essential for T cell proliferation, differentiation, and survival, and is required nTREG cell development in the thymus (1, 11). The IL-2 receptor (i.e. CD25) is composed of an alpha, beta, and gamma chain; together they chains form the high affinity IL-2 receptor (12). On

naïve T cells, the IL-2Rb and g chains are constitutively expressed having an intermediate affinity for IL-2. Upon antigen presentation on TCR, T cells rapidly express IL-2Ra on its surface which forms a complex with IL-2Rb and g to obtain the high affinity IL-2 receptor (14). In contrast, Treg cells constitutively express IL-2Ra, which allow them to consume IL-2 to maintain Foxp3 expression (1). Our cohort of patients and healthy subjects have similar proportions of IL-2 producing CD4 T cells (~30%), and this is also observed when skin-homing T cells are stratified. Here, IL-2 may be contributing to the development and maintenance of Treg cells, but also potentiating survival of other T helper cells (TH2, TH1, TH17) in untreated patients and patients treated with dupilumab.

Contrary to our findings, Bakker et al. observed increased IFN-γ producing cells in AD vs healthy subjects, with no changes detected in patients followed dupilumab treatment[171]. We observed less IFN-γ production from CLA^+CD4^+ T cells in AD patients compared to healthy subjects. However, following treatment with dupilumab, CLA^+CD4^+Th1 cells produced significantly higher levels of IFN-γ compared to untreated AD patients, regaining MFI per cell that is similar to healthy subjects. A T_H1/T_H2 balance is required to maintain protective immunity and not be predisposed to autoimmune or allergic diseases[210]. Our data suggests that dupilumab may reset or normalize IFN-γ production per cell, thereby slightly restoring the T_H1/T_H2 deviation and contributing to improved disease symptoms. Recently, a study assessed the clinical relapse rate of AD patients following discontinuation of dupilumab (after 6 months of treatment)[172]. The exacerbation group (7/22) experienced relapse after ~ 15.6 ± 2.4 weeks , while the remission group (15/22) remained in remission for 40.5 ± 4.6 weeks after discontinuation[172]. Investigating the changes that occur in T cell subsets during remission and at relapse is critical to identify the cellular drivers of disease reactivation. Some limitations of this work include the lack of ability to

monitor medication adherence on a per patient basis. Although this was a cross-sectional study, the homogeneity within each cohort provided the ability to detect differences within T helper cell subsets. While the adult patients included in this study are representative of cohorts included in multinational clinical trials, larger cohorts are needed to fully understand the range of immune responses that may be found in specific ethnic populations and/or age sub-groups. In addition, while EASI scores were similar between untreated AD patients and baseline EASI scores of the dupilumab treated cohort (i.e. similar disease severity at baseline), our study had different cohorts of patients between untreated and dupilumab treatment (unmatched). Therefore, a longitudinal study would be required to follow the same set of patients from baseline to remission to identify changes in the immune landscape.

Thus, our study reveals that despite long-term treatment with dupilumab, AD patients have residual and persistent levels of IL-13 producing T_H2 cells compared to healthy subjects. While these patients are under remission, the factors perpetuating the maintenance of this T cell subset remains to be clarified.

To determine the skin-associated molecular factors promoting IL-13^+ T_H2 expansion in AD, we used our *in vitro* model to induce IL-13^+ T_H2 cells in an inflammatory milieu recapitulating cutaneous environmental AD disease factors. Our model employs TSLP, a skin-associated alarmin produced by keratinocytes, that is strongly implicated in the generation and expansion of T_H2 cells in AD, and *S. aureus* derived enterotoxin B, given that 75-90% of AD lesional skin is known to be colonized with *S. aureus,* which primarily produces SEB compared to other enterotoxins in AD skin[211-214].

Intracellular cytokine staining revealed that TSLP+SEB was sufficient to expand IL-13^{+} CD4 and CD8 T cells from AD and healthy subjects. Conventionally, *in vitro* T_H2 differentiation requires artefactual antibodies including anti- IFN-γ and essential cytokines IL-4 and IL-2. Here, we demonstrate that our novel *in vitro* model, consisting of TSLP and SEB, is capable of generating type 2 T cells responses without artefactual TH1 blockade, foreign antigens, and expensive purification of DCs.

While no differences in IFN-γ proportions between stimulatory conditions were observed, AD patients appear to have decreased IFN-γ^{+}CD4^{+} T cells compared to healthy subjects, which is similar to our *ex vivo* findings observed in untreated AD patients, compared to healthy subjects. In addition, we demonstrate that the combination of TSLP+SEB synergistically increases the level of IL-13 production (MFI) from AD patients, compared to untreated control or each factor alone. This suggests SEB superantigen activation of T cells in parallel with TSLP would promote IL-13 production, to further exacerbate disease in AD patients. We also show that these expanded T_H2 cells express T_H2 transcription factors (pSTAT6, GATA3), and makers of memory (CD45RO), activation (HLA-DR), and TH2-associated costimulatory factors (ICOS, OX40). Our *in vitro* model promotes the expression of co-stimulatory receptors ICOS and OX40, which are important factors in the development of T_H2 cells, as seen in AD patients[177]. Currently, there are two monoclonal antibodies (rocatinlimab and amlitelimab) in trials targeting OX40:OX40L axis; both have demonstrated clinical improvement (reduction in EASI) from baseline compared to placebo, which were safe and tolerated by patients [215, 216]. Further, stimulation with TSLP+SEB can increase the expression of master transcription factors pSTAT6 and GATA of T_H2 cells, allowing us to further investigate these cells in a disease context without the use of artefactual antibodies. Finally, our *in vitro* findings are consistent with our *ex vivo* data where AD patients had elevated levels of

activated T cells and memory cells compared to healthy subjects, suggesting that our can capture key features found in AD patients blood.

Multiple *in vitro* models of AD are now available to investigate disease etiology, however, they pose challenges as a single model cannot recapitulate both immune dysregulations and epidermal barrier alterations[217].One study demonstrated that TSLP induced increased IL-4 production from skin-homing T_H2 cells of adult AD patients (peripheral blood) compared to healthy subjects [42]. As IL-4 is an essential cytokine required for T_H2 differentiation, and not produced by dendritic cells, TSLP-induced IL-4 production rapidly promotes this process[175]. Another study showed that PMBCs, from children with AD, cultured with SEB produced high levels of IFN-γ and IL-4 as measured by ELISA[218]. To our knowledge, this is the first work demonstrate the combinatory effects of TSLP and SEB stimulation on cytokine production from AD patients PBMCs. Recently, a SEB-activated coculture model of purified CLA^+ T cells and lesional epidermal cells showed increased IL-13 production from skin-homing T cells [78]. In our cohort, SEB alone was able to induce IL-13 production from few AD patients.

Whether TSLP+SEB can induce IL-13 production from skin-homing SEB-specific Vβ T cells of AD patients must further be investigated in our model, since this will identify whether SEB-specific Vβ T cells are the major source of IL-13 in AD and further serve as a therapeutic target. Immune regulation mediated by Treg cells can also be hindered by superantigen activation as skin-homing $CD4^+FOXP3^+$ Treg cells were found to exert T_H2 properties through production of more IL-5, but less IL-10 [219]. Investigations on the impact of TSLP+SEB on Treg cell function must be performed. Limitations of this *in vitro* data includes a small sample size with variation, therefore, additional patients must be included into the cohort. In addition, while we observed an increase in cell number following TSLP+SEB treatment, it would be important to address initial

numbers of T_H2 cells at the beginning of the 7-day culture. Finally, we collected supernatants from *in vitro* cultures (4 conditions), and will perform a multiplex ELISA to determine the levels of secreted cytokines.

CONCLUSION

In this present study, we aimed to investigate whether type 2 responses persist in adult AD patients following long-term treatment with dupilumab. Using multiparametric flow cytometry, we sought to characterize T helper cell populations, specifically IL-13^+T cells, found *ex vivo* in peripheral blood of AD patients and patients treated with dupilumab. We also examined skin-derived factors that may contribute to T_H2 cell increase in AD patients. Our results demonstrate that activated HLA-DR^+ and memory $CRTH2^+$ T_H2 cells are elevated in AD patients, and persist following dupilumab treatment. Importantly, our data demonstrate persistent IL-13 production in AD patients after long-term treatment with dupilumab. T_H1 cytokine production was also increased in patients after dupilumab treatment, back to baseline levels. In addition, we showed that TSLP+SEB is sufficient to expand IL-13^+CD4^+T cells from AD patients. Our findings provide definite evidence on residual disease activity after long-term treatment (2.28 years) with dupilumab, contributing to the current gap in literature regarding potential mechanisms of disease relapse. This study will have strong implications in drug development of novel therapeutic targets to prevent disease relapse in adult patients with uncontrolled moderate-severe AD.

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