T_H17 cell

and its associated cytokines in

atopic dermatitis

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ABSTRACT

Atopic dermatitis (AD) is a common inflammatory skin disease. Autoimmunity has been suggested to play a role in the pathogenesis of AD. The recently described T_H17 cells are reported to be involved in the pathogenesis of some autoimmune diseases. We examined the expression of the TH17-associated cytokines in AD patients and explored the presence of this T cell subset particularly in chronic lesions. AD patients (acute and chronic) were recruited together with a comparable group of normal subjects. Skin biopsy specimens were taken from each. The expression of IL-17A, IL-17F, and IL-22 were studied using immunocytochemistry. Identification of IL-17A/F-producing T lymphocytes was confirmed by immunofluorescence. Using laser capture microdissection (LCM), we isolated mononuclear inflammatory cells and investigated the expression of T_H17-associated cytokines mRNA by quantitative real time PCR. We detected significantly higher numbers of IL-17A, and IL-17F immunoreactive cells in AD (especially in chronic) cases compared to controls. We successfully isolated mononuclear inflammatory cells from skin of chronic AD lesions by LCM and have demonstrated the expression of IL-17A and IL-17F mRNA, similarly. Our data suggest that T_H17-associated cytokines are highly expressed in chronic AD lesions. These cytokines might be implicated in the pathogenesis of AD, especially chronic lesions.

ABRÉGÉ

L'auto-immunité a été proposé de jouer un rôle dans la pathogenèse de la cette maladie. Il a été rapporté que les cellules T_H17, récemment décrites, participent à la pathogénèse de certaines maladies auto-immunes. Nous avons examiné l'expression des cytokines associées aux T_H17 chez des patients atteints de dermatite atopique et ainsi exploré la présence de cette sous-cellule T en particulier dans les lésions chroniques. Les patients atteints de la DA (aiguë et chronique) ont été recrutés avec un nombre égale de sujets normaux. Des biopsies de la peau ont été prises à partir de chaque groupe. L'expression de l'IL-17A, et IL-17F a été étudié par immunocytochimie. L'identification des Lymphocytes T productrices de l'IL-17A/F a été confirmée par immunofluorescence. Les cellules mononuclées inflammatoires ont été isolées par microdissection (LCM), sur lesquelles, nous avons étudié l'expression de l'ARNm des cytokines associées T_H17 par PCR quantitative en temps réel (RT-PCR). Nous avons constaté de manière significative un nombre plus élevé de cellules immunes-réactives IL-17A, et IL-17F dans la maladie DA (en particulier dans les cas chroniques) par rapport aux contrôles. Nous avons réussi à isoler les cellules mononuclées inflammatoires de la peau des lésions chroniques AD par LCM et on a démontré l'expression de l'IL-17A et IL-17F. Nos données suggèrent que les cytokines T_H17 associés sont fortement exprimées dans les lésions chroniques de DA. En conclusion, ces cytokines pourraient être impliquées dans la pathogenèse de la maladie dermatite atopique, en particulier chronique.

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CHAPTER 1

INRODUCTION

1.1 ATOPIC DERMATITIS

1.1.1 NOMENCLATURE AND DEFINITION

The nomenclature of atopic dermatitis (AD) has been a matter of uncertainty, for more than a century. Since its description as a form of "prurigo diasthésique" by Ernest Besnier in 1892, contributors to the literature have attributed more than 20 different names to this disease. The term "neurodermatitis" was coined, based on Jacquet's erroneous hypothesis that it is "an itch that rashes". Together with Freud's theories, this led to decades of mis-conceptualization of AD as a disease of psychological instability, until newer studies made this notion obsolete. The concept of allergic association and "atopy", emerging in the 1920s, led Wise and Sulzberger, in 1933, to create the term "atopic dermatitis", to signify its association with respiratory atopy. It is still in use, interchangeably with "atopic eczema" (1). While the word eczema is derived from a Greek word (***ekzeō***) meaning to effervesce or literally to "boil over"; signifying the histopathological hallmark of the disease: spongiosis, the word atopy is derived from another Greek word (***ατοπία*** atopos) meaning out of the place; indicating the widespread nature of the disorder (2). However, there is no general consensus of a definition of the term "atopy". The European Academy of Allergology and Clinical Immunology (EAACI) defines it as a personal and/or familial tendency to become sensitized and produce IgE antibodies in response to ordinary exposures to allergens (3). In contrast,

extending it beyond the limits of IgE is worthy. So, atopy can be defined as a genetically predisposed diathesis (i.e. constitutional predisposition) to a variety of environmental stimuli (irritants, allergens, and microbes), manifesting as exaggerated responses (eg, bronchoconstriction, IgE production, vasodilatation, pruritus). The impact of such extension of the definition on systematic clinical research is great. Furthermore, the "atopic" term was not flawless, as it led to another misconception strictly linking the disease with allergy, resulting in consequent over enthusiasm to study that link, instead of concentrating on the mechanisms underlying the pathological changes. In spite of the lack of convincing evidence for a role of IgE in the development of AD lesions, many researchers have been sticking to the thought that allergy is somehow involved (4).

On the other hand, the terminology of AD is also controversial. Different names have been used at different times and different countries. The EAACI coined a new term, "atopic eczema/dermatitis syndrome" (AEDS), which encompasses two types; allergic (IgE-associated) and non-allergic. They recommended reserving the term AD for the allergic, IgE-associated forms of the diseases (3). Later on, it has been suggested to substitute the term AEDS by the term ***eczema***. The latter is further divided into atopic or non atopic, based on the presence or absence of IgE-associated reaction, respectively (5). AD is a highly pruritic, chronic inflammatory skin disease that usually begins early in life and is characterized by eczematous lesions, xerosis (dry skin), and lichenification (thickening of the skin and increase in skin markings). AD is often considered as the cutaneous manifestation of a systemic disorder; the atopic triad, also comprising asthma, and allergic rhinitis, seen in approximately 80% of people affected with AD. These conditions are characterized by elevated serum IgE levels and peripheral eosinophilia (6).

1.1.2 EPIDEMIOLOGY

Interest in this disease has increased by reports of its growing prevalence and the significant drawbacks it may have on quality of life. The difference in designs of various studies assessing the prevalence of AD may make it difficult to compare their results. However, considerable growth since initial description is clear; more than twenty fold in some industrialized societies, over the last decades (7). Although many investigators have reported ongoing increase, some recent studies have suggested stable figures of AD (8).

Furthermore, the lifetime prevalence of AD is markedly variable between different countries, and even within the same nation. However, as a rule, the prevalence of AD in urban areas within industrialized countries is much higher than in agricultural regions in developing nations, such as China, Eastern Europe, and rural Africa. For instance, prevalence ranges from less than 1% in Tanzanian children 7 to 18 years old living in rural areas (9) to about 32% by age 10 in a dry, inland Australian town (10). Moreover, ethnic variations have been well documented but not universally accepted. The frequency of the disease has been reported to be higher in black Caribbean children (11).

Although AD is an infancy and childhood disease, it might occur in or continue during adulthood. It might begin during the first 6 months, first year of life, or before the age of 5 years in about 45%, 60%, and 85% of children, respectively (12). However, its prevalence in adults is not well defined. While a highly selected group in Japan showed point prevalence in adults to be 3% (13), a recent US study reported it to be as low as 0.8% (14). While some reports have indicated slight female predominance, differences related to gender are not confirmed yet (15).

Moreover, the prevalence of AD has been reported to correlate with socioeconomic class. Higher rates of the disease have been reported from Switzerland and Britain (16). This might be due to more thorough medical care, better immunization programs, more allergenic home atmosphere, and smaller family size (17).

Regarding the consequences of AD, they are literally serious. It negatively affects all aspects of family health; social, personal, emotional, and financial. Care for a child with AD of moderate or severe degree is more stressful to family than taking care for diabetes mellitus type 1 (18). Itching is habitually underscored in terms of the troubles it might produce. However, pruritus has the strongest negative impact on quality of life, often resulting in deprivation from sleep, disturbed growth, and disruption of social development and recreational activity as well as loss of employment, worries about appearance, feelings of guilt and frustration as a result of the disease affecting the child, and time-consuming treatment (19). Of all childhood dermatoses, only scabies impacts a child's quality of life more than AD (20). However, scabies is a treatable skin infestation which is time-limited, as most patients would get rid of it after a short course of treatment. The consequences of total sleep deprivation on human health are well recognized, but recently, recurrent, partial sleep disturbance (the type of disturbance commonly associated with AD) has been reported to cause significant neuropsychiatric illness (21). Economically speaking, AD is a costly disease that clearly causes a major financial load to both individual families and national health care systems. Taken together the direct and indirect costs of AD are significant and likely comparable to other diseases with large annual economic burdens such as asthma and psoriasis. A recent Canadian estimate is in the range of 1.4 billion $ a year (22).

Traditionally, AD is mostly considered a problem of early childhood. However, prevalence studies have suggested that the decline in AD frequency throughout childhood is not as sharp as generally proposed. On the contrary, incidence appears to drop considerably in the late childhood period (23).

Williams and his colleague have reviewed the literature on the natural history of AD and concluded that "the quality of the studies done so far is generally poor in terms of small sample size, lack of disease definition at inception and follow-up, length of follow-up being too short, heavy losses to follow-up, lack of adjustment for recurrent disease and selection bias in the initial cohort" (24). On the other hand, most of the well designed, large studies of AD imply that almost 60% of cases of childhood AD are either clear or free of symptoms early on adolescence (25), with estimated clearance rates varying greatly, ranging from 17% (26) to 92% (27). However, recurrence during adulthood is quite common (28) Disease recurrence can be predicted by association with poor prognostic factors. This includes early onset, severe extensive disease earlier in life, personal history of asthma or hay fever, a family history of AD and living in urban areas (28).

Moreover, long term follow-up revealed a subset of children with AD who go on to develop adult hand eczema, particularly in the context of occupational irritants. Risk factors include a history of hand dermatitis before the age of 15, persistent adult AD, adult xerosis, and a history of irritant exposure (25).

1.1.3 CLINICAL DIAGNOSIS

Although clinical manifestations rely on age, the main symptom in all cases is intense pruritus. While acute lesions show all the signs of eczema, chronic lesions typically exhibit skin lichenification. Both lesions have a typical distribution pattern depending on the patient's age, with face and extensors being involved in children, and flexural surfaces in adults. The disease course is marked by flares and remissions (29).

As we lack pathognomic laboratory tests, diagnosis of AD mainly depends on the broad identification of constellations of features. Moreover, establishing reliable and valid diagnostic criteria is also of utmost importance for epidemiological, aetiological, and therapeutic studies' results and reproducibility. In 1980 Hanifin and Rajka structured a set of diagnostic criteria (Table 1). Later on, different lists of diagnostic criteria for AD have been proposed (Table 2). Hanifin and Rajka's have been considered to be the most utilized, being used in more than forty percent of published clinical trials. However, consistency in the use of diagnostic criteria for AD is missing. In more than 20% of the clinical trials available in the literature, criteria used to diagnose AD were not specified. Moreover, validation studies reviewed, lack unified methodological quality. A recent systematic review of validation studies found the U.K. diagnostic criteria to be the best and recommended its use; it is simple and can be used by non-physicians (30). However, it lacks the necessary precision for clinical or genetic ascertainment (6). In contrast, a recent simplified set of criteria fitting different ages and ethnicities was developed by an American Academy of Dermatology consensus conference (31). Overall, the ideal set of diagnostic criteria is not yet found.

Table (1) Hanifin and Rajka criteria for the diagnosis of atopic dermatitis (166):

"***Must have three or more major features***

Pruritus

Typical morphology and distribution

Facial and extensor involvement in infants and children

Flexural lichenification or linearity in adults

Chronic or chronically relapsing dermatitis

Personal or family history of atopy (eg, asthma, allergic rhinitis, atopic dermatitis)

Plus three or more minor features

Xerosis

Ichthyosis / palmar hyperlinearity / keratosis pilaris

Immediate (type I) skin test reactivity

Elevated serum IgE

Early age of onset

Tendency toward cutaneous infections and impaired cell-mediated immunity

Tendency toward nonspecific hand or foot dermatitis

Nipple eczema

Cheilitis

Recurrent conjunctivitis

Dennie-Morgan infraorbital fold

Keratoconus / Anterior subcapsular cataracts

Orbital darkening

Facial pallor / facial erythema

Pityriasis alba

Anterior neck folds

Itch when sweating

Intolerance to wool and lipid solvents

Perifollicular accentuation

Food intolerance

Course influenced by environmental/emotional factors

White dermographism/delayed blanch"

Table (2) Criteria lists used to diagnose AD (30)

Criteria list Requirements	Number of criteria
Hanifin and Rajka diagnostic criteria, 1980	3 major + 3 minor
Kang & Tian diagnostic criteria, 1989	1 basic + 3 minor
Schultz-Larsen criteria, 1992	≥ 50 points
Lillehammer criteria, 1994	Visible eczema + 4 minor
U.K. diagnostic criteria, 1994	Pruritus + 3 minor
ISAAC questionnaire, 1995	Score ≥ 3
Japanese Dermatology Association criteria, 1995	All 3 features
Criteria of Diepgen, 1996	≥ 10 points
Millennium diagnostic criteria, 1998	Allergen-specific IgE + 2 principal
Danish Allergy Research Centre (DARC), 2005	3 features

In contrast, objective assessment of the severity of AD is of utmost importance in daily practice, specially to evaluate the disease improvement while and after being treated. Too many published outcome measures are available in the literature to evaluate AD. Unfortunately, in most cases they are not tested properly. A recent systematic review evaluated twenty different names of disease severity and concluded that only SCORAD (SCORing Atopic Dermatitis), EASI (Eczema Area and Severity Index), and POEM (Patient-oriented Eczema Measure) currently perform adequately. The authors recommended that these scales should be used in future studies (31).

1.1.4 HISTOPATHOLOGY

The histologic features of AD depend largely on the duration of the skin lesion. Interestingly, uninvolved or clinically normal-appearing skin of patients with AD is histologically abnormal. Basically, there is mild hyperkeratosis and a sparse perivascular cellular infiltrate consisting primarily of T_H2 lymphocytes (33, 34).

Acute lesions are characterized by marked spongiosis (intercellular edema) of the epidermis together with a light epidermal infiltrate consisting mostly of T lymphocytes. In the dermis, there is a marked perivascular inflammatory infiltrate, consisting predominantly of T lymphocytes. The lymphocytic infiltrate is composed mainly of memory T cells bearing CD3, CD4, and $CD45RO^+$. Macrophages are occasional. Eosinophils, basophils, and neutrophils are rarely present. Mast cells, in various stages of degranulation, are present in normal numbers (33, 34).

On the other hand, chronic lichenified lesions have hyperplastic epidermis with elongated rete ridges, prominent hyperkeratosis, and minimal spongiosis. In addition, there is an increased number of IgE-bearing Langerhans cells in the epidermis, and a dermal mononuclear cell infiltrate dominated largely by macrophages. The number of mast cells is increased compared to controls and they are generally fully granulated. Increased numbers of eosinophils are also observed (33, 34).

1.1.5 PATHOPHYSIOLOGY

Although AD is a widespread problem, its etiology and pathogenesis have not been fully elucidated yet. The pathophysiology of AD involves a multifaceted interplay between a mixture of susceptibility genes, neurologic, psychologic, and immunologic factors, as well as various endogenous and exogenous triggers like host environment and infectious agents (35). Although understanding the immunopathology of AD is still defective, a lot has been done by investigating the cell types and inflammatory mediators in AD skin lesions. AD is immunologically complex, involving interaction of multiple cell types; like T cells, dendritic cells, macrophages, keratinocytes, mast cells, and eosinophils (36).

At least two types of AD have been identified: an extrinsic (affecting 55–84 % of patients), and a less common intrinsic, which affects 16–45%, depending on the country and the criteria for definition. Both have associated eosinophilia. The extrinsic one has IgE-mediated sensitization against both inhaled and food allergens, and/or associated allergic rhinitis / asthma. T cells, expressing CLA (cutaneous lymphocyte-associated antigen), produce higher levels of T_H2 cytokines, including IL-4 and IL-13, which stimulate IgE isotype switching. They also produce IL-5, essential for eosinophil development and survival. However, the level of IFN-γ is abnormally low. In contrast, the intrinsic subtype frequently starts later in life (>20 years) and lacks sensitization of IgE to air or food allergens. Concomitant IL-4 and IL-13 production in the skin is less marked, but increased peripheral blood levels indicate the predominance of a T_H2 immune response (37).

AD has a high familial occurrence with a strong maternal influence, with a 2-fold higher risk for a child to develop AD in cases where one parent is affected and a 3-fold increase if both of them are suffering. Parental AD grants a considerably more chance to offspring to get AD, with higher incidence of severe affection than does parental asthma or allergic rhinitis. This implies the existence of genes specific to AD. Moreover, usually the disease has very early age of onset, and twin studies have shown high concordance rates of 77% in monozygotic twins and 15% in dizygotic twins. Taken together, these findings indicate that a complex genetic background plays an important role in AD development. No single gene can be accused. Rather, AD development would likely depend on the interaction of several genes with various environmental factors (38). To date, two different approaches have been used to identify these potential genes. The first is based upon genome-wide screens identifying broad regions of the genome linked with AD phenotype. This "linkage analysis" is followed by "positional cloning" studies. No substantial overlap has been detected between the results of the four genome-wide screens for AD, done so far, in four different populations (39). Moreover, most of these associations might not give effective identification of specific genes when examined in more detail. In contrast, the other approach "candidate gene studies" is used to narrow the focus. It is restricted to a single, already known, gene locus and investigates the association of its polymorphisms with AD phenotype. Potential atopy genes include components of the allergic response. Many loci have been identified. Overall, genetic studies done to date focused more on IgE/T_H2 associations than on AD specifically (40).

However, an exceptionally major breakthrough in understanding the genetic basis of AD came when researchers documented that 2 common polymorphisms in the filaggrin

gene (FLG) represent strong predisposing factors for that common disease. FLG produces filaggrin, a key component of the granular cell layer of the epidermis in human skin, which serves as a template for the assembly of the cornified envelope. Deficiencies in that protein leads to defective skin barrier function. This emphasizes the role of the barrier defect in AD and explains its constant association with xerosis (41). The fact that filaggrin deficiency is only present in certain proportion of the AD patients, and those affected outgrow their disease dictates that other players are involved. An appropriate skin barrier function is established by the interaction of cytoskeletal proteins (eg, filaggrin), intercellular lipids (eg, ceramides), and a set of epidermal proteases (eg, the stratum corneum chymotryptic enzyme). Defects in each of these components has been shown to be a possible pathogenetic factor in the causation of barrier function defect, and consequently of AD. For instance, ceramides, the major water-retaining molecules in the extracellular space of the cornified envelope, are reduced in the skin of AD patients, whether lesional or not (42). Such defects in barrier function ultimately leads to a dual effect: increased trans-epidermal water loss as well as increased antigen absorption. While the former leads to skin dryness, a hallmark of the disease even affecting non-involved skin, that stimulated investigating the impairment of skin barrier function as a cause of AD, the latter is assumed to contribute to the cutaneous hyperreactivity characteristic of the disease (43).

An interesting question, which is a matter of strong debate right now, is about the possibility that a genetically determined defect affecting the epidermal barrier could be the primary cause in AD pathogenesis. Researchers supporting this theory claim that this defect is an important requirement for the entrance of large protein environmental

allergens into the epidermis, and their subsequent uptake by antigen-presenting cells, for eventual production of an effective T-cell response. Some of these allergens can stimulate the production of proinflammatory cytokines, shift the immune response in a T_H2 direction, or both. On the other hand, certain proinflammatory cytokines have been reported to modulate the expression of genes of the epidermal barrier, such as FLG (44).

The role of immunity in AD was raised as early as 1906, when von Pirquet and Schick described allergy. Since then, this disease has been largely considered an immunologically-mediated disease. This concept is supported by many observations. Patients suffering primary disorders involving immunodeficiency of T cell often have high serum levels of IgE, eosinophilia, and eczematous skin lesions markedly similar to AD. In Wiskott-Aldrich syndrome, successful bone marrow transplantation is followed by correction of the immunologic defect with subsequent disappearance of the rash. Additionally, non-atopic recipients getting bone marrow transplants from atopic donors have been shown to suffer positive immediate skin tests as well as atopic manifestations after successful grafting. findings imply that AD is mediated by cells derived from the bone marrow– (45).

The initial mechanisms that induce skin inflammation in patients with AD are unknown. The cascade might be induced by neuropeptides, irritation, or pruritus, inducing scratching. Proinflammatory cytokines might be released from keratinocytes. Furthermore, the whole process could represent a series of T-cell-mediated reactions to allergens, either in the disturbed epidermal barrier or in food, independent on IgE. In addition, dysfunction of the epidermal barrier represents an obligation for various

allergens to penetrate into the skin and subsequent stimulation of dendritic cells leading to T_H2 polarization in the skin, and even in other organs like the lungs (46).

Both arms of the immune systems; the innate and the adaptive, are implemental in the pathogenesis of AD. Patients display obvious defective innate immune reactions, leading to increased vulnerability to various types of infections whether viral, bacterial or fungal. The quite high rate of Staphylococcus aureus colonization of the skin in AD patients, compared with only 5% colonization in healthy people represents a clear illustration of that situation (47). Moreover, patients with AD are predisposed to eczema herpeticum and eczema vaccinatum (48). Such findings have been explained by the fact that the inflammatory micromilieu initiated by cytokines in AD down-regulates families of antimicrobial peptides like defensins, cathelicidins and dermcidin in the skin of patients suffering the disease (46). Accumulating evidence also documents the abnormality of the adaptive immune system, including its different components. Figure (1) illustrates our current understanding of the immune dysregulations in AD.

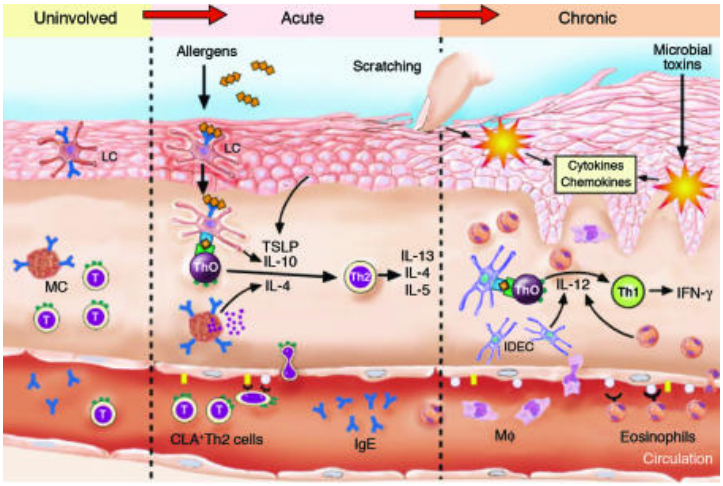

Figure (1). Immunologic pathways in AD.
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Aside from their function in the innate immune response, dendritic cells (DC) are also crucial in the initiation of adaptive immunity. The latter is also controlled by lymphocytes, both T and B, in addition to many cytokines and chemokines. Epidermal DC populations described in AD lesions are CD1a+ and involve the myeloid DCs: Langerhans' cells (LCs) and inflammatory dendritic epidermal cells (IDECs). Both types express elevated levels of the high affinity receptor for IgE, on their surface (Fc epsilon receptor I; FcεRI). Such finding could not be detected in healthy skin and could be either due to high serum levels of IgE detected in patients with AD or an impaired phenotype producing TGF-β, as both situations have been reported to be involved in *in vitro* stabilizating FcεRI on LC and IDEC. The clinical relevance of FcεRI-positivity is documented by its necessity to incite an eczematous reaction after application of aeroallergens in the atopy patch test. This relation may be explained by the decreased skin barrier function in AD patients, where varied allergens with diverse specificities could easily penetrate into the epidermis. The FcεRI-bound IgE molecules on epidermal LCs would take up and process such allergens, before targeting them into the histocompatibility class II molecules (49).

Furthermore, FcεRI-activated LCs also increase the migratory capacity of T_H0 monocytes. The latter represent the precursor cells of IDEC. Chemotactic signals, including monocyte chemotactic protein 1 (MCP-1) and interleukin-16 are essential in this process. In turn, FcεRI-activated IDEC convert T_H0 into IFN-γ-producing T cells (T_H1) that secrete IL-12 and -18. They also serete big amounts of pro-inflammatory cytokines and chemokines. This includes IL-1α/β, IL-6 and TNF-α (tumour necrosis factor-α), and may thus augment the inflammatory immune response in the AD skin. This

mostly indicates that IDEC could be significantly concerned in the change of the T_H2 into the T_H1 immune reaction. This gives a clue that LC and IDEC function in a different way in the pathogenesis of AD. While FcεRI+ LCs are critical in the initiation of the reaction, where T_H2 cells predominate, IDECs might be more crucial later on, when T_H1 become involved to a larger extent (50).

IgE-bearing CD1a+ DCs that have captured antigen/allergen may either activate memory T_H2 lymphocytes directly in atopic skin, or expand the pool of systemic T_H2 lymphocytes after migration into the draining lymph nodes by driving T_H0 into IL-4-producing T cells. They also secrete chemokines, like IL-16, MCP-1 (monocyte chemoattractant protein 1) / CCL2, TARC (thymus and activation–regulated chemokine) / CCL17, and MDC (macrophage-derived chemokine)/ CCL22. Such products might lead to the attraction of other pro-inflammatory cells into the sites of inflammation within the skin. It has been shown that in lesional skin of AD, there was a significant increase in the number of CD1a+ DC within the epidermal layer compared with the controls, reflecting the augmented antigen-presenting function of these cells (51).

Another type of DCs; plasmacytoid dendritic cell (PDC), has been found to be less prevalent in AD lesions compared with IDECs, possibly due to decreased expression of skin-homing molecules such as cutaneous lymphocyte antigen and the L-selectin, CD62L, with subsequent apoptosis. This selective lack of PDCs (that normally produce IFN-α and –β) in AD lesions together with lower level of type-I IFN upon stimulation, may be one of the reasons for the high susceptibility of AD patients to viral skin infections (49).

Among cells, T lymphocytes have a pivotal role in the etiopathogenesis of AD. Many studies documented that both in humans and in animal models. T cells in the affected skin switch on effector cytokines and induce the activation and apoptosis of keratinocytes (52). Although the T_H1/T_H2 paradigm has been a main addition to our understanding of immunologic mechanisms, the actual processes may be more complicated. AD patients may develop a biphasic helper T-cell pattern. T_H2 cytokines predominate early in the acute stage. Over time, a switch to a more T_H1-like profile occurs in chronic cases, with higher levels of IFN-γ. Although significant increase in cells expressing mRNA for IL-4, IL-5, and IL-13 is evident in acute and chronic lesions of AD, IFN-γ mRNA expression is similar to normal skin. Nonetheless, cells expressing mRNA for IL-4 and IL-13 are significantly fewer in chronic versus acute lesions. In the mean time, cells expressing mRNA of IL-5 and IL-12 are significantly increased in chronic cases compared to acute. Such findings mean that maintaining chronic inflammation in skin lesions of AD could be associated with the predominant expression of IL-5 and IL-12. The latter may lead the switch from T_H2 dominance during the acute phase of the disease to more T_H1 involvement during the chronic stages (53).

Lately, a new subset has been added to the CD4+ T helper cell family. This novel fellow, the T_H17, has been identified based on the ability of T_H17 cells to produce IL-17A, and IL-17F. Of special note, T_H17 cells have also been associated to the progress of autoimmune disease, a function until that time ascribed to IFN-γ and T_H1 cells (54). Few reports investigated the association between IL-17 and AD, indicating that IL-17 expression is upregulated in AD compared with healthy controls skin (55-59). See section 1.2.1 on T_H17.

T regulatory cells (T_{reg}) represent another subset of the CD4+ T cell family that has been shown to be involved in AD. Compared to healthy control subjects and asthmatics, AD patients have more CD4+CD25+ T_{reg} cells in peripheral blood (60). Moreover, immunohistochemistry of chronic AD skin lesions shows dense perivascular infiltrates of CD25+ cells in the papillary dermis. On the other hand, FoxP3+ cells are found in the dermis, perivascularly and interstitially, in the epidermis and along the dermoepidermal junction. In contrast, skin of healthy controls show weaker staining with either CD25 or FoxP3 (61). Therefore, a defect in T_{reg} cells does not seem to be implemental in the pathogenesis of AD.

The role of eosinophils in the pathogenesis of AD has been debated. Some it has been suggested that eosinophil degranulation occurs in the skin (62). However, other groups claim that it might take place in the blood, bone marrow, or both (63). Increased levels of circulating eosinophils and eosinophil granule proteins have been reported both in sera (64) and in urine (65) of AD patients. Furthermore, those levels correlate with disease activity and response to therapy for the disease. Acute exacerbations of AD are associated with increased blood levels of IL-5, a T_H2-derived cytokine that induces formation, activation and chemotaxis of eosinophils. Moreover, eosinophil chemotaxins, e.g. eotaxin, increase in the peripheral blood of AD patients (66). The ultimate result is eosinophil extravasation through selectins and their recruitment to the skin (67). The presence of high levels of eosinophil cationic protein, major basic proteins and eosinophil-derived neurotoxin in AD skin lesions represents an evidence of eosinophil degranulation and eosinophil cytolytic degeneration, inspite of the low number of intact eosinophils (68).

The localization (homing) of the pathogenic inflammatory cells to the sites of inflammation in the skin in AD is likely to be regulated, as in various inflammatory diseases, by the expression of specific cell adhesion molecules (CAM; receptors) on these cells. The level of these CAM is higher in the peripheral blood of AD patients. The main adhesion molecules essential in the process of T cells homing to inflammatory sites involve ICAM-1 (intercellular adhesion molecule), VCAM-1 (vascular cell adhesion molecule), α-6 integrin, and IL-8 (69, 70). However, the most important CAM in this regard is CLA (cutaneous lymphocyte-associated antigen) that interacts with E-selectin, a vascular endothelial cell surface antigen on inflamed dermal postcapillary venules, to guide circulating T lymphocytes to areas of skin inflammation, and identifiess a group of circulating memory T cells, on experiencing foreign pathogens or allergens. Higher numbers of activated CLA+ T cells are reported in the blood of patients with AD (71).

Moreover, chemokines play a major role in determining this process. Increased expression of IL-16 attracting $CD4^+$ T cells has been demonstrated in acute lesions. Furthermore, the CC chemokines MCP-4 (*m*onocyte *c*hemotactic *p*rotein-4) / CCL13, eotaxin / CCL11, and RANTES (*r*egulated on *a*ctivation, *n*ormal *T* cell *e*xpressed and *s*ecreted) / CCL5 have been shown in lesional skin of AD patients. They mainly help in the attraction of $CCR3^+$ eosinophils and T_H2 cells (72). Moreover, the keratinocytes chemokines are also involved in this process of DC activation. The MDC/CCL22 (*m*acrophage-*d*erived *c*hemokine) and TSLP (thymic stromal lymphopoetin), produced by keratinocytes, have been demonstrated to be increased in AD (73, 74). These chemokines contribute to the activation of CD11c+ DC. The ultimate result is the production of such chemokines as TARC/CCL17 (thymus activation-regulated chemokine) and favouring

the careful employment of CCR4+ TH2 cells into the skin. Recently, it has been reported that MIP-4 (macrophage inflammatory protein)/CCL18 is the most highly expressed chemokine in AD skin (75). It is regulated by *Staphylococcus aureus* products and claimed to have an important role in the initiation and amplification of skin inflammation. Furthermore, the interaction of the skin-associated chemokine CCL27 and its receptor CCR10 has been shown to mediate the chemotactic response of skin-homing T cells in vitro and in vivo (75a).

The deviated immune response in AD can be observed both on systemic and local levels. Systemic elevation of T_H2 immune response has been detected, indicated by higher frequency of allergen-specific T cells that can produce IL-4, -5 and -13, with lower figures of T-cells producing capable of production of IFN-γ, (53). IL-4 has been shown to be essential in IgE isotype switch. However, IL-5 attracts eosinophils and prolongs their survival. It is likely that this might explain the increased IgE serum levels and the peripheral blood eosinophilia observed in many patients suffering AD (76). T_H2 predominance in those patients might be related to apoptosis of circulating T_H1 cells observed in these cases (52). Moreover, high numbers of circulating regulatory T cells (Treg) that show normal in vitro immunosuppressive function have been demonestrated. Induction of such Tregs with Staphylococcus aureus superantigen led to a loss of immunosuppressive function in patients suffering AD, which may be involved in the augmentation of T cell activity in this disease (60).

Locally, high amounts of mRNA for IL-4, IL-5 and IL-13 are detected in both affected and unaffected AD skin. Moreover, in acute AD skin lesions, mRNA for T_H1 cytokines such as IFN-γ and IL-12 were not significantly present. In contrast, chronic AD

skin lesions demonstrated increased levels of mRNA for IFN-γ, IL-5, IL-12 and granulocyte–macrophage colony-stimulating factor (GM-CSF). These cytokines prolong the survival of eosinophils, macrophages, monocytes and LC (53, 77).

This is the basis for the hypothesis of a biphasic mode of AD, with an acute phase, predominantly T_H2-mediated and a chronic one, mostly T_H1-dependent. This notion has got more support by the results of the atopy patch test; where aeroallergens are applied on the skin to cause eczematoid reactions in sensitized patients (78).

Several factors are suggested to precipitate immune dysregulation leading to AD.

Stress-induced immunomodulation is distorted in AD patients, even though the exact mechanisms are not well established. This might be due to neuroimmunologic factors. Substance P has not only been found in the plasma of AD patients but also was shown to correlate positively with the disease activity. Moreover, high levels of brain-derived growth factor (BDGF) have been detected in both sera and plasma of AD patients. BDGF decreases eosinophil apoptosis but increases eosinophils chemotaxis (79).

Food allergens produce rashes in about 40% of children with moderate to severe disease. Moreover, it is well accepted that food might worsen AD through both allergic and nonallergic hypersensitivity mechanisms. Foods like egg, milk, wheat, soy, and peanut are amongst the most disreputable. Patients frequently outgrow food allergy after the age of 3 years (80).

Autoallergens could also play a role in the pathophysiology of AD. Although the immune dysregulation can be initiated by environmental allergens, the process of allergy

can continue thereafter autonomously. Skin damage in severe chronic AD might expose de novo epitopes that act as endogenous antigens. These autoallergens might start immune responses mediated by either IgE or T cells (81).

Furthermore, **infection** has been claimed to be implemental in the causation of AD. Most AD patients are colonized with *S aureus* and have flares of their skin lesions after staph infection. Moreover, treatment of these patients with antibiotics with a good antistaph coverage, can lead to amelioration of skin lesions. Superantigens, stimulating activation of T cells and macrophages, represent an essential mechanism of the staph action in AD lesions. Specific anti-staphylococcal superantigens IgE antibodies correlate with the severity of skin disease. Moreover, superantigens are responsible for corticosteroid resistance. This complicates the process of treating staph-infected patients. In contrast, treatment with topical corticosteroids or tacrolimus decreases *S. aureus* counts in AD skin. This implies that skin inflammation in AD increases *S. aureus*-skin binding (82).

Another explanation of increased prevalence of skin infections in AD stems from the fact that AD skin has been found to be deficient in antimicrobial peptides. The latters are essential for host defense against different microorganisms like viruses, bacteria and fungi. Remarkable upregulation of T_H2 cytokines in AD might be involved in this process. Taken together with the lower levels of proinflammatory cytokines, like TNF-α and IFN-γ, this could clarify the pathophysiology of increased susceptibility to skin infections in AD patients compared with psoriatic patients (83).

Moreover, patients with AD have also an increased tendency toward disseminated infections with herpes simplex that might be linked to the severity of atopy (48). In addition, it has been shown that the Malassezia species, an opportunistic yeast, might be a contributing factor in the pathogenesis of AD skin lesions (84).

Worth mentioning, AD has been related to respiratory atopy. The term "atopic march" refers to the sequential development of AD, food allergy, asthma, and allergic rhinitis. AD has been shown as a risk factor for future development of allergic airway disease in longitudinal studies; however, the mechanisms are not yet clear (85). Animal models have clarified that in contrast to sensitization by respiratory or intraperitoneal routes, allergen sensitization through the skin yields several-fold elevated levels of IgE. Moreover, inhalational challenge with protein antigen, after cutaneous sensitization, leads to pulmonary inflammation and increased responsiveness to methacholine challenge (58, 86). Collectively, these findings suggest that skin disruption in AD, might represent an avenue for antigens, with subsequent sensitization, leading to allergic responses in other organs, far beyond the skin.

1.1.6 Management:

Successful **management** of AD requires a multifaceted approach. ***Avoidance*** of irritants and specific immunologic stimuli is a prerequisite to any successful treatment of AD (87). As mentioned before, aeroallergens and foods, might either induce the AD or trigger the itch-scratch cycle perpetuating it,. ***Hydration*** of the skin using emollients to repair the impaired barrier function of the skin is another key in managing AD. "Ceramide-dominant" emollients have been shown to result in amelioration of skin lesions when added to standard regimens in children with severe resistant AD. Reduced transepidermal water loss and improved stratum corneum integrity have also been evident (88). The addition of ***antibiotics*** to anti-inflammatory therapy leads to better results upon treating skin disease in cases of *S. aureus* colonization or infection (89).

However, the cornerstone in successful control of AD on the long-run is the use of effective ***anti-inflammatory*** therapy. *Topical glucocorticoids* remain the basis in this regard, showing efficacy to control both acute and chronic phases of skin inflammation. They produce their anti-inflammatory action mainly through regulation of corticosteroid-responsive genes, which is done using two major actions. The first is called “transactivation”, inducing gene transcription, which is mainly responsible for the unwanted side effects of glucocorticoids disturbing its use in maintenance therapy. The second one, transrepression, inhibits transcriptional activity of different genes encoding proinflammatory proteins such as cytokines, chemokines, and adhesion molecules. This is the major route underlying glucocorticoids anti-inflammatory mechanisms (90).

Relatively recently, the FDA approved *topical calcineurin inhibitors*, as tacrolimus and pimecrolimus, to treat AD. These act by binding macrophilin with high affinity, inhibiting the activity of calcineurin; a calcium-dependent phosphatase. This ultimately suppresses the activation of a number of key effectors involved in the pathogenesis of AD, including T cells and mast cells. Short- and long-term, multicenter, blinded, vehicle-controlled trials, in both adults and children, comparing both drugs with corticosteroids, have documented both topical tacrolimus and pimecrolimus to be effective and safe, with minimal side effects, (91). However, being immunosuppressors raises concerns about their long-term effects. In February 2005, the FDA announced serious worries about the potential long-term drawbacks of these products and issued a 'public health advisory' (92). On the other hand, the American Academy of Dermatology Association Task Force reviewed the important figures and concluded that "there is no causal proof that topical calcineurin inhibitors cause lymphoma or nonmelanoma skin cancer." (93). On the other hand, even though systemic absorption of such products is minimal, there is a necessitt for thorough observation to exclude the possibility that increased viral skin infections and even skin cancers might emerge when such agents are used on long-term basis(94).

Cyclosporin A, a potent *systemic calcineurin inhibitor*, has been used to treat AD. Several studies have shown its efficacy in different age groups with harsh, recalcitrant AD. Nonetheless, toxicity, above all affecting the kidneys, decreases chances of its use for long periods. On the contrary, preliminary data indicate that a related product, oral pimecrolimus, might enjoy a higher safety index than either systemic cyclosporin or tacrolimus. This could make it more satisfactory to some AD patients. Antimetabolites, including mycophenolate mofetil, a purine biosynthesis inhibitor, methotrexate, and

azathioprine, have also been used for recalcitrant AD, but potential systemic toxicities restrict their use and require close monitoring (95).

The use of UV light therapy has proven successful in treating chronic irresponsive AD. Key cells, eg LCs and keratinocytes, represent ideal targets for the UV. The ultimate result is suppression of cytokine production and downregulation of activation markers as HLA-DR and IL-2 receptor on CLA^+T cells. High-dose UVA1 therapy has been demonstrated to be more effective than the classical UVA/UVB therapy that appears less effective for acute exacerbations. In contrast to the latter that acts primarily in the epidermis, the former suppresses LC migration out of the epidermis and significantly reduces dermal IgE-binding cells, including mast cells, LCs, and DCs (96).

Allergen-specific desensitization has been claimed to have clinical benefits in AD. However, there was no consistent efficacy of immunotherapy compared to placebo in the treatment of AD in double-blinded, controlled trials. Recently, a humanized IgG_1 monoclonal antibody against IgE, omalizumab, has been developed. It binds to and blocks an epitope in the IgE-CH_3 region. This particular area is necessary for binding to the high-affinity FcεR on both mast cells and basophils. The biologic, omalizumab has been successful in allergic asthma and rhinitis. Hence, it might counteract the effects of IgE in AD too. Nevertheless, the elevated levels IgE in serum, in cases of AD might reduce the efficacy of this product. Even though, it could have a job in combating food-induced AD. A cohort of patients allergic to peanut treated with omalizumab, showed significant increase of of the peanut sensitivity threshold on oral food challenge. This implies a potential defense against unintended ingestion of the food allergen (97).

Actually, we are in real need for new, more effective and meanwhile safer therapeutic approaches to both prevent evolution of AD to more severe forms of this common skin problem and stopping what is known as ‘atopic march’, leading to the progress to asthma. The advancement of novel, goal -directed treatment options relies on an expanding understanding of the cellular and molecular features of AD. This comprises better descriptions for the different clinical phenotypes of AD, including recognition of the susceptibility genes producing different forms of AD and characterization of the relative role of immunoregulatory anomalies and structural defects in skin barrier that might underlie the development of AD skin. Most of the innovative advancements target components of the allergic inflammatory response. This might involve modulating cytokines, inhibiting inflammatory cell employment, and blocking activation of T-cell (98).

1.2 T HELPER CELL, SUBSET 17 (T_H17)

1.2.1 DIFFERENTIATION, PROLIFERATION AND FUNCTIONS

Upon antigen challenge, in the presence of appropriate cytokine milieu, naïve T cells are directed into a variety of differentiation pathways, to produce a highly heterogeneous population of effector/memory cells. The innovative work of Mossman and Coffman in 1986 dichotomized the $CD4^+$T helper (T_H) cells into two distinct subsets; T_H1 and T_H2; each has its own signature cytokines. While the former lineage evolved to enhance eradication of intracellular pathogens, and is a potent activator of cell-mediated immunity, the latter evolved to enhance elimination of parasitic infections and is implemental in humoral immunity (99). Furthermore, these scientists also had enough foresight to predict the existence of additional subsets. However, we would have been waiting for two decades for the groundbreaking discovery of the recently designated T_H17 cell that triggered major revisions of the established paradigms in T cell biology. This lineage has been considered independently as a consequence of a series of novel experiments showing IL-23 rather than IL-12 to be instrumental in autoimmunity (100) and IFNγ and IL-4 to be inhibitory of the IL-17-producing effectors (101). Thus, the basis of the molecular mechanism in the development of T_H17 cells has been found, and is illustrated in figure (2). Available data reveals a species-specific milieu. The presence of IL-6 in the presence of TGF-β is needed for differentiation of T_H17 in mice. However, such T cells lack pathogenic activity, since they also secrete an anti-inflammatory cytokine, IL-10 (102). On the other hand, IL-1β, IL-6, and IL-21, but not TGF-β are

essential for human T_H17 cell commitment. In fact, it has been claimed that TGF-β inhibits IL-17 production in human cells (103).

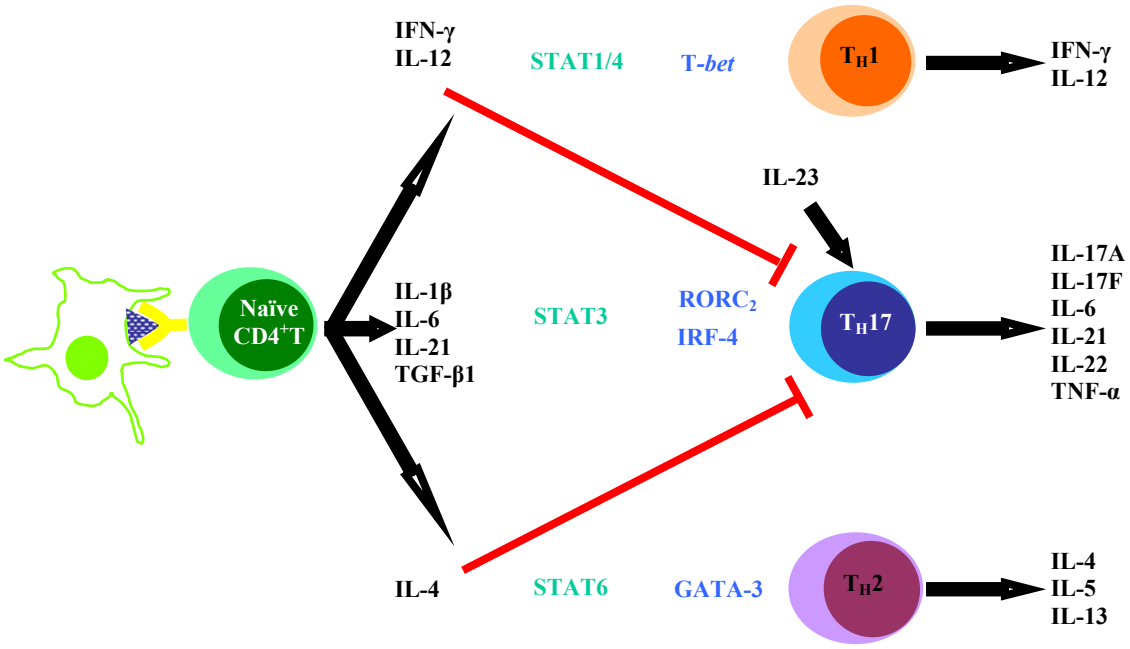


Figure (2). Current understanding of T_H17 cell pathways.

IL-6 has been shown to be vital for the differentiation of naïve T cells into T_H17, signaling through the STAT3 pathway. However, while such induction is transient, the co-presence of IL-1β is essential, changing it into a sustained one. IL-1R is homologous to TLR, and its activation cascades down a pathway quite similar to that of TLR (104). Meanwhile, IL-21, a relatively new member of the IL-2 cytokine family, is considered the lineage-specific cytokine for this subset, where it promotes differentiation to the subsets that originally produced it while simultaneously inhibiting other pathways. Moreover, it serves as an autocrine regulator of IL-17 production (105).

On the other hand, our understanding of the role of IL-23 in T_H17 subset development has changed over time. Even though in vivo reports indicated a crucial role of this cytokine in the induction of T_H17, IL-23 was shown to be dispensable for in vitro lineage commitment. This might be due to the fact that IL-23R upregulation is dependent on IL-21 that acts via STAT3 and RORγt. Hence, it was proposed to primarily function in the expansion or survival of already committed T_H17 cells (106). On the other hand, the three cytokines IL-6, IL-21, and IL-23 activate JAK and STAT3. The latter appears to be a critical regulator of T_H17 differentiation at more than one level (107). However, STAT3 activation is not enough alone. ROR-γt is identified as a master regulator or lineage-specific transcription factor of T_H17 cell subset although unlikely to play solo, fully directing the functional differentiation of the subset (108). Another transcription factor required for gene expression in this process is IRF4 (interferon regulatory factor 4), known as a key inducer of GATA3 expression and T_H2 lineage commitment. Most recently, however, evidence has been given that IRF4 has a definite role in *in vitro* T_H17 differentiation and in the development of experimental automimmune encephalitis *in vivo* (109). Remarkably, this is a rare example of a factor required for both T_H17 and T_H2 commitment, which contrasts with the previously recognized links with T_H17 and T_H1 development via IL-23 (101) and T_H17 and T_{reg} development via TGF-β1 (110).

In terms of cytokine production, it is agreed now that T_H17 is characterized by the production of IL-17A, IL-17F, IL-6, IL-21, and IL-22, IL-26, and TNF-α (111). **IL-17A** was originally cloned from activated T cells, and described by Rouvier et. al. and named CTLA8 (112). It has been then renamed IL-17 and, more recently, IL-17A. Subsequently,

five more family members were revealed and named as IL-17B-F. Unique to this family is the presence of five distinct cysteine residues. Among the six IL-17 cytokine family members, the expression and functions of IL-17A, IL-17F, and IL-17E (IL-25) are more explored (113). IL-17A, the prototypic family member, is a disulfide-linked homodimeric glycoprotein, consisting of 155 amino acids. Recently, IL-17A was shown to be the defining cytokine of T_H17. Although produced by "adaptive" T cells, IL-17A promotes expansion and recruitment of innate immune cells such as neutrophils. This could be done by the gamma-delta T cell population, another highly significant source of IL-17 that is likely to be enriched at mucosal and epithelial surfaces such as the gut, lung, and skin, leading to immediate recruitment of neutrophils, providing defense responses until adaptive immune cells are recruited to combat remaining pathogens. On the other hand, it also cooperates with TLR ligands, IL-1β, and TNF-α to enhance inflammatory reactions and stimulate production of β-defensins and other antimicrobial peptides. IL-17A stands in the middle of several complex disorders that incorporate both innate and adaptive immune pathomechanisms and need thorough revision to exploit defensive and diminish harmful host effects (114).

On the other hand, **IL-17F**, another IL-17 family member, is a protein containing 153 amino acids, and has 55% homology with IL-17A, more so than with any other members of the IL-17 family, whereas IL17E (IL-25, produced by cells other than T_H17) is the most distant (17%). It has not received as much attention as IL-17A mainly due to initial lack of reagents to measure IL-17F, but more data on its expression and role are now gradually accumulating (115). Moreover, interleukin IL-17A/IL-17F heterodimers have been identified in activated human $CD4^+$ Tcells (116).

IL-17A and *IL-17F* are syntenic both in mice and humans. Contrary to other IL-17 cytokine family genes present on different chromosomes, they are located on the same one (chromosome number 1mouse and number 6 in humans). This indicates that the regulatory regions may exist in the IL-17A/F common locus. In fact, the promoters and regions of IL-17A and IL-17F genes undergo harmonized chromatin alterations, corresponding to those identified in the T_H2 cytokine genes loci (113). Also, both IL-17A and IL-17F are typically produced by T_H17 cells, whereas the other IL-17 family members, IL-17B, IL-17C, and IL-17D, are products of non-T cell origin (117). Moreover, IL-17A and IL-17F stimulate multiple pro-inflammatory products. This involves chemokines, cytokines, and metalloproteinases, from several different cell lines including epithelial and endothelial cells, as well as smooth myocytes, and fibroblasts.

IL-17 receptor (IL-17RA) was the first IL-17R to be described. It is a type I transmembrane protein consisting of a 293 amino acid extracellular domain, a 21 amino acid transmembrane domain, and a long 525 amino acid cytoplasmic tail. The IL-17RA mRNA is widely upregulated in variety of organs including liver, kidneys, spleen, and lungs, as well as in isolated cells like fibroblasts, epithelial cells, mesothelial cells, and different myeloid cells from mice and rats. IL-17RA binds human IL-17A with relatively higher affinity compared with IL-17F. The addition of IL-17RC was shown to result in fully intact IL-17A signaling. It has been documented that IL-17A, IL-17F, or the heterodimer of both can bind and signal through the same receptor complex (IL-17RA/IL-17RC), although with a different affinity (118). Data available in the literature suggest that both the soluble receptors or anti-receptor monoclonal antibodies could be used to specifically antagonize each of these interleukins (119).

Much less is known about **IL-22** and **IL-26**, IL-10 family members, made predominantly by T_H17 cells. IL-22 acts directly on epithelial and some fibroblast cells in peripheral tissues (in the skin, kidney, respiratory, and digestive systems, outside of the immune system) and promotes innate, non-specific immunity, inducing *an acute phase response in vivo and chemokines and metalloproteinases in* vitro (120). Moreover, the role of the IL-22 binding protein in modulating IL-22 effects is uncertain. Limited data are available on IL-26 biology other than being involved in targeting colon epithelium and in mucosal immunity (121).

Another interesting issue is the topic of T_H17 control. IL-2 is influential in this respect. Knock-out mice deficient in IL-2 had both increased serum levels of IL-17 and excess T_H17 cells in lymphoid organs as well as in the skin (122). In addition, the anti-inflammatory cytokine, IL-10, is indispensable in regulating the T_H17 through suppression of IL-12p40 (a component of both IL-12, and IL-23), IL-1β, and IL-6 (123). Moreover, IL-17E (IL-25) is a potent inhibitor of T_H17 cells. This could be due to induction of T_H2 cells and its signature cytokines (124). Furthermore, IL-27, produced by dendritic cells and macrophages, has been shown to inhibit T_H17 cells through STAT1 signaling (125). However, the factors downstream of this point are still mysterious. It seems that further understanding of the mechanisms involved in T_H17 inhibition will provide a valuable insight on how to regulate this lineage (126).

Most important is the function of that new T helper subset. Under physiological conditions, the primary role of T_H17 is to bridge the innate and the adaptive arms of the immune system by stimulating the mobilization and *de novo* generation of neutrophils by

G-CSF. This has been proven in some extracellular pathogens binding mucosal surfaces. T_H17 infiltration in asthmatic airways is an example of how these cells link T cell activity (adaptive) with neutrophilic inflammation (innate). However, T_H17 are most infamous for their pathological involvement in a range of autoimmune diseases. (115).

A strong association between T_H17 subset and chemokine system has been agreed. IL-17 induces an array of chemokines, including CXCL-1, -2, -5, -8, CCL-2, and CCL-20, leading to recruitment of T cells, B cells, monocytes and neutrophils, all of which populate the inflammatory sites (127). More recently, up-regulation and stable expression of CCR6 was confirmed on T_H17 cells (128). This is a chemokine receptor that has been suggested to be essential in the regulation and initiation of the immune response. It is expressed on lung dendritic cells, B cells, and effector and memory T cells. Its ligand, CCL20/MIP-3α, liver and activation-regulated chemokine, is made by airway epithelial cells and mast cells in the lungs (129). This gives the possibility of a new therapeutic hope by blocking this pathway. Accordingly, specifically targeting these cells could be a logical method to manage chronic inflammatory diseases. This is the rationale of thoroughly investigating the inhibitory pathways of this lineage. IFN-γ and IL-4 could antagonize initial steps of T_H17 differentiation. However, once committed, this subset becomes resistant to such effect. To further complicate the picture, a new class of IL-17/IFNγ "double producers" have been described very recently in addition to the "classic" T_H17 or IL-17-single producers. They share the features of T_H1 and T_H17. This novel subset exists *in vivo* in humans, but not mice, and can be induced in vitro by stimulating T_H17 in the presence of IL-12, thus raising new issues on the T_H17 developmental and/or functional relationship with T_H1 (126).

Additionally, it has been shown that T-*bet*, the master transcription regulator of T_H1 inhibits both neutrophilic inflammation mediated by T_H17 cell and eosinophilic inflammation mediated by T_H2, in the airways. As a result, the dysfunction of T-*bet* might also be involved in the pathogenesis of severe asthma, where accumulation of both neutrophils and eosinophils in the airways is a major characteristic of that disease. On the other hand, T-*bet* has been recently proved to enhance expression of IL-23R (130). Thus, recognizing whether T-*bet* is positively or negatively driving T_H17 is worthy.

1.2.2 T_H17 in some allergic diseases

The link between AD and asthma has been highlighted before. Although asthma has been classically thought of as a T_H2 disease, there is significant evidence that suggests an important role for T_H17 in the pathogenesis of asthma. This sounds very promising as if it proves true, it will have ultimate consequences on our understanding of AD and, subsequently the way we treat it.

IL-17A has been shown to be expressed in asthma. Sputum samples as well as BAL fluids from asthmatic patients have been shown to contain elevated levels of IL-17A compared with control subjects (131). Patients with allergic asthma also had elevated levels of IL-17A in plasma compared with non-asthmatic control subjects (132). Again, the level of IL-17A in bronchial biopsies correlated with the disease severity and was significantly decreased after corticosteroid treatment. Taken together, these imply a contribution of IL-17A to the pathogenesis of asthma (133). Most recently, T_H17 cells were demonstrated in asthmatic bronchial biopsies (134).

There is evidence that IL-17A might promote allergic asthma through several effects in the lung, as well as systemically. One of the critical functions is its effect on neutrophils. *In vitro* stimulation of human bronchial fibroblast cells with IL-17A produces IL-6, IL-8, and GRO-α (131). The latters are known chemoattractants for neutrophils. In contrast, the former is a neutrophil-activating cytokine. The increased expression of IL-17A in the lung during asthma may explain the increased accumulation and activation of lung neutrophils. In the same way, using IL-17A to activate cultured human airway smooth muscle cells produced IL-8 and IL-6, which was enhanced by IL-

1β and TNF-α, respectively (135, 136). Similarly, incubation of human airway smooth muscle cells with IL-17A led to production of eotaxin-1 (CCL11). Synergism between IL-17A and IL-1β/TNF-α was also detected in the stimulation of IL-8 and IL-6 production by human lung epithelial cells (137). Moreover, IL-17A up-regulates the expression of a number of CXCR2 chemokines including CXCL8, CXCL1, and CXCL6 as well as the neutrophil survival factors GM-CSF and G-CSF from the epithelium of the airways (138). This might indicate that T_H17 lineage is essential in induction and maintenance of neutrophilic inflammation seen in severe asthma. additionally, IL-17A can function synergistically with inflammatory mediators like TNF-α, produced by viral infections to increase these effects (139).

Furthermore, IL-17A, through IL-6, was also shown to induce the expression of the mucin genes, MUC5B and MUC5AC, by human bronchial epithelial cells. An effect that has not been produced by the T_H2 cytokines IL-4, IL-9, and IL-13 (140). Since many of the inflammatory effects of T_H17 cells are attributed to the expression of IL-17A, one might extrapolate this to suggest a definite role of this T_H subset in asthma.

Another proof documenting the role T_H17 cells contribute to human allergic asthma comes from observation of *Dermatophagoides farinae* (Df)-restimulated T-cell clones from the peripheral blood. Stimulation of PBMCs using anti-CD3 and anti-CD28 antibodies induced comparable levels of IL-17A from control subjects, non-atopic asthmatics, and allergic asthmatics. However, only the atopic asthmatic group had IL-17A following Df-mediated activation (141).

Interestingly however, the production of IL-17A was not restricted to $CD4^+$ T_H cells in the sputum and bronchial alveolar lavage (BAL) of asthmatic patients, but IL-17A was also detected in airway eosinophils by immunocytochemistry (131). Most recently, it has been claimed that IL-17A is mainly produced by macrophages but not T_H17 in allergic inflammation related to asthma in an animal model (142).

Taken together, the above data suggests that IL-17A plays an important pathologic role in the development of asthma. This highlights the potential of interference with the activity of IL-17 as an effective tool in treating asthma. It has been shown before that glucocorticoids can block the upregulation of cytokines and chemokines by human bronchial fibroblasts and epithelial cells, *in vitro*. Moreover, it has also been shown that treatment with oral steroid decreased the levels of IL-17A detected by immunocytochemistry in bronchial biopsies (143). On the other hand, there is growing evidence that **IL-17F**, the other major product of T_H17 cell, plays a significant role in the pathogenesis of asthma. Increased expression of the *IL17F* (but not *IL17A*) gene has been shown in patients with allergic asthma in response to allergen challenge (144). Moreover, overexpression of IL-17F enhanced antigen-induced allergic inflammation, including pulmonary neutrophilia (through the induction of CXC chemokines), airway responsiveness, goblet cell hyperplasia, and mucin gene expression in an animal model of asthma. All contribute to allergic inflammation and airway remodelling (145). IL-17F has also been demonstrated to stimulate various cytokines, chemokines, and adhesion molecules including IL-6, TGF-β (transforming growth factor-β), IL-8, GROα (growth-regulated oncogene-alpha), ENA-78 (Epithelial cell-derived neutrophil-activating peptide 78), MCP-1 (monocyte chemoattractant protein-1), G-CSF (granulocyte-colony forming

factor), GM-CSF (granulocyte macrophage-colony forming factor) and intracellular adhesion molecule-1 (ICAM-1), in airway epithelial and endothelial cells, as well as in fibroblasts (146).

Moreover, in tissue extracted from the lungs IL-17F transduced mice exhibited significantly high levels of inflammatory cytokines and chemokines. This includes IFN-γ, IL-6, inflammatory protein 10 (IP-10), and insulin-like growth factor-1. Lung fibrosis and airway remodelling have been known to result from some of these molecules. This suggests a probable role of IL-17F in the process of airway remodelling accompanying chronic inflammation occurring in certain lung diseases, as asthma and COPD. Of interest, a mutation in the gene coding IL-17F has been demondtrated to be associated with human asthma and COPD (147).

More recently, mice deficient in IL-17F, but not IL-17A, have been shown to have defective airway neutrophilia in response to allergen challenge. What is more interesting is that, in an asthma model, even though IL-17A deficiency decreased T_H17 upregulation, IL-17F–deficient mice displayed increased T_H2 cytokine making and eosinophil job. This shows that IL-17F is an essential key of inflammatory responses that looks to operate in a different way from IL-17A in both immune physiology and pathology (148).

In addition, it has been shown that bronchial epithelial cell is a new cell source of IL-17F. Epithelial IL-17F may act on bronchial epithelial cells to induce several cytokines, chemokines and adhesion molecule in an autocrine manner. Moreover, glucocorticoids decrease the expression of IL-17F gene and protein in the lungs. Therefore, the blockade of IL-17F expression, and its downstream effects, appears to be sensitive to classic anti-

inflammatory drug therapy, which may suggest an attractive pharmacotherapeutic strategy for modulation of airway inflammatory diseases (149). Taken together, these findings suggest that IL-17F may cause and perpetuate airway inflammation.

Furthermore, although IL-17F expression has been described to be regulated by the same cytokine network and transcription factors as IL-17A, different ratios of IL-17A and IL-17F expression have been found in different T cell populations both *in vitro* and *in vivo*. This suggests differential cytokine expression in differentiated T_H17 cells. Furthermore, analysis of animals deficient in either of the two genes has revealed their distinct functions in inflammatory response. At this point particular point of time, we do not know what explains this differential regulation. Moreover, the biological or pathological importance of this instruction is also uncertain (148).

In summary, both IL-17A, IL-17F (the major products of T_H17) are expressed in the lungs of asthmatics and play a role in the pathogenesis of that common disease, at least in certain subset of patients. Therefore, further investigations confirming the presence of T_H17 cells in the airways in asthma patients and clarifying the mechanisms underlying their prototype cytokines are worthy, since these cells would present a novel therapeutic opportunity to reduce neutrophilic inflammation in the lung.

1.2.3 T_H17 in non-allergic diseases

Psoriasis is a common chronic inflammatory disease of the skin, affecting 1-3% of the population. The disease is characterized by the presence of erythematous, silvery scaly, well-demarcated plaques. It causes major suffering and agony for patients. In some conditions, the disease is associated with psoriatic arthritis, involving chronic inflammatory joint changes. It is characterized by epidermal hyperplasia, dermal angiogenesis, and being infiltrated by lymphocytes, neutrophils, monocytes and dendritic cells (DCs). Overall, psoriasis is largely understood as a T-cell-mediated disease (150).

For long time, widespread evidence has been demonstrating a substantial involvement of T_H1 cytokines in the condition. This initial understanding was largely based on increased expression of the p40 subunit of IL-12. However, the discovery that the p40 subunit is also a part of IL-23, has changed a lot of our thoughts about many diseases, originally attributed to T_H1. Accordingly, several studies have shown that the common p40 subunit and the IL-23-specific p19 subunit, not the IL-12-specific p35 subunit, are highly expressed in monocytes and DCs in psoriatic skin lesions. Moreover, recent data reveals a potent contribution of T_H17-associated cytokines (151).

Additional evidence came from those investigators who studied skin biopsies of psoriatic plaques and demonstrated increased levels of TGF-β1, IL-6, IL-15, IL-17, IL-22, and IL-23 (152-156). Moreover, direct detection of elevated numbers of T_H17 cells have also been documented in psoriatic lesions (157). Furthermore, the mRNA and protein levels of IL-6 were increased in the peripheral blood of psoriatic patients. IL-22 is also elevated in blood, and the level correlates with psoriasis severity. IL-22R, expressed

on epithelial cells, is known to regulate the proliferation and differentiation of keratinocytes (158).

On the other hand, peripheral blood mononuclear cells from patients with psoriasis have increased mRNA for CCR6 and skin homing CLA^+ T cells express high levels of CCR6. In addition, lymphocytes from psoriasis patients respond to very low levels of CCL20 and have a greater chemotactic response than those from healthy controls. CCR6 and CCL20 are also markedly upregulated in psoriatic skin lesions. Since human T_H17 cells express the chemokine receptor CCR6 and its ligand CCL20, this gives another clue to the role of TH17 in the pathogenesis of psoriasis (159).

More interesting, however, is that inhibition of the p40 subunit (shared between IL-12 and IL-23) has been shown effective in treating psoriasis. It led to improvement in psoriasis area-and-severity index (PASI; a measure of overall psoriasis severity and coverage, commonly-used in clinical trials for psoriasis treatments) that increased in a dose-dependent manner, which was associated with a decrease in the mRNA levels of several pro-inflammatory cytokines, including the p19 subunit of IL-23 (160). Furthermore, TNF-α induces APCs to secrete IL-23 and upregulate the Th17 cell response. Etanercept (a soluble TNF receptor) was effective in treating psoriatc patients, producing significant improvements in PASI, epidermal hyperplasia, and inflammatory infiltrate. It also rapidly down regulated expression of many T_H17-associated cytokines, including IL-17, IL-22, IL-6, IL-1β, IL-23, as well as CCL20 and anti-microbial peptides. In contrast, IFN-γ and other T_H1 effector molecules were not down regulated until very

late in disease resolution. This has been interpreted as an evidence for a much more essential role of T_H17 in psoriasis pathogenesis than that of T_H1 (161).

Meanwhile, data gathered from experimental models of psoriasis adds more support to this view, and furthers our understanding of that topic. The T_H17-associated cytokines; IL-23, IL-22, and IL-17, have been demonstrated to induce both the inflammation and epidermal hyperplasia associated with psoriatic plaques. The role of IL-23 in psoriasis has been confirmed by *in vivo* studies done in mice. When injected into the skin of the ears, IL-23 induced epidermal hyperplasia and inflammatory cellular infiltration similar to psoriasis, which was mediated by TNF-α, IL-22, IL-17A, and IL-17F. Moreover, IL-22 appears to be a primary downstream mediator of psoriatic acanthosis (162). Additionally, IL-6 has been shown to be expressed in high levels in psoriatic skin and stimulates proliferation of cultured human keratinocytes and invasion of macrophages and T-cells (163). Moreover, TGF-β1 was proven to initiate T-cell and fibroblast activation as well as angiogenesis and neovascularisation (164). Finally, IL-15 is essential in inducing angiogenesis, immune cell recruitment, and keratinocytes activation. Psoriasis resolved upon blockade of IL-15 biological activity in a xenograft mouse model (165).

In conclusion, new evidence imply that both T_H1 and T_H17 cell lines have an evident role to play in psoriasis. Inhibition of either of them significantly ameliorates that disease. It looks like that the connection between T_H1, T_H17 cells, and psoriasis will continue to be a topic of major concern for dermatologists and immunologists over coming years.

1.3 STUDY OBJECTIVES

1. Demonstrate the presence of T_H17-associated cytokines in skin from AD versus controls.
2. Show the differential expression of these cytokines in acute and chronic AD.
3. Identify the population of T_H17 cells in both acute & chronic AD lesions.

1.4 STUDY HYPOTHESIS

TH17 cell and its associated cytokines are important mediators in AD, particularly in chronic lesions.

CHAPTER 2

MATERIALS AND METHODS

2.1 SUBJECT RECRUITMENT

Our study comprised 17 (10 acute and 7 chronic) atopic dermatitis patients and 10 healthy individuals with no history of skin disease. The atopic dermatitis patients fulfilled the diagnostic criteria of Hanifin and Rajka (166). Two 2-mm punch biopsies were collected from noninfected acute (less than 3 days) and chronic (2 weeks) lesional skin, based on clinical appearance. Diagnoses were confirmed histologically, and there were no cases of diagnostic discordance. Punch skin biopsy specimens were also obtained from the 10 non-atopic healthy volunteers. Skin samples were submerged immediately in 10% buffered-formalin and embedded in paraffin (for immunohistochemical studies) or snap frozen in OCT (for immunofluorescence or laser capture microdissection (LCM) studies). None of these patients had other skin pathologies, and none had been previously treated with oral corticosteroids. None of the patients had received systemic corticosteroids or cyclosporine previously, and none had received topical corticosteroid or calcineurin inhibitors for a period of at least one week before enrollment. The study was approved by the institutional review board at National Jewish Medical and Research Center (Denver, CO), and all subjects gave written informed consent prior to participation in these studies.

2.2 IMMUNOHISTOCHEMISTRY

Immunocytochemistry is the identification of cellular or tissue constituents (antigens) by means of antigen antibody interaction. This allows the precise examination of aspects of cell function and chemical composition and their relationship to our perception of cell and tissue morphology. It greatly enhanced our understanding of disease processes. Many methods are available for immunocytochemistry. They can be generally divided into three categories, depending on the labeling method (Figure 3). The direct method, which is the simplest, involves application of a labeled antibody directly to the tissue preparation. However, this method lacks sensitivity due to the low signal amplification. On the other hand, in the indirect approach, the primary antibody is unlabeled and is identified by a labeled secondary antibody raised to the immunoglobulin of the species providing that primary. The sensitivity of this technique is definitely higher, and can be further enhanced by applying a third step, a labeled antibody raised against the species in which the second antiserum has been raised. A third approach is to use an unconjugated, bridging secondary antibody between the primary antibody and the label detection reagent. This could be either an enzyme-anti-enzyme complex (e.g. peroxidase-anti-peroxidase; PAP) or an avidin-biotin enzyme complex (167).

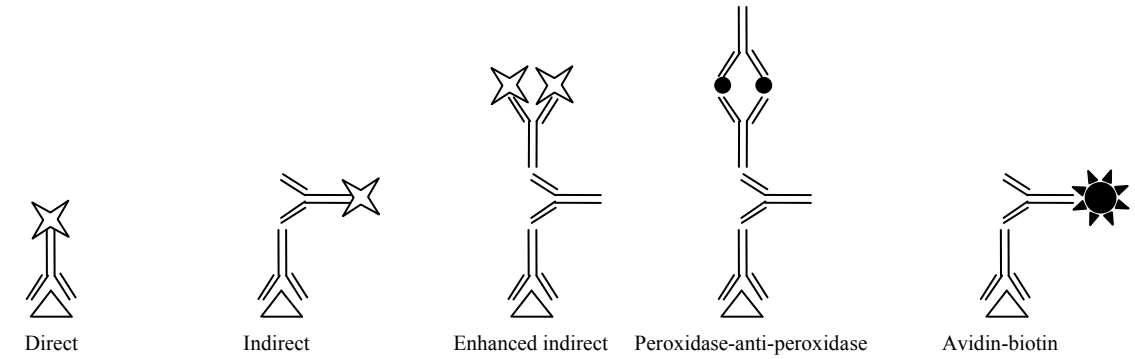
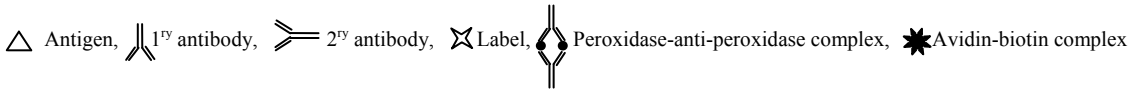


Figure (3). Illustration of different methods of immunohistochemistry.



Staining was performed using a peroxidase-based method. Sections of 5 μm in thickness were de-paraffinated in xylene, dehydrated in ethanol, and washed in PBS. After permeabilization with Triton (0.2% in PBS), tissues were immersed in a solution of hydrogen peroxide (5% in PBS). The sections were then washed 3 times for 5 minutes in PBS before incubating with Universal Blocking Solution (DakoCytomation, Mississauga, Ontario, Canada) for 20-30 minutes. Tissue sections were immunostained with polyclonal antibodies raised in goat against the cytoplasmic domain of IL-17A or the cytoplasmic domain of IL-17F, at concentrations of 1.0 μg/mL and 0.67 μg/mL, respectively (both from R&D Systems, Minneapolis, Minn). Biotinylated-secondary antibodies, raised in rabbit (DakoCytomation), were applied at concentrations of 1:100. In order to confirm specificity of the IL-17A and IL-17F immunostainings, sections were also stained with normal goat IgG served as an isotype control for goat-derived primary antibodies (IL-17A and IL-17F). The reactions were developed using Strept AB Complex/HRP and DAB substrate according to manufacturer's instructions (DakoCytomation). Slides were examined and images were acquired by a BX51 Olympus microscope attached to a CoolSNAP-Pro color digital camera (Carsen Group, Markham, Ontario, Canada) using the Image Pro-plus 4.0 system (Media Cybernetics, Silver Spring, MD). Scoring of epidermal staining (which refers to the percentage of the epidermis that stained positively) was performed blindly. The scoring system was as follows: 0 = no staining, 1 = 0-12.5%, 2 = 12.5-25%, 3 = 25-37.5%, 4 = 37.5-50%, 5 = 50-62.5%, 6 = 62.5-75%, 7 = 75-87.5%, and 8 = 87.5-100%. Dermal inflammatory cell staining for each antibody was reported as the number of positively-stained inflammatory cells/mm^2 of subepidermal area (excluding hair follicles, glands, blood vessels, and adipose tissue).

2.3 Laser Capture Microdissection

Laser capture microdissection (LCM) is a technique for isolating specific cells of interest from sectioned tissue. Using special software, a thin section of tissue is viewed, under a microscope, and individual cells are identified. The operator can then select the cells of interest using instrument software. A laser beam can be fired to activate a transfer film on a cap placed on the tissue sample, fusing the film with the underlying cells of choice. The cells are then lifted off the thin tissue section, leaving all unwanted cells behind (168).

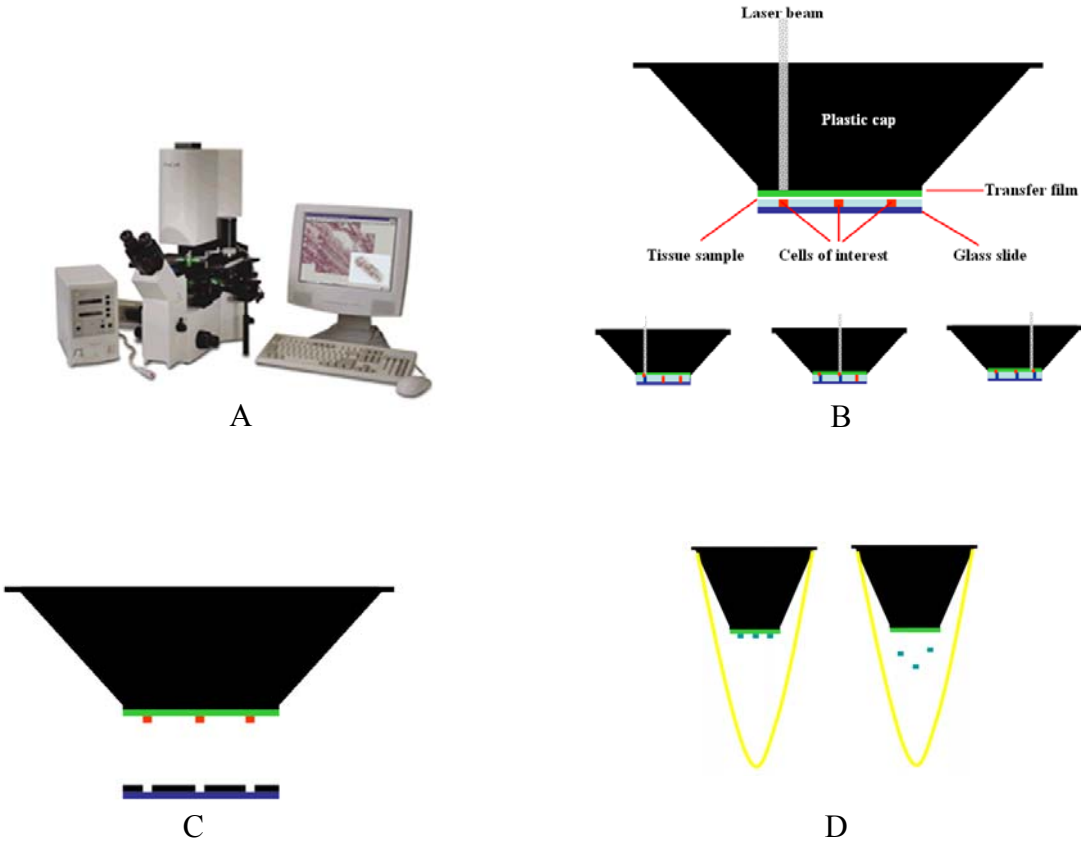


Figure (4): The PixCell II Laser Capture Micro Dissection System. A. The system, B. An illustration of sequential capturing of the cells of interest, C. The targeted cells attached to the caps, D. The caps, containing the cells moved into the RLT lysis buffer.

LCM was performed on frozen sections using PixCell II system (Arcturus Engineering Mountain View, CA), according to instructions of the manufacturer (HistoGene TM LCM Frozen Section Staining Kit). Briefly, 8-μm thick cryosections prepared from biopsies were kept frozen in -80ºC. Before LCM, they were stained with Meyer's hematoxylin and eosin, air-dried, dehydrated through graded alcohols and xylene, to prevent the activation of endogenous RNase in the tissues. The interval between the staining of the slides and completion of microdissection was 1-2 hours. The inflammatory cells were carefully captured using the PixCell laser microscope. During this process mononuclear inflammatory cells were obtained from frozen sections of 4 acute and 3 chronic atopic dermatitis patients. Numbers of cells varied from 400 to 1000. Cellular material captured on HS LCM Capsure Caps, and digested in guanidine-thiocyanate-containing RLT lysis buffer (RNAeasy kit Qiagen, Mississauga, ON, Canada), supplemented with β-mercaptoethanol (1% V/V). Samples were stored at -80ºC until RNA isolation.

2.4 RNA extraction and reverse transcription

RNA was extracted using the RNAeasy micro kit according to the manufacturer's instructions (Qiagen) (Figure 5). RNA was eluted in 12 µl of RNase-free water.

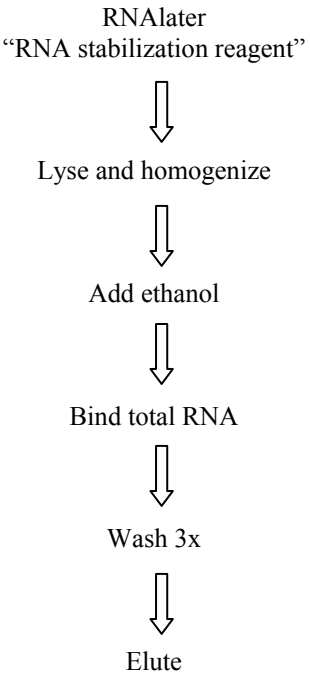


Figure (5):
Steps of RNA extraction

Total RNA of each sample was subjected to reverse transcription (Figure 6). cDNA was generated in a 30 µl reaction tube. RNA was reverse-transcribed using oligo(dT)12-18 primers in the presence of RNAguard (both from Amersham Pharmacia Biotech) and Superscript II reverse transcriptase (Invitrogen Corporation, Burlington, ON, Canada), according to the manufacturer's instructions.

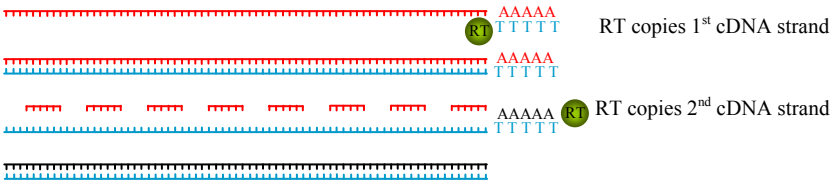


Figure (6): Conversion of mRNA to cDNA by reverse transcriptase (RT)

2.5 Real-time quantitative PCR

After reverse transcription, the relative quantification of mRNA of GAPDH, IL-17A, and IL-17F was done by real-time polymerase chain reaction (Figure 7), using the LightCycler PCR (Roche Diagnostics, Laval, Quebec, Canada), QuantiTect SYBRGreen PCR mastermix (Qiagen), according to the instructions of the manufacturer. mRNA-specific primer sets were purchased from Invitrogen, and the sequences are as follows: IL-17A sense primer: 5′-CTCAGGCTTCCTTTGGAGATT-3′; IL-17A antisense primer: 5′- ATCAGCTCCTTTCTGGGTTGT-3′; IL-17F sense primer: TTCCAAAAGCCTGAGAGTTG; IL-17F antisense primer: CCAGTGTAGGAACTTGGGCT; GAPDH (glyceraldehyde-3-phosphate dehydrogenase) sense primer: 5′-AGCAATGCCTCCTGCACCACC-3′; GAPDH antisense primer: 5′-CCGGAGGGGCCATCCACAGTC-3′. The PCR conditions were as follows: one cycle at 95°C for 15 minutes, then 40 cycles of denaturation (95°C for 15 seconds), annealing (59°C, 20 seconds) and extension (72°C, 30 seconds). After PCR, one cycle of denaturation was done to generate melting curve of the amplified products. The primers resulted in amplification of 130, 199, and 136 bp specific amplicons, respectively. PCR reactions were performed in a 20 µl volume containing 7 µl water, 2 µl cDNA, 10 µl SYBR green PCR master mix and 1 µl of each primer. The PCR products were migrated and visualized on a 1% agarose gel containing 0.2g/ml ethidium bromide.

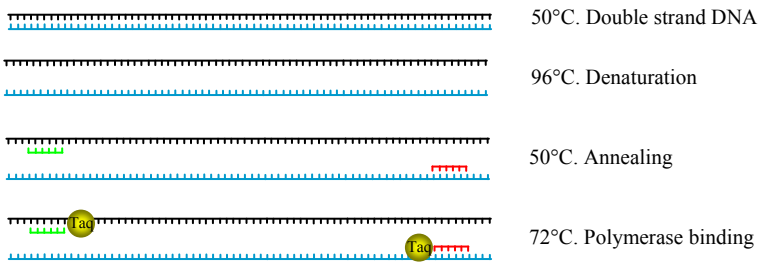


Figure (7): Steps of QRT-PCR

2.6 Immunofluorescent staining

Frozen sections were retrieved from -80ºC, defrosted in foil wrap, washed in PBS, fixed in a 4% paraformaldehyde solution, washed again. After rehydration, tissue was permeabilized using 0.2% Triton X-100 to improve the penetration of the antibodies. After washing, nonspecific binding of the antibody was prevented by incubating the slides with 1% BSA in PBS. Slides were concomitantly incubated overnight at 4ºC with 2 μg/mL polyclonal Goat-antihuman IL-17A or IL-17F (R&D Systems) and monoclonal mouse-antihuman CD3 antibodies (BD Biosciences, Mississauga, ON, Canada). Slides were extensively washed before incubation with a 1/300 dilution of Alexa Fluor 488 labeled donkey anti-goat (Invitrogen). After thorough washing, a second incubation with a 1/300 dilution of Alexa Fluor 555 labeled goat anti-mouse (Molecular probes, Eugene, OR). Nuclei were visualized by staining with 2 μg/mL Hoechst 33342 (Molecular Probes), and slides were examined with a BX51 Olympus epifluorescence microscope attached to a CoolSNAP-Pro color digital camera (Carsen). Colocalization of different markers was performed using Adobe Photoshop® 10.0 (San Jose, CA).

2.7 Statistical analysis

Statistical analysis of the results was performed by using Kruskal-Wallis nonparametric ANOVA, and subsequently, post hoc comparisons were made by using Dunn's multiple comparison tests. The coefficients of correlation were calculated from the Pearson moment coefficient and corrected for multiple comparisons by using the Bonferroni correction factor (Systat version 8.0; SASS, Chicago, Ill). Results were considered as statistically significant when the p values were less than 0.05.

CHAPTER 3

RESULTS

3.1 Histological and immunocytochemical analysis of skin tissue samples from subjects with atopic dermatitis showed increased IL-17A and IL-17F.

Biopsy specimens from acute AD lesions demonstrated varying degrees of epidermal hyperplasia with focal spongiosis. Perivascular infiltrates were also prominent. In contrast, chronic AD lesions were characterized by acanthosis with minimum epidermal infiltration.

All biopsy specimens from acute and chronic AD skin lesions displayed higher numbers of IL-17A-immunopositive cells (37.71 ± 26.48 and 51.79 ± 40.56 cells/mm^2, respectively) compared with normal controls (16.69 ± 16,55 cells/mm^2) (**Figure 8a, b and c, Figure 10a**). However, it was interesting to note that the numbers of IL-17A-positive cells were significantly higher only in chronic tissue, compared with healthy skin samples. Moreover, the numbers of IL-17A cells were higher in chronic compared to acute lesions (51.79 ± 40.56 cells/mm^2 and 37.71 ± 26.48). Biopsy specimens from acute and chronic AD skin lesions showed increased numbers of IL-17F-immunopositive cells (29.97 ± 14.37 and 169.4 ± 154.4 cells/mm^2, respectively) compared with normal controls (24.53 ± 26.74 cells/mm^2 (**Figure 9a, b and c, Figure 10b**). Here again, although acute patients showed a trend toward an increase in the numbers of subepidermal IL-17F+ cells, only chronic AD patients came out statistically significant from controls. The numbers of IL-17F cells are higher in chronic than in acute lesions (169.4 ± 154.4 versus 29.97 ± 14.37).

3.2 LCM-based demonstration of T_H17-associated cytokine expression in AD skin

To further the evidence of IL-17A and IL-17F expression in the dermis of AD patients, we used the state-of-the-art technique of LCM to pick up mononuclear cells from the dermis (**Figure 11a, b, c**) and then used the QRT-PCR to assess the level of mRNA of IL-17A and IL-17F in these cells. As depicted by means of quantitative PCR melting peaks, we demonstrated that GAPDH was expressed by inflammatory cells picked up by LCM from all atopic dermatitis cases (**Figure 12a**). In contrast, IL-17A (**Figure 12b**) was expressed in one case and IL-17F (**Figure 12c**) by three others. To confirm the results, gel electrophoresis was done on the PCR products, which demonstrated clear proof of the presence of the unique bands relevant to these cytokines in two different samples (**Figure 13**).

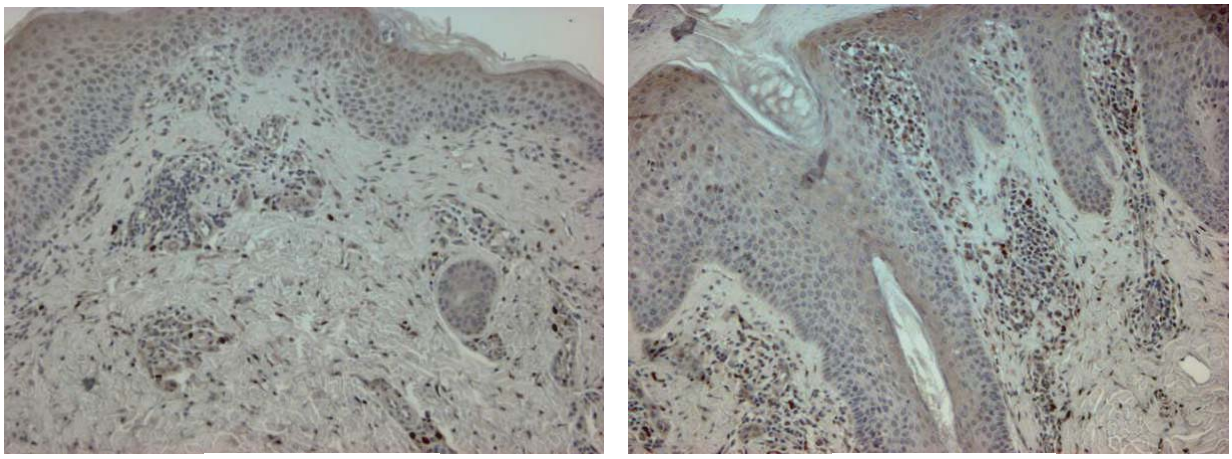
3.3 Immunofluorescence-based evidence for the presence of TH17 cells in AD

Consistent with our immunohistochemical findings of IL-17A/IL-17F-positive cells in the dermis of paraffin-embedded AD skin sections, our immunofluorescence studies of frozen sections from atopic skin confirmed the expression of IL-17A and IL-17F in the dermis of AD patients. To document the cell source of IL-17A and IL-17F cytokines, double staining for CD3 and IL-17A/IL-17F revealed that there is a colocalization of these proteins in AD dermis. We were able to co-localize Hoechst 33342 (Molecular Probe, Invitrogen, CA) nuclear stain™ (**Figure 14a**), with Alexa Fluor™ 488-labeled, goat anti-human IL-17A antibody (green) (**Figure 14b**), and Alexa Fluor™ 555–labeled, mouse anti-human CD3 antibody (red) (**Figure 14c**), resulting in a merge of yellow color (**Figure 14d**). Such co-localization brings evidence of the presence of T cells expressing IL-17A and IL-17F. However, one should notice that not all IL-17A cells displayed CD3-

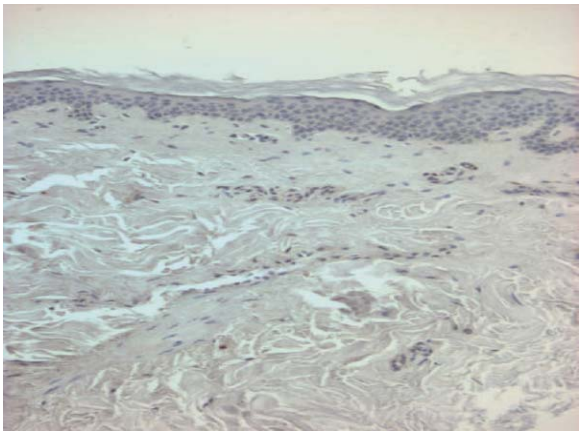
immunoreactivity, which suggests that other cells (either resident or infiltrating) of the skin express IL-17A. It is important to note that all patients showed positive staining for IL-17A and IL-17F. However, 4 control subjects were negative for IL-17A, and 3 were negative for IL-17F.

3.4 Evidence for IL-17F expression by skin keratinocytes

Interestingly, immunocytochemistry staining for IL-17F showed immunoreactivity in keratinocytes. Cells from both acute and chronic AD skin lesions showed strong staining for IL-17F compared to controls (**Figure 15a, b and c; respectively**). We were also able to confirm expression of IL-17F mRNA in cDNA samples isolated from atopic skin keratinocytes by LCM and subsequent real-time quantitative PCR (**Figure 16**, depicting melting peaks of q PCR amplifications).



A **B**



C

Figure (8) Immunohistochemical detection of IL-17A in patients with atopic dermatitis (AD). Tissue samples from patients with acute and chronic AD as well as controls were processed for immunocytochemical detection of IL-17A (a, b and c, respectively), as detailed in the Materials and Methods section. Results are representative of four independent stainings. Numbers of subjects per group were as follows: acute and chronic AD (n=10 and 7, respectively), controls (n=10). Photomicrographs taken at 200X.

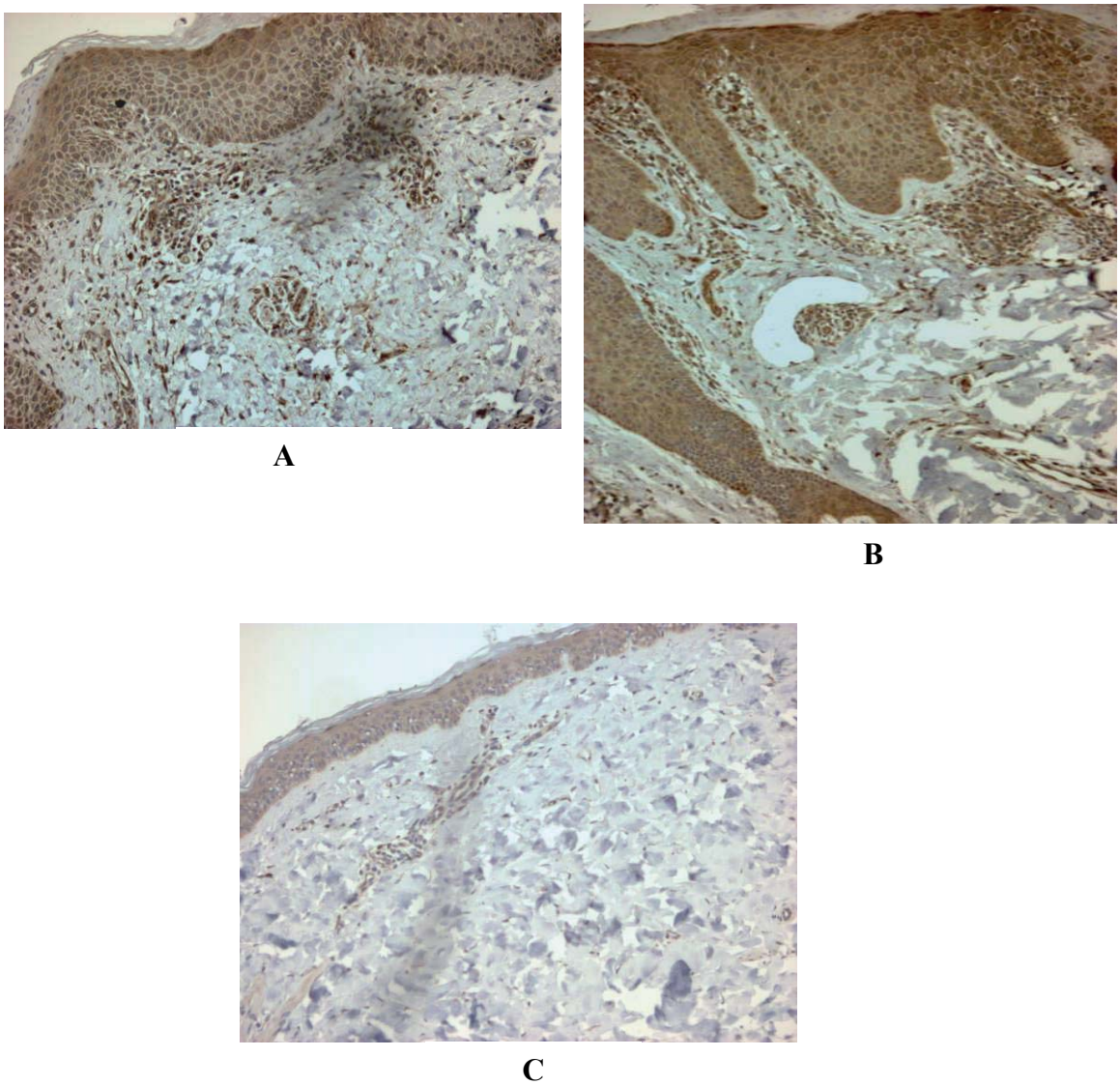
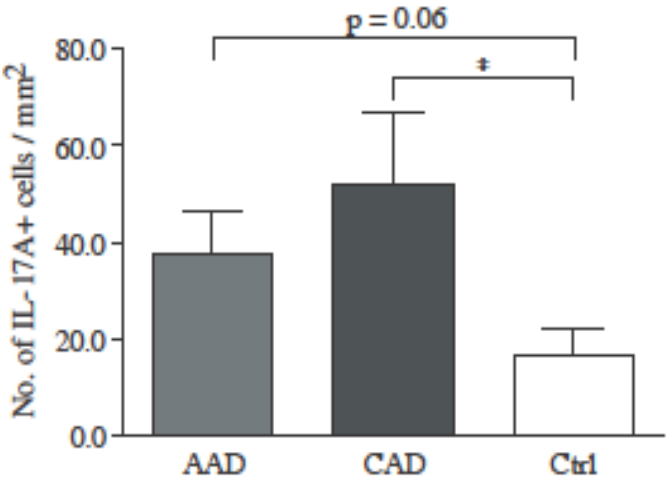


Figure (9): Immunohistochemical detection of IL-17F in patients with atopic dermatitis (AD). Tissue samples from patients with acute and chronic AD as well as controls were processed for immunocytochemical detection of IL-17F (a, b and c, respectively) as detailed in the Materials and Methods section. Results are representative of four independent stainings. Numbers of subjects per group were as follows: acute and chronic AD (n=10 and 7, respectively), controls (n=10). Photomicrographs taken at 200X.

A



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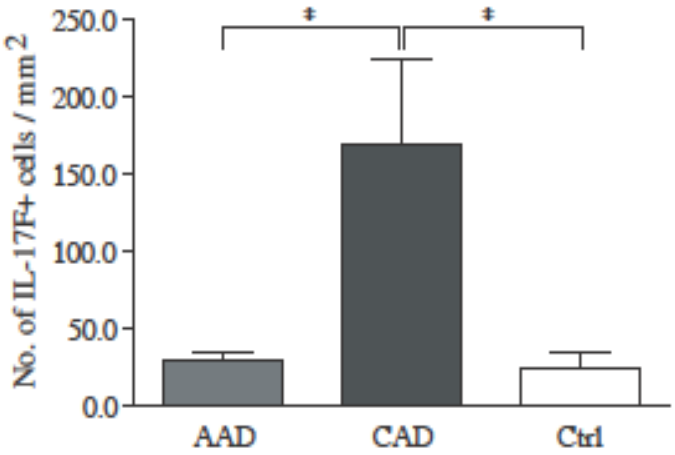
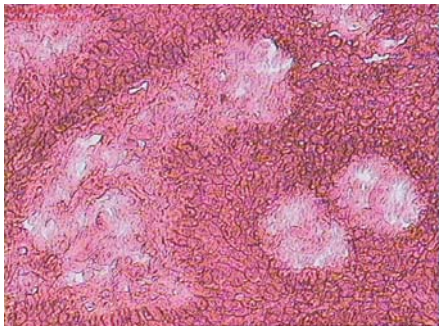


Figure (10): Quantitative assessment of the numbers of IL-17A- (a) and IL-17F (b)-immunoreactive cells infiltrating the dermal compartment of skin lesions from subjects with acute AD, chronic AD or from healthy skin specimens. Cells were blindly scored and the data are presented as the numbers of immunoreactive cells per mm^2 of subepidermal surfaces. * indicates $p<0.05$ using ANOVA and Dunn's Multiple Comparison Test.

A



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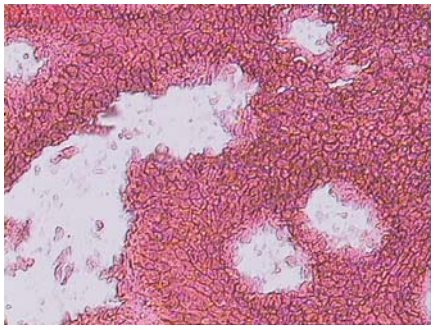

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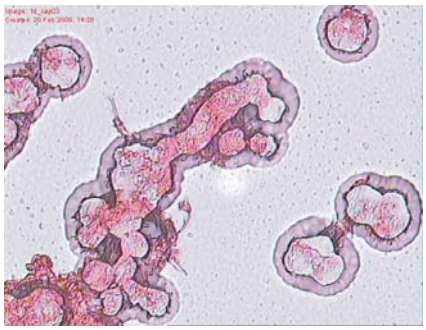

Figure (11): Laser capture microdissection (LCM) / real time-quantitative polymerase chain reaction (RT-qPCR) evidence of IL-17A & F in dermal mononuclear cells. Atopic skin tissue sections before (A) and after (B) LCM of mononuclear cells, as well as caps (C).

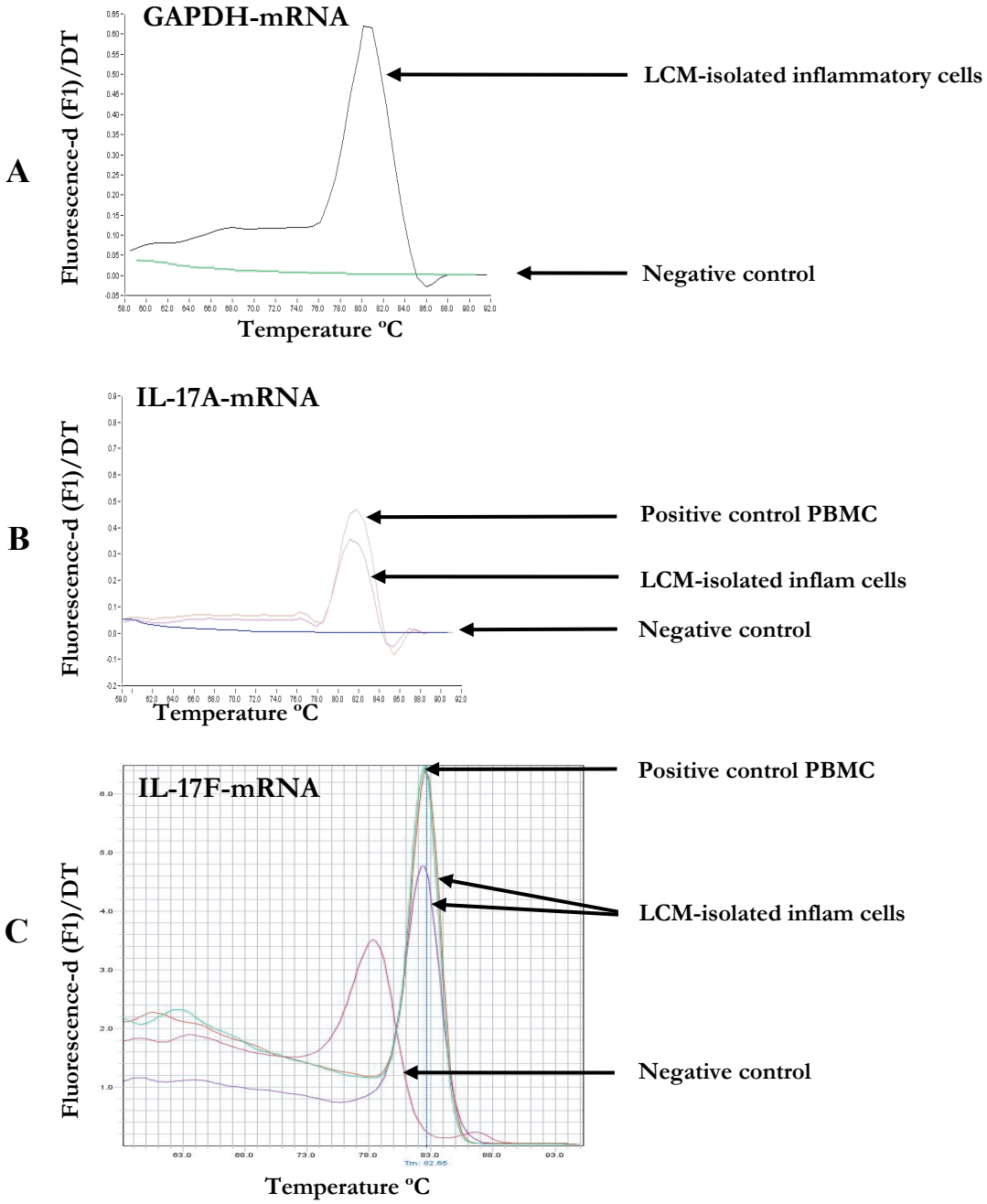

Figure (12): Laser capture microdissection (LCM) / real time-quantitative polymerase chain reaction (RT-qPCR) evidence of IL-17A & F in dermal mononuclear cells. qPCR Amplification curves for GAPDH (A), IL-17A (B) and IL-17F (C).

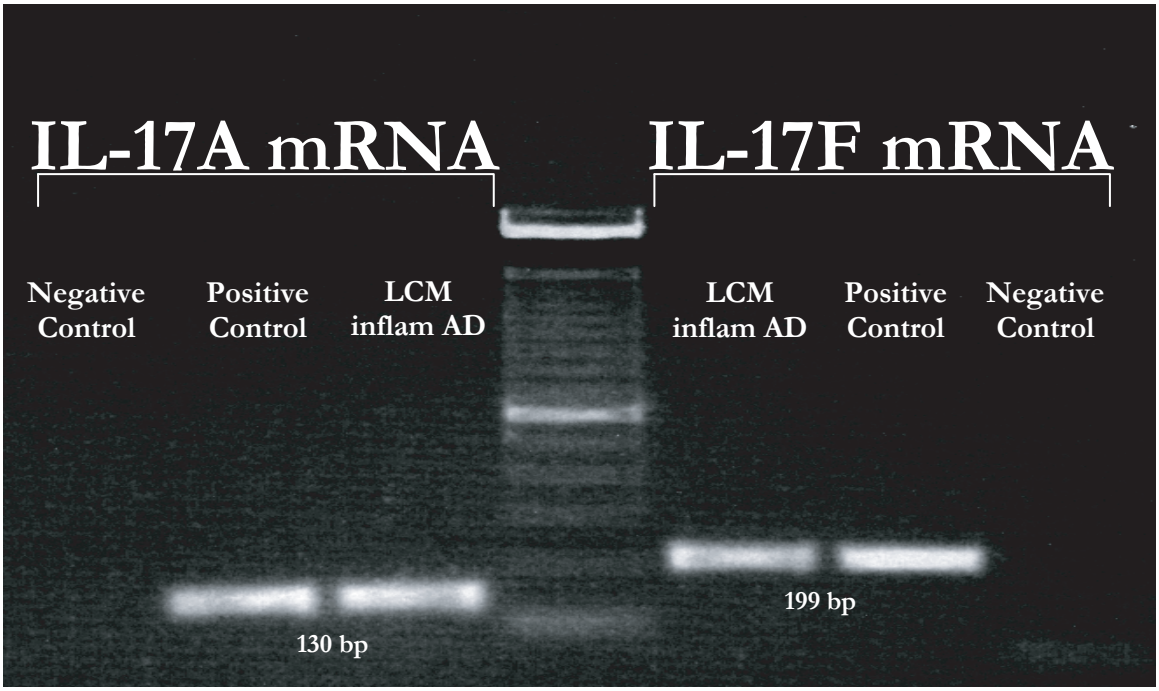

Figure (13): Laser capture microdissection (LCM) / real time-quantitative polymerase chain reaction (RT-qPCR) evidence of IL-17A & F in dermal mononuclear cells. Size distribution patterns of products of electrophoretic separation of DNA strands on 1% agarose gel.

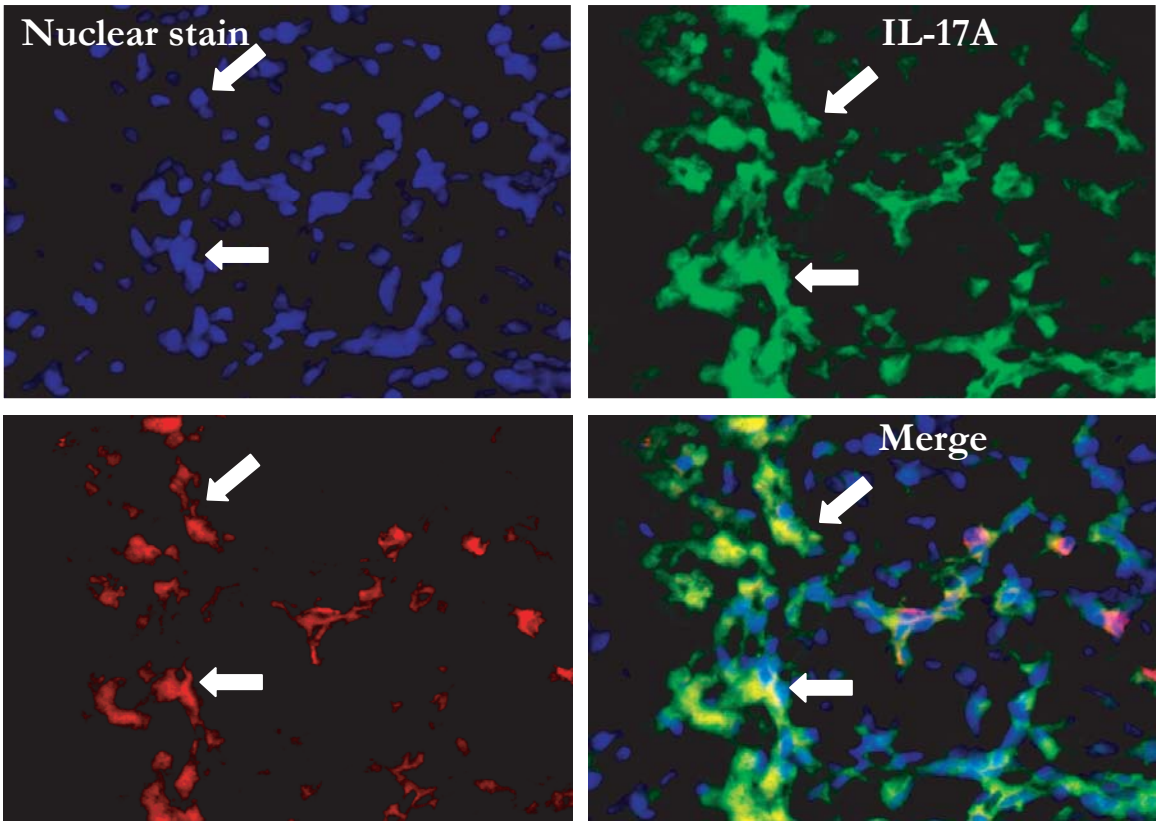

Figure (14): Immunofluorescent localization of T helper type 17 cells in atopic dermatitis tissues. This was done using double immunofluorescence staining of frozen sections, for CD3 (red staining, arrow) and IL-17A/IL-17F (green staining, arrow), and Hoechest (blue). Cells expressing both IL-17+CD3+ (merge) show yellow color (arrows). Tissues were analyzed by immunofluorescence microscopy and staining shows one staining representative of three.

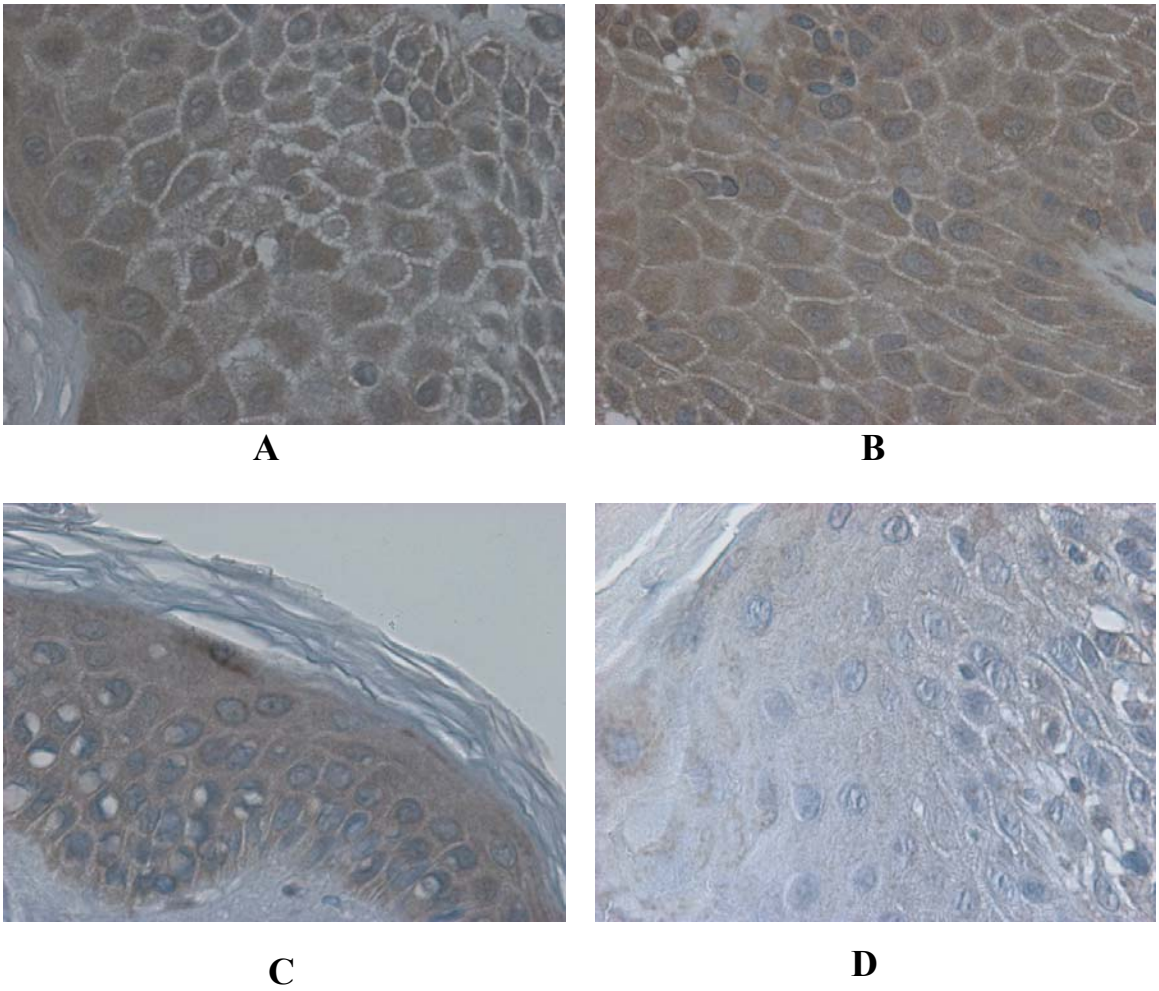

Figure (15): Immunohistochemical detection of IL-17F in Keratinocytes. (A) Skin tissue section from acute AD patient showing diffuse staining of the epidermis (400x). (B) More intensive staining seen in chronic AD epidermis (400x). (C) Skin tissue sections from healthy controls (400x). (D) No staining with IgG isotype (400x).

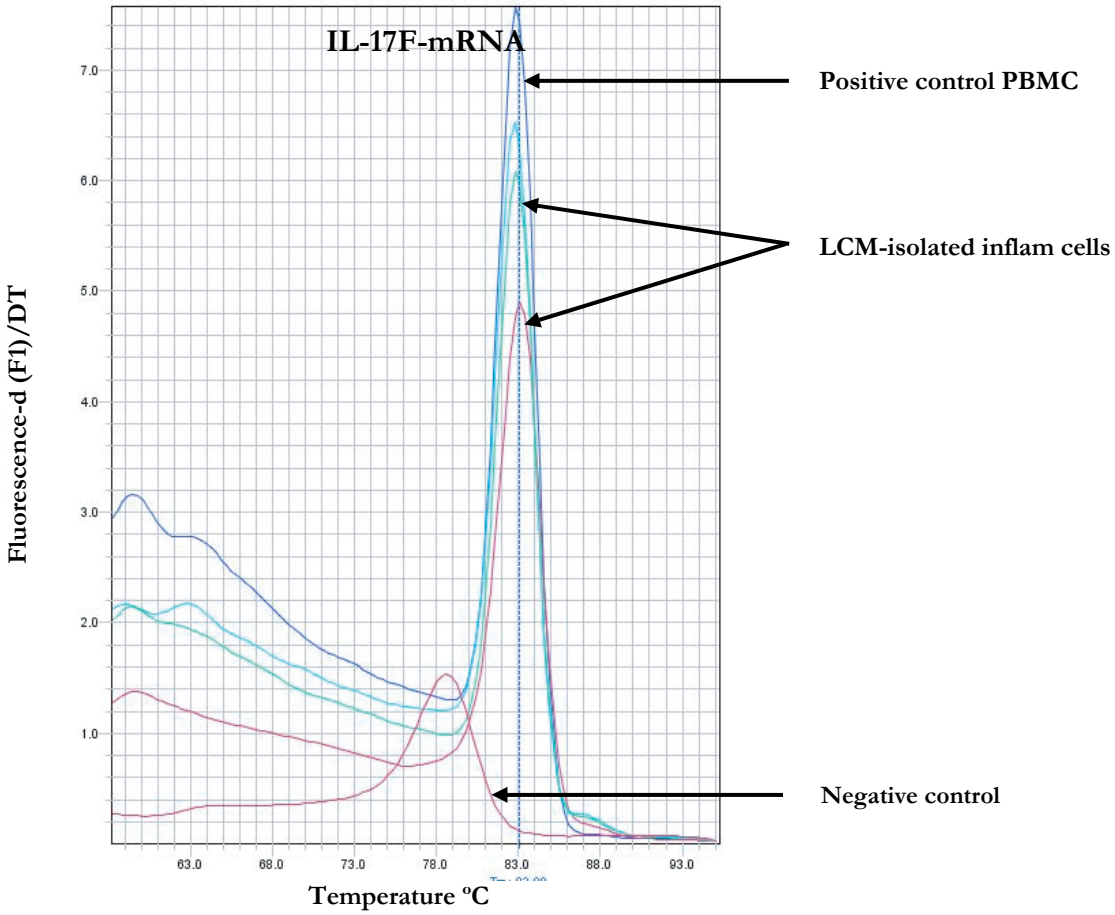

Figure (16): Laser capture microdissection (LCM) / real time-quantitative polymerase chain reaction (RT-qPCR) evidence of IL-17F in keratinocytes. qPCR Amplification curves for IL-17F.

CHAPTER 4

DISCUSSION

Although atopic dermatitis (AD) was originally regarded as a T_H2-mediated disease, this was challenged by strong evidence that the development of AD skin lesions results from initial activation of T_H2 cells in the lymph nodes followed by T_H1-type cells in the lesions during the chronic phase (169). Interestingly, another T_H2-mediated allergic disease, allergic asthma, was shown to be associated with elevated levels of T_H17-cytokines, the most cited being IL-17A (170). Moreover, T_H17 cytokines (IL-17A, along with IL-22 and IL-23) have been shown crucial to the pathogenesis of psoriasis, which has similarities to AD and was previously considered as a T_H1-associated disorder (171). Traditionally, acute AD lesions have been more known to be T_H2 dominated disease, whereas the inflammatory profile of chronic ones has been described as a mixture of T helper cell phenotypes (172). Taken together; these findings raise questions about possible involvement of T_H17 cells in AD as well. Furthermore, although many studies investigated the presence of T_H17-associated cytokines (mainly IL-17A) in skin inflammatory disorders, including AD (54-58), no one to date has directly established the presence of T lymphocytes committed to the T_H17 phenotype in skin tissue from human subjects with atopic dermatitis.

The first evidence for IL-17 cytokine expression in the skin of AD patients came from Toda and collaborators, who detected IL-17 (subsequently, more formally identified as IL-17A) in skin lesions from AD patients. However, no significant correlation between the expression of IL-17 and the number of T cells was detected (55). Koga and associates

also performed immunohistochemical analysis of skin lesions and reported increased numbers of IL-17A-immunoreactive cells in skin lesions from subject with acute AD compared with both healthy subjects and those with chronic AD (56). In the present study, the numbers of IL-17A and IL-17F were found to be increased in both chronic and acute AD compared with healthy controls, although only chronic were significantly higher than controls. Moreover, the observation that not all cells expressing IL-17A positivity displayed CD3 immunoreactivity suggest that the marked increase in IL-17A expression in AD lesions is not restricted to T cell compartment.

However, our data suggests that IL-17A- and IL-17F-expressing cells are more present in chronic diseases than in acute lesions which contrasts with the previous literature (55, 56) establishing increased numbers of IL-17A-immunoreactive cells in the skin of subjects with acute versus chronic. As an attempt to explain such discrepancies, one should refer to investigations on IL-17A expression in the context of another atopic disorder, bronchial asthma. In this disease, studies demonstrated an association between increased expression of IL-17A, severity of asthma, and histological evidence of remodeling (e.g. collagen deposition) (173). Molet et al (174). also showed that IL-17A induces dermal fibrosis through an indirect effect as it would stimulate epithelial cells and fibroblasts to secrete pro-fibrotic cytokines like IL-6 and IL-11. Tissue fibrosis and the extent of remodeling are well-established features of chronic and severe inflammatory disorders like asthma. Therefore, it is somehow logical to find more IL-17 cytokines in chronic skin lesions, which features stronger evidence for remodeling, TGF-β1 and IL-11 expression than acute skin specimens (55, 175).

Recently, Guttman-Yassky and her colleagues (57) demonstrated elevated IL-17A and IL-17F mRNA in homogenized skin samples taken from patients with psoriasis and atopic dermatitis compared to controls, although only tissues from psoriatic subjects came out significantly different from controls. These results coincide with our observations regarding IL-17A and IL-17F mRNA detected among dermal mononuclear cells isolated from AD skin lesions by laser capture microdissection (LCM). The LCM technique overcomes the short comings encountered during molecular studies due to the presence of different cells in the biopsies to be studied, often "contaminating" the "minority" cell of interest. To our knowledge, this is the first report using LCM to study an inflammatory skin disease, although it has been used in skin cancer research (176). These data are in line with experiments conducted in a murine model of atopic dermatitis. In this study, He et al., used mice epicutaneously-sensitized with ovalbumin to study skin inflammation and demonstrated that both IL-17A and F transcript levels were increased, along with IL-4 and IL-13 but not IFN-γ (58).

In order to confirm the presence of T_H17-committed cells in skin lesions from AD, we performed fluorescent immunostainings specific for IL-17A- and IL-17F-producing cells, while also expressing T lymphocyte surface markers. This approach revealed co-localization of IL-17A and F together with CD3 expression, which clearly demonstrates infiltration of the dermis by T_H17 cells in AD. These findings are in line with another recent study (59). Using a tissue digestion-based procedure, Eyerich and collaborators assessed the cytokine production profile of T cell clones expanded after they were isolated from digested AD skin lesions. Their data suggested that less than 10% of cells were able to produce IL-17A (by flow cytometry) upon *in vitro* stimulation. However, IL-

17A secretion failed to be enhanced by *Dermatophagoides pteronyssinus* (Der p 1) extract to which patients were clinically reacting (59). This study also revealed that 50% of IL-17A-producing T cells were pure T_H17 cells, whereas one third of cells co-expressed either IL-4 (T_H2/T_H17 cells) or IL-4 and IFN-γ (T_H0/T_H17 cells). Moreover, a minority co-produced IL-17A and IFN-γ, which is in line with the existence T_H1/T_H17 cells reported by others (177). On another hand, Koga et al., using flow cytometry, investigated IL-17A-positive circulating T lymphocytes and suggested that $CD3^+$ $CD8^-$ T lymphocytes (e.g. $CD4^+$ T cells) are a major source of IL-17A. Interestingly, they reported increased numbers of circulating IL-17A-producing $CD8^-$ T cells in severe atopic dermatitis subjects compared to healthy controls (56).

Rather than considering IL-17A as the main TH17-associated marker with regards to the pathogenesis of AD, our data also supports a role of IL-17F. This cytokine has not received as much attention as IL-17A until recently, mainly due to initial lack of reagents to measure it. Like IL-17A, IL-17F induces multiple pro-inflammatory mediators, including chemokines, cytokines, and metalloproteinases, from epithelial and fibroblast cells that play a significant role in the pathogenesis of asthma, including airway remodelling (57). Several studies reported increased expression of IL-17F in psoriasis (178, 179). For example, Wilson et al (179). detected significantly higher levels of IL-17A and IL-17F mRNA levels in homogenized psoriatic skin lesions compared to non-lesion skin, whereas both psoriatic samples were higher compared with normal controls. To the best of our knowledge, our study is the first to report an increased expression of IL-17F in the dermis of AD patients (both acute and chronic) compared with controls.

Furthermore, the number of IL-17F-expressing cells was not only increased among dermal mononuclear cells, but we also report the expression of IL-17F in keratinocytes of patients with AD. In addition to forming the physical barrier between the organism and its environment, keratinocytes form a proactive tissue with chemical, biochemical and immunological properties. Permeability barrier disruptions taking place in psoriasis and atopic dermatitis are good examples of keratinocyte stimuli triggering their production of pro-inflammatory mediators including TNF-α, IL-1 and IL-6, the latter being involved in T_H17 cell differentiation (180). As another example illustrating the involvement of T_H17 cytokines in AD, the production of anti-bacterial peptide psoriasin (S100A7) was shown to be increased in AD skin lesions, while IL-17A also enhanced its expression by keratinocytes (181). Furthermore, keratinocytes were also shown responding to IL-17F themselves (182), which suggests an autocrine feedback loop. Finally, IL-17F expression by keratinocytes is also in line with studies documenting the expression of IL-17F by airway epithelial cells. So far, two studies documented an increased IL-17F expression by bronchial epithelial cells in a murine model of allergic airway inflammation (183) and in the lung of asthmatic subjects (184), respectively. Moreover, on the genetics perspective, a single study investigated T_H17-associated single nucleotide polymorphisms (SNPs) in association with AD. Nonetheless, IL-17F SNPs failed to be associated with clinical manifestation of psoriasis vulgaris and atopic dermatitis in a Japanese population (132).

To further our understanding of the role of T_H17 in atopic diseases, the unique study of He and his associates demonstrated that T_H17 cytokines could represent a link between AD and asthma (58). In a murine model, epicutaneous sensitization with OVA was able to produce an experimental model of AD and to excite a local, as well as a systemic IL-

17 response. Further inhalation challenge of OVA- sensitized mice induced IL-17 and CXCL2 expression and neutrophil influx in the lung along with bronchial hyper-reactivity, which were reversed by blocking of IL-17.

In conclusion, our study supports a potential role for T_H17 cells and their cytokines in the pathophysiology of atopic dermatitis. In addition to confirming the current literature linking IL-17 cytokine expression to inflammation seen in acute AD skin lesions, our data further suggests roles for both IL-17A and IL-17F in the skin remodeling observed in chronic AD. Our data also demonstrate that keratinocytes could themselves be a therapeutic target as they also express IL-17F in AD. Our findings should stimulate trials to further our understanding of the clinical importance of T_H17 cells, allowing us to sense the role of each cytokine independently, and to eventually design optimal cell- or cytokine-targeted therapies. Further studies will also aim at determining whether IL-17 (A&F)-producing T cells also express cytokines of the T_H1 and/or T_H2 family as suggested by others.

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