UNIVERSITAT DE BARCELONA

# Role of CLA+T lymphocytes in the mechanisms triggering psoriasis

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PhD Program in Biomedicine

# *Role of CLA+ T lymphocytes in the mechanisms triggering psoriasis*

Thesis submitted by

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to qualify for the Doctorate degree by the University of Barcelona

Thesis director and tutor:

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Barcelona, March 2021

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*Uno no sabe lo que sabe*
*hasta que puede enseñar a otros.*

*Es de importancia para quien*
*desee alcanzar una certeza*
*en su investigación,*
*el saber dudar a tiempo.*
- Aristóteles

A Kika,
por enseñarme,
inspirarme y
acompañarme,
siempre.

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Después de casi 4 años desde que comenzara este proyecto, ha llegado el momento de cerrar esta importante etapa en mi vida y, para ello, quiero empezar por reconocer y agradecer a cada una de las personas que me han acompañado, de una forma u otra, durante este proceso.

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A la Carmen de 2017, por no quedarte con las ganas, hacer las maletas y lanzarte a intentarlo.

¡Lo conseguiste!

# ABSTRACT

Psoriasis is a common immune-mediated inflammatory skin condition affecting around 2% of the population worldwide. Different disease subtypes have been described regarding their specific clinical phenotype: chronic plaques, inverse, guttate, pustular and erythrodermic. Disease onset occurs from a complex interaction between the immune system (mostly T cells) and environmental factors (like microbes), along with a susceptible genetic background, resulting in increased secretion of cytokines, governed by the IL-17/IL-23 axis, that enhance the inflammatory loop leading to the appearance of cutaneous lesions. The skin, besides being an effective physical barrier that protects us from external agents, accommodates diverse types of immune cells. Importantly, the extravasation of T cells from blood to cutaneous tissue is tightly regulated by the expression of specific adhesion molecules and their ligands on both T cells and dermal blood vessels respectively. The Cutaneous Lymphocyte-associated Antigen (CLA) identifies the subset of effector memory T cells with cutaneous tropism. CLA-expressing cells represent more than 90% of skin infiltrating T cells but, since these cells are able to recirculate between blood and tissue, a proportion of them can be found in circulation, thus being a useful tool with translational value for the study of T cell-mediated skin conditions. Our group has developed an *ex vivo* model of psoriasis based on the coculture of circulating $CLA^+$ T cells with autologous epidermal cells obtained from lesional biopsies in the same patient. In this thesis, this model is used to discuss how circulating $CLA^+$ T cells respond to relevant disease triggers such as microorganisms (*Streptococcus pyogenes* and *Candida albicans*) and lesional pro-inflammatory cytokines (IL-15 and IL-23) in plaque and guttate forms of psoriasis, as well as the association between cytokine response to microbes *in vitro* and patients exposure to the same microorganism, measured by the presence of specific antibodies in plasma. First, the study of *S. pyogenes* (SE)-specific antibodies revealed increased IgA response in both plaque and guttate psoriasis compared to healthy controls and atopic dermatitis patients. Interestingly, increased anti-SE IgA levels were reported in patients despite no history of s*treptococcal* infection. Of note, anti-SE IgA levels positively correlated with $CLA^+$ T cell dependent IL-17 response *in vitro*. Next, humoral response against *C. albicans* (CA) was assessed and plaque psoriasis patients showed increased anti-CA IgA and IgG compared with guttate psoriasis and controls. T cell cytokines induced after stimulation with CA were evaluated in supernatants revealing raised Th17 and Th9 responses in psoriasis, specifically in the $CLA^+$ T cell subset. Interestingly, anti-CA IgA levels and IL-17F/A responses were directly associated in $CLA^+$ and $CLA^-$ T cells cocultures from plaque psoriasis only. Because patients with increased anti-CA IgA may carry pathological peculiarities, a broad proteomic profile was evaluated in plasma samples of plaque psoriasis patients reporting raised presence of proteins associated to anti-microbial humoral response that could link psoriasis and periodontitis. Finally, the influence of lesional pro-inflammatory cytokines IL-15 and IL-23 was examined in our model and revealed the synergistic induction of IL-17F and IL-17A cytokines by $CLA^+$ T cells in cocultures from psoriasis but not healthy controls. This synergy required the presence of autologous epidermal cells, although it did not depend on skin-resident memory T cells, and was mainly mediated by activated $CD4^+$ $CLA^+$ T cells. In summary, this work has characterized novel mechanisms related to triggering factors of psoriasis and confirms that the subset of skin-tropic $CLA^+$ memory T cells particularly are key inducers of IL-17 cytokines upon activation by these triggers, which may contribute to the feedforward inflammatory loop and perpetuate psoriasis pathogenesis.

# INDEX

# LIST OF FIGURES

# LIST OF TABLES

# LIST OF ABBREVIATIONS

**AD**: Atopic dermatitis
**ADAMTSL5**: A disintegrin-like and metalloprotease domain containing thrombospondin type 1 motif-like 5
**AMP**: Anti-microbial peptides
**APC**: Antigen presenting cell
**ASO**: Anti-streptolysin O antibody
**BCA**: B cell-specific chemokine
**BCR**: B cell receptor
**Breg**: Regulatory B cell
**CA**: *Candida albicans* extract
**CCL**: CC-chemokine ligand
**CCR**: CC-chemokine receptor
**CGRP**: Calcitonin gene-related peptide
**CMC**: Chronic mucocutaneous candidiasis
**CXCL**: CXC-chemokine ligand
**CXCR**: CXC-chemokine receptor
**CLA**: Cutaneous lymphocyte-associated antigen
**CTACK**: Cutaneous T cell attracting chemokine
**DC**: Dendritic cell
**dDC**: Dermal dendritic cell
**DNA**: Deoxyribonucleic acid
**EGF**: Epidermal growth factor
**EMA**: European Medicines Agency
**EOP**: Early onset psoriasis
**EPI**: Epidermal cell suspension from biopsies
**FDA**: Food & Drug Administration
**FGF**: Fibroblasts growth factor
**GP**: Guttate psoriasis
**GWAS**: Genome-wide association studies
**hBD2**: Human beta defensin 2
**HC**: Healthy controls
**HEV**: High endothelial venule
**HLA**: Human leukocyte antigen
**IBD**: Inflammatory bowel disease
**ICAM**: Intercellular adhesion molecule
**IFN**: Interferon
**Ig**: Immunoglobulin
**IL**: Interleukin
**ILC**: Innate lymphoid cell
**IMQ**: Imiquimod
**iNOS**: Inducible nitric oxide synthase
**iNKT**: Invariant natural killer T cell
**Jak**: Janus kinase
**KC**: Keratinocyte
**K17** : Keratin 17
**LC**: Langerhans cell
**LFA**: Lymphocyte function-associated antigen
**LOP**: Late onset psoriasis
**mAbs**: Monoclonal antibodies
**mDC**: Myeloid dendritic cell
**MHC**: Major histocompatibility complex
**Mϕ**: Macrophages
**NETs**: Neutrophils extracellular traps
**NF-κB**: Nuclear factor-κB
**NK**: Natural killer cell
**PASI**: Psoriasis area severity index
**PBMCs**: Peripheral blood mononuclear cells
**pDC**: Plasmacytoid dendritic cell
**$PLA_2$**: Phospholipase A2
**PP**: Plaque psoriasis
**PsA**: Psoriatic arthritis
**PSGL**: Platelet selectin ligand
**PSORS**: Psoriasis susceptibility loci
**RNA**: Ribonucleic acid
**SAg**: Superantigens
**SALT**: Skin-associated lymphoid tissue
**SE**: *Streptococcus pyogenes* extract
**SNP**: Single nucleotide polymorphism
**Spe**: Streptococcal pyrogenic exotoxins
**Spp**: species
**STAT**: Signal transducers and activators of transcription
**TARC**: Thymus- and activation-regulated chemokine
**Tc**: T cytotoxic cell
**TCR**: T cell receptor
**TGF**: Transforming growth factor
**Th**: T helper cell
**TIP-DC**: Tumor necrosis factor and inducible nitric oxide synthase producing dendritic cell
**TLR**: Toll-like receptor
**TNF**: Tumor necrosis factor
**$T_{RM}$**: Tissue resident memory T cell
**VEGF**: Vascular endothelial growth factor
**VCAM**: Vascular cell adhesion molecule
**VLA**: Very late antigen

# INTRODUCTION

# 1. Skin Immune System

For many years, the connection between the fields of immunology and dermatology has been theorized, based on the occurrence of specific cutaneous inflammatory and allergic reactions. By virtue of numerous researches, the existence of the Skin-Associated Lymphoid Tissues (SALT) was first proposed by Dr J. W. Streilein (Wayne Streilein 1983), a concept that has evolved to our times in many aspects (Egawa and Kabashima 2011). This first chapter will discuss the main components of the cutaneous immune system, how lymphocytes develop skin-tropism and the concept of immune surveillance in the skin.

## 1.1 Cellular components and tissue organization

The skin is the largest organ in the human body, broadly understood as a protective shield against external agents, whose integrity must remain unbroken to maintain its functionality. However, besides being a fundamental physical barrier, the cutaneous tissue shelters different cellular components with immunomodulatory functions thus constituting an active barrier too (Figure 1). The skin can be structurally divided into three main layers which are, from the outer to the inner side: the epidermis, the dermis, and the hypodermis.

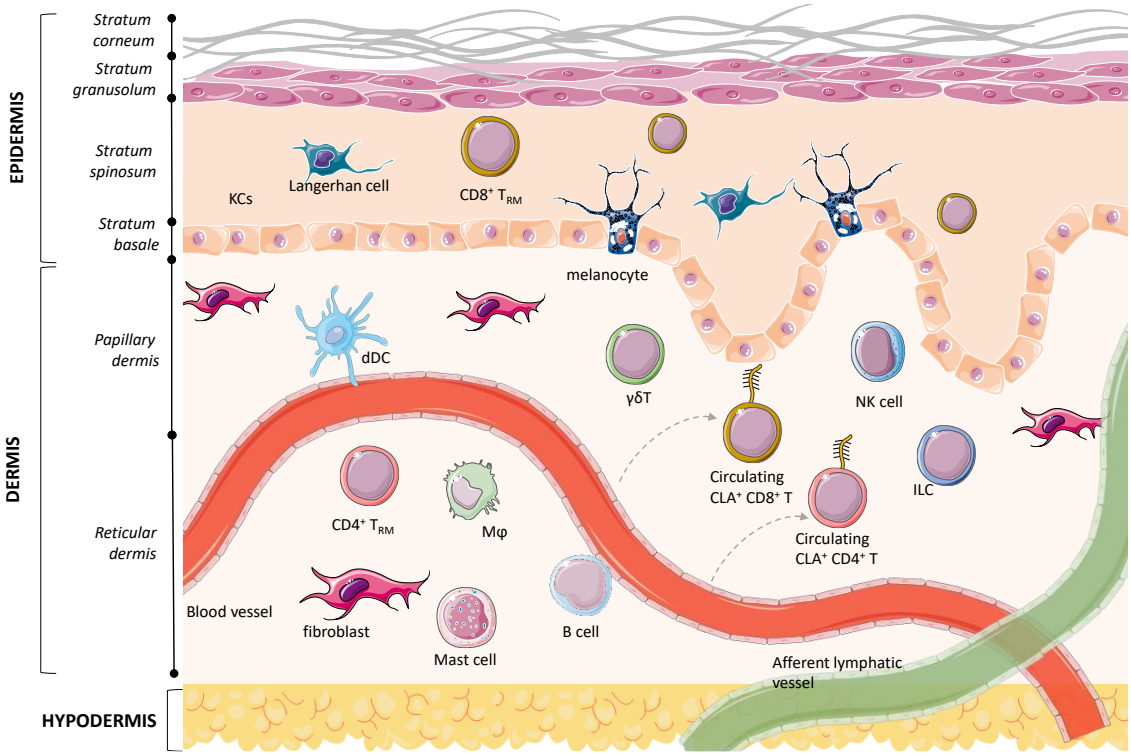



**Figure 1. Skin structure and distribution of immune cells.** The epidermis is composed of layers of keratinocytes (KCs), with melanocytes and Merkel cells intersected in the inner sheet, as well as Langerhans cells (LCs) and $CD8^+$ resident memory T ($T_{RM}$) cells present at intermediate layers. Instead, the dermis is mainly composed of connective tissue produced by fibroblasts, and includes dendritic cells (dDCs), macrophages, innate lymphoid cells (ILCs), natural killer (NK) cells, γδ T cells, scarce B cells and both $CD4^+$ and $CD8^+$ recirculating and resident memory T cells. Finally, the hypodermis mainly contains adipose tissue.

The epidermis is primarily composed of keratinocytes (KCs), which are less differentiated at inner the *stratum basale* and become more keratinized towards the outer *stratum corneum*. Melanocytes and Merkel cells are present at the internal layer, whereas in the intraepithelial sites there are specialized dendritic cells, known as Langerhans cells (LCs), and lymphocytes, mainly $CD8^+$ tissue-resident memory T ($T_{RM}$¡**Error! Marcador no definido.**) cells. Beneath the epidermal layer and tightly connected by a basement membrane is the dermis, mainly integrated by connective tissue produced by dermal fibroblasts. Structurally divided into a superficial and a deep thicker region, named *papillary* and *reticular* respectively, it contains classical dendritic cells (dDCs), both recirculating and tissue resident memory lymphocytes (mostly $CD4^+$ and regulatory T cells), interspaced B cells, non-conventional γδ T cells, mast cells, macrophages (Mϕ), innate lymphoid cells (ILCs) and natural killer (NK) cells. Located within this reticular region are also the blood and afferent lymphatic vessels, from which hematopoietic immune cells are constantly entering and leaving the tissue. Finally, there is the hypodermis that mainly contains adipose tissue. The functional coordination of all these cellular mediators is crucial for maintaining cutaneous homeostasis. Instead, altered, or inadequate cell function leads to defective immune responses ultimately causing diverse skin disorders.

## 1.2 Skin homing and memory T cells recirculation

Lymphocytes in the blood extravase to different peripheral tissues in a specific manner. Distinct combinations of selectins, integrins and chemokines receptors at the lymphocyte surface, as well as the presence of their ligands at the target tissue, sharply control the molecular mechanisms of cell trafficking. The expression of these "homing receptors" by antigen-experienced T cells occurs after priming at the tissue draining lymph nodes (Figure 2). After leaving the thymus, naïve T cells constantly circulate through secondary lymphoid organs where antigen presenting cells (APCs) may display their specific antigen. Following activation, these cells differentiate into effector memory T cells and acquire the capacity to enter peripheral tissues. Their function and tissue tropism will be determined by local environmental mediators at the lymph node and, most importantly, cellular interactions with tissue specific dendritic cells. Afterwards, memory T lymphocytes expressing distinct homing receptors return to blood circulation through the lymphatic system, and finally enter the site of antigen encounter, therefore activating the immune response where needed (Agace 2006; Fu et al. 2016).

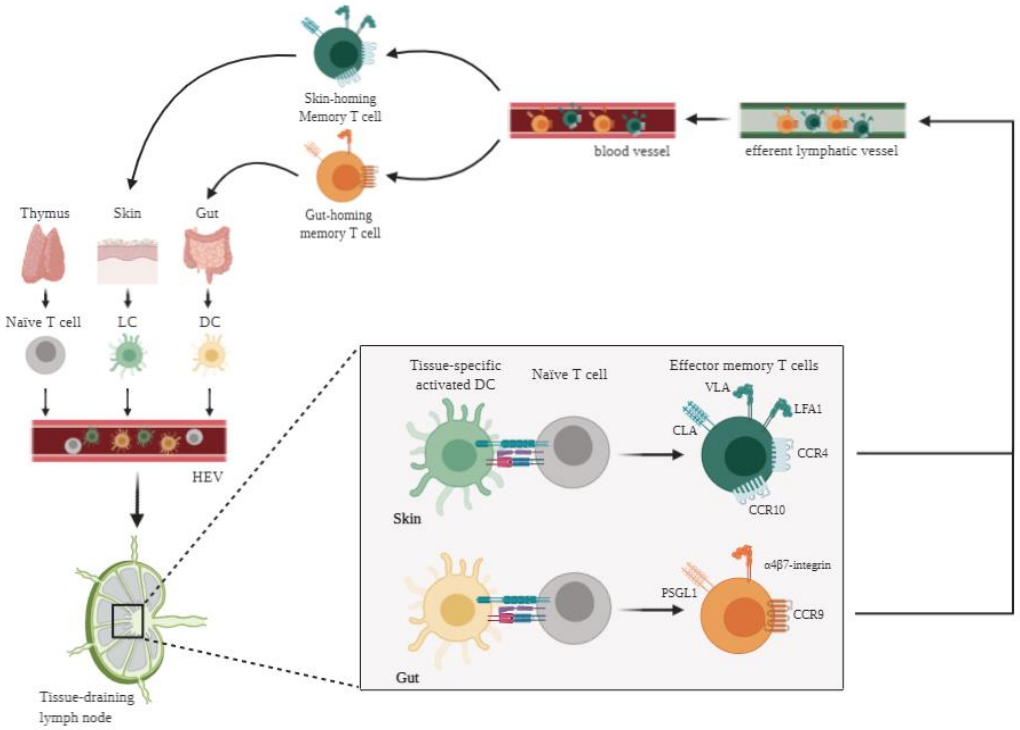



**Figure 2. Memory T cell generation and induction of homing receptors at lymph nodes.** Once they leave the thymus, naïve T cells circulate through secondary lymphoid organs entering via high endothelial venules (HEV). Tissue-specific activated dendritic cells (DCs) also enter their proximal draining lymph node, where they activate naïve T cells recognizing the antigen they are presenting. This interaction results in generation of effector memory T cells with specific tropism for different extra-lymphoid tissues. Expression of selective tissue-homing receptors depends mainly on tissue-derived dendritic cells but also lymph node environment. Finally, effector memory T cells expressing specific homing receptors enter the general circulation, through efferent lymphatic vessels, and will extravase to their target extra-lymphoid tissue expressing the ligands for their specific homing receptors. LC; Langerhans cells. CLA; Cutaneous Lymphocyte-associated Antigen. VLA; Very Late Antigen; LFA1; lymphocyte function-associated antigen-1. CCR; CC-motif Chemokine Receptor. PSGL1; P-Selectin Glycoprotein Ligand-1. Adapted from Agace, 2006 and created in biorender.com

The expression of Cutaneous Lymphocyte-associated Antigen (CLA) identifies the subset of effector memory T cells with skin tropism. It is present in more than 90% of cutaneous infiltrating T cells but in less than 20% of T cells in other peripheral tissues. Interestingly, a proportion of CLA-expressing T cells can be found in circulation, around 15% of human circulating T cells (Picker et al. 1993). The CLA molecule is a carbohydrate modification of platelet selectin ligand-1 (PSGL-1) mediated by the fucosyltransferase VII (Fuhlbrigge et al. 1997). Its expression is induced on $CD45RO^+$ memory T cells at skin draining lymph nodes (Picker et al. 1993) and it binds both endothelial-cell and platelet selectins (E-selectin and P-selectin) molecules expressed on postcapillary venules in the skin. Also, CLA epitope has been found on the sialomucin CD43, constitutively expressed on T cells, but it showed E-selectin binding activity only (Fuhlbrigge et al. 2006). Additional molecules are required for $CLA^+$ T cells extravasation into the skin (Figure 3). Very late antigen (VLA, also known as $\alpha_4\beta_1$-integrin) and lymphocyte function-associated antigen-1 (LFA1) integrins expressed on T cells interact with vascular cell adhesion molecule-1 (VCAM1) and intercellular adhesion

molecule-1 (ICAM1) respectively, present on cutaneous endothelial cells (Santamaria-Babi et al. 1995). Likewise, CCL17/TARC (Thymus- and activation-regulated chemokine) produced by dendritic cells, endothelial cells, keratinocytes and fibroblasts (Chong et al. 2004) and CCL27/CTACK (cutaneous T-cell-attracting chemokine) constitutively expressed by basal keratinocytes (Homey et al. 2002), displayed on the skin post capillary venules, attract $CLA^+$ T cells which express their respective receptors, CC-chemokine receptors 4 and 10 (CCR4 and CCR10).

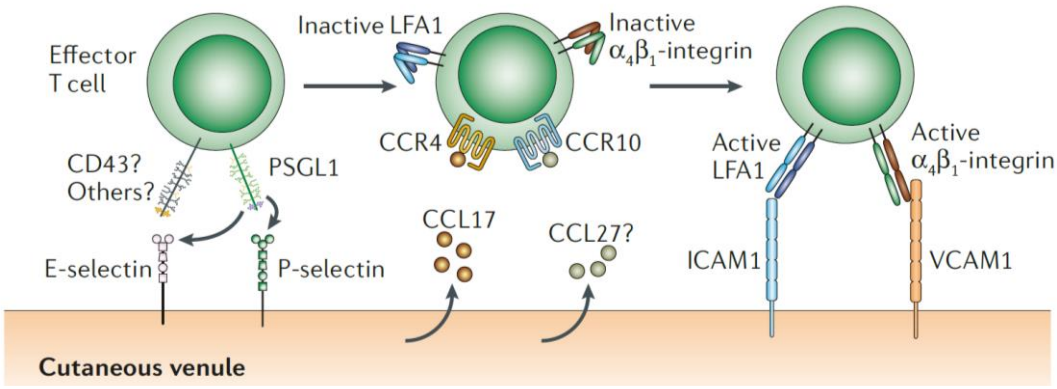



**Figure 3. Molecules implicated in skin homing.** Extravasation of skin-homing lymphocytes occurs as the result of a coordinated multistep process. First, transitory tethering and rolling of effector memory T cells on endothelium is mediated by CLA binding to E/P-selectins on the cutaneous venules. Also, CLA epitope present on CD43 molecules binds exclusively to E-selectin. This initial attachment facilitates T cell exposure to chemokines present on the endothelial cells surface, as well as the activation of integrins present at T cell membrane. Then, CCL17/TARC and CCL27/CTACK chemokines further attract $CLA^+$ T cells expressing their receptors CCR4 and CCR10, respectively. Finally, activated LFA1 and VLA/$\alpha_4\beta_1$-integrins on lymphocytes firmly bind to their ligands, ICAM1 and VCAM1, expressed on postcapillary venules. These settlements induce lymphocytes arrest on the epithelium, followed by their transmigration into cutaneous tissue. From Agace 2006.

Given the relevance of these skin-homing molecules in T cell trafficking towards cutaneous tissue, the therapeutic potential of their blockade was explored in T cell mediated skin conditions, such as psoriasis or atopic dermatitis. Efalizumab, a monoclonal antibody targeting the CD11a subunit of LFA-1 molecule, proved clinical improvement associated to decreased epidermal and dermal T cell infiltration together with increased circulating lymphocyte counts in moderate-to-severe psoriasis (Gottlieb et al. 2002). Raised numbers of $CLA^+$ memory T cells were observed during efalizumab treatment in psoriasis (Vugmeyster et al. 2004) and atopic dermatitis (Harper et al. 2008) patients. This lymphocytosis effect might be explained by the impairment of LFA-1/ICAM-1 transendothelial migration of $CLA^+$ T cells towards the skin, but also by the accumulation of lesion infiltrating $CLA^+$ T cells leaving the cutaneous tissue through lymphatic drainage (Ferran et al. 2013b). Such phenomenon is of clinical relevance, since skin relapse may occur after treatment discontinuation, as observed by Johnson-Huang *et al* in psoriasis (Johnson-Huang et al. 2012). All in all, anti-LFA-1 mechanism of action supports the idea of effector memory $CLA^+$ T cells recirculation capacity between skin and blood during cutaneous inflammation.

## 1.3 The concept of immune surveillance of the skin

Initially, the concept of cutaneous immune surveillance, understood as the constant transit of lymphocytes through blood, lymphoid organs, and non-lymphoid tissues looking for foreign or malignant antigens, explained the immune defence of the skin. However, after the SALT was depicted, this approach evolved giving value to both skin resident cells (KCs, LCs, mast cells, dDCs or Mϕ), that are innate sentinels for danger signals, and circulating lymphocytes that continually patrol the organism in maintaining cutaneous homeostasis, as well as to activate adaptive immune responses against infections or malignancies. Hence, the interplay of the innate and adaptive immune systems is crucial for the induction and maintenance of effective immune surveillance.

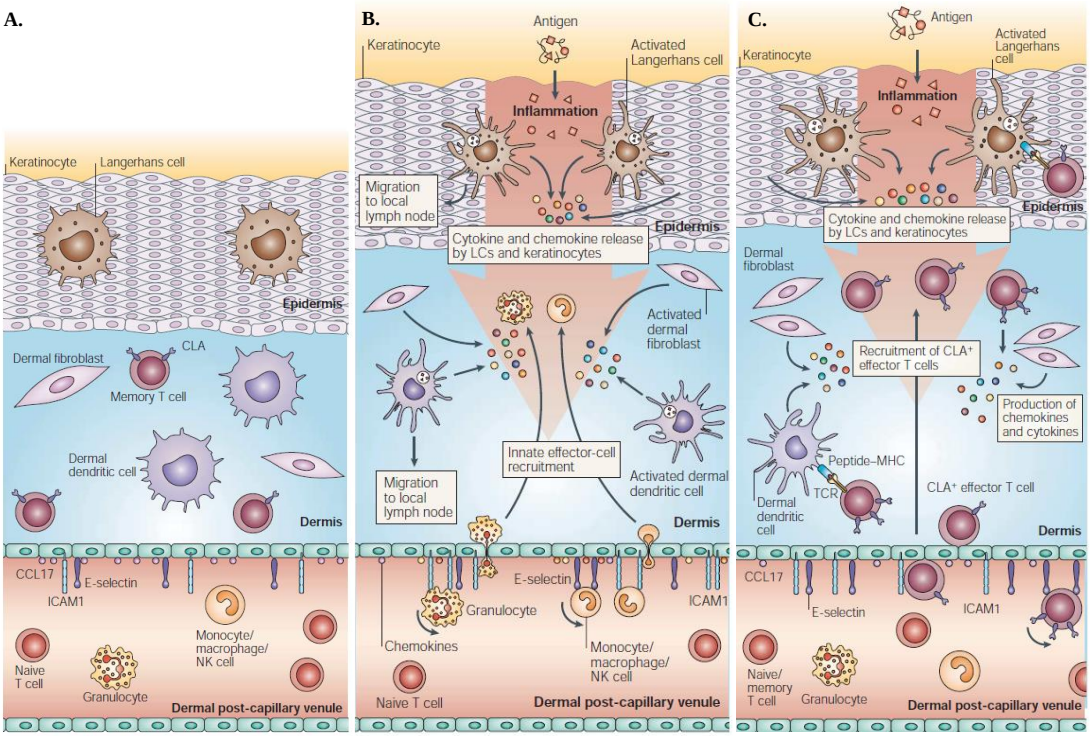



**Figure 4. The mechanisms of immune surveillance at cutaneous tissues. (A)** At non-inflamed skin, constitutive low expression levels of E-selectin, CCL17 and ICAM1 allows extravasation of skin-homing memory T cells to maintain tissue homeostasis. **(B)** After cutaneous injury, epidermal KCs and LCs activate and secrete chemokines and cytokines that stimulate dermal fibroblasts, dDCs and other immune cells. This innate response, through activation of NF-κB pathway, eventually increases the expression of adhesion molecules at dermal post-capillary venules, enhancing effector cells recruitment to the skin, whereas activated LCs and dDCs migrate to local lymph nodes. **(C)** Antigen-experienced T cells, primed at cutaneous draining lymph nodes and expressing skin homing molecules (CLA, CCR4, LFA1), extravase from blood stream towards cutaneous tissues, where they continue to move to the site of inflammation in response to chemotactic gradients. Finally, $CLA^+$ T cells can return to the general circulation through lymphatic vessels. From Kuuper and Fuhlbrigge 2004.

At steady state, dermal post-capillary venules constitutively express low levels of E-selectin, CCL17 and ICAM1, supporting the migration of memory T cells expressing CLA, CCR4 and LFA1 into non-inflamed skin (Figure 4a) (Weninger et al. 2000). Together with this baseline emigration of skin-tropic T cells, epidermal KCs and LCs, and dermal mast cells, DCs and

Mϕ, constitute an early warning system able to secrete anti-microbial peptides, chemotactic proteins, and cytokines promptly upon any cutaneous injury (infectious, malignant, or mechanic). The stimulation of this innate immune response ultimately leads to activation of the NF-κB pathway, that is a major link with the adaptive immunity, resulting in enhanced expression of endothelial adhesion molecules (as mentioned before E-selectin, P-selectin, ICAM1, VCAM1) and distinct chemokines and cytokines that actively increase leukocyte recruitment to the skin (Figure 4b). Next, adaptive immune surveillance can be understood in three steps: first, naïve T cell are activated by APCs in the specialized environment of skin-draining lymph nodes; second, skin-homing antigen-specific effector T cells -namely effector memory $CLA^+$ T cells- migrate to the site of antigen encounter; and finally, long-term central memory T cells are generated, potentially able to migrate to lymph nodes throughout the body, thus ensuring rapid responses even if the same antigen is recognized at a different site (Figure 4c). Importantly, skin recruited and activated $CLA^+$ T cells can enter lymphatic vessels and return back to the general circulation (Chong et al. 2004; Fu et al. 2016), which is vitally important for the study of T cell-mediated inflammatory skin conditions, as will be reviewed in the following section.

## 2. Circulating $CLA^+$ T cells in immune-mediated skin inflammation

The fact that $CLA^+$ T cells recirculate between skin and blood is key to their value in translational research, so they have been proposed as peripheral biomarkers in T cell-mediated skin conditions for several reasons (Ferran et al. 2013b). First, they can be easily isolated from blood samples, which are more feasible than skin biopsies for patients in daily clinical practice. Also, skin tropic $CLA^+$ T cells specific for relevant antigens or allergens in cutaneous pathologies have been described, most of which are summarized in Table 1. Finally, changes in $CLA^+$ T cells phenotype (cytokine profile or activation markers) and numbers in circulation have been associated to clinical features, such as disease severity, and treatment response in distinct T cell-mediated cutaneous diseases such as psoriasis, atopic dermatitis, vitiligo or alopecia areata (de Jesús-Gil et al. 2021) (included in APPENDIX II: supporting publications). Relevant examples illustrating this are compiled in Table 2. In summary, it can be stated that circulating $CLA^+$ memory T cells reflect the immune abnormalities found in cutaneous diseases. Therefore, the analysis of this unique subset of T lymphocytes, primed for skin homing and traceable through the expression of the CLA molecule on their membrane, in peripheral blood samples provides the opportunity to better understand different human T cell mediated skin diseases, like psoriasis.

**Table 1. Antigen-specific CLA$^+$ T cells described in human skin pathologies.** Adapted from Santamaria-Babí 2004 and de Jesús-Gil C et al 2021.

| Disease | CLA$^+$ cell | Antigen | Reference |
|---|---|---|---|
| *Atopic dermatitis* | CD45RO$^+$ T cells | *Dermatophaogides pteronyssinus* | Santamaria-Babi L *et al.* J Exp Med 1995 |
| | PBMCs | *Staphylococcal* enterotoxin A and B | Davison A *et al*. Clin Exp Immunol. 2000 |
| *Allergic eczema* | CD3$^+$ T cells | Casein | Abernathy-Carver KJ *et al.* 1995 |
| *Contact dermatitis* | CD45RO$^+$ T cells | Nickel | Santamaria-Babi L *et al.* J Exp Med 1995 |
| | CD4$^+$ T cells | Piperacillin | Sullivan A *et al*. J Allerfy Clin Immunol. 2018 |
| *Herpes simplex* | CD8$^+$ T cells | Herpes Simplex Virus-2 | Koelle DM *et al.* J Clin Invest. 2002 |
| *Psoriasis* | CD8$^+$ T cells | Keratin peptides sharing sequence with streptococcal M protein | Johnston A *et al.* Clin Exp Immunol. 2004 |
| | CD3$^+$ T cells | *Streptococcus pyogenes* | Diluvio L *et al*. J Immunol. 2006 |
| | CD45RO$^+$ T cells | *Streptococcus pyogenes* | Ruiz-Romeu E *et al*. J Allergy Clin Immunol. 2016<br>Ruiz-Romeu E *et al.* J Invest Dermatol. 2018 |
| *Vitiligo* | CD8$^+$ T cells | Melan-A | Ogg GS *et al.* J Exp Med. 1998 |

**Table 2. Circulating CLA$^+$ T cells clinical and functional activities in T-cell mediated cutaneous conditions in humans.** Adapted from Ferran et al 2013b and de Jesús-Gil et al 2021.

| Disease | Clinical and functional roles in humans | Reference |
|---|---|---|
| *Acute graft versus host disease* | Increased CLA$^+$ Tregs at engraftment is associated with prevention of skin aGVHD | Engelhardt BG *et al*. Bone Marrow Transplant. 2011 |
| *Alopecia Areata* | CLA$^+$ Th2/Tc2 and Th22 are significantly increased compared to healthy controls, whereas frequency of CLA$^+$ Treg is decreased | Czarnowicki T *et al*. Allergy. 2018 |
| *Atopic dermatitis* | CLA$^+$ T cells are *in vivo* activated (HLA-DR$^+$) and spontaneously produce IL-4[1] and IL-13[2] able to induce IgE in autologous B cells | [1]Santamaria-Babi L *et al.* J Exp Med 1995<br>[2]Akdis M *et al.* J Imunol 1997 |
| | Increased percentage of *Staphylococcus aureus* superantigen-responsive CLA$^+$ T cells | Torres MJ *et al*. Clin Exp Allergy. 1998 |
| | CLA$^+$ T cells are increased under stress in atopic dermatitis patients | Schmid-Ott G *et al.* J Allergy Clin Immunol. 2001 |
| | Pruritus associated cytokine; IL-31 is exclusively produced by CLA$^+$ T cells | Sonkoly E *et al.* J Allergy Clin Immunol. 2006 |
| | CGRP neuropeptide directly activates CLA$^+$ T cells and increase IL-13 production | Antúnez C *et al.* Br J Dermatol. 2009 |
| | Expansion of CLA+ Th2/Tc2[1] response in children and adults, and Th22/Tc22[2] response only in adults | [1]Czarnowicki T *et al*, J Allergy Clin Immunol. 2015 ; 136 (4)<br>[2]Czarnowicki T *et al*. J Allergy Clin Immunol. 2015 ; 136 (1) |
| *Non immediate cutaneous reaction to drugs* | CLA$^+$ Th2 cells, secreting IL-4 and IL-13, are increased in drug rash with eosinophilia and systemic symptoms (DRESS) | Teraki Y *et al.* Dermatology. 2017 |

**Table 2. Continued**

| Disease | Clinical and functional roles in humans | | Reference |
|---|---|---|---|
| *Lichen Planus* | Accumulation of CLA+ T cells in buccal mucosa in oral lichen planus, as well as in epidermis in cutaneous lichen planus | | Walton LJ *et al.* J Oral Pathol Med. 1997 |
| *Psoriasis* | Circulating $CD8^+$ $CLA^+$ T cells correlates with disease severity | | Sigmundsdottir H *et al.* Clin Exp Immunol. 2001 |
| | *Streptococcus pyogenes* tonsillar infection generates $CLA^+$ T cells that migrate to psoriatic cutaneous lesions | | Diluvio L *et al.* J Immunol. 2006 |
| | Circulating $CD3^+$ $CLA^+$ and $CD4^+$ $CLA^+$ T cells are activated ($HLA\text{-}DR^+$) and increased[1], and inversely correlate with PASI and BSA[2] in acute but not chronic psoriasis | | [1]Pont-Giralt et al J Invest Dermatol. 2006<br>[2]Ferran M et al, Eur J Dermatol. 2008 |
| | Decreased number of circulating $CLA^+$ $V\gamma9\delta2^+$ T cells, inversely correlated with PASI and were normalized after successful treatment | | Laggner U *et al.* J Immunol. 2011 |
| | Psoriasis lesions actively recruit $CLA^+$ T cells by producing chemokines such as CCL20, CCL27, CXCL8, CXCL9, CXCL10, CXCL11[1] and CXCL16[2]. | | [1]Mabuchi T *et al.* J Dermatol Sci. 2012<br>[2]Günther C *et al.* J Invest Dermatol. 2012 |
| | $CLA^+$ T cells, but not CLA-, secrete Th1/Th17 cytokines upon activation with *S. pyogenes* extract | | Ferran M *et al.* J Invest Dermatol. 2013 |
| | Reduction of circulating CLA+ T cells associated to clinical efficacy after anti-TNF-α[1,2] and balneophototherapy[3,4] | | [1]Jokai H *et al.* Exp Dermatol. 2013<br>[2]Cordiali-Fei P *et al.* Mediators Inflamm. 2014<br>[3]Holló P *et al.* J Dermatol Sci 2005<br>[4]Eysteinsdóttir JH *et al.* J Dermatol Sci. 2019 |
| | *S. pyogenes* preferentially triggers IL-9 in CLA+ T cells | | Ruiz-Romeu E *et al.* J Invest Dermatol. 2018 |
| | $CLA^+$ $CD4^+$ T cells tend to recirculate and patrol the skin compartment, whereas $CLA^+$ $CD8^+$ T cells tend to accumulate and stay as resident memory T cells | | Diani M *et al.* Clin Immunol. 2017 |
| *Scleroderma* | $CD8^+$ $CLA^+$ T cells accumulate in lesions and produce IL-13 and cytotoxic granules | | Fuschiotti P *et al.* Arthritis Rheum. 2012 |
| *Skin tumors* | Anti-tumoral T cells | Down-regulation of adhesion molecules on endothelial cells reduces $CLA^+$ T cell infiltration in metastatic malignant melanoma[1], squamous cell carcinoma[2] and basal cell carcinoma[3] favoring tumor progression | [1]Weishaupt C *et al.* Clin Cancer Res. 2007<br>[2]Clark RA *et al.* J Exp Med. 2008<br>[3]Verhaegh M *et al.* Eur J Dermatol 1998 |
| | Malignant T cells | Primary cutaneous T cell lymphoma requires co-expression of CLA and skin-specific chemokines for skin infiltration | Jokai H *et al.* Pathol Oncol Res. 2012 |
| | | $CLA^+$ $CCR4^+$ $CCR7^-$ expression guide malignant cells mainly towards skin in Mycosis fungoides, whereas co-expression of CLA, CCR4 and CCR7 explains cutaneous and lymph node infiltration in Sézary syndrome | Campbell JJ *et al.* Blood. 2010 |
| *Vitiligo* | Highest frequency of IFN-γ and IL-9 producing $CLA^+$ $CD8^+$ T cells, but also $CLA^+$ $CD4^+$ T cells | | Czarnowicki T *et al.* J Allergy Clin Immunol. 2019 |
| | Stressed keratinocytes produce CXCL9 enhancing $CLA^+$ $CD8^+$ T cell recruitment to skin | | Ahn T *et al.* J Invest Dermatol. 2020 |

# 3. Psoriasis: an immune-mediated inflammatory cutaneous disease

Our understanding of psoriasis disease has evolved substantially over the last decades as a result of years of translational research that have transformed the field of immunodermatology. This chapter reviews the main clinical and molecular features underlying psoriasis pathogenesis that are known so far, the impact of the disease on the population and how the evolution of our knowledge on psoriasis has occurred, also noting which subjects remain to be fully understood.

## 3.1 Epidemiological aspects

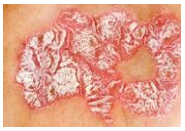
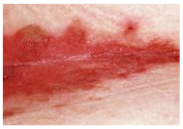
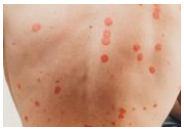
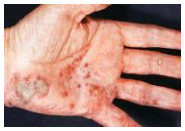
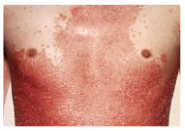
Psoriasis is a common, chronic, immune mediated skin condition affecting about 2% of the population worldwide (Parisi et al. 2020; World Health Organization 2016). Its prevalence is influenced by ethnicity and geographical location, being most frequent among Caucasians in Europe -particularly in Scandinavia- and North America, whereas rarely present in Asians, Africans, or Native Americans. There is no clear gender predilection and disease may initiate at any age, being more frequent in adults, but a bimodal age of onset is generally observed: early onset psoriasis (EOP, type I), starting at or before 40 years of age, and late onset psoriasis (LOP, type II), presenting after 40 years of age (Henseler and Christophers 1985). Differences in immunocytochemical, demographic and clinical features between EOP and LOP have been reported, with important implications for better management of the two types of disease (Theodorakopoulou et al. 2016). Notably, psoriasis is accepted as a disabling non-contagious disease and people with visible lesions face important stigma in their daily life.

## 3.2 Types of psoriasis

Regarding the clinical manifestations, psoriasis may present in heterogeneous and diverse forms showing distinct lesion morphology, distribution, and anatomical localization. *Plaque psoriasis* (also, *psoriasis vulgaris*) is the most common type, representing more than 80% of psoriasis diagnosis. Lesions, often itchy and painful, can appear anywhere on the body as raised, inflamed and scaly patches. It is often accompanied by nail affectation, which can be discolored, pitting, or separated from the nail bed. *Guttate psoriasis* affects about 8% of people living with psoriasis. Characterized by small papules, it frequently starts at an early age, and develops suddenly. Guttate form often clears spontaneously or after topical treatment, but in some patients the disease becomes chronic and worsen into the plaque-type, a matter that cannot be predicted in the clinic yet. Other subtypes of psoriasis include inverse or intertriginous, pustular and erythrodermic. Prevalence, lesion description and typical localization of each subtype are summarized in Table 3. Although molecular mechanisms are generally shared among different types of psoriasis, inflammatory responses shaping psoriasis clinical spectrum vary from high IL-17 responses in plaque psoriasis to predominant IL-36 responses observed in pustular forms (Billi et al. 2019). These singularities in the cytokine network may be crucial in outlining their

different clinical manifestations, as well as for their specific and effective treatment. Concerning this thesis, only plaque and guttate forms of psoriasis will be addressed.

**Table 3. Principal features of the different psoriasis subtypes.** Prevalence indicates predominance among all psoriasis diagnostics. Images obtained from the National Psoriasis Foundation website.

| *Psoriasis form* | **Plaque or *vulgaris*** | **Inverse or intertriginous** | **Guttate** | **Pustular** | **Erythrodermic** |
|---|---|---|---|---|---|
| *Prevalence* | > 80% | ~ 25% | ~ 8% | ~ 3% | ~ 2% |
| *Type of lesions* | Raised, inflamed and scaly patches | Smooth and shiny, lacking scales due to the moist environment | Small, round spots | White, pus-filled, painful bumps surrounded by inflamed or discolored skin | Intense redness and shedding of skin layers in large sheets |
| *Typical localization* | Anywhere, generally symmetrically. Often on the scalp, knees, elbows and torso. | Skin folds: armpits, groin, under the breasts. Often coexists with another type of psoriasis elsewhere. | Usually on arms, legs and torso. Less common on face, ears, scalp. | Generalized (GPP): large areas of the body. Palmoplantar (PPPP): palms of the hands and/or soles of the feet. Acropustulosis: tips of the fingers and/or toes (rare). | Nearly the whole body (can be life-threatening). |
| *Clinical phenotype* |  |  |  |  |  |

## 3.3 Triggering psoriasis: from genes to environment

As a complex disease, there is not a unique and clear cause associated with psoriasis onset thus far, so the initial trigger of the disease can be only understood as the particular combination of genetic and environmental factors, with the eventual implication of autoantigens.

Among the complicated genetic nature of the disease, the most important role is played by the human leukocyte antigen (HLA)-Cw*06:02 allele, located at the Psoriasis Susceptibility locus 1 (PSOR1), which has been attributed up to 50% of the disease heritability in different ethnicities. Its presence correlates with early onset, positive family history and more severe course of the disease, and the risk of developing psoriasis is increased in homozygous versus heterozygous individuals. In all, genetic association studies revealed up to 15 psoriasis susceptibility loci, with more than 80 candidate genes (Table 4). Nonetheless, alongside PSORS1, weak but relevant linkage was only observed for PSORS2 (affecting the NF-B signaling pathway) and PSORS4 (comprising genes involved in epidermal development and maturation) (Nedoszytko et al. 2020). Interestingly, genetic variants are associated with psoriasis phenotypes in a particular manner. Whereas HLA-Cw*06:02 is mostly related to chronic plaque and guttate psoriasis, pustular subtypes often present mutations on *IL-36RN*, *CARD14* and *APIS3* genes (included in the PSORS2, 14 and 15) but are less associated to PSORS1. More recently, genome-wide association studies (GWAS) identified numerous psoriasis genetic risk markers, most of which are involved

in adaptive and innate immunity, as well as skin barrier function, proving their primary role in the pathogenesis of psoriasis (Nedoszytko et al. 2020). Withal, they merely explain less than one third of psoriasis cases, suggesting exceptional value for the environmental influence on disease development.

**Table 4. Psoriasis susceptibility loci, chromosome location and candidate genes revealed by linkage studies.** From Nedoszutko et al. 2020

| Loci | Chromosome | Candidate genes |
|---|---|---|
| PSORS1 | 6p21.33 | *HLA-C* |
| PSORS2 | 17q25.3 | *CARD14* |
| PSORS3 | 4q | *NFKB1, CFI, KIAA1109, IL2, IL21, IL21-AS1, BBS12* |
| PSORS4 | 1q21 | *HFE2, FLG, LCE3C, LCE3B, LCE3A, LCE3E, LCE2C, LCE1C, LCE1A, SMCP, IVL, SPRR2C, SPRR2G, LELP1, PRR9, LOR, PGLYRP3, PGLYRP4, S100A9* |
| PSORS5 | 3q21 | *SLC12A8* |
| PSORS6 | 19p13 | *BSG, SMARCA4, OR7A10* |
| PSORS7 | 1p | *TNFRSF9. TNFRSF1B, KAZN, IGSF21, PAX7, CAPZB, IFNLR1, RUNX3, AZIN2, CSMD2, OMA1, IL23R, GNG12-AS1, LRRC7, AK5, SPATA1, DDAH1, GBP6, KIAA1107, CEPT1, DENND2D, PTPN22* |
| PSORS8 | 16q | *CYLD, NOD2, FTO, CDH8, SMPD3, CDH3, IL34, MLKL, CMIP, CDH13, SLC38A8, MBTPS1, WFDC1, KIAA0513* |
| PSORS9 | 4q31-q34 | *RNF150, DCHS2, MSMO1, SPATA4* |
| PSORS10 | 18p11.23 | |
| PSORS11 | 5q31.1-q33.1 | *RAD50, IL13, IL4, STK32A, TNIP1* |
| PSORS12 | 20q13 | *SPATA2, RNF114, CYP24A1* |
| PSORS13 | 6q21 | *TRAF3IP2* |
| PSORS14 | 2q14.1 | *IL36RN* |
| PSORS 15 | 2q36.1 | *AP1S3* |

Despite the genetic background does not explain all psoriasis cases, the strong risk association to the HLA-Cw*06:02 suggested an autoimmune nature of psoriasis, by presence of autoreactive T cells restricted to this HLA molecule. This hypothesis led to several studies trying to identify potential autoantigens, which have resulted in several molecules proposed so far, that will be briefly discussed below. Protein autoantigens include keratin 17 (K17), cathelicidin (LL-37) and ADAMTSL5. Keratin 17 is a cytoskeletal protein whose increased expression, induced by IL-22 and IL-17 cytokines, is considered a hallmark of psoriasis (de Jong et al. 1991). Due to its sequence homology with the M protein from group A streptococci, that closely associated with psoriasis development and will be discussed in following chapters, K17 may be recognized by T cells due to molecular mimicry leading to an important IFN-γ response (Gudmundsdottir et al. 1999; Johnston et al. 2004). Subsequent studies have demonstrated that K17 peptides induce T cell proliferation in an HLA-Cw*06:02-dependent manner (Yunusbaeva et al. 2018). Later, $CD4^+$ and $CD8^+$ T cells recognizing LL-37 were detected in circulation and psoriatic lesions, which induced IFN-γ and, only $CD4^+$, IL-17 upon activation (Lande et al. 2014). This antimicrobial peptide, secreted by immune cells (neutrophils, mast cells) and keratinocytes after injury or infection, is overexpressed in psoriatic lesions LL-37 and their complexes with self-

RNA/DNA molecules are known to activate plasmacytoid dendritic cells (pDCs) via toll-like receptors (TLR)-7/9, inducing type I interferon signaling (Ganguly et al. 2009; Lande et al. 2007). More recently, melanocyte-derived ADAMTSL5 was identified to activate intraepidermal $CD8^+$ T, inducing the expression of IFN-γ and IL-17A (Arakawa et al. 2015). Besides protein antigens, psoriatic skin also harbors lipid-specific T cells recognizing phospholipase $A_2$ ($PLA_2$)-derived products presented by $CD1a^+$ Langerhans cells, inducing IL-22 and IL-17 upon activation (Cheung et al. 2016). For a long time, increased $PLA_2$ activity has been reported in psoriatic lesions and absent in healthy skin but its relevance in genetically susceptible individuals is yet unclear. Notwithstanding all these autoantigen candidates, autoreactive T cells have been found in some but not all moderate-to-severe plaque psoriasis patients and, although they may participate in the initial steps of lesion formation, their role in psoriasis immunopathogenesis needs to be clarified (Ten Bergen et al. 2020; Hawkes et al. 2017).

Along with genetic susceptibility variants and autoantigens, numerous studies revealed the association of distinct environmental factors to psoriasis onset or flare-ups. Skin trauma, caused by cuts, scrapes, insect bites or severe sunburn, may result in new lesions, which is known as Koebner phenomenon since first described by Heinrich Koebner in 1876 (Köbner 1876). Smoking, excessive alcohol intake and stress are also defined as psoriasis triggers (Roszkiewicz et al. 2020). Notably, particularly in women, hormonal changes may affect psoriasis development and severity, being more frequent during puberty and menopause, when hormone levels fall, while improving during pregnancy, due to increased estrogen and progesterone (Ceovic et al. 2013). Similarly, certain medicines (principally lithium, β-blockers, nonsteroidal anti-inflammatory drugs, and antimalarial agents) and microorganisms (including bacteria, fungi, and viruses), in which this thesis has been mainly focused, can incite or exacerbate psoriatic lesions (Fry and Baker 2007).

### 3.3.1 The role of *Streptococcus pyogenes* in psoriasis

Since first reported over a century ago (Winfield 1916), β-hemolytic streptococci are widely recognized to be implicated in psoriasis pathogenesis. Initially, streptococcal infection of the upper respiratory tract 1-2 weeks prior to the debut of skin lesions was reported in over 60% of guttate psoriasis patients (Norrlind 1955). Presence of the microorganism in guttate psoriasis patients' throat cultures, as well as high anti-streptolysin O antibodies (ASO) in their serum, have been reported ever since (Hossain et al. 2013). Within chronic plaque psoriasis patients, *Streptococcus pyogenes* infections were tightly related to patients with early disease onset, positive family history and presence of HLA-Cw6, -B57 and -B13 alleles (which are generally known as type I psoriasis), with predominant role of predisposing HLA over age of onset (Wisenseel et al. 2002). Additionally, plaque psoriasis patients also

reported increased incidence of sore throat and positive β-hemolytic streptococci (M protein carriers) throat swabs cultures than healthy individuals, which are associated to important exacerbation of psoriatic lesions (Gudjonsson et al. 2003). Increased *S. pyogenes* specific IgG are also observed in serum from psoriasis patients compared with controls (El-Rachkidy et al. 2007). Notably, several studies reported significant clinical improvement of psoriasis after tonsillectomy, thus supporting a role for this microbe in pathogenesis of the disease (Rachakonda et al. 2015; Thorleifsdottir et al. 2012). Interestingly, group A *Streptococcus* extra- and intracellular biofilms have been reported in tonsillectomy specimens from psoriasis (Allen et al. 2018). Biofilm formation may explain the recurrent relapses over time, due to lack of penetration of anti-streptococcal agents leaving a source of antigen exposure, along with the clinical improvement after tonsillectomy. Therefore, among HLA-Cw6 positive psoriasis patients, *S. pyogenes* tonsillar infection may contribute to the initiation and/or exacerbation of cutaneous lesions in both guttate and plaque forms of disease (Thorleifsdottir et al. 2016).

Given this well-established clinical association, the subsequent question that arose was: how are streptococcal throat infections linked to skin manifestations at a molecular level? Skin-homing molecule CLA is found in 5-10% of T cells and dendritic cells in tonsils and peripheral lymph nodes (Picker et al. 1990). Besides, bacterial superantigens (SAg), including group A streptococcal pyrogenic exotoxins A and C (Spe-A and C respectively), have been proved to induced CLA expression on circulating T cells, partially dependent on IL-12 induction (Leung et al. 1995a; Sigmundsdóttir et al. 2003). These toxins are able to activate T cells expressing a particular T cell receptor (TCR)-Vβ chain in an unrestricted manner, inducing a polyclonal T cell activation and large cytokine release. Hence, the first hypothesis focused on streptococcal SAgs as the triggering factor of psoriasis. In fact, overrepresentation of $V\beta2^+$ T cells, specially activated by SPEC, was observed in skin biopsies from chronic plaque and guttate psoriasis (Leung et al. 1995a; Lewis et al. 1993). Sequence analysis revealed extensive junctional region diversity in both $CD4^+$ and $CD8^+$ T cells, further supporting the idea of SAg polyclonal activation rather than antigen-specific T cell response (Leung et al. 1995b). Additionally, increased expression of TCR $V\beta2^+$ was detected in CLA+ peripheral blood lymphocytes from chronic plaque psoriasis patients compared to healthy controls (Davison et al. 1999). Almost simultaneously, an alternative hypothesis originated from sequence analysis of TCR repertoires in psoriatic skin, which revealed marked oligoclonal expansion compared to healthy individuals (Menssen et al. 1995). This observation suggested that antigen-specific T cell expansion was involved in psoriasis pathogenesis and recurrence of lesions, which was further supported by additional studies (Prinz et al. 1999; Vollmer et al. 2001). Remarkably, identical clonal TCR

rearrangement was observed in psoriatic lesions and tonsillar $CLA^+$, but no $CLA^-$, T cells, again linking streptococcal sore throat with psoriatic lesions at cutaneous level (Diluvio et al. 2006).

At this point, identifying the streptococcal derived antigen responsible for this clonal T cell expansion was the coming step. Consecutive studies pinpointed that perpetuation of psoriatic lesions may be due to cross-reactive immune responses between pathogenic streptococcal antigens and self-components sharing a similar structure, a mechanism known as molecular mimicry. Homologies between streptococcal M proteins, a virulence factor shared between psoriasis-associated β-hemolytic *streptococci*, and keratins, which are filament proteins of the epithelia, were reported (McFadden et al. 1991). Thereunder, psoriasis patients showed increased T cell response for homologous M protein and keratin determinants (Gudmundsdottir et al. 1999; Sigmundsdottir et al. 1997). Specially, $CD8^+$ $CLA^+$ T cells from psoriasis patients with positive HLA-Cw*06:02 allele highly responded to keratin peptides compared to healthy individuals (Johnston et al. 2004). To a lesser degree, patients positive for HLA-DRB1*04 and/or *07, which are also significantly related to psoriasis, showed strong $CD4^+$ T cell cytokine response to keratin 17 determinants too (Shen et al. 2005). Instead, $CD4^+$ T lymphocytes preferentially reacted to streptococcal peptidoglycans presented by dermal macrophages (Baker et al. 2006), which complements the altered immune response to *Streptococci spp.* observed in psoriatic skin.

As a whole, antigen presenting cells in the tonsils and lymph nodes may present various streptococcal determinants to T cells, while streptococcal SAgs may enhance CLA expression on the resultant effector memory T cells at the same time (Figure 5A). These skin-homing effector memory T cells will be actively recruited to cutaneous tissue under inflammatory conditions. Within the epidermis, tonsillar induced $CD8^+$ $CLA^+$ T will be activated upon recognition of self-keratins, which present sequence homologies with streptococcal M protein. Conversely, most of the *Streptococcus*-specific $CD4^+$ $CLA^+$ T cells induced will also recognize streptococcal determinants, including proteoglycans, and expand clonally within the dermal compartment. The activation of both $CD8^+$ and $CD4^+$ $CLA^+$ T cells will lead to cytokine secretion, ultimately inducing keratinocytes hyperproliferation and lesion formation, as well as further recruitment of innate and adaptive immune cells that contribute to the lesional pro-inflammatory environment (Figure 5B). (Prinz 2001; Valdimarsson et al. 2009).

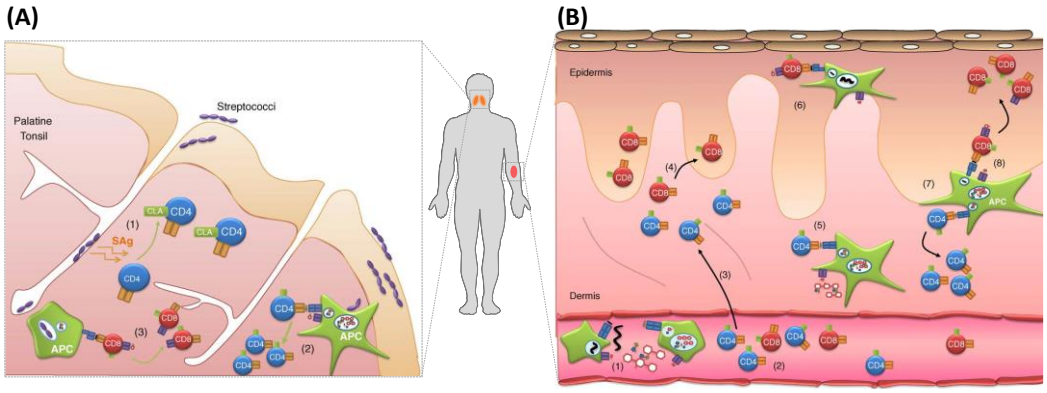



**Figure 5. Linking streptococcal throat infection and psoriasis development. (A)** Step 1: Infections by Streptococcus spp producing superantigens enhance CLA expression on tonsillar T cells. Steps 2 and 3: At the same time, antigen presenting cells (APC) process streptococcal components -including M-protein (black) and proteoglycan (red)- and promote maturation of naïve T cells towards streptococcal specific $CD4^+$ (blue) and $CD8^+$ (red) T cells. **(B)** Steps 1 and 2: APCs containing streptococcal components and streptococcal specific $CD4^+$ and $CD8^+$ T cells expressing CLA are actively recruited to the skin. Steps 3 and 4: effector $CD4^+$ T cells migrate into the dermis, while $CD8^+$ T cells into the epidermis. Step 5: streptococcal components are presented mainly in the dermis to $CD4^+$ T cells. Step 6: keratin peptides cross-reacting with streptococcal M protein are mostly presented in the epidermis to $CD8^+$ T cells. Steps 7 and 8: APCs at the dermal-epidermal junction can present antigens to both dermal $CD4^+$ T cells via HLA-II molecules and epidermal $CD8^+$ T cells via HLA-I molecules. The activation of effector memory T cells results in a cytokine-mediated tissue reaction, inducing keratinocyte hyperproliferation and recruitment of innate immune cells. Adapted from Valdimarsson et al. 2009.

### 3.3.2 *Candida albicans* and psoriasis

Unlike for *Streptococcal* infections, the link between *Candida* species (spp.) and psoriasis development is less well-understood. Albeit *Candida* spp. are part of our commensal mycobiota, which is the fungal component of our microbiota, at cutaneous and mucosal tissues, they frequently cause opportunistic infections when dysbiosis or deregulation of the immune system occurs. In psoriasis, the exacerbation of skin lesions correlates with cutaneous *Candida albicans* infections (Fry and Baker 2007) but the exact underlying mechanism probably due to the recognition of *Candida albicans* antigens or superantigens, remains to be fully elucidated. Interestingly, it has been recently demonstrated how topical preexposure to *C. albicans* exacerbates psoriasiform skin inflammation in the imiquimod (IMQ) mice model, inducing a gene signature that resembles more of the human lesional psoriatic skin than when IMQ is applied alone (Hurabielle et al. 2020). Importantly, this work demonstrated that the mere presence of *C. albicans* enhanced cutaneous inflammation, without the need of an active infection, and pointed out neutrophils and *C. albicans*-primed Th17 cells as the main cellular mediators.

The parallels between skin immunity against fungi (Figure 6) and psoriasis pathogenic mechanisms, which will be further addressed below, are straightforward. Innate and adaptive immune responses against *C. albicans* revolve around IL-17 response, which is crucial since mutations altering the IL-17 pathway lead to chronic mucocutaneous candidiasis (CMC) in

humans (Puel 2020). Initially, *C. albicans* binding to host pattern recognition receptors (PRRs) induces the secretion of Th17 polarizing cytokines (IL-1β, IL-6, IL-23, and TGF-β) and other pro-inflammatory cytokines (TNF-α, IL-12, IL-18 and type I IFN) by DCs, macrophages and keratinocytes among others. Besides Th17 cells, type 3 innate lymphoid cells (ILC3s), γδ T cells and neutrophils constitute important sources of IL-17 to address *C. albicans* infection, as it is the formation of neutrophils extracellular traps (NETs) that prevent fungal dissemination. IL-17, along with TNF-α, acts on keratinocytes inducing the secretion of AMPs (LL37 or cathelicidin, β-defensins and S100 proteins) and chemoattractants to recruit neutrophils and other inflammatory immune cells, that will be further activated by IL-17 at cutaneous tissue (Kashem and Kaplan 2016). Additionally, circulating skin-tropic $CLA^+$ and IL-9 producing Th cells have been described to preferentially respond to *C. albicans* in healthy humans (Schlapbach et al. 2014), whereas IL-9 together with mast cells are key mediators of *C. albicans* commensalism and pathogenesis in the gut (Renga et al. 2018).

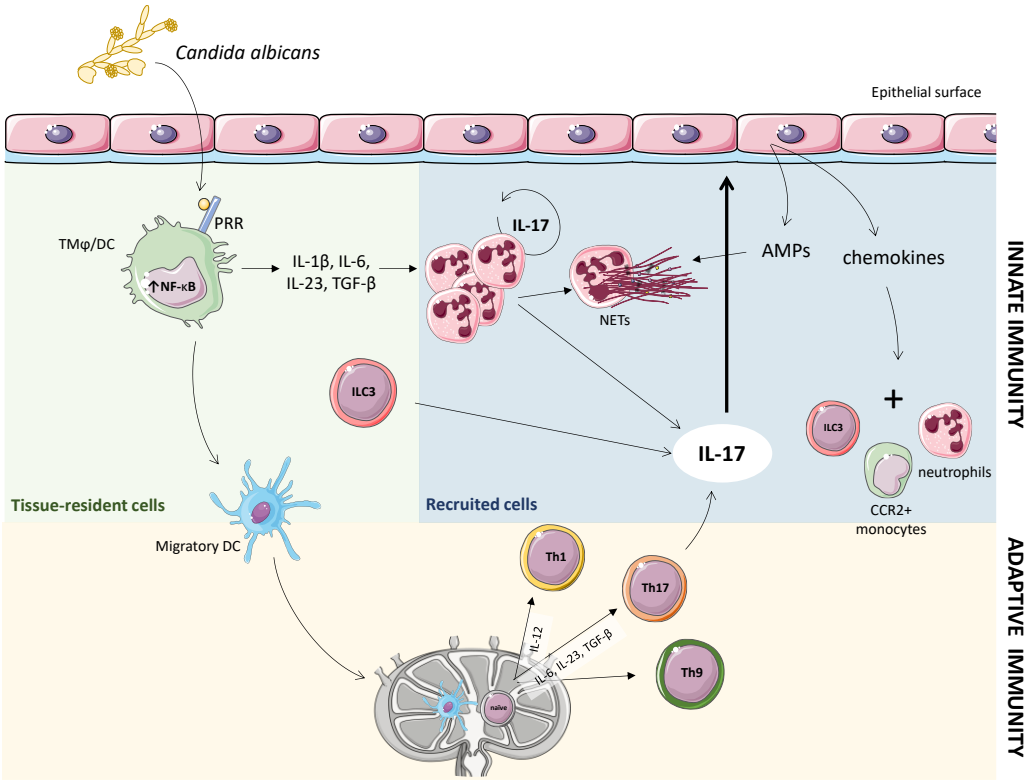



**Figure 6. Antifungal immunity at epithelial surfaces.** Initially, *Candida* penetrates the tissue and binds pattern recognition receptors (CLR, TLR) on tissue resident-macrophages and dendritic cells (DC), which will phagocyte it and activate ultimately inducing the secretion of pro-inflammatory cytokines and chemokines, to recruit neutrophils and monocytes from the circulation. Activated neutrophils will secrete IL-17, which will activate them in an autocrine manner, and release neutrophil extracellular traps (NETs), preventing fungal dissemination. Additional innate sources of IL-17 are ILCs and γδ T. Subsequently, DCs migrate to lymph nodes where they induce antigen-specific Th cells that will be recruited back to the site of antigen encounter. Th17 cells will produce IL-17, enhancing neutrophils recruitment and activation, as well as the secretion of antimicrobial peptides (LL-37, β-defensins and S100 proteins) by epithelial cells. Whereas Th1 cells will induce IFN-γ that further activates innate phagocytic cells. Adapted from (Carvalho 2017).

In humans, differences in the presence of *Candida* in psoriatic versus healthy skin have not been proved, which is likely explained by the increased presence of AMPs and IL-17 cytokines at psoriatic lesions that may control *Candida* colonization of the skin. However, a recent meta-analysis confirmed significantly increased prevalence of *Candida* spp. in mucosal membranes from plaque psoriasis patients when compared with healthy controls (Pietrzak et al. 2018). This association, together with the above-mentioned study in animal models, further supports the presumption that fungal colonization and/or infection may contribute to plaque psoriasis pathogenesis. Even so, whether psoriasis patients are more susceptible to *Candida* spp. or psoriasis disease predispose *Candida* colonization remains uncertain, as it is the role of this fungus in psoriasis subtypes other than plaque psoriasis.

## 3.4. Immunopathogenesis

Our understanding of the pathogenic mechanisms underlying psoriasis has drastically evolved over the last years, simultaneously to the better comprehension of the immune system and the discovery of distinct subsets of T cells and cytokines that have been proved to be key mediators of the disease. Currently, psoriasis is no longer considered to be caused exclusively by hyperproliferation of keratinocytes, but it is defined as a complex disease resulting from the interplay between the immune system and the cutaneous tissue, as will be detailed hereunder.

### 3.4.1 Evolution of pathogenic concepts

According to the histopathological signs characteristic of psoriatic lesions, keratinocyte hyperplasia was initially considered the main cause of disease and this theory endured for many decades. Key histological features of psoriasis are epidermal thickening (acanthosis), incomplete differentiation of keratinocytes with retention of the nucleus by corneocytes (parakeratosis) and thickening of the *stratum corneum* (hyperkeratosis). In the inner side, epidermal rete ridge elongates entering the dermal papillae, and hyperplastic and dilated blood vessels are found underneath (Figure 7) (Lowes et al. 2007; Sabat et al. 2007). Beside this structural changes, neutrophils aggregates are also a major histopathological aspect of psoriatic lesions, which are known as Munro's microabscesses (intracorneal) or spongiform pustules of Kogoj (subcorneal) depending on their location. Interestingly enough, this collection of neutrophils is not seen in seborrheic dermatitis, helping in the differential diagnosis of two dermatological affections with similar appearance in the scalp.

It was not until the early eighties when increased leukocyte infiltration of psoriasis lesions was detected (Bos et al. 1983). Subsequent histological studies revealed an uneven distribution of T cell subsets within the skin. Epidermal layers are dominated by $CD8^+$ T cells, whilst a mixture of $CD4^+$ and $CD8^+$ T cells can be found within dermal tissue, with $CD4^+$ T cell prevalence. From then on, the role of T cells in the initiation and persistence of

psoriasis pathogenesis was pointed out after several observations. First, inhibition of T cell function resulted in successful treatment of cutaneous lesions. Cyclosporin A, anti-CD4 antibodies and a fusion protein IL-2-difteria toxin fusion protein were some of the T cell-targeted drugs showing clinical improvement of psoriatic skin (Ghoreschi et al. 2007). Second, patients with psoriasis receiving a hematopoietic stem cell transplant from a non-psoriatic donor showed lesions improvement (Adkins et al. 2000), whereas, healthy patients receiving bone marrow from a psoriatic donor frequently developed psoriasis afterwards (Gardembas-Pain et al. 1990). Finally, engraftment of uninvolved skin from psoriasis patients on immunodeficient mice, together with injection of autologous immune cells, resulted in psoriasis-like alterations of the transplanted skin. However, this phenomenon was not observed when healthy skin and blood were tested (Wrone-Smith and Nickoloff 1996).

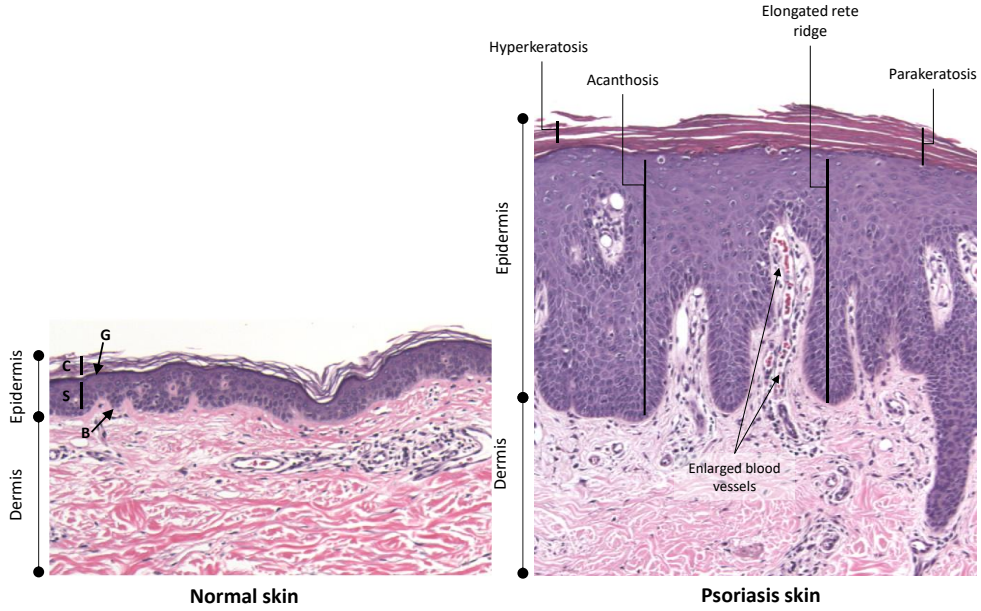



**Figure 7. Histopathological features of psoriatic skin.** Compared to healthy cutaneous tissue, psoriatic epidermal layer shows important thickening (acanthosis) with elongated rete ridges, along with thicker stratum corneum (hyperkeratosis) and impaired keratinocytes differentiation to corneocytes that retain the nucleus (parakeratosis). Besides, the granular layer is greatly reduced or absent. Within the dermal layer, blood vessels are increased, enlarged, and located in the papillary dermal region between the epidermal rete. B: basal layer; C: corneal layer; G: granular layer; S: spinous layer.

Additionally, dendritic cells activity is generally increased in psoriatic skin, but different subtypes exert distinct functions. Myeloid dendritic cells ($CD11c^+$; mDCs) are classified into immature (also known as inflammatory mDCs or TIP-DCs) and mature resident DCs (Zaba et al. 2009). The former is the most prevalent subtype in psoriatic skin, being the source of relevant pro-inflammatory cytokines involved in epidermal hyperplasia and Th17 cell differentiation, such as TNF-α, iNOS, IL-20, IL-6, and IL-23. The latter, although not increased in the lesions, is likely responsible for antigen presentation to cutaneous T cells. Additionally, plasmacytoid dendritic cells ($CD11c^-$ $BCA\text{-}2^+$; pDCs), which are also present

at high numbers in the dermis, induce an important type I IFN signature upon activation and are thought to be essential in the first stages of psoriasis pathogenesis (Gilliet et al. 2004; Nestle et al. 2005).

Notably, the role of B cells in psoriasis pathogenesis has been overlooked, probably due to their scarce presence within cutaneous tissue, but there are few studies that illustrate how they might contribute to the disease. First, and most interestingly, B cell depletion with rituximab (a monoclonal antibody targeting CD20) has been reported to aggravate or trigger psoriasis in humans (Kersh and Feldman 2018), which has been associated to the reduction of IL-10-producing regulatory B cells (Breg, also known as transitional B cells) that have been shown to prevent psoriasiform inflammation in IMQ (Yanaba et al. 2013) and IL-23 (Mizumaki et al. 2021)-induced mice models. However, the presence of B cells ($CD19^+$) in psoriatic lesions is considered rare, despite some have reported them increased in lesional skin from non-arthritic plaque (Mahmoud et al. 1999) or erythrodermic psoriasis (Lu et al. 2016), and, although psoriasis patients have showed altered frequencies of circulating B cell subsets, still no clear consensus can be reached as diverse studies have demonstrated increased (Hayashi et al. 2016; Kahlert et al. 2019), decreased (Czarnowicki et al. 2016) or equivalent (Thomas et al. 2019) numbers of Breg in psoriasis compared with healthy individuals. Instead, several analyses generally agree on the increased presence of IgA in plasma from untreated psoriasis patients (Kahlert et al. 2019; Laurent et al. 1981; Öztürk et al. 2001; Thomas et al. 2019), but there is no evident explanation for this phenomenon yet. Of note, a reported case of psoriasis in a patient with lack of B cells and immunoglobulins, due to a hereditary common variable immunodeficiency, point outs that the role of B cells in psoriasis may be rather secondary for disease pathogenesis (Thomas et al. 2019). Since the relevance of skin-associated B cells in tissue homeostasis and inflammation is settling into place (Debes and McGettigan 2019); the involvement of B cells in psoriasis is, without doubt, a matter that needs further investigation.

### 3.4.2 Current model of psoriasis pathogenesis

Nowadays the hallmarks of psoriasis pathogenesis are chronic and sustained inflammation with uncontrolled keratinocyte proliferation and up-normal differentiation. The current understanding of the pathogenic mechanisms of disease is based on the interaction between epidermal keratinocytes and cells form the innate and adaptive immunity, an interplay that is essentially driven by pro-inflammatory molecules with the IL-23/IL-17 axis playing a critical central role, as proved by the clinical efficacy of their blockade in patients; and other mediators that may somehow contribute to the initiation and maintenance of the disease, like IFN-α, IFN-γ and IL-22 (Figure 8).

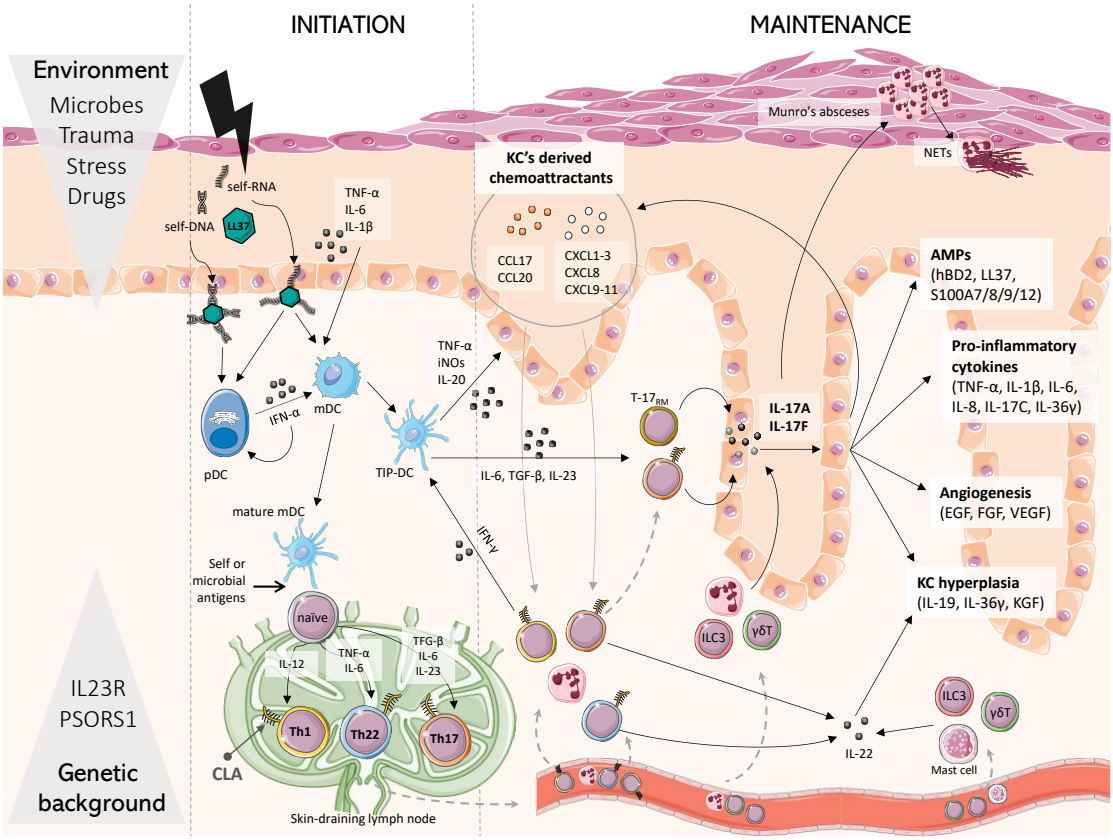



**Figure 8. Current model of psoriasis pathogenesis.** The combination of environmental factors and a susceptible genetic background triggers a succession of events leading to the development of psoriatic lesions. In the initiation phase, damaged keratinocytes (KC) release self-nucleic acids and LL-37, which bind together in complexes that will activate plasmacytoid dendritic cells (pDC), inducing IFN-α secretion, and myeloid dendritic cells (mDC). Activated inflammatory DCs (TIP-DC) will further secrete proinflammatory cytokines (TNF-α, IL-20), Th17-inducing cytokines (IL-6, TGF-β IL-23) and nitric oxide radicals (iNOs), while mature mDCs will migrate to skin-draining lymph nodes, where they will present either self or microbial antigens to naïve T cells, inducing their differentiation into skin-homing ($CLA^+$) Th1, Th17 or Th22 cells. Th1-dependent IFN-γ supports TIP-DCs activation. Maintenance of psoriatic lesions depends on both resident and circulating $CLA^+$ T cells, actively recruited by epidermal chemoattractants, essentially guided by the IL-23/IL-17 axis. IL-17A and IL-17F, mainly produced by Th17 but also Tc17 cells, γδT cells, ILC3 and neutrophils, will impact on KCs further inducing the secretion of chemokines, antimicrobial peptides (AMPs. hBD2: human beta-defensin 2; LL-37 and S100A proteins) and pro-inflammatory cytokines, as well as proliferation- and angiogenic-stimulating factors (EGF: endothelial growth factor; KGF: keratinocyte growth factor; FGF: fibroblast growth factor; VEGF: vascular endothelial growth factor). Likewise, epidermal hyperplasia is promoted by IL-22, released from Th22, Th17, γδT cells, ILC3 and mast cells. Adapted from Di Meglio P et al 2011.

Despite the exact initial trigger is still known, it is postulated that the combination of a susceptible genetic background and environmental factors results in different pathogenic events leading to lesion formation. In the initiation phase, damaged keratinocytes secrete the anti-microbial peptide (AMP) cathelicidin (LL-37) which binds to self-RNA/DNA fragments and subsequently activate plasmacytoid dendritic cells (pDCs) via TLR7/9 respectively (Ganguly et al. 2009; Lande et al. 2007). This interaction results in increased type I IFN release which further stimulate pDCs and, in combination to LL-37-self-RNA/TLR8 complexes, also activate the two types of myeloid dendritic cells (mDCs) in the

dermis. On the one hand, pro-inflammatory mDCs which actively secrete TNF-α and iNOS (supporting the pro-inflammatory milieu), IL-20 (involved in epidermal hyperplasia) and IL-6, IL-12, and IL-23 (promoting Th17 differentiation). On the other hand, mature mDCs that migrate to skin-draining lymph nodes to present either self or microbial antigens to naïve T cells, which will evolve to effector memory T cells with skin tropism, thus expressing the CLA. Despite skin resident memory T cells ($T_{RM}$) are described to be essential for the initiation of lesions, as proved in the AGR129 xenotransplantation model (Boyman et al. 2004); active recruitment of skin-homing effector memory T cells is likely required for the perpetuation of cutaneous inflammation (Valdimarsson et al. 2009). A matter that is supported by the close correlation between the frequency of circulating $CLA^+$ T cells and disease severity in psoriasis (Sigmundsdóttir et al. 2001). Next, the maintenance of psoriasis lesions is essentially guided by the IL-23/IL-17 inflammatory environment. Within the lesion, cellular sources of IL-17 include αβ-T cells (both $CD4^+$ and $CD8^+$, resident and recruited), γδ-T cells, group 3 innate lymphoid cells (ILC3) and neutrophils, while the role of mast cells and NK cells is still controversial (Sato et al. 2020). IL-17 cytokines activate keratinocytes to secret a wide variety of chemokines increasing $CLA^+$ T cell (CCL20) and neutrophils (CXCL1, 2, 5, 8, 11) recruitment; cytokines (TNF-α, IL-17C, IL-36γ, IL-19, IL-6); proliferative and angiogenic factors (EGF, VEGF, FGF); together with AMPs (hBD2, LL-37 and S100A7/8/9/12). Additionally, IL-17 actively recruit neutrophils that accumulate at the epidermis and generate neutrophils extracellular traps (NETs), which contains self-RNA/DNA/AMPs complexes further amplifying the pro-inflammatory milieu (Herster et al. 2020). Other important cytokines include IL-22, produced by Th22, Tc22, γδ-T cells, ILC3 and mast cells, which is mainly involved in epidermal hyperplasia by impairing KCs differentiation; and IL-9, which supports Th17 survival (Ruiz-Romeu et al. 2018) and angiogenesis *in vitro* (Singh et al. 2013).

The autoimmune versus autoinflammatory nature of psoriasis is still under debate, albeit the pathogenic view of psoriasis has clearly shifted from a bare skin disease to a cutaneous condition related to systemic inflammation. In this regard, as for other immune-mediated inflammatory diseases, psoriasis is generally associated with a large variety of concomitant conditions that contribute to the disease burden. The clearest example is the occurrence of psoriatic arthritis (PsA), which affects around 30% of patients with chronic psoriasis. PsA has a complex etiology, is characterized by asymmetric oligoarthritis, nail disease, enthesis and/or dactylitis, and in most severe cases can lead to patient disability. Other important comorbidities include cardiovascular disease (myocardial infarction and stroke), metabolic disorders (obesity, non-alcoholic fatty liver disease, dyslipidemia and diabetes), Crohn's disease and depression (Di Meglio and Nestle 2017; Samotij et al. 2020). Alike in atopic

dermatitis, the concept of "psoriatic march" has emerged to encompass the interplay between psoriasis and its comorbidities, that substantially impact on the overall quality of life of patients (Boehncke et al. 2011). Because the psychosocial concerns of psoriasis go far beyond skin manifestations, with systemic and maintained inflammation behind its root, the better we comprehend the molecular basis of this psoriatic march the better it will be addressed in everyday clinical practice.

### 3.4.3 The IL-23/IL-17 axis in psoriasis pathogenesis

Currently it is well established that psoriasis immunopathogenic mechanisms are governed by the dysregulation of the IL-23/IL17 axis, which has been also proved essential for some of the above-mentioned inflammatory disorders often concomitant to psoriasis disease (Gooderham et al. 2018). Here, the main features and functions of these cytokines orchestrating the pro-inflammatory loop behind psoriasis pathogenesis will be outlined (Figure 9).

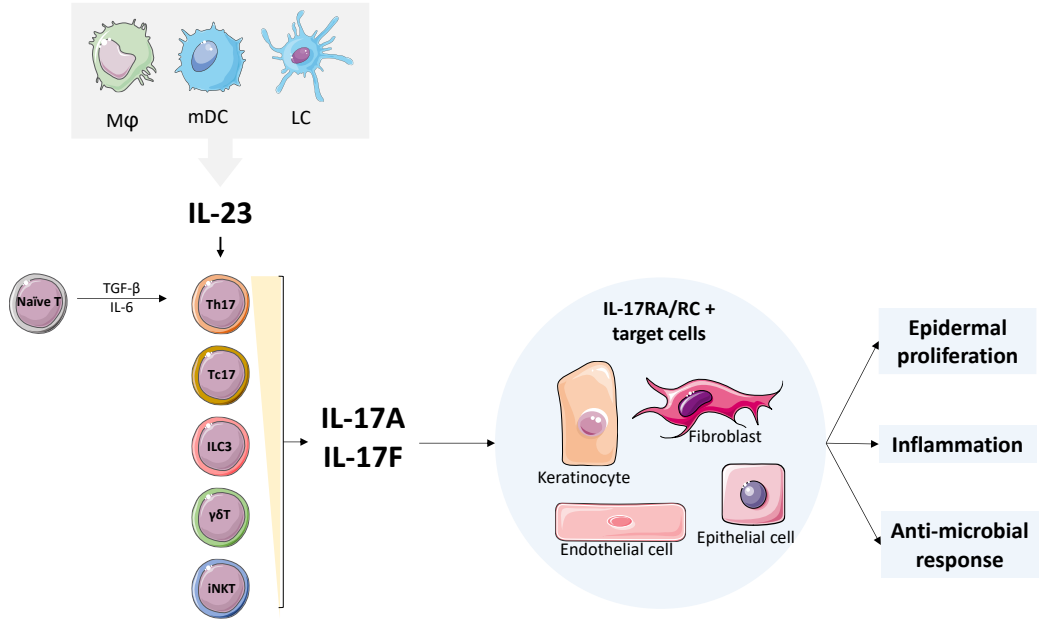



**Figure 9. Schematic overview of the role of IL-23 and IL-17 in psoriasis.** Activated antigen presenting cells such as macrophages (Mϕ), myeloid dendritic cells (mDCs) and Langerhans cells (LC) secrete IL-23 that, along with TGF- and IL-6, is essential for Th17 differentiation. Other sources of IL-17 cytokines comprise cytotoxic T cells (Tc17), innate lymphoid cells (ILC3), γδ-T cells and invariant natural killer-like T cells (iNKT), which are more or less dependent on IL-23 as represented by the yellow triangle. Effector cells expressing the IL-17RA/RC receptor are primarily keratinocytes, fibroblast, endothelial and epithelial cells, that upon IL-17A/IL-17F activation will induce epidermal proliferation, contribute to the environmental inflammation with chemokines and cytokines, and secrete anti-microbial peptides.

Since first described in the early 2000s, IL-23 is widely known as the "master regulator" of Th17 cell development. Its structure is composed by the unique p19 subunit and the p40 subunit, which is shared with IL-12, and it is mainly secreted by activated antigen presenting cells (APCs) under inflammatory conditions. Th17 differentiation cascade is initiated by TFG-β and IL-6, which upregulate the transcription factor ROR-γt on naïve T cells leading

to the expression of IL-17A and the receptor for IL-23 (IL-23R). Subsequent binding of IL-23 to its receptor triggers Jak2/STAT3 signalling pathway enhancing ROR-γt and IL-17 expression, which are key for Th17 expansion and perpetuation. Besides, IL-23, along with IL-1β, can induce and activate IL-17-producing ILC3 and γδ-T cells, both increased in psoriatic skin and thus supporting the IL-17 proinflammatory loop (Sutton et al. 2009; Teunissen et al. 2014; Villanova et al. 2014).

The IL-17 family comprises six structurally related cytokines (IL-17 from A to F) which act as homodimers, except for IL-17A and IL-17F that can form heterodimers together. Their receptors are composed by heterodimeric combinations of five different subunits (IL-17RA-E) which are widely expressed in human tissues, mainly in non-hematopoietic cells such as epithelial and endothelial cells, keratinocytes, fibroblasts, synoviocytes and osteoblasts. Of note, IL-17A and IL-17F homodimers and heterodimer signal through the same receptor consisting of the IL-17RA and IL-17RC subunits. At a functional level, IL-17 immunity is essential for anti-fungal defense and microbiota homeostasis, as well as in wound healing or epithelial proliferation in healthy skin. As mentioned before, cellular sources of IL-17 cytokines other than Th17 have been identified and comprise cells from both innate and adaptive immune systems, some of which secrete IL-17 in an IL-23-independent manner (Keijsers et al. 2014).

The relevance of IL-23 in psoriasis arose from the finding of its increased expression in psoriatic lesions (Lee et al. 2004; Piskin et al. 2006), along with the identification of single nucleotide polymorphisms (SNPs) in IL-23R associated to psoriasis susceptibility (Nair et al. 2008), and was further supported by psoriasis-like inflammation induced by overexpression of IL-23 in mice models (Chan et al. 2006). Within the IL-17 family, IL-17A is singled out as the most important mediator in psoriasis (Brembilla et al. 2018), but its principal homologue, IL-17F, has been also proved to be increased in lesional skin and blood from psoriasis patients (Johansen et al. 2009; Soderstrom et al. 2017). However, the undeniable central role of IL-23 and IL-17 in psoriasis pathogenesis was singled out by the clinical efficacy achieved with therapies targeting these cytokines, a matter that will be further discussed in the following chapter.

## 4. From bench to bedside: the translational revolution

Numerous cytokines have been described to be increased in the lesions and/or bloodstream of psoriasis patients, therefore being considered potential therapeutic targets. Since the late nineties, the use of humanized monoclonal antibodies (mAbs) have been widely explored for the treatment of autoimmune and oncologic disorders, as they can be selectively directed to block a specific molecule, representing a clinically useful tool for precision medicine. In the design of this targeted

mAbs for psoriasis treatment, the challenge arises when trying to select the exact cytokine within the complex network initially implicated in pathogenesis of the disease (Figure 10) (Chiricozzi et al. 2018; Tsai and Tsai 2017). Some biologics targeting several of these promising candidates have been withdrawn due to the lack of clinical efficacy in psoriasis, usually measured as the PASI-75 response that implies a 75% or greater improvement of the PASI score at baseline. Anti-IFN-α (Bissonnette et al. 2010), anti-IFN-γ (Harden et al. 2015), anti-IL-20 (Gottlieb et al. 2015), anti-IL-8 or anti-IL-22 (Tsai and Tsai 2017) mAbs are some examples of discontinued studies in early phase clinical trials for psoriasis.

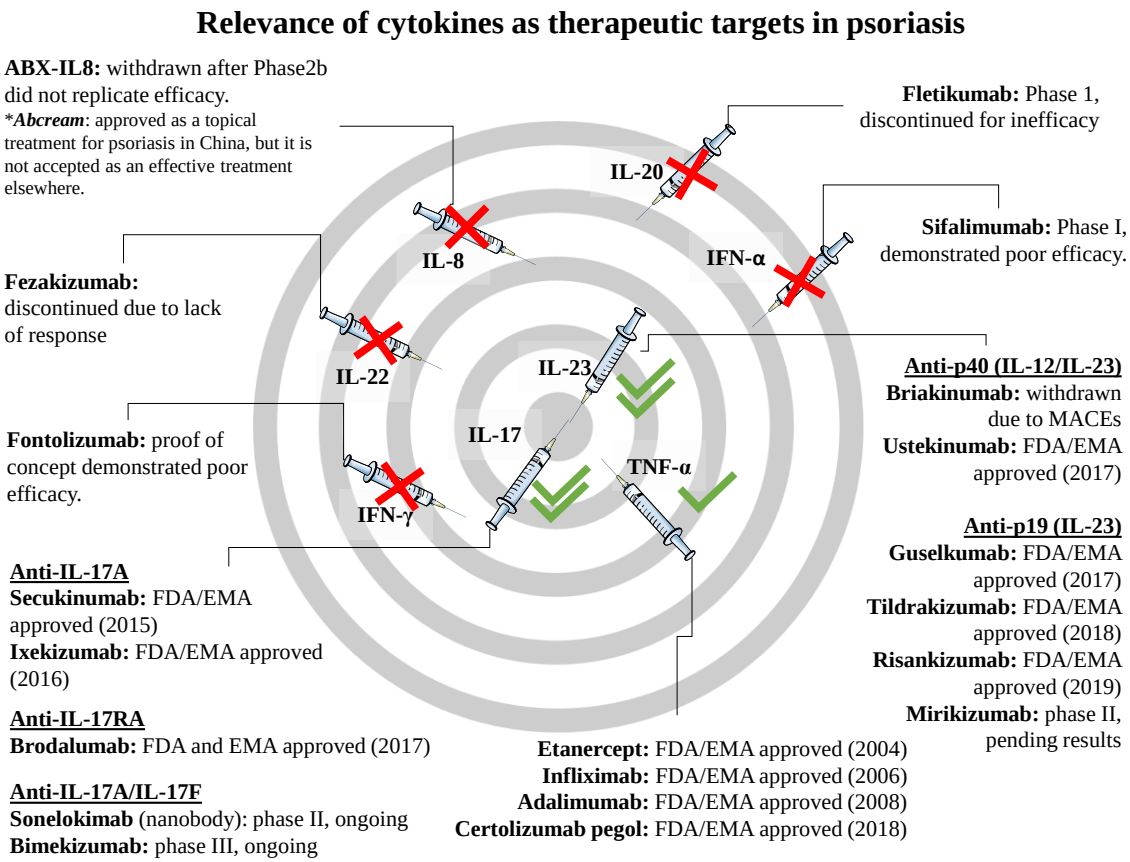



**Figure 10. Relevance of cytokines as therapeutic targets in psoriasis.** The dartboard shows the best targets for psoriasis treatment, based on the clinical efficacy of their blockade in patients. In the center IL-17 and IL-23, which have proved to achieve important clinical responses with almost complete clearance of skin lesions. Moving away from the center the clinical efficacy diminishes. Closer to the center is TNF-α, whose blockade was first approved to treat psoriasis with relatively good clinical response rate. Further from the center are IFN-γ, IL-22, IL-8, IFN-α and IL-20, whose neutralization led to poor or absent efficacy in the clinic, despite being somehow involved in the pathological mechanisms underlying psoriasis. FDA: U.S. Food & Drugs Administration; EMA: European Medicines Agency. Adapted and updated from Chiricozzi A et al 2018.

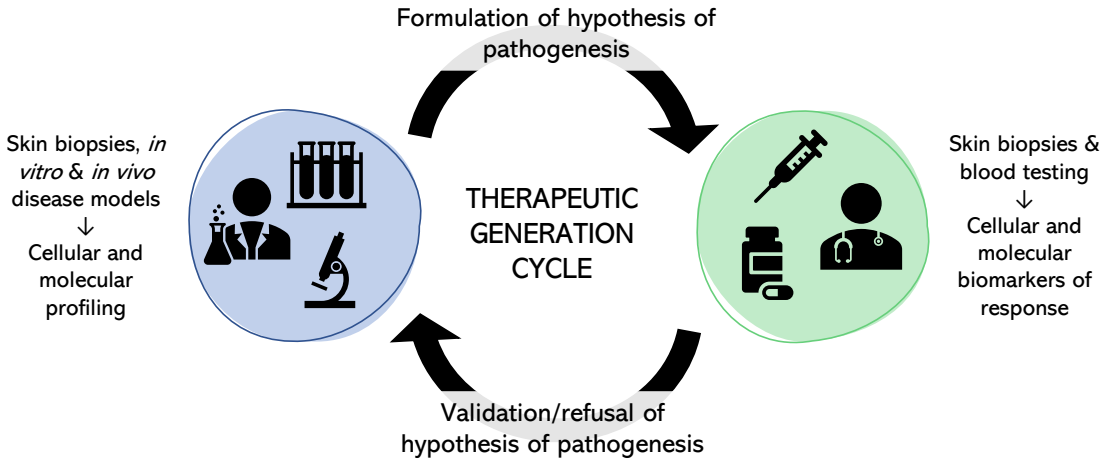
Moving towards relevant mediators of psoriasis, TNF-α inhibitors have proved long term efficacy and safety profiles in both psoriasis and PsA. Since their approval, they are still used in the clinic and biosimilars are currently available, increasing the scope of application (Rendon and Schäkel 2019). TNF-α blockers effectiveness is likely to be due to the neutralization of TNF-α direct actions as a pro-inflammatory cytokine, but most importantly the indirect suppression of IL-

23/IL-17 axis, as TNF-α synergizes with IL-17 to activate keratinocytes and also induces IL-23 production by dendritic cells (Yost and Gudjonsson 2009). Of note, some patients treated with TNF-α blockers developed "paradoxical psoriasis" as a result of dysregulated type I IFN response (Conrad et al. 2018; Toussirot and Aubin 2016).

However, more promising results have been obtained when directly targeting the IL-23/IL-17 axis. Already approved IL-17 inhibitors, addressed against IL-17A (secukinumab, ixekizumab) or the receptor IL-17RA (brodalumab), have shown excellent clinical response, often achieving more than 70% of PASI90 (Ghoreschi et al. 2021). More recently developed bimekizumab, a dual antibody targeting IL-17A and IL-17F, has shown impressive clinical response, even reporting complete clearance, compared to both placebo and ustekinumab (dual IL-12/23 inhibitor targeting their common subunit p40) (Gordon et al. 2021; Reich et al. 2021). Also, IL-17 blockers succeed after TNF-α inhibitors failure and are effective for PsA and spondyloarthropathies. Nonetheless, special attention should be paid since IL-17A/F neutralization can exacerbate Inflammatory Bowel Disease (IBD) and increase the rate of *Candida* infections in patients (Kolli et al. 2019).

Finally, several specific IL-23 inhibitors, targeting the p19 subunit, have been approved for psoriasis treatment in recent years (guselkumab, risankizumab and tildrakizumab). These have shown exceptional PASI-90 response in up to 70-80% of patients, coming to PASI-100 in about 50% of them, with longer time of sustained remission (Ghoreschi et al. 2021). Remarkably, unlike anti-IL-17A/F mAbs, IL-23 neutralization carries lower risk of IBD or candidiasis as it preferentially reduces the stimulation of pathogenic Th17 cells, but spares alternative IL-23-independent sources of IL-17 that contribute to maintain intestinal epithelial homeostasis (Lee et al. 2015).

The rapid validation or refusal of distinct cells and cytokines as presumed therapeutic targets has shaped the evolution of psoriasis pathogenic model through the past years. Today, and after several of these "therapeutic generation cycles" (see the chart on Figure 11), the IL-23/IL-17 axis is considered the main pathogenic mechanism of psoriasis. Because of the experience gained over time, psoriasis is a clear example of how translational research has revolutionized the knowledge on pathophysiological mechanisms and therapies in dermatology. Even so, pro-inflammatory cytokines that are present at psoriatic lesions may influence the IL-23/IL-17 axis in different ways. Therefore, a better understanding of these cytokines interactions is needed to continue modeling the molecular pathogenic mechanisms of the disease, which could ultimately have a positive impact on patient's care.




| Formulation of pathogenic model | Therapeutic target | Clinical efficacy (PASI75) | Validation/refusal of formulated models |
|---|---|---|---|
| Simple T cell model | Broad T cell | 20-40% | Validated but deficient |
| Polar T cell model (IL-12/Th1) | IFN-γ | Lack of efficacy | Refused model |
| Innate immunity model (it's not T cells) | IFN-α /IL-8/IL-20 | Lack of efficacy | Refused models |
| Complex T cell model (Th1, Th17 &Th22) | IL-22 | Lack of efficacy | Refused model |
| | TNF-α | 40-60% | Validated but still deficient |
| | IL-12/IL-23 (p40) | ~ 70% | |
| IL-23/IL-17 axis model (IL-23-induced IL-17 production) | IL-17/IL-17R | 80-90% and moving to PASI90/100 | Validated as key pathogenic mechanism |
| | IL-23 (p19) | | |

**Figure 11. The therapeutic generation cycle in psoriasis.** Laboratory research works on psoriasis revealed molecules related to mechanisms involved in psoriatic lesions, leading to the formulation of hypothesis explaining the disease pathogenesis. Based on these models, proposed therapeutic targets were addressed in clinical trials. Analysis of achieved clinical efficacy, initially measured as PASI75 (meaning a reduction of 75% of the PASI score at baseline), either validated or refuted the proposed pathogenic models and therapeutic targets. Consecutive cycles of this process led to a better understanding of psoriasis pathogenesis, essentially focused on the IL-23/IL-17 axis as the key driver of psoriatic inflammation. Adapted from a conference presentation by Dr. JG Krueger in 2017.

## 5. Modeling psoriasis

Before reaching human clinical trials, much research must be done using distinct models that try to recapitulate disease mechanisms and phenotypes in a more feasible way for testing distinct hypothesis, but the development of these models of human disease is not always easy.

Psoriasis is not observed in animals other than humans, which implies a formidable challenge to pursue models of the disease. Even with this initial complexity, several mouse models have been developed resembling human psoriasis to a greater or lesser extent. These include genetically engineered models (both transgenic and targeted mutations), spontaneous models, major histocompatibility complex (MHC)-mismatched allografts, xenografts using non-lesional psoriatic skin, which are the most similar to human disease but implies considerable technical difficulties, and the more accessible drug induced models, among which the induction of

psoriasiform inflammation with IMQ, an agonist of the TLR7, is widely used (Luo et al. 2020). Despite none of the models can represent psoriasis pathogenesis thoroughly, they are essential and help us to understand the roles of specific mediators and signaling pathways. However, significant divergence in the role of specific cytokines as inducers of skin inflammation has led to wrong target design for therapeutic interventions. In this regard, whereas skin inflammation in human psoriatic lesions is mostly induced by IL-17, epidermal hyperplasia and inflammation is largely mediated by IL-22 in mouse models (Figure 12) (Krueger 2012). This difference translated into the very effective clinical response of IL-17 blockers, but poor effectiveness of IL-22 neutralization, in humans. Similarly, despite neutrophils are the major source of IL-17A in human psoriatic lesions, IMQ-induced psoriasis model does not reflect this matter, as the main IL-17A producers in this model are γδT cells instead (Pantelyushin et al. 2012).

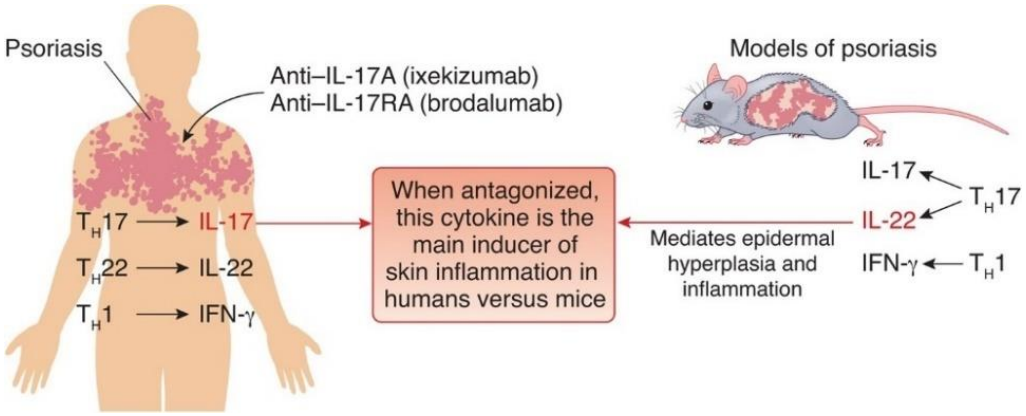



**Figure 12. Different mediators of skin inflammation in human psoriasis and mouse models.** Human cutaneous inflammation is primarily driven by IL-17, whilst IL-22 is the key driver of epidermal hyperplasia in mouse models of psoriasis. These differences are further illustrated by the excellent clinical response of IL-17 neutralization but the lack of clinical efficacy of IL-22 inhibitors in humans, despite its effectiveness in pre-clinical trials (Krueger 2012).

Furthermore, chronic inflammatory diseases, like psoriasis, are very heterogeneous in terms of clinical manifestation, rate of progression and presence of associated comorbidities. For these reasons, the use of *in vitro* models based on human samples, that better represent patients' singularities, are preferred for translational purposes (Florian et al. 2020).

## 5.1.An *ex vivo* model of psoriasis: principles and description of the model

Considering the limitations of non-human models of psoriasis and the need to prove disease mechanisms in humans, our group has established an *ex vivo* model of psoriasis which reproduces the interactions between T lymphocytes and epidermal cells in the context of clinically relevant triggers. It is based on the coculture of circulating effector memory $CLA^+$ or $CLA^-$ T cells with autologous epidermal cells from lesional skin (referred as $CLA^+$ T/EPI and $CLA^-$ T/EPI). These cocultures are then activated with an extract of *S. pyogenes* (SE), consisting of a mixture of sonicated bacteria isolated from throat swabs of psoriasis patients, which represents a relevant disease trigger (Figure 13). This system allows not only the

evaluation of the immune response generated by this trigger but also the analysis of the effector functions in the context of patients' clinical features, such as type of psoriasis, disease severity, age of onset, or genetic background (HLA-Cw*06:02). Typically, T lymphocytes in cocultures comprised around 80% of $CD4^+$ and 20% of $CD8^+$ T cells, both in $CLA^+$ or in $CLA^-$ T cell conditions, and after SE activation increased number of T cells is observed, but the proportion of $CD4^+$ / $CD8^+$ T cells is maintained.

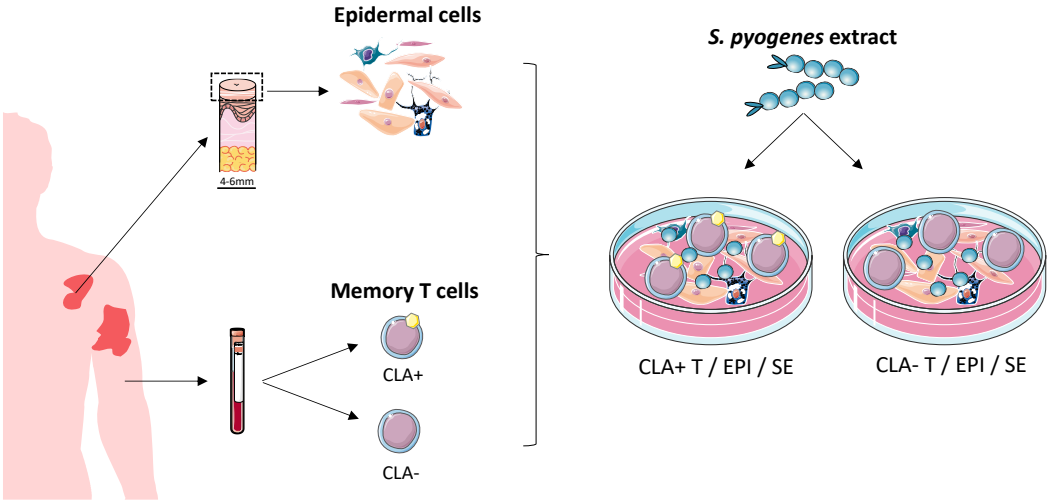



**Figure 13. *Ex vivo* model of psoriasis.** Epidermal cells isolated from lesional punch biopsies are cocultured with autologous circulating $CLA^+$ memory T cells, isolated from peripheral blood, and activated with *S. pyogenes* extract (SE), as disease trigger. Cocultures of epidermal cells with $CLA^-$ memory T cells are used as a control for other tissue tropism.

## 5.2.Characterization of the immune response

In psoriasis patients, the activation of $CLA^+$ T/EPI coculture with SE led to the expression and secretion of Th1, Th17 and Th22 cytokines and epidermal chemokines (CXCL8-11); but this response was not observed neither in SE-activated $CLA^-$ T/EPI cocultures of patients nor in any condition for healthy controls. Interestingly, SE-induced expression of relevant cytokines was positively associated to anti-streptolysin O (ASO) titters in patients and SE-activated $CLA^+$ T/EPI supernatants induced epidermal hyperplasia *in vivo* (Ferran et al. 2013a), highlighting the capacity of our model to induce hallmarks of psoriasis response *ex vivo*. Further studies have demonstrated that guttate psoriasis patients with positive HLA-Cw*06 and flares associated to previous pharyngitis display a stronger Th17 response to *S. pyogenes* in $CLA^+$ T/EPI cocultures, which is able to up-regulate IL-17-induced genes (hBD2, S100A7 or lipocalin) and down-regulate epidermal barrier genes (filaggrin and loricrin) in normal human keratinocytes (Ruiz-Romeu et al. 2016b). Additionally, supernatants from SE-activated $CLA^+$ T/EPI cocultures induced the expression of ZC3H12A, encoding the RNase monocyte chemotactic protein-induced protein 1 (MCPIP1), in treated keratinocytes mainly through IL-17A (Ruiz-Romeu et al. 2016a). Importantly, MCPIP1 expression is up-regulated in psoriatic skin and modulated after successful treatment with IL-17 inhibitors. More recently, our group

proved *S. pyogenes* and *C. albicans*-dependent secretion of IL-9 in $CLA^+$ T/EPI cocultures, which contributes to IL-17A production and $CLA^+$ T cells survival *in vitro*. Despite IL-9 induction by *S. pyogenes* was similarly reported in guttate and plaque psoriasis patients, higher cytokine induction has observed in parallel to PASI and ASO peaks (Ruiz-Romeu et al. 2018) (included in APPENDIX II: supporting publications).

In this thesis, that started with a review of the $CLA^+$ T cell response to microbes in psoriasis (de Jesús-Gil et al. 2018) (included in APENDIX II: supporting publications), we continue to exploit this *ex vivo* model of psoriasis to better understand how environmental triggers of the disease affect the effector functions of skin homing $CLA^+$ T cells and their possible association with clinical features from plaque and guttate psoriasis patients.

# AIM AND OBJECTIVES

The main objective of this thesis is to study the activity of circulating $CLA^+$ T lymphocytes in the context of environmental triggers of psoriasis pathogenesis, in particular disease associated microorganisms and pro-inflammatory cytokines present at cutaneous lesions. On that purpose, the specific objectives that we set up are:

1. Characterize the exposition to *Streptococcus pyogenes* and *Candida albicans* in psoriasis patients, and their relationship with specific $CLA^+$ T cell response *in vitro* in different clinical profiles.
    1.1. Detect levels of IgG and IgA recognizing *S. pyogenes* and *C. albicans* in plasma from psoriasis patients through an indirect customized ELISA.
    1.2. Study the possible association between Igs in plasma with clinical parameters of disease evolution, such as Psoriasis Severity Area Index (PASI), Anti-Streptolysin O antibodies (ASO), response to treatment or length of disease.
    1.3. Assess the possible connection between Igs in plasma with Th17, Th1 and Th9 cytokines secreted by $CLA^+$ T cells cocultures after stimulation with *S. pyogenes* or *C. albicans* extracts *in vitro* in the same patient.
2. Define the influence of the pro-inflammatory psoriatic environment on $CLA^+$ T cells function, particularly studying the role of IL-23 and IL-15.

The scheme shown in Figure 14 delineates the holistic approach of this thesis to achieve the above-mentioned objectives.

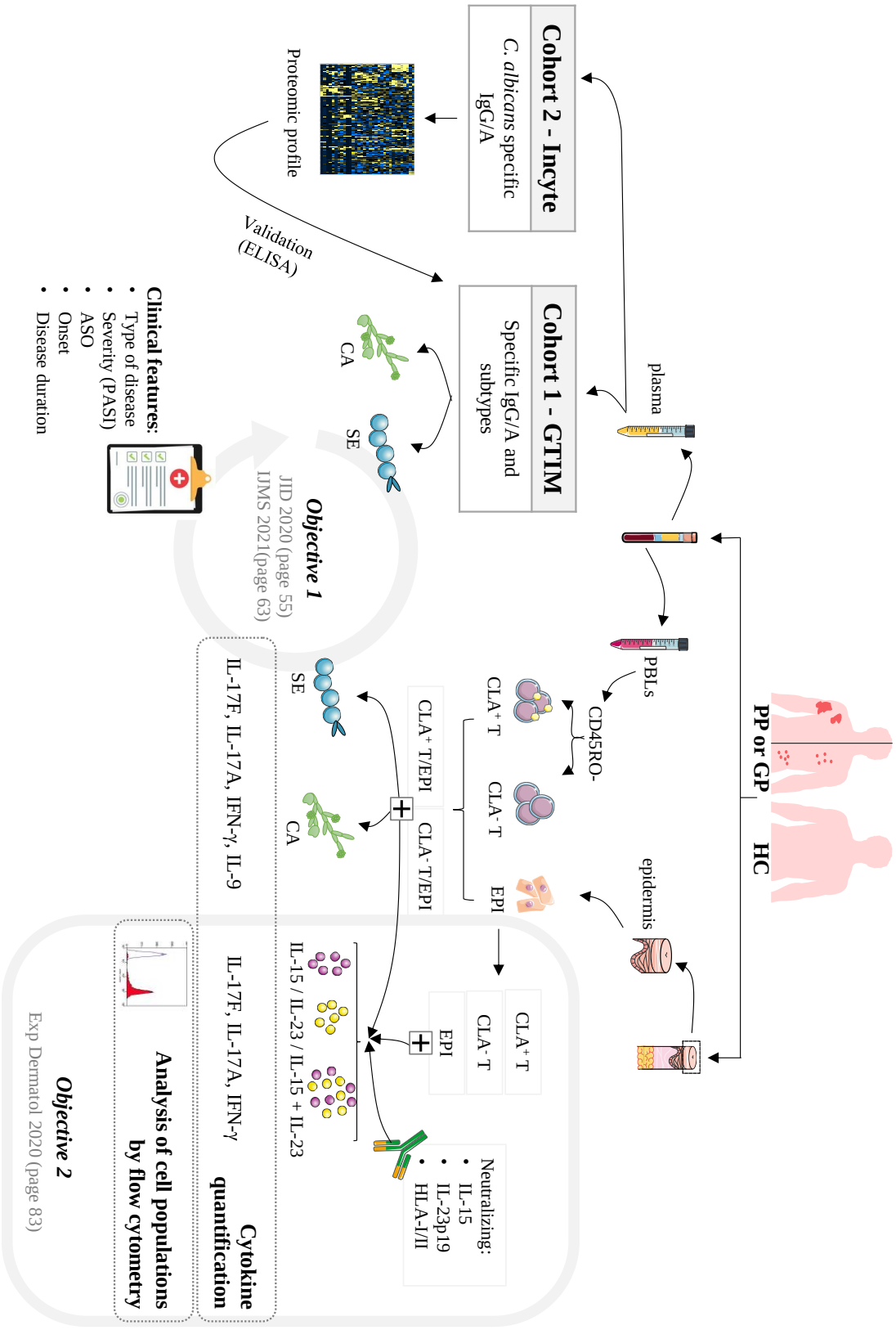



**Figure 1414. Scheme illustrating the holistic approach of this thesis**.
Most of the experiments take advantage of cellular components isolated from blood (memory $CLA^+$ / $CLA^-$T cells) and skin biopsies (epidermal cells) from psoriasis and healthy subjects. Additionally, plasma samples were obtained to study the presence of specific antibodies. From the middle to the left side of the figure, the studies carried out to characterize microbial (*S. pyogenes* and *C. albicans*) exposition and their association with specific $CLA^+$ T cell response *in vitro* are represented (*Objective 1*). The right side shows the characterization of the pro-inflammatory cytokines IL-15 and IL-23 in the context of our *ex vivo* model of psoriasis (*Objective 2*). PP: plaque psoriasis, GP: guttate psoriasis; HC: healthy controls; SE; *Streptococcus. pyogenes*; CA: *Candida albicans*.

# PUBLICATIONS

# I. Specific IgA and CLA$^+$ T cell IL-17 Response to *Streptococcus pyogenes* in Psoriasis

ORIGINAL ARTICLE

## Specific IgA and CLA$^+$ T-Cell IL-17 Response to *Streptococcus pyogenes* in Psoriasis

Carmen De Jesús-Gil[1], Lidia Sans-de San Nicolás[1], Ester Ruiz-Romeu[1], Marta Ferran[2], Laura Soria-Martinez[3], Anca Chiriac[4], Antonio Celada[5], Ramon M. Pujol[2] and Luis F. Santamaria-Babí[1]

*Streptococcus pyogenes* tonsillar infection is well known to trigger and exacerbate psoriasis lesions in both guttate and plaque forms of the disease. Although mucosal and cutaneous tissues are closely involved in psoriasis pathology, the interaction between their specific immune responses has not been deeply explored. This work aims to address and characterize the presence of humoral responses against *S. pyogenes* in patients with psoriasis and its putative association with cytokine responses detected in vitro in our psoriasis ex vivo model, based on the coculture of cutaneous lymphocyte-associated antigen$^{+/-}$ T cells with autologous epidermal cells. Patients with psoriasis presented increased IgA response to *S. pyogenes* when compared with control subjects. In patients with plaque psoriasis, despite being negative for anti-streptolysin O antibody titer, IgA plasma levels against *S. pyogenes* correlated with cutaneous lymphocyte-associated antigen$^+$ T-cell–dependent IL-17F response in vitro. No association is observed for IgG levels in plaque psoriasis. Similar association is observed for IgA anti-*S. pyogenes* extract and IL-17A in patients with guttate psoriasis. We propose *S. pyogenes*–specific IgA as a potential new perspective for better understanding the role of *S. pyogenes* in psoriasis development.

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### INTRODUCTION

Psoriasis is a chronic inflammatory T-cell–mediated skin disease, consequence of a combination of genetic and environmental factors (Hawkes et al., 2017). The role of microorganisms in psoriasis development has been discussed for years, and several bacteria, fungi, and viruses have been associated with the either the onset or new flares of the disease (Fry et al., 2013). *Streptococcus pyogenes* tonsillar infection is the strongest environmental factor linked to trigger and/or to exacerbate psoriasis skin lesions, not only in guttate form of the disease (Norrlind, 1955) but also in chronic plaques (Cohen Tervaert and Esseveld, 1970; Thorleifsdottir et al., 2017; Wardrop et al., 1998). Tonsillectomy has proved to ameliorate psoriatic symptoms in patients, even leading to disease clearance in some cases (Thorleifsdottir et al., 2017). Patients with early onset of psoriasis are more likely to develop flares after an upper respiratory tract infection, to have a family history psoriasis, and to present higher disease severity (Theodorakopoulou et al., 2016). These patients with early onset of psoriasis tend to require systemic treatments, being more likely to receive biologics and suggesting that streptococcal throat infection somehow conditions psoriasis development and the clinical management of the disease. However, the exact pathogenetic links between *S. pyogenes* tonsillar infection and psoriasis are not fully elucidated. Streptococci are able to be internalized and to survive in tonsillar cells as intracellular structures. This immune evasion mechanism allows *S. pyogenes* to remain within the organism, giving rise to a reservoir of persistent putative pathogenic antigens (Österlund et al., 1997). The well-established association between *S. pyogenes* sore throat and psoriasis has led to the hypothesis that cutaneous lesions are mediated by T cells originated in the tonsils that later migrate to the skin, where they activate and secrete proinflammatory cytokines (Valdimarsson et al., 2009). This hypothesis is further supported by the demonstration of identical *TCRVB* gene rearrangements in cutaneous and tonsillar T cells isolated from the same patient (Diluvio et al., 2006).

There is a subset of memory T cells whose activity is confined to cutaneous tissue, which are identified by the expression of the cutaneous lymphocyte-associated antigen (CLA). During cutaneous inflammation, these skin-homing T cells are able to recirculate between skin lesions and blood and are considered peripheral biomarkers of human T-cell–mediated cutaneous diseases (Ferran et al., 2013). Patients with psoriasis have increased levels of CLA$^+$ T cells expressing IL-23 receptor in the blood and tonsils (Sigurdardottir et al., 2013). Variations in the number and phenotype of circulating CLA$^+$ T cells are closely related to improved clinical outcome of patients with psoriasis (Thorleifsdottir et al., 2012). Our group has established a

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*Abbreviations: ASO, anti-streptolysin O antibody; CLA, cutaneous lymphocyte antigen; PBS, phosphate buffered saline; SE, Streptococcus pyogenes extract; Th17, T helper type 17*

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psoriasis ex vivo model based on the coculture of circulant $CLA^+$ or $CLA^-$ T cells and autologous epidermal cells, isolated from skin biopsies from patients with lesional psoriasis, which are then activated by a *S. pyogenes* extract (SE), leading to specific IL-17 response in $CLA^+$ but not $CLA^-$ T-cells cultures (Ferran et al., 2013; Ruiz-Romeu et al., 2018, 2016).

As considered a T-cell–mediated disease, the study of the cellular immune response in psoriasis has been widely approached. Nevertheless, few results addressing the humoral immune response against *S. pyogenes* in patients with psoriasis have been undertaken. It has been reported that patients with plaque psoriasis had increased plasma IgG levels recognizing *S. pyogenes* heat shock proteins (Pérez-Lorenzo et al., 2003), specifically the 60 kDa protein named rHSP60Sp, compared with patients with guttate psoriasis and controls (Cancino-Díaz et al., 2004), which were associated with higher psoriasis area and severity index and anti-streptolysin O antibody (ASO) titer. More recently, nontreated patients with chronic plaque psoriasis showed increased blood levels of IgG against secreted *S. pyogenes* proteins but not cellular components compared with control subjects (El-Rachkidy et al., 2007). Notably, the palatine tonsils are key components of the mucosal immune system at the oropharyngeal tract and are commonly infected by streptococci in psoriasis. Although mucosal and cutaneous tissues are closely involved in psoriasis pathology, the interaction between their specific immune responses has not been deeply explored. The role of IgA as the major antibody participating in humoral mucosal immunity may be of great interest in psoriasis, despite that the presence of B cells is not required in psoriasis pathogenesis (Thomas et al., 2019). Thomas et al. (2019) showed increased IgA blood levels in patients with plaque psoriasis that positively correlated with IgA producing plasma cells ($CD19^+$, $CD24^-$, $CD38^+$, and $CD138^+$) and psoriasis area severity index. However, less is known about the presence of specific IgA against *S. pyogenes* in those patients. This work aimed to address and characterize the presence of mucosal immune response against *S. pyogenes* in patients with psoriasis and its putative association with the IL-17A, IL-17F, IFN-γ, and IL-9 cytokine response detected in vitro in our psoriasis model.

## RESULTS

### Patients with psoriasis show elevated anti-SE IgA compared with control subjects

To study the psoriasis-specific humoral immune response against a relevant microbial trigger of the disease such as *S. pyogenes*, plasma from patients with psoriasis (n = 62), psoriatic arthritis (n=13), atopic dermatitis (n = 17), and healthy controls (n = 21) were collected and analyzed by ELISA against the microorganism extract. Patients with psoriasis have higher IgA levels against SE than individuals with atopic dermatitis and controls but similar to patients with psoriatic arthritis (Figure 1a). However, differences in anti-SE IgG plasma levels are not detected within patients with psoriasis, psoriatic arthritis, atopic dermatitis, and healthy controls subjects (Figure 1c). According to their subtype of disease, patients with plaque (n = 34) and guttate (n = 28) psoriasis show increased anti-SE IgA levels both relative to patients with atopic dermatitis and control subjects (Figure 1b and d). Nonetheless, neither patients with plaque nor guttate psoriasis showed higher anti-SE IgG plasma levels than control subjects (Figure 1d). Notably, anti-SE IgG was detected in plasma from many control subjects at similar levels to those in patients with psoriasis, whereas anti-SE IgA was hardly detectable in controls.

### Increased anti-SE IgA levels in ASO negative plaque psoriasis

ASO titer is an easy and frequently used tool to assess recent group A *Streptococcus* infection. Considering that 71.43% of

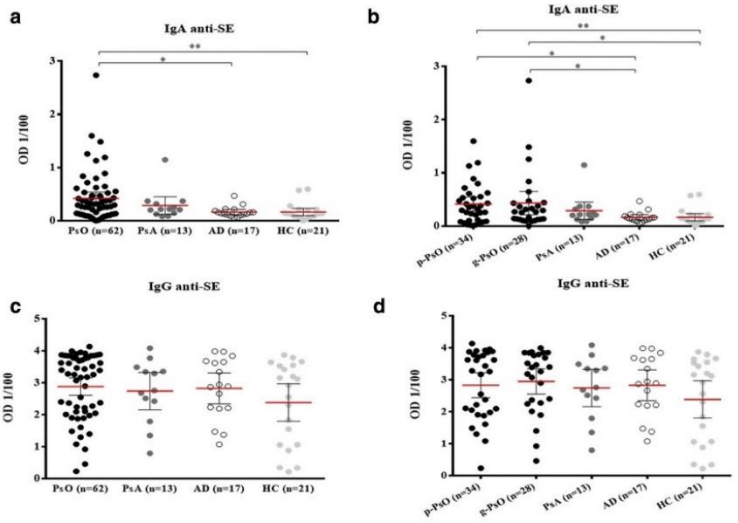


**Figure 1. Patients with psoriasis show increased plasma IgA levels against *S. pyogenes* extract.** Specific IgA and IgG recognizing SE were detected through ELISA in plasma collected from blood of patients with psoriasis (n = 62), PsA (n = 13), and AD (n = 17) and HCs (n = 21). OD of 1:100 diluted plasma is reported. Patients with psoriasis (**a**) IgA and (**c**) IgG levels against SE are shown. According to their subtype of disease **(b, d)**, g-PsO and p-PsO show differential anti-SE Ig profile. Statistics lines are represented as mean with 95% confidence interval. Mann-Whitney test was used to compare two different groups (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$). AD, atopic dermatitis; g-PsO, guttate psoriasis; HC, healthy control; OD, optical density; p-PsO, plaque psoriasis; PsA, psoriatic arthritis; PsO, psoriasis; SE, *S. pyogenes* extract.

**Table 1. Clinical Features of Patients with Guttate and Plaque Psoriasis**

| Clinical Parameters | | Plaque Psoriasis (n = 34) | Guttate Psoriasis (n = 28) | *P*-value |
|---|---|---|---|---|
| ASO | | 99.36 (67.74) | 442.3 (260.9) | *** |
| PASI | | 14.58 (6.55) | 7.10 (3.14) | *** |
| Length of disease (mo) | | 26.69 (62.73) | 2.71 (7.12) | ** |
| Age of onset, y | | 37.46 (14.33) | 27.19 (11.98) | * |
| HLA Cw6 n (%) | Positive | 11 (32.35) | 22 (78.57) | NA |
| | Negative | 14 (41.18) | 2 (7.14) | NA |
| | Unknown | 9 (26.47) | 4 (14.19) | NA |
| Flare associated to Streptococcal infection n (%) | Yes | — | 20 (71.43) | NA |
| | No | 34 (100) | 3 (10.71) | NA |
| | Unknown | — | 5 (17.86) | NA |

Mean values ± (standard deviation) or total number (%) are shown. Mann-Whitney test was used to compare numerical variables (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$).

Abbreviations: ASO, anti-streptolysin O antibody titer; NA, not assigned; PASI, Psoriasis Area Severity Index.

guttate psoriasis flares in our first cohort were associated to *S. pyogenes* infection and they present elevated ASO titer (Table 1), increased anti-SE IgG plasma levels were expected in these patients (n = 28). Higher anti-SE IgA levels than those of control subjects were also observed. However, although our cohort of patients with plaque psoriasis (n = 34) showed negative ASO titer (<200 U/ml) and their flares were not associated to clinical signs of *S. pyogenes* infection (Table 1), we were able to detect statistically significant increased anti-SE IgA levels in plasma compared with controls. In addition, patients with plaque psoriasis showed a more severe disease according to the psoriasis area and severity index (mean [± standard deviation] value of 14.58 [6.55] vs. 7.10 [3.14] in guttate psoriasis, $P < 0.0001$), longer clinical evolution (26.69 [62.73] months vs. 2.71 [7.12], $P < 0.01$), slightly later onset of the disease, and less association to the presence of HLA-Cw6 allele (Table 1) than individuals with guttate psoriasis. Altogether, our results indicate that some patients with plaque psoriasis have been exposed to *S. pyogenes*, despite having negative clinical signs of recent infection and that elevated anti-SE IgA levels may be a sign of chronic immune response against *S. pyogenes*.

**Psoriasis patients anti-SE IgA plasma levels, but not IgG, correlate with $CLA^{+-}$–dependent IL-17 response in vitro**

Next, we wanted to see whether anti-SE IgA or IgG plasma levels were associated with in vitro response to SE activation in cocultures of $CLA^{+/-}$ T cells together with autologous epidermal cells. For that purpose, cytokines such as IL-17A, IL-17F, IFN-γ, and IL-9 were quantified in culture supernatant from some patients with plaque psoriasis (n = 27), guttate psoriasis (n = 26), and healthy control subjects (n = 14). A significant direct correlation ($r = 0.501$; $P = 0.0056$) is established between IgA anti-SE plasma levels and $CLA^+$ T-cell–dependent IL-17F response to *S. pyogenes* in patients with plaque psoriasis (Figure 2a). This association is not observed for $CLA^-$ T-cell IL-17F response. A direct correlation is also observed between IgA anti-SE and IFN-γ induction in both $CLA^+$ and $CLA^-$ T-cell cultures (Figure 2c) for the plaque form of the disease. However, there is not a clear association between IgA anti-SE plasma levels and IL-17A and IL-9 responses in vitro for patients with plaque psoriasis (Figure 2b and d). In patients with guttate psoriasis, we observed distinct patterns of association between IgA anti-SE and cytokines induced in culture. In contrast, there is no association with neither IL-17F nor IFN-γ (Figure 2e and g) observed for plaque psoriasis. By contrast, there is a clear direct correlation between IgA anti-SE and $CLA^+$ T cell–dependent IL-17A induction ($r = 0,586$; $P = 0.0017$) but not in $CLA^-$ T cells (Figure 2f) and also, a direct correlation with IL-9 levels for both $CLA^+$ and $CLA^-$ T cells (Figure 2h). Notably, no correlation was found with IgG anti-SE plasma levels (Supplementary Table S1). Altogether, plasma levels of anti-SE IgA—but not IgG—from patients with plaque psoriasis revealed in vitro IL-17 response of $CLA^+$ T cell and epidermal cell cocultures stimulated with SE.

**Anti-SE IgA response in patients with psoriasis is associated to type 1 IgA**

Having proved the presence of IgA recognizing *S. pyogenes* in plasma from patients with psoriasis, even in those with nonassociated sore throat infection, we sought to explore the putative source of mucosal immune response against these microorganisms by analyzing IgA subtypes. Plasma from patients with psoriasis was then analyzed by a similar ELISA but using anti-human IgA1 or IgA2 as secondary antibodies. We observed that patients with psoriasis showed higher anti-SE IgA1 than IgA2 subtype in plasma (Figure 3a). Although the optical density values detected were lower than those for IgA, positive and negative controls proved that antibodies worked in each ELISA (Supplementary Figure S1). When looking at the distribution of specific IgA1 and IgA2 against *S. pyogenes* between the different forms of the disease, we observe that both patients with plaque (Figure 3b) and guttate psoriasis (Figure 3c) have slightly increased levels of anti-SE IgA1 compared with IgA2.

## DISCUSSION

*S. pyogenes* infection can influence psoriasis development and evolution (De Jesús-Gil et al., 2018; Norrlind, 1955). Our results showed that IgA against *S. pyogenes* is present in plasma from patients with plaque and guttate psoriasis and its levels are directly associated to $CLA^+$ T cell–dependent IL-17 response in our ex vivo model of the disease that correlates with the clinic (Ruiz-Romeu et al., 2018).

We found that our cohort of patients with plaque psoriasis, with no history of streptococcal-mediated tonsillitis and negative ASO titer, had developed humoral response against *S. pyogenes*. Despite what other clinical parameters may indicate, anti-SE IgA levels found in the plasma of patients with plaque psoriasis proved that these patients have been exposed to this microorganism. Although B cells are not indispensable for psoriasis development, as proved by a case of full psoriasis vulgaris phenotype in a patient with common variable immunodeficiency (Thomas et al., 2019), the presence of anti-SE IgA response may condition disease development or progression. In patients with plaque psoriasis, values for IgA-SE correlated with $CLA^+$ T cell–mediated IL-17F response in vitro. However, higher but not significantly

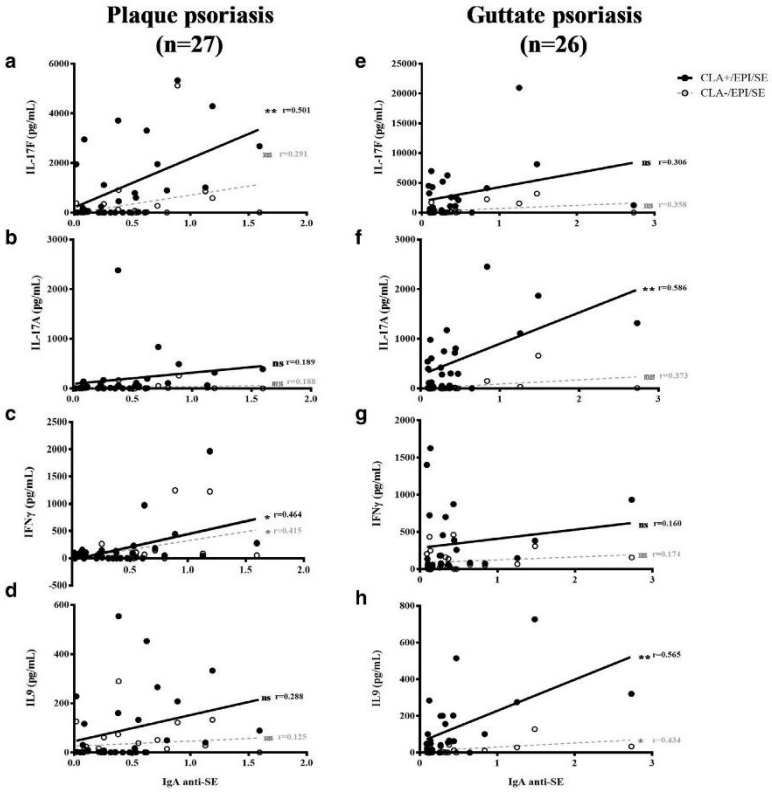


**Figure 2. Patients with psoriasis anti-SE IgA plasma levels correlate with CLA$^+$ dependent–IL-17 response in vitro.** (**a, e**) IL-17F, (**b, f**) IL-17A, (**c, g**) IFN-γ, and (**d, h**) IL-9 concentrations were measured in culture supernatants after 5 days of *S. pyogenes* stimulation and correlated to levels of anti-SE IgA in plaque (n = 27) and guttate (n = 26) psoriasis. Statistics lines are represented as linear regression and Pearson *r* values are indicated. *P*-values are represented as $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$. CLA, cutaneous lymphocyte antigen; SE, *S. pyogenes* extract.

increased anti-SE IgG blood levels were detected in patients with plaque psoriasis than control subjects, even though previous studies showed increased blood levels of IgG recognizing secreted *S. pyogenes* proteins (Cancino-Díaz et al., 2004; Pérez-Lorenzo et al., 2003), probably because of the different source of antigen used to determine IgG against *S. pyogenes*. Increased levels of IgA anti-SE were also detected in patients with guttate psoriasis when compared with healthy individuals, but these were directly correlated with IL-17A secretion by CLA$^+$ T cells in vitro after *S. pyogenes* stimulation.

Although the association between IL-17 and IgA in psoriasis has not been previously reported to our knowledge, the link between IL-17 and Igs has been previously described in animal models. IL-17 deficient (IL-17$^{-/-}$) mice showed impaired Ig response in allergic (Nakae et al., 2002) and autoimmune (Nakae et al., 2003) disease models. Despite molecular mechanisms underlying this effect remain to be fully understood, in vitro studies suggest that IL-17 may be indirectly involved in antibody production by enhancing B-cell activators by other cells (Shibui et al., 2012) and that IL-17A/IL-17RA axis modulates B-cell migration within the germinal centers (Ferretti et al., 2016). Most of the evidence about the association between T helper type 17 (Th17) and IgA in vivo derives from studies of the intestinal immunity, in which Th17 cells have proved to be relevant for IgA isotype switch and secretion (Cao et al., 2012; Hirota et al., 2013). Christensen et al. (2017) have recently demonstrated how parentally primed Th17 cells induce antigen specific IgA in the lungs from immunized mice, confirming the link between Th17 cells and IgA responses in the airways. Regarding the published data, we hypothesized that long-term exposure to *S. Pyogenes*–activated Th17 cells could induce IgA synthesis and secretion in the tonsils. However, proving the molecular mechanism behind this remains a limitation of this study.

The tonsils from patients with psoriasis are more frequently infected by group A *Streptococcus* than those from control subjects, and it often precedes the appearance of psoriatic lesions in skin. Recently, the development of group A *Streptococcus* extra- and intracellular biofilms has been reported in tonsillectomy specimens from psoriasis, supporting the microbial role in the pathogenesis of the disease (Allen et al., 2018). Biofilm formation within the tonsils may be responsible for the recurrent relapses over time because anti-streptococcal agents are not able to penetrate them and may explain why not all patients present upraised ASO titers, leading to a lack of *S. pyogenes* infection serum markers (Kim et al., 2010). Because of the relevant role of the palatine

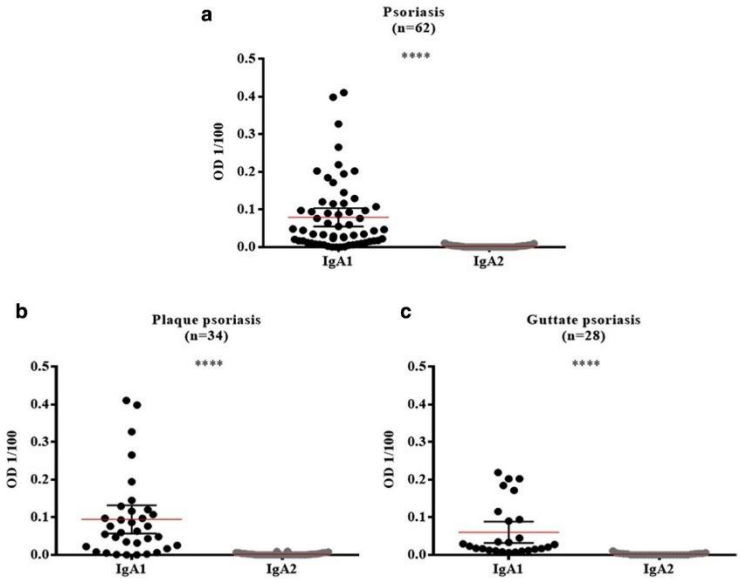


**Figure 3. Anti-SE IgA response in patients with psoriasis is associated to type 1 IgA.** Specific IgA1 and IgA2 recognizing SE were detected through ELISA in plasma collected from blood of patients with psoriasis (n = 62). OD of 1:100 diluted plasma is reported. (**a**) In patients with psoriasis, IgA1 levels against SE proved to be higher than IgA2. This preferential anti-SE IgA1 over IgA2 response is maintained for patients with (**b**) plaque and (**c**) guttate psoriasis. Statistics lines are represented as mean with 95% confidence interval. Mann-Whitney test was used to compare two different groups (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$). OD, optical density; SE, *S. pyogenes* extract.

tonsils in *S. pyogenes* infection in psoriasis and because they are key components of the mucosal immune system, we believed the study of IgA against this microorganism in patients would be of great interest. Notably, analyzing the presence of *S. pyogenes* in the tonsils from our cohort of patients and comparing the results with those of IgA anti-SE in plasma remain a limitation of our study. Our results postulate anti-SE IgA blood levels as a new parameter of exposition of *S. pyogenes*, which is also present in patients with negative ASO titers, whose disease is currently considered to be independent of *S. pyogenes* infection.

Previously published data from our group showed $CLA^+$ T-cell–dependent Th17 responses after *S. pyogenes* activation in vitro in patients with psoriasis (De Jesús-Gil et al., 2018; Ruiz-Romeu et al., 2016). We found a direct correlation between IgA anti-SE and IL-17F (for plaque psoriasis) or IL-17A (for guttate psoriasis) responses in vitro when $CLA^+$ T cells were cocultured with autologous epidermal cells and activated with SE. Considering that IgA anti-SE was detected in plasma from patients with no history of streptococcal throat infection and negative ASO titer, we thought that these patients could have been exposed to this microorganism in an alternative manner. For that reason, type 1 and type 2 IgA against *S. pyogenes* were analyzed. Notably, although IgA1 is the most common subtype present in circulation, the fact that IgA response was type 1—and type 2 was completely absent—may indicate that the origin of this humoral immune response took place in the upper respiratory tract (Pakkanen et al., 2010). During disease development, increased levels of $CD4^+$ and $CD8^+$ $CLA^+$ T cells expressing IL-23 receptor are found in the blood and tonsils (Sigurdardottir et al., 2013). Coexistence of *S. pyogenes* and $CLA^+$ T cells in the tonsils lead to the hypothesis that cutaneous lesions are mediated by T cells initially originated in the tonsils that then migrate to the skin, where the proinflammatory environment induces their activation and therefore, secretion of IL-17 cytokines (Valdimarsson et al., 2009). Our findings support this linkage between mucosal tissue (palatine tonsils), microbe (*S. Pyogenes)*, and skin immune response ($CLA^+$ T cells).

Although mean values of IgA anti-SE optical density are similar in patients with plaque and guttatepsoriasis, the number of patients whose values are over this mean are higher in patients with plaque guttate psoriasis. This uneven distribution may reflect the differences between these two types of disease. Guttate psoriasis is an acute form, closely related to *S. pyogenes* upper respiratory tract infection and generally associated with better prognosis; however, some patients evolve to chronic plaque form of the disease. Few long-term follow-up studies have attempted to identify patients with guttate psoriasis who are likely to progress to chronic forms of the disease according to clinical and laboratory data, but no clear parameters have been established to date (Ko et al., 2010; Martin et al., 1996; Pfingstler et al., 2016). We hypothesized that the presence of elevated anti-SE IgA levels in patients with guttate psoriasis could help track the progression toward plaque form of the disease. However, long-term follow-up studies are required to confirm this idea. By contrast, plaque psoriasis flares can also be exacerbated by *S. pyogenes* infection (Cohen Tervaert and Esseveld, 1970; Wardrop et al., 1998), and tonsillectomy has been proved to be an effective treatment for the cutaneous lesions in some patients (Thorleifsdottir et al., 2017). ASO antibody titer is an easy and frequently used tool to assess group A *Streptococcus* infection, which begin to increase around 1 week and peak 3–6 weeks after infection (Gerber et al., 2009). Nonetheless, the course of plaque

psoriasis disease can take several months or even years, leading to negative ASO titers that may cover the presence and relevance of *S. pyogenes* infection in those patients. Previous studies reported that secretory IgA–coated *S. pyogenes* increased in chronic tonsillitis, whereas IgG-coated pathogen levels increased in acute forms and remained equal despite disease duration (Lilja et al., 1999). These findings support our observation of anti-SE IgA in patients with psoriasis.

## MATERIALS AND METHODS

### Patients

This study was performed with human samples and in accordance with the Declaration of Helsinki. A total of 62 patients with non-treated psoriasis and 21 healthy individuals were enrolled. All participants contributed voluntarily and provided written informed consent, and human material collection has been approved by the Comité Ético de Investigación Clínica from the Parc Salut Mar (Hospital del Mar, Barcelona). Psoriatic samples were from patients with guttate and plaque lesions, without any age or sex restriction. Patients who received any systemic treatment for the last 6 weeks were excluded to not obtain underestimated cellular activation. Patients with psoriasis and healthy subjects underwent two skin biopsies, which were punched in lesional skin of patients with psoriasis and a blood extraction. In addition, plasma samples from patients with psoriatic arthritis (n = 13) and atopic dermatitis (n = 17) were analyzed as examples of other chronic inflammatory diseases.

### ELISA against *S. pyogenes*

SE was as previously described by Baker et al. (1991). Briefly, group A beta-hemolytic *Streptococcus* was isolated from throat swabs of patients with psoriasis and cultured in liquid Todd-Hewitt medium (SIGMA-Aldrich, St Louis, MO) for 24 hours at 37 °C, followed by four washes with phosphate buffered saline (PBS). Finally, bacteria were adjusted to 1 mg protein/ml, sonicated, and maintained in sterile conditions. SE was diluted in coating buffer (50 mM sodium bicarbonate in ultrapure water, pH = 9.6) to a final protein concentration of 5 μg/ml and incubated for 3 hours at 37 °C. The same extract was used for all described experiments. Wells were then washed five times with PBS, blocked with 5% skimmed milk powder in PBS overnight at 4 °C, and then washed again five times in PBS-Tween 0.05%. Plasma samples were diluted 100-fold in PBS–1% skimmed milk, added to coated wells, and incubated 2 hours at 37 °C. Wells were again washed five times with PBS-Tween and incubated with alkaline phosphatase–labeled goat anti-human IgA, IgA1, IgA2, or IgG (SIGMA-Aldrich) diluted 1:4,000 in PBS–1% skimmed milk for 90 minutes at 37 °C. After five more washes with PBS-Tween 0.05%, p-nitrophenyl phosphate substrate (SIGMA-Aldrich) was incubated for 30 minutes at room temperature, and finally, enzymatic reaction was stopped by adding sodium hydroxide 3M solution. Plates were read within the next 30 minutes at 405 nm and 570 nm for background signal extraction, according to suppliers' instructions. The titer of reactive IgA, IgA1, IgA2, or IgG was taken as the value of optical density$_{405nm}$ signal after subctraction of background signal (optical density$_{570nm}$) and negative control well signal, incubated with only PBS–1% skimmed milk. Positive control wells were coated with human IgA/G isotype controls (Invitrogen, Waltham, MA), incubated with PBS as primary antibody and the corresponding secondary antibody before substrate addition.

### Circulating memory T cell and epidermal cell isolation

Peripheral blood mononuclear cells were isolated by Ficoll gradient (GE Healthcare, Princeton, NJ) and, after subsequent immunomagnetic separations (Miltenyi Biotech, Bergisch Gladbach, Germany), memory CD45RA$^-$ CLA$^+$ and CLA$^-$ T cells were purified as previously described (Santamaria Babi et al., 1995). Punch skin biopsies (4–6 mm) were incubated overnight in dispase (Corning, Bedford, MA) at 4 °C, then the epidermal sheet was peeled off from the dermis. The epidermis was cut in smaller pieces and incubated in trypsin solution (Biological Industries, Kibbutz Beit Haemek, Israel) for 15 minutes at 37 °C. Equal volume of RPMI media (SIGMA-Aldrich) containing 10% of fetal bovine serum (Gibco, Grand Island, NY) was added to inhibit trypsin action. Epidermal tissue was then mechanically disaggregated by gently pipetting up and down. Epidermal cell suspension was transferred to fresh media (RPMI, 10% fetal bovine serum, 1% penicillin-streptomycin [SIGMA-Aldrich]), and the remaining tissue leftovers were discarded.

### Cultures and pathogen activation

Ex vivo cocultures consisted of $5 \times 10^4$ CLA$^+$ or CLA$^-$ T cells plated together with $3 \times 10^4$ autologous epidermal cells (CLA$^+$/epidermal cell suspension or CLA$^-$/epidermal cell suspension, respectively), in 96-well flat-bottom plates (SIGMA-Aldrich), in the culture media described above. Cocultures were left untreated or activated with SE at 1 μg/ml final well concentration. After 5 days of culture, supernatants were collected and kept frozen at −20 °C for later cytokine quantification.

### Cytokine quantification

Multiplex fluorescent bead-based immunoassays were used to measure IL-17A and IFN-γ (Diaclone SAS, Besançon, France) and IL-17F (BD Biosciences, Franklin Lakes, NJ) concentration in collected culture supernatants. IL-9 concentration was quantified by using precoated ELISA kits (BioLegend, San Diego, CA).

### Statistical Analysis

Data are generally represented as the mean and 95% confidence interval. Differences between two groups were analyzed by the Mann-Whitney test. Differences were considered significant at a $P$-value $< 0.05$ and represented by symbols as follows: $^*P < 0.05$; $^{**}P < 0.01$; and $^{***}P < 0.001$.

### Data availability statement

No datasets were generated or analyzed during the current study.

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### CONFLICT OF INTEREST

The authors state no conflict of interest.

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## AUTHOR CONTRIBUTIONS

Conceptualization: CdJG, ERR, LSB, MF, RMP; Data Curation: JG, LSdSN, ERR, LSM, MS, PG, AC; Formal Analysis: CdJG, LSM, ERR, MS, PG, AC; Funding Acquisition: LSB, MF, RMP; Investigation: CdJG, LSM, LSdSN, ERR; Methology: CdJG, ERR, MS, PG, AC; Project Administration: LSB; Resources: MF, MS, AC, PG, RMP, LSB; Supervision: LSB; Validation: CdJG, ERR, LSB; Visualization: CdJG, ERR; Writing - Original Draft Preparation: CdJG, LSB; Writing - Review and Editing: CdJG, LSB.

## SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at www.jidonline.org, and at https://doi.org/10.1016/j.jid.2019.12.022.

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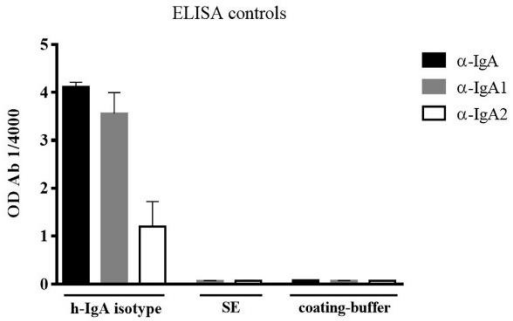


**Supplementary Figure S1. Positive and negative controls in our handmade ELISA.** Plates were coated with human IgA isotype as positive control, then incubated with PBS-1% milk and finally with secondary antibodies targeting IgA, IgA1, and IgA2. For negative controls, plates were coated with *S. pyogenes* extract or only coating buffer, then incubated with PBS-1% milk and finally with enzyme-conjugated secondary antibodies. PBS, phosphate buffered saline.

**Supplementary Table S1. Correlation between IL-17F, IL-17A, IFN-γ, and IL-9 Responses In Vitro and Plasma Igs against *S. Pyogenes* from Patients with Plaque and Guttate Psoriasis and Healthy Controls**

| | | | Plaque Psoriasis (n = 29) | | Guttate Psoriasis (n = 26) | | Healthy Controls (n = 14) | |
|---|---|---|---|---|---|---|---|---|
| **Cytokine** | **Culture Condition** | **Statistic** | **IgA-SE** | **IgG-SE** | **IgA-SE** | **IgG-SE** | **IgA-SE** | **IgG-SE** |
| IL-17F | CLA+/EPI/SE | Pearson *r* | 0.5011 | 0.0234 | 0.3154 | 0.2742 | −0.308 | 0.0004 |
| | | *P* value | 0.0056 | 0.9018 | 0.1165 | 0.1753 | 0.3288 | 0.9990 |
| | CLA-/EPI/SE | Pearson *r* | 0.2908 | −0.0759 | 0.3663 | 0.2449 | 0.4681 | 0.1487 |
| | | *P* value | 0.1259 | 0.6953 | 0.0657 | 0.2279 | 0.1248 | 0.0221 |
| IL-17A | CLA+/EPI/SE | Pearson *r* | 0.1894 | 0.189 | 0.5859 | 0.359 | −0.0667 | 0.2251 |
| | | *P* value | 0.3251 | 0.367 | 0.0017 | 0.071 | 0.8209 | 0.4392 |
| | CLA-/EPI/SE | Pearson *r* | 0.1874 | −0.087 | 0.3732 | 0.208 | 0.2114 | 0.1944 |
| | | *P* value | 0.3293 | 0.655 | 0.0604 | 0.308 | 0.4681 | 0.5054 |
| IFN-γ | CLA+/EPI/SE | Pearson *r* | 0.4643 | 0.005 | 0.1603 | 0.116 | −0.0346 | −0.1731 |
| | | *P* value | 0.0112 | 0.718 | 0.4340 | 0.089 | 0.9066 | 0.5540 |
| | CLA-/EPI/SE | Pearson *r* | 0.4149 | 0.005 | 0.1739 | 0.070 | 0.4917 | 0.0757 |
| | | *P* value | 0.0252 | 0.703 | 0.3956 | 0.191 | 0.0742 | 0.7970 |
| IL-9 | CLA+/EPI/SE | Pearson *r* | 0.2787 | −0.018 | 0.5650 | 0.4242 | −0.0571 | −0.0366 |
| | | *P* value | 0.1432 | 0.9247 | 0.0026 | 0.0308 | 0.8464 | 0.9012 |
| | CLA-/EPI/SE | Pearson *r* | 0.1250 | 0.1637 | 0.4338 | 0.3760 | 0.1359 | 0.2818 |
| | | *P* value | 0.5182 | 0.3961 | 0.0268 | 0.0584 | 0.6431 | 0.3291 |

Pearson *r* and *P*-values are indicated for each culture condition.

# II. Interplay between Humoral and CLA$^+$ T Cell Response against *Candida albicans* in Psoriasis

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*Article*

# Interplay between Humoral and CLA$^+$ T Cell Response against *Candida albicans* in Psoriasis

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**Abstract:** *Candida albicans* (CA) infections have been associated with psoriasis onset or disease flares. However, the integrated immune response against this fungus is still poorly characterized in psoriasis. We studied specific immunoglobulins in plasma and the CA response in cocultures of circulating memory CD45RA$^-$ cutaneous lymphocyte antigen (CLA)$^+$ T cell with autologous epidermal cells from plaque and guttate psoriasis patients (cohort 1, $n = 52$), and also healthy individuals ($n = 17$). A complete proteomic profile was also evaluated in plaque psoriasis patients (cohort 2, $n = 114$) regarding their anti-CA IgA levels. Increased anti-CA IgA and IgG levels are present in the plasma from plaque but not guttate psoriasis compared to healthy controls. CA cellular response is confined to CLA$^+$ T cells and is primarily Th17. The levels of anti-CA IgA are directly associated with CLA$^+$ Th17 response in plaque psoriasis. Proteomic analysis revealed distinct profiles in psoriasis patients with high anti-CA IgA. C-C motif chemokine ligand 18, chitinase-3-like protein 1 and azurocidin were significantly elevated in the plasma from plaque psoriasis patients with high anti-CA levels and severe disease. Our results indicate a mechanism by which *Candida albicans* exposure can trigger a clinically relevant IL-17 response in psoriasis. Assessing anti-CA IgA levels may be useful in order to evaluate chronic psoriasis patients.

**Keywords:** psoriasis; *candida albicans*; IgA; CLA; IL-17

## 1. Introduction

Psoriasis is a T cell-mediated skin disease resulting from epithelial and immunological cells' interactions [1]. Psoriasis onset is influenced by genetic and environmental factors, particularly infections. Psoriasis flares have been attributed to *Candida albicans* infections [2]. Several studies have confirmed increased *Candida albicans* colonization of the oral [3–7] and gut [3,7] mucosa in psoriasis patients compared to healthy individuals. Whereas some authors revealed an association between *C. albicans* existence at mucosal

sites and psoriasis severity [3,5,7], others did not find a correlation [4,8,9]. Likewise, the presence of *Candida albicans* at cutaneous levels is still controversial. Most skin mycobiome studies have not revealed differences between psoriasis and healthy individuals' Candida spp. levels [4,10–16]. However, Sarvtin T. and colleges found elevated *C. albicans* in lesional skin from the trunk compared to either adjacent normal skin or healthy controls' skin [6]. Furthermore, Salem I. et al. recently reported the presence of higher *C. albicans* in non-lesional skin swabs [17].

Currently, the evidence of microbes' presence in psoriatic patients is focused on complex DNA-based technologies [18]. Specific immunoglobulins assess environmental microorganism exposure. The isotypes observed in immunoglobulins can elucidate the mechanisms of antigen encounters [19]. Initial studies of anti-Candida antibody levels did not observe differences between patients with psoriasis and healthy individuals [10,20]. Recently, however, Liang YS et al. showed increased anti-whole cell antigen IgG and decreased anti-soluble antigen IgA and IgM in serum from psoriasis patients versus healthy individuals [21]. However, Sarvtin MT et al. reported decreased anti-*Candida* IgM, IgA and IgG levels in psoriasis patients compared to controls [6]. As such, the humoral response against *Candida albicans* in psoriasis patients remains controversial.

The IL-23/IL-17/IL-22 axis protects us against *Candida albicans* [22], but it also plays a key role in psoriasis development. The study of *Candida albicans* in psoriasis patients is very limited and mainly studied in total peripheral blood mononuclear cells [23–25]. Schlapbach et al. reported IL-9 induction by *C. albicans* specifically on skin-tropic T helper cells from healthy donors, along with increased IL-9$^+$ cells in psoriasis lesions [26]. Likewise, previous data from our group described the increased induction of IL-9, IL-17A and IFN-$\gamma$ by CLA$^+$CD4$^+$ T cells after *C. albicans* activation in four psoriasis patients compared to healthy individuals [27]. Investigating CD45RA$^-$CLA$^+$ memory T cell-induced IL-17 responses to a microorganism with microbe-specific immunoglobulins levels with clinical features can help to understand how microorganisms might modulate psoriasis pathogenesis from a more holistic view. We have recently demonstrated the relevance of this approach for *Streptococcus pyogenes*, showing that bacteria specific-IgA and CLA$^+$ T cell IL-17 response are associated in psoriasis patients [28].

In this study, we investigated humoral and cellular immune responses against *Candida albicans* in psoriasis patients, comparing plaque and guttate forms. The association between antibodies and cytokine responses was also assessed. Finally, we examined the proteomic profile in plasma from plaque psoriasis patients. Altogether, our data illustrate the heterogeneity of *Candida albicans* exposure in psoriasis, which might reflect the pathological mechanisms.

## 2. Results

### *2.1. Description of Patients and Controls Samples Used in Different Experiments*

A total of 166 psoriasis patients and 17 healthy individuals were enrolled. Psoriasis patients were classified in cohort 1 ($n$ = 52), consisting of both plaque and guttate forms of the disease, and cohort 2 ($n$ = 114), which included only plaque psoriasis patients. Complete humoral and cellular responses against *Candida albicans* were assessed in cohort 1, as well as ELISAs to validate differentially expressed proteins. Cohort 2 was only studied for *C. albicans*-specific IgA levels and the complete proteomic profiling in plasma. Detailed information of the source and experiments performed with each cohort can be found in Supplementary Table 1. The inclusion and exclusion criteria for each cohort are detailed in the materials and methods section.

### *2.2. Plaque Psoriasis Patients Have Increased Levels Candida Albicans-Specific IgA and IgG*

Plasma samples from psoriasis cohort 1 ($n$ = 52) and healthy ($n$ = 17) individuals were analyzed. The clinical features of plaque and guttate psoriasis patients from cohort 1 are summarized in Table 1. The specific IgA and IgG for *Candida albicans* in plasma samples

were tested by ELISA (referred as anti-CA IgA or IgG). The levels of anti-CA IgA and IgG were significantly increased in the plasma from plaque psoriasis patients compared to guttate psoriasis and healthy controls, which presented similar anti-CA IgA and IgG levels (Figure 1a,d). IgA and IgG subtypes were also assessed. Both anti-CA IgA1 and IgA2 were significantly increased in plaque psoriasis patients in comparison to guttate psoriasis and healthy individuals (Figure 2b,c), whereas no differences were observed between guttate psoriasis and controls. Interestingly, anti-CA IgA and IgA2 levels positively correlated with disease duration only in plaque psoriasis (IgA Spearman r: 0.4435, $p$ value = 0.03; IgA2 r = 0.4082, $p$ value = 0.0477, data not shown). Anti-CA IgG1, IgG2 and IgG3 were detected in both psoriatic groups and healthy individuals (Figure 1e–g), whereas anti-CA IgG4 was not detectable (Figure 1h). Nonetheless, only anti-CA IgG3 levels were significantly increased in plaque psoriasis compared to controls (Figure 1g). Additionally, we used a commercial diagnostic kit for the quantitative determination of IgA antibodies against *C. albicans* in human plasma, revealing current infection. Both psoriasis and healthy individuals presented negative antibody titer (< 8 U/mL), indicating no fresh *C. albicans* infection (Figure S1). Interestingly, plaque psoriasis patients showed significantly higher anti-CA titer compared to healthy controls, supporting our results.

**Table 1.** Clinical features of psoriasis patients in cohort 1 according to the subtype of disease.

| | | **Plaque** | **Guttate** | ***p* value** |
|---|---|---|---|---|
| **Number of patients** | | 31 | 21 | NA |
| **Age (mean ± SD)** | | 46.65 (11.97) | 32.65 (10.92) | *** |
| **PASI (mean ± SD)** | | 13.74 (5.99) | 7.23 (2.91) | *** |
| **HLA-Cw*6 % (*n*)** | **Positive** | 32.26 (10) | 85.71 (18) | NA |
| | **Negative** | 61.29 (19) | 14.29 (3) | NA |
| | **UK** | 6.45 (2) | - | NA |

Mann–Whitney test was used to compare quantitative variables, $p$ values are indicated as ns: $p > 0.05$; ***: $p < 0.001$. NA = not assigned. Bold: the clinical features.

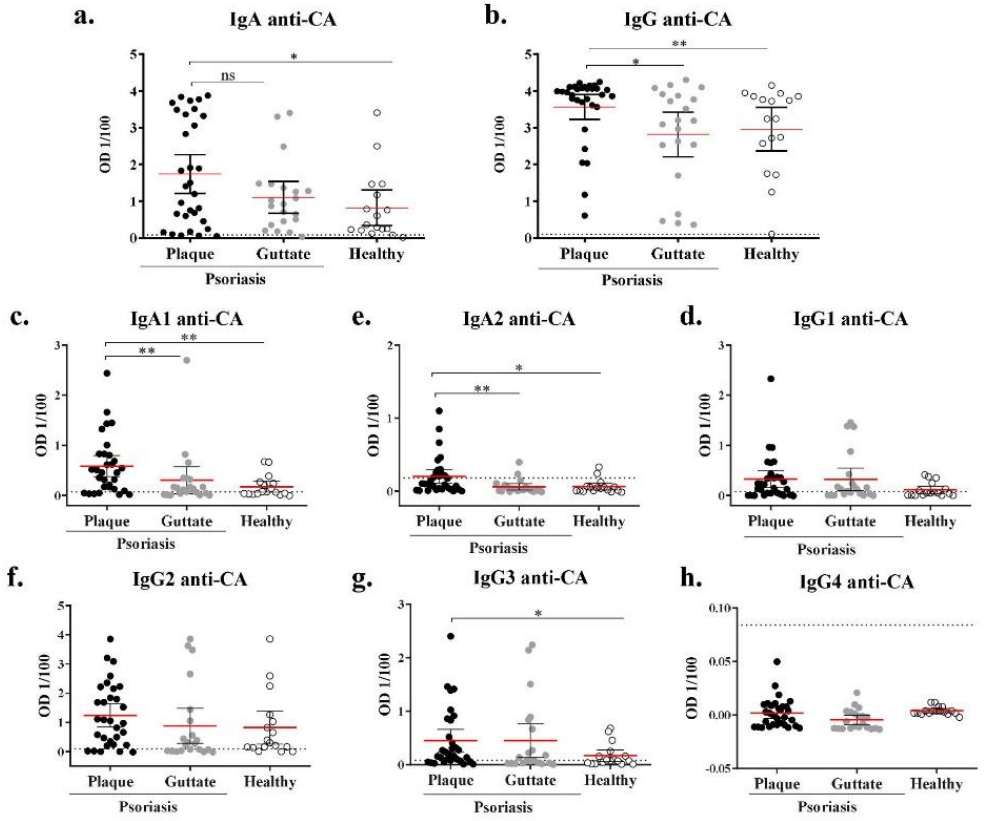


**Figure 1.** Plaque, but not guttate, psoriasis patients present increased IgA-1, IgA-2 and IgG-3 against *Candida albicans* compared to healthy controls. Specific immunoglobulins recognizing *C. albicans* cellular antigens (CA) were detected through ELISA in plasma collected from the blood of plaque psoriasis ($n = 31$, ●), guttate psoriasis ($n = 21$, ●) and healthy controls ($n = 17$, ○). Net OD of 1/100 diluted plasma is reported. Plasma levels of IgA (**a**), IgA-1 (**b**), IgA-2 (**c**), IgG (**d**), IgG1 (**e**), IgG2 (**f**), IgG3 (**g**) and IgG4 (**h**) against CA are shown. Statistics lines are represented as mean with 95% confidence interval. Dotted lines indicate the mean background signal. A Mann–Whitney test was used to compare between groups, and $p$ values are indicated as: ns: $p > 0.05$; *: $p < 0.05$; **: $p < 0.01$.

### *2.3. Candida Albicans-Induced Th17 and Th9 Responses Are Confined to CLA+ T-cells and Dominated by IL-17F*

*C. albicans* cellular response was assessed in psoriasis cohort 1 ($n = 52$) and healthy controls ($n = 17$). Cocultures of CD45RA$^-$ memory CLA$^+$ or CLA$^-$ T cells with autologous epidermal cells were left untreated or activated with *C. albicans* extract. After 5 days of culture, cytokines were quantified in stimulated supernatants. Plaque and guttate psoriasis patients showed significant CLA$^+$ T cells-dependent CA-mediated induction of IL-17F, IL17A, and IL-9 compared to unstimulated CLA$^+$T/EPI and CA-stimulated CLA$^-$T/EPI cocultures (Figure 2a–c). Notably, CA-induced IL-17F and IL-17A levels in CLA$^+$T cells were significantly higher in cocultures from guttate psoriasis compared to those of plaque psoriasis. CA induction of IFN-γ was also higher in guttate psoriasis, whereas IL-9 induction was slightly increased in plaque psoriasis, but not significantly differently. Cytokine responses were also detected in healthy controls' CLA$^+$T/EPI cocultures after CA activation. Only IL-17F and IL-17A responses were significantly associated with CLA$^+$ T cells when

compared to CA-stimulated CLA⁻T/EPI coculture in healthy subjects; however, no substantial differences were found when compared to the CLA⁺T/EPI untreated condition. Interestingly, levels of CA-induced IL-9 by CLA⁺T/EPI cocultures were significantly higher in psoriasis than in healthy individuals (Figure 2d). Additionally, single cultures of epidermal cells as well as CLA⁺ and CLA⁻ T cells were left untreated and stimulated with CA for 5 days. IL-17A and IFN-γ induction was measured in culture supernatants. The IL-17A response was almost null even in CA-stimulated conditions (Figure S2a). Similarly, although low IFN-γ levels were detected in CA-stimulated conditions, still this response was similar to the one found in untreated cultures (Figure S2b). Therefore, only when CLA⁺ T cells are cultured in the presence of autologous epidermal cells is increased cytokine response to *C. albicans* stimulation observed.

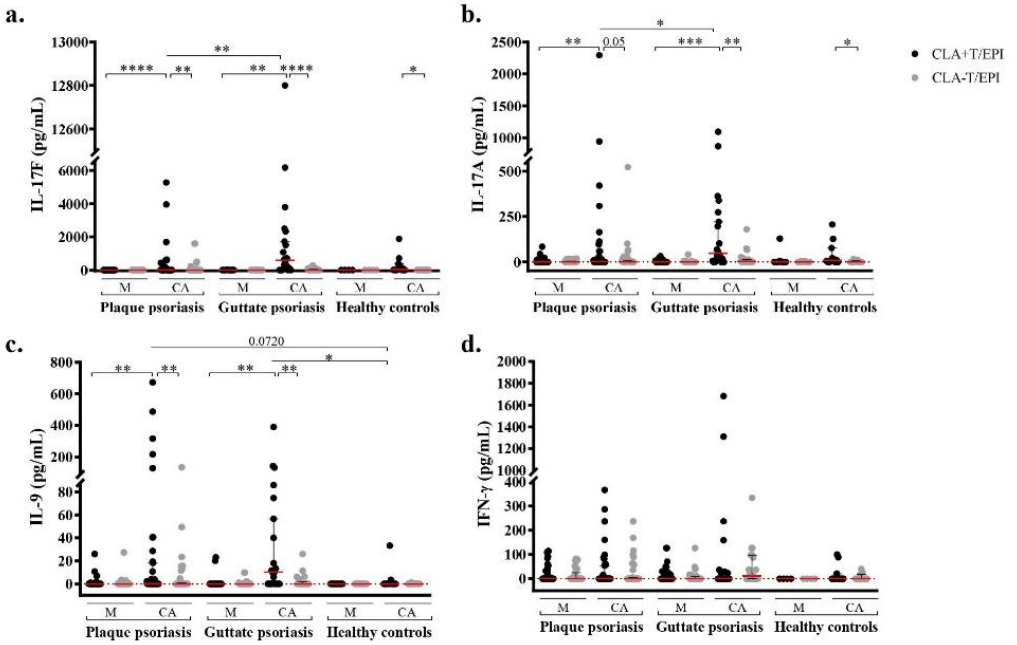


**Figure 2.** Th17 and Th9 responses to *C. albicans* are confined to CLA⁺ T cells. IL-17F (**a**), IL-17A (**b**), IL-9 (**c**) and IFN-γ (**d**) levels were measured in culture supernatants from CLA⁺ or CLA⁻ T cells cocultured with autologous epidermal cells (EPI) after 5 days, in basal conditions (M) and stimulated with *C. albicans* (CA), in plaque psoriasis ($n = 31$), guttate psoriasis ($n = 21$) and healthy controls ($n = 12$). Column bars are represented as mean with 95% confidence interval. Wilcoxon test was used to compare two conditions within psoriasis or controls (double-pointed line), whilst Mann–Whitney test was used to compare psoriasis versus controls (single-pointed line). For both, $p$ values are indicated as ns: $p > 0.05$; *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$; ****: $p < 0.0001$.

### *2.4. Candida Albicans' Specific IgA Plasma Levels and IL-17 T cell Responses Are Directly Associated in Plaque Psoriasis Patients*

Correlations between anti-CA IgA or IgG and the different measured cytokines were assessed in psoriasis cohort 1 ($n = 52$) and healthy controls ($n = 17$). Only for plaque psoriasis patients were levels of anti-CA IgA positively associated with IL-17F and IL-17A responses (Table 2), in CLA⁺ T cells/EPI but also in CLA⁻ T cells/EPI cocultures. This association was not observed for guttate psoriasis (data not shown) or healthy controls, or for specific IgG (Table 2). Additionally, disease duration was significantly correlated with CA-induced CLA⁺ T cell dependent IL-17F and IL-17A, and CLA⁻ T cell dependent IL-17F

production in plaque psoriasis ($CLA^+$ T-IL-17F: r = 0.5569, $p = 0.0047$; $CLA^-$ T-IL-17F: r = 0.4112, $p = 0.0459$ and $CLA^+$ T-IL-17A: r = 0.5873, $p = 0.0026$).

**Table 2.** Correlation of anti-CA IgA or IgG and cytokine responses to *Candida albicans* in $CLA^+$ or $CLA^-$ T cells cocultured with autologous epidermal cells. Spearman ϱ and $p$ values are indicated (ns: $p > 0.05$; *: $p < 0.05$; **: $p < 0.01$).

| | | | Anti-CA IgA | | | Anti-CA IgG | | |
|---|---|---|---|---|---|---|---|---|
| | **Coculture Condition** | **Cytokine** | **Spearman ϱ** | ***p* value** | | **Spearman ϱ** | ***p* value** | |
| **Plaque psoriasis ($n = 31$)** | $CLA^+$T/EPI | IL-17F | **0.3735** | **0.0385** | * | −0.0842 | 0.6525 | ns |
| | | IL-17A | **0.4798** | **0.0063** | ** | 0.0349 | 0.8521 | ns |
| | | IL-9 | 0.2363 | 0.2006 | ns | 0.1250 | 0.5028 | ns |
| | | IFN-γ | 0.1872 | 0.3219 | ns | 0.0116 | 0.9515 | ns |
| | $CLA^-$T/EPI | IL-17F | **0.5028** | **0.0039** | ** | −0.0267 | 0.8864 | ns |
| | | IL-17A | **0.4714** | **0.0074** | ** | 0.0625 | 0.7383 | ns |
| | | IL-9 | 0.2558 | 0.1724 | ns | 0.1183 | 0.5264 | ns |
| | | IFN-γ | −0.0073 | 0.9690 | ns | −0.0589 | 0.7573 | ns |
| **Healthy controls ($n = 12$)** | $CLA^+$T/EPI | IL-17F | −0.4496 | 0.1681 | ns | 0.0458 | 0.9015 | ns |
| | | IL-17A | −0.4307 | 0.1622 | ns | −0.0392 | 0.9061 | ns |
| | | IL-9 | −0.5691 | 0.0591 | ns | 0.0734 | 0.8288 | ns |
| | | IFN-γ | −0.2203 | 0.5015 | ns | −0.3885 | 0.2227 | ns |
| | $CLA^-$T/EPI | IL-17F | 0.2197 | 0.5192 | ns | 0.2197 | 0.5192 | ns |
| | | IL-17A | −0.1706 | 0.5948 | ns | 0.2538 | 0.4246 | ns |
| | | IL-9 | −0.2527 | 0.4394 | ns | 0.0161 | 0.9697 | ns |
| | | IFN-γ | −0.0275 | 0.9394 | ns | 0.4773 | 0.1258 | ns |

### *2.5. Proteomic Profile of Plasma from Psoriasis Patients according to Anti-CA IgA Levels*

Specific IgA was assessed in plasma samples from psoriasis cohort 2 ($n = 114$). Based on the upper limit of the 95% confidence interval and the maximum optical density (OD) signal of anti-CA IgA from healthy individuals, patients were stratified in the following groups: low (OD < 1.5), intermediate (OD = 1.5–3) and high (OD > 3). Broad proteomic expressions of 1012 proteins were compared between groups using a generalized linear model. Table S2 shows the differentially expressed proteins between low and high groups ordered by fold change. Positive fold change values represent higher protein levels in the high IgA-Candida group. A total of 27 proteins differed significantly between the high and low groups using a raw $p$ value. Of these, two proteins yielded >30% of values below the limit of detection and should be interpreted with caution. Although no significant differences remained after false discovery rate (FDR) $p$ value correction, some proteins of potential interest were identified and selected for further validation in samples from cohort 1 ($n = 52$) and healthy individuals ($n = 17$). Four proteins were found with increased presence in plasmas with high anti-CA IgA (eosinophil cationic protein (RNASE3/ECP), chitinase-3-like protein 1 (CHI3L1), azurocidin (AZU1) and C-C motif chemokine 18 (CCL18)), and two that were found to be decreased (Follistatin (FST) and Fas-ligand (FSLG)). Plasma levels of CCL18, CHI3L1 and AZU1 were significantly increased in plaque compared to guttate psoriasis (Figure 3a–d). CHI3L1 levels were significantly higher in plaque but not guttate psoriasis when compared to controls (Figure 3b). Similar results were obtained for CCL18, although differences between plaque psoriasis and healthy individuals did not reach statistical significance (Figure 3a). Conversely, AZU1 and RNASE/ECP protein levels were significantly higher in both plaque and guttate psoriasis compared to healthy subjects (Figure 3c,d). Plaque psoriasis patients showed significant positive correlation between CCL18 levels and PASI, as well as between CHI3L1 levels and age of onset (Figure 3e,f), whereas a strong negative correlation was found for

CHI3L1 and disease duration (Figure 3g). In psoriasis patients, we also found significant direct correlations between levels of CCL18, CHI3L1 or AZU1 and PASI, as well as age of onset (Figure S3). No differences were observed for FST and FSLG (data not shown).

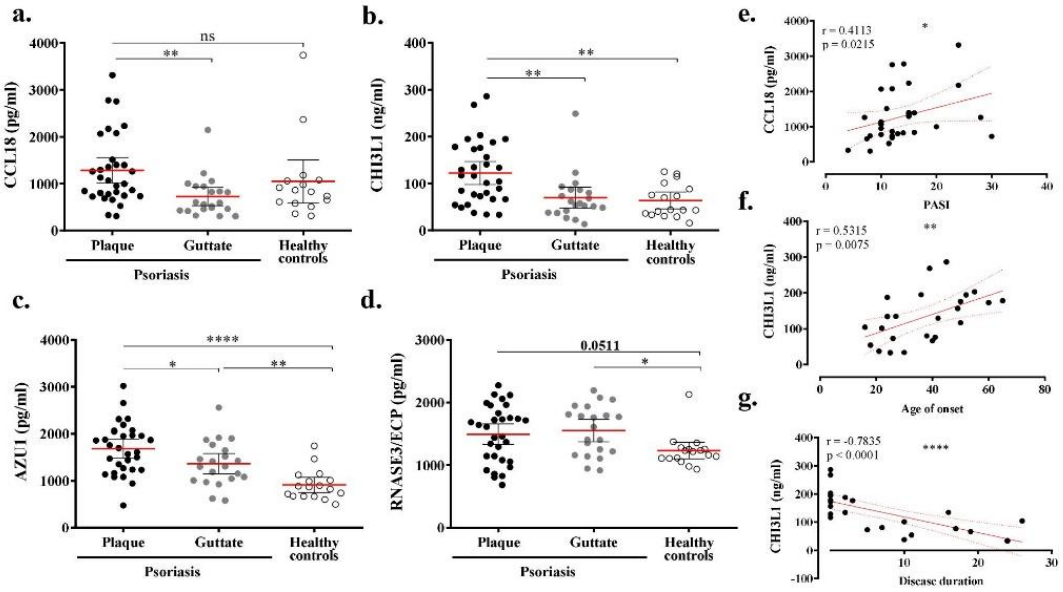


**Figure 3.** AZU1, CCL18, CHI3L1 and AZU1 are significantly increased in plasma from plaque psoriasis compared to guttate or/and healthy controls. CCL18, CHI3L1, AZU1 and RNASE3/ECP levels were assessed by ELISA in plasmas from plaque psoriasis ($n$ = 31, ●), guttate psoriasis ($n$ = 21, ●) and healthy individuals ($n$ = 16, ○). (**a**–**d**) Comparison of levels detected between plaque and guttate psoriasis versus controls. In plaque psoriasis patients, relevant correlations between (**e**) CCL18 and disease severity (PASI), as well as CHI3L1 and age of onset (**f**) or disease duration (**g**), were found. A Wilcoxon test was used to compare two conditions within psoriasis or controls. Spearman test was used for correlations. Spearman coefficient (r) and $p$ values ($p$) are indicated as ns: $p > 0.05$; *: $p < 0.05$; **: $p < 0.01$; ****: $p < 0.0001$.

## 3. Discussion

Microorganisms can affect psoriasis presentation and patient's natural history [2,29]. While lymphoid B-cells are dispensable for psoriasis development, B-cell dysregulation and increased IgA has been shown in psoriasis [30]. Our results showed that non-treated plaque psoriasis patients present increased plasma levels of *Candida*-specific IgA that correlate with the IL-17 response to this fungus in vitro.

To evaluate *Candida albicans* exposure in psoriasis patients, fungus-specific immunoglobulins in plasma were assessed. Significantly elevated levels of IgA and IgG against *Candida albicans* were found in the plasma from plaque psoriasis patients, particularly those with higher disease severity, compared to healthy controls. Current studies of *Candida albicans*-specific immunoglobulins in psoriasis are controversial. One study reported increased anti-whole cell antigen IgG but decreased anti-soluble antigen IgA and IgM in psoriasis vulgaris versus healthy controls [21]. More recently, generally reduced anti-CA immunoglobulins plasma levels in psoriasis vulgaris were shown, with no association with disease severity or duration [6]. Importantly, 76% of their psoriasis cohort had mild to moderate forms of the disease, which may explain the low levels of antibodies detected compared to our results. To better characterize anti-*C. albicans* antibody response, different subtypes of IgA and IgG were assessed. Both anti-CA IgA1 and IgA2 plasma levels were increased in plaque psoriasis patients compared to guttate psoriasis and controls.

Human monomeric IgA subclasses are predominantly secreted by IgA-producing cells in the bone marrow, but also in the spleen, synovial tissue and gingiva [31]. Specifically, IgA1-secreting cells are present in the upper orogastric tract, whereas IgA2-secreting cells predominate in the lower gastrointestinal tract [19]. B-cells activated in the gut-associated lymphoid tissue (GALT) may be home to the marginal zone of the spleen [32]. Therefore, the commensal or pathogenic-specific IgA produced by these GALT activated-plasma cells may be secreted into the bloodstream. Despite being a commensal fungus, the presence of higher levels of *Candida albicans* in both oral and gut samples from psoriasis patients is widely demonstrated [33]. Altogether, our results identify both the oral and gut mucosa as sites of encounter with *C. albicans* and IgA production. However, we cannot exclude the possible existence of additional sources of CA-specific IgA in psoriasis, since its prevalence in cutaneous tissues is not well-defined, and skin-homing IgA1- and IgA2-secreting cells have also been described [19].

Memory CLA$^+$ T cells participate in cutaneous immune responses due to their skin-homing properties [34]. A preferential CLA$^+$ T cell response to *Candida albicans,* dominated by IL-17F, was found in psoriasis but also in control subjects. Although preferred *C. albicans* induction of IL-9 on CLA$^+$ T cells has already been shown in healthy donors [26], we observed significantly increased CA-induced IL-9 response in psoriasis patients compared to controls. We found differentiated T-cell responses to *C. albicans* stimuli between plaque and guttate psoriasis, indicating increased cytokine responses in the latter. This finding is of special interest since the IL-23/Th17 axis drives and maintains psoriasis pathogenesis [1] and *Candida albicans* may fuel the IL-17/Th17 loop, thus perpetuating psoriasis. Of note, a recent study observed that responses to commensal skin fungi, such as *Candida albicans,* enhance psoriasiform Th17 inflammation in the imiquimod-induced psoriatic mice model [35].

Interestingly, we reported different humoral and cellular responses to *Candida albicans* plaque and guttate psoriasis. Whereas *C. albicans*-specific antibody levels are increased in the former, the T-cell response to *C. albicans* extract is higher in the latter. This may be due to disparities in the course of each type of disease. While guttate psoriasis occurs in a more acute way and generally has better prognosis, plaque psoriasis is usually more severe, harder to control, and therefore, presents a more chronic duration. Plaque psoriasis patients are more likely to receive immunosuppressive treatment intermittently and for longer periods of time, which would cause microbiome dysbiosis, leading to increased *C. albicans* colonization and an increased presence of specific IgA in plasma. Nevertheless, because guttate psoriasis occurs more abruptly, skin and blood samples are usually taken closely to the appearance of the flare, wherein pro-inflammatory CLA$^+$ T cells in circulation are actively recruited to the cutaneous tissue. Conversely, in plaque psoriasis patients, the recirculation of pro-inflammatory T-cells may diminish or rebalance over time, resulting in a continuous flow of minor cells towards cutaneous lesions. This distinct presence of active pro-inflammatory T-cells in circulation may explain the substantial response to CA extract in cocultures from guttate psoriasis patients.

The link between Th17 and IgA has been extensively studied in animal models [36,37] and humans. Although the molecular mechanisms underlying this effect are not fully understood, Shibui A et al. suggested an indirect role for IL-17 in antibody production, possibly by enhancing B-cell activators by other immune cells [38], whilst Ferreti E and colleagues demonstrated how B-cell migration within germinal centers is modulated by the IL-17A/IL-17RA axis [39]. A direct correlation between anti-CA IgA levels and IL-17 cytokines responses is found for both CLA$^+$ and CLA$^-$ T only in cocultures from plaque psoriasis patients. Recently, Wilson et al. reported the presence of antibody-secreting cells (ASC) in healthy skin, secreting mainly IgM but also IgA and IgG, which are important for maintaining tissue homeostasis [40]. The role of skin-associated B-cells in homeostasis has also been recently reviewed [41], highlighting their interplay with other skin-resident immune cells. We hypothesized that skin-associated B-cells may present *Candida albicans* peptides to antigen-specific CLA$^+$ T inducing its activation and fungus-specific antibody

secretion. Recently published data from our group demonstrated a similar correlation between *S. pyogenes*-specific IgA and $CLA^+$ T-cell IL-17 response in psoriasis [28]. However, despite the direct correlation of the in vitro cytokine response to each antigen, no effect of the association between psoriasis patients' exposure to *S. pyogenes* and *C. albicans* on IgA/G levels has been observed (data not shown). Nonetheless, IL-17 response by $CLA^-$ T cells may contribute to *C. albicans*-specific IgA generation at extracutaneous sites, as mentioned above.

Patients with high anti-CA IgA levels may carry pathophysiological peculiarities, creating disease heterogeneity. Despite the fact that differentially expressed proteins were not statistically significant after FDR correction, which remains a limitation of our study, patients with high anti-CA IgA displayed generally increased levels of proteins involved in antimicrobial humoral response, cell chemotaxis and inflammatory immune response. Human RNASE3 is a cytotoxin with high anti-candida activity [42]. CHI3L1, which is regulated by IL-17F/A [43,44], has also shown anti-candida properties [45]. Azurocidin, secreted by neutrophils, acts against *Candida albicans* [46], and has been reported as a biomarker for periodontal disease [47]. CCL18 mediates $CLA^+$ memory T cells homing towards skin [48]. Its expression is not only upregulated in lesions from atopic dermatitis and psoriasis [49,50], but also gingival biopsies from periodontitis [51]. Increased CCL18, CHI3L1 and AZU1 levels were detected in plasmas from plaque psoriasis, but we could not confirm their association with anti-CA IgA levels, which remains a limitation of the study. Our data support evidence linking the presence of *C. albicans*, psoriasis pathogenesis and periodontal disease [52–54]. Nonetheless, longitudinal follow-up studies are required to better establish a causal relationship.

We believe psoriasis patients' increased cellular and humoral responses to *C. albicans* appear as a consequence of increased exposure to this fungus. Th17 response is fundamental for fighting against *Candida* spp. infection [55], as it is key for psoriasis immunopathogenesis. Therefore, we hypothesize that our observation could be related to treatment approaches for psoriasis. Effective therapies aim to reduce Th17 response in patients that may facilitate fungal colonization and the subsequent promotion of IL-17 responses, thus fueling psoriasis pathogenesis in a vicious circle. This hypothesis deserves careful analysis in future clinical studies. On the other hand, a predisposing background to increased response to *C. albicans* in psoriasis cannot be ruled out. Single nucleotide polymorphisms on IL-17 and IL-23 related genes, described as psoriasis genetic risk makers, could be associated to altered responses to *Candida* spp., a matter that remains unexplored to our knowledge.

In summary, the presence of IgA against *Candida albicans* in plasma from patients with plaque psoriasis without clinical signs of infection identifies subjects that have been exposed to this microbe, which preferentially activates skin-homing $CLA^+$ T cells to secrete IL17F and IL17A, two cytokines that are clinically demonstrated to be relevant in psoriasis immunopathogenesis. We consider that *Candida albicans* exposure may affect psoriasis evolution, and eventually response to therapies. Assessing anti-CA IgA levels may be beneficial to better evaluate and stratify psoriasis patients.

## 4. Materials and Methods

### *4.1. Patients*

All participants contributed voluntarily and provided written informed consent, and human material collection has been approved by the corresponding Ethical Committees. Psoriasis cohort 1 included non-treated patients with plaque or guttate lesions, without any age or sex restriction. Exclusion criteria included any systemic treatment during the 4 weeks prior to the study or any topical treatment during the last 2 weeks. Psoriasis cohort 2 comprised baseline plasma samples from the Clinical Trial NCT00778700, which included plaque psoriasis involving 2 to 20% body surface area, and those aged from 18 to 75 years. Exclusion criteria included lesions solely involving intertriginous areas, the

scalp or the face, pustular psoriasis or erythroderma, systemic therapy, being currently on other topical agents or UVB within 2 weeks of the first dose of study medication, having started or discontinued therapy within 2 months of screening with agents that can exacerbate psoriasis, or currently receiving systemic triazole antifungals.

### 4.2. ELISA

ELISAs were performed as previously described [28]. The antibodies are listed in Supplementary Table 2. As the negative control of the experiment, CA-coated wells were incubated with PBS-1% skimmed milk instead of plasma dilution. The titer of reactive anti-CA antibody was taken as the $OD_{405nm}$ after background signal ($OD_{570nm}$) and negative control well signal subtraction, referred to as net OD 1/100. As a positive control of the technique, the wells were coated with human IgA/G isotype controls (Invitrogen, Carlsband, CA, USA), incubated with PBS as the primary antibody and a corresponding secondary antibody before substrate addition.

### 4.3. Circulating Memory T-cell and Epidermal Cell Isolation

Peripheral blood mononuclear cells were isolated by Ficoll gradient (GE Healthcare, Princeton, NJ, USA) and, after subsequent immunomagnetic separations (Miltenyi Biotech, Bergisch Gladbach, Germany), memory CD45RA $CLA^{+}$ and $CLA^{-}$ T cells were purified as previously described [56]. Punch skin biopsies (4–6 mm) were incubated overnight in dispase (Corning, Corning, NY, USA) at 4 °C, then the epidermal sheet was peeled off from the dermis. The epidermis was cut in pieces and incubated in trypsin (Biological Industries, Kibbutz Beit Haemek, Israel) for 15 min at 37 °C. Epidermal tissue was then mechanically disaggregated by gently pipetting and the cell suspension was transferred to fresh culture media (RPMI, 10% FBS, 1% penicillin-streptomycin (SIGMA-Aldrich, St. Louis, MO, USA)).

### 4.4. Co-Cultures

The ex vivo cocultures consisted of $5 \times 10^4$ $CLA^{+/-}$ T-cells plated together with $3 \times 10^4$ autologous epidermal cells at the same time ($CLA^{+}$T/Epi or $CLA^{-}$T/Epi, respectively), seeded together simultaneously in 96-well flat-bottom plates (SIGMA-Aldrich, St. Louis, MO, USA), in the culture media described above. Cocultures were left untreated or activated with CA extract (Stallergenes Greer, Lenoir, NC, USA) reconstituted at 800 µg/mL in sterile water (SIGMA-Aldrich). CA extract was used at 20 µg/mL final well concentration. After 5 days of culture, supernatants were collected and kept frozen at −20 °C for later cytokine quantification.

### 4.5. Cytokine Quantification

Multiplex fluorescent bead-based immunoassays were used to measure IL-17A and IFN-γ (Diaclone SAS, Besançon, France) and IL-17F (BD Bioscience, Franklin Lakes, NJ, USA) concentration in collected culture supernatants. IL-9 concentration was quantified by using pre-coated ELISA kits (Biolegend, San Diego, CA, USA).

### 4.6. Proteomic Study

Anti-CA IgA plasma levels were measured in 114 plasma samples collected at baseline from psoriasis cohort 2. Patients were stratified into high (>3 OD, $n = 61$), intermediate (1.5–2.9 OD, $n = 26$), and low (<1.5 OD, $n = 27$) IgA-Candida. Broad proteomic analysis of plasma samples was conducted by OLINK Proteomics ($n$ = 1012 proteins) (Watertown, MA, USA) and compared between groups using a generalized linear model. For technical information go to www.olink.com/downloads. Least-squared mean differences were used to compare high vs. negative groups. Statistical analyses were conducted in Array Studio version 10.1. Three of the differentially expressed proteins were selected for validation in a different cohort of patients (cohort 1, $n = 52$) and healthy individuals ($n = 16$) via ELISA

(Fine test, Wuhan Fine Biotech Co., Ltd., Wuhan, China): RNASE3, AZU1, CHI3L1, CCL18, FST and FSLG.

### *4.7. Statistical Analysis*

GraphPad Prism software (Version 8, GraphPad Software Corporation, San Diego, CA, USA) was used for statistical analysis and graphical representation. Data are generally represented as the mean and 95% confidence interval (CI). Differences between two conditions within the same group were analyzed by Wilcoxon test and represented by double pointed lines. Differences between two groups were analyzed by the Mann–Whitney test and represented by single pointed lines. Differences were considered significant at a $p$ value of less than 0.05 and represented by the following symbols: (ns): $p > 0.05$; (*): $p < 0.05$; (**): $p < 0.01$ and (***): $p < 0.001$. The correlations were examined using Spearman's rank correlation, and the Spearman's coefficient and $p$ value are indicated for each test.

**Supplementary Materials:** The following are available online at www.mdpi.com/1422-0067/22/4/1519/s1, Figure S1: quantitative measurement of anti-CA IgA plasma levels with a commercial ELISA kit validate our results, Figure S2: *Candida albicans* cytokine response is not induced in single cultures of epidermal or $CLA^{+/-}$ T cells, Figure S3: Correlations between CCL18, Chi3L1 and AZU1 plasma levels with disease severity and onset in psoriasis, Table S1: Detailed information on samples from psoriasis and healthy individuals, Table S2: List of antibodies and concentrations used in the ELISA, Table S3: Differentially expressed proteins in psoriasis patients with low versus high anti-CA IgA levels.

**Author Contributions:** conceptualization, C.d.J.-G., E.R.-R., M.F., R.M.P. and L.F.S.-B.; data curation, C.d.J.-G., E.R.-R. and S.O.; formal analysis, C.d.J.-G. and S.O.; funding acquisition, M.F., R.M.P. and L.F.S.-B.; investigation, C.d.J.-G., L.S.-d.S.N., E.R.-R., L.S.-M., I.G.-J. and S.O.; methodology, C.d.J.-G., E.R.-R. and L.F.S.-B.; project administration, L.F.S.-B.; resources, M.F., A.C. (Anca Chiriac), J.M.C.-S., J.M.F.-A., S.O., M.D.H., R.M.P. and L.F.S.-B.; supervision, M.D.H., R.M.P. and L.F.S.-B.; validation, C.d.J.-G. and L.F.S.-B.; visualization, C.d.J.-G.; writing—original draft, C.d.J.-G. and L.F.S.-B.; writing—review and editing, C.d.J.-G., S.O., A.C. (Antonio Celada), M.D.H., R.M.P. and L.F.S.-B. All authors have read and agreed to the published version of the manuscript.

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**Institutional Review Board Statement:** The study was conducted according to the guidelines of the Declaration of Helsinki and approved by the Comité Ético de Investigación Clinica (CEIC) del Hospital del mar (nº2016/6614/I on 20 January 2017).

**Informed Consent Statement:** Informed consent was obtained from all subjects involved in the study.

**Data Availability Statement:** The data presented in this study are available on request from Incyte Corporation. Incyte Corporation (Wilmington, DE, USA) is committed to data sharing that advances science and medicine while protecting patient privacy. Qualified external scientific researchers may request anonymized datasets owned by Incyte for the purpose of conducting legitimate scientific research. Researchers may request anonymized datasets from any interventional study (except Phase 1 studies) for which the product and indication have been approved on or after 1 January 2020 in at least one major market (e.g., US, EU, JPN). Data will be available for request after the primary publication or 2 years after the study has ended. Information on Incyte's clinical trial data sharing policy and instructions for submitting clinical trial data requests are available at: https://www.incyte.com/Portals/0/Assets/Compliance%20and%20Transparency/clinical-trial-data-sharing.pdf?ver=2020-05-21-132838-960.

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**Conflicts of Interest:** S.O. and M.D.H. are employees and shareholders of Incyte Corporation. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results. The other authors declare no conflict of interest.

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## SUPPLEMENTARY MATERIAL

**Figure S1. Quantitative measurement of anti-CA IgA plasma levels with a commercial ELISA kit validate our results.** Plasma from psoriasis (n=52, ●) and healthy controls (n=15, ○) were tested in *Candida albicans* IgA ELISA kit from IBL-international according to manufacture instructions. Differences in anti-CA IgA titters quantified with the commercial kit were analysed according disease type: plaque (n=31) and guttate (n=21). Dotted line indicates the limit of detection for positive results on *Candida albicans* fresh infection. Mann-Whitney test was used to compare two groups and p values are indicated as *: $p<0.05$.

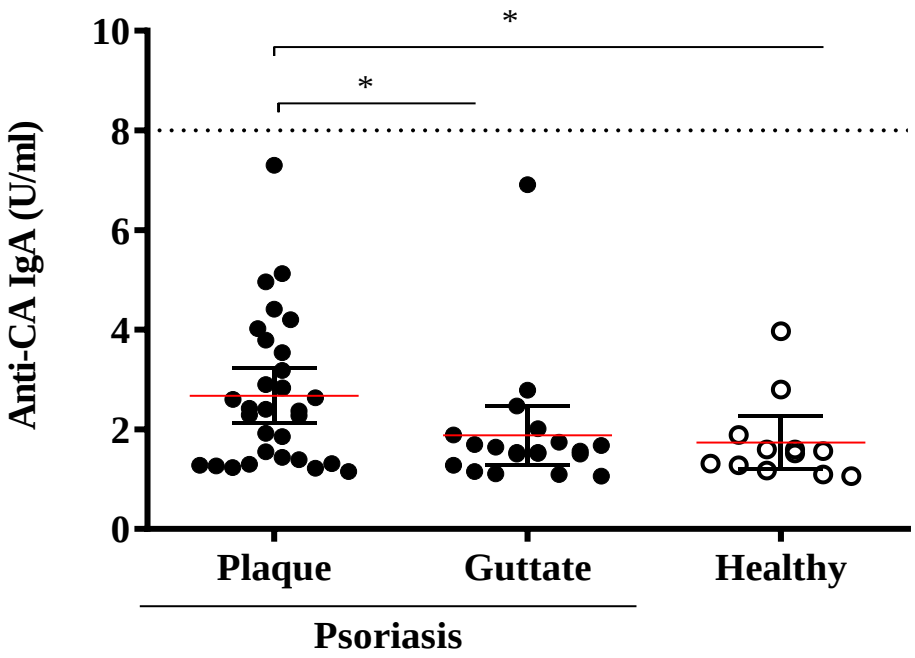

**Figure S2. *Candida albicans* cytokine response is not induced in single cultures of epidermal or CLA$^{+/-}$ T cells.** Single cultures of CLA+ T cells, CLA$^{-}$ T cells and epidermal cells were left untreated or stimulated with *C. albicans* extract for 5 days before cytokines were quantified in supernatants (n=4). Levels of IL-17A (a) and IFN-γ (b) were measured. Data are presented as single points for each patient and median (red line).

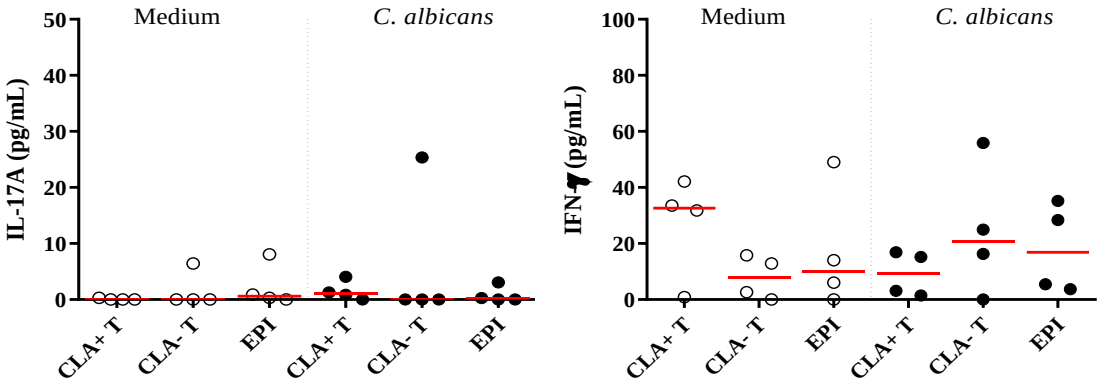

**Figure S3. Correlations between CCL18, CHI3L1 and AZU1 plasma levels with disease severity and onset in psoriasis.** Levels of CCL18, CHI3L1 and AZU1 were quantified by commercial ELISA kits on plasma from psoriasis patients (n=52). Association with disease severity, in terms of PASI (a-c), and onset (d-f) was assessed by spearman correlation. Spearman coefficient (r) and p values (p) are reported for each condition.

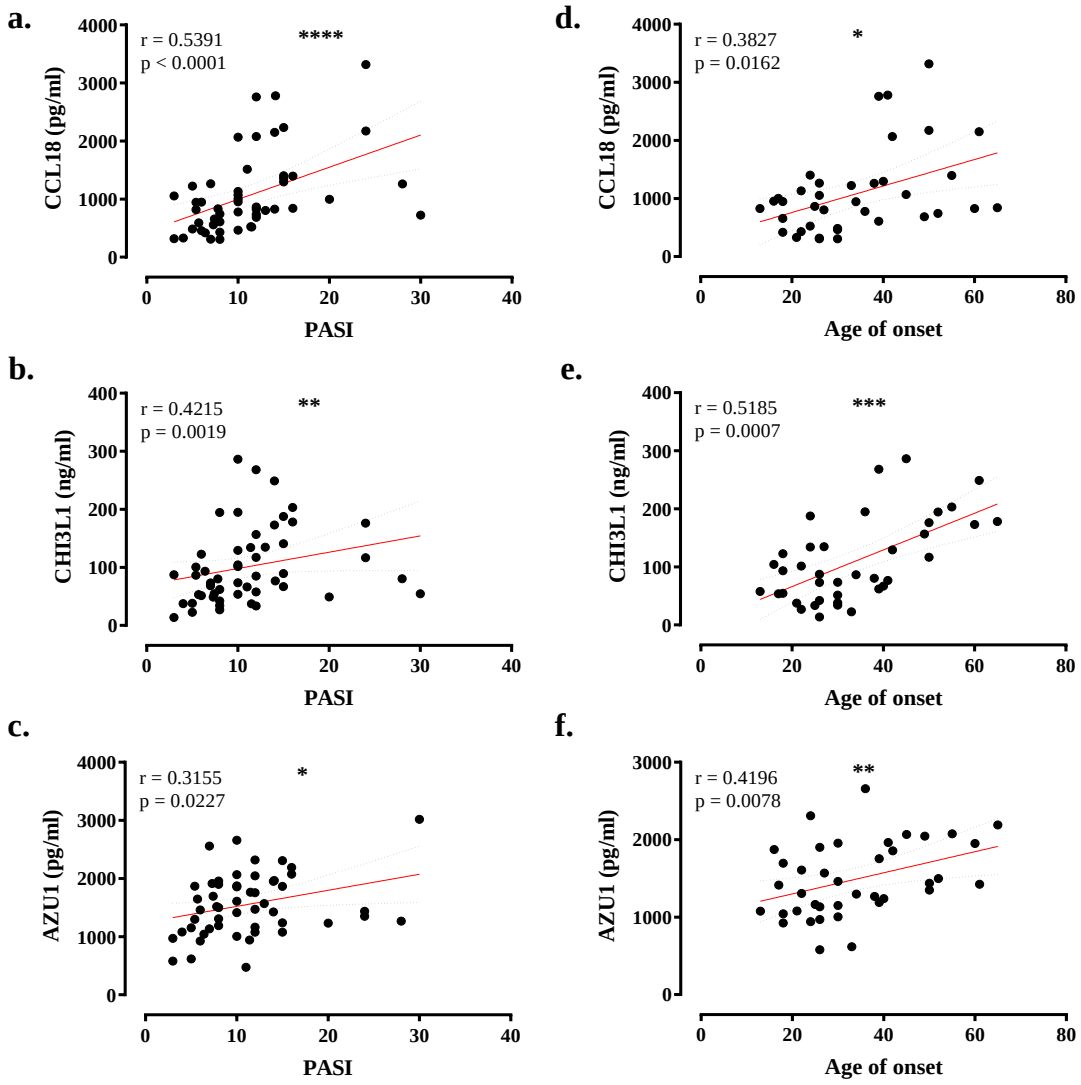

**Table S1. Detailed information on samples from psoriasis and healthy individuals**. Biopsies from psoriasis patients were performed in skin lesions.

| | | Sample type | | Experiments | Hospital / Institution |
|---|---|---|---|---|---|
| | | **Biopsy + blood** | **Plasma** | | |
| **Psoriasis (n=166)** | *Cohort 1* | 52 | 52 | • Anti-CA / SE ELISAs<br>• CLA+/- T cells and EPI cocultures<br>• ELISAs for validating proteomic results | - Hospital del Mar, Barcelona (Spain).<br>- Hospital Arnau de Vilanova de Lleida, Lleida (Spain).<br>- Iasi (Rumania) |
| | *Cohort 2* | - | 114 | • Anti-CA IgA ELISA<br>• Proteomic profiling | Incyte Corporation, Clinical Trial NCT00778700 |
| **Healthy controls (n=17)** | | 12 | 17 | • N=17 Anti-CA/SE ELISAs<br>• N=12 CLA+/- T cells and EPI cocultures<br>• N=17 ELISAs for validating proteomic results | Hospital del Mar, Barcelona (Spain). |

**Table S2. List of antibodies and concentrations used in the ELISA.**

| Antibody name | Company | Reference | Dilution |
|---|---|---|---|
| Anti-human IgA - Alkaline Phosphatase antibody produced in goat | SIGMA-Aldrich | A9669 | 1:4000 |
| Mouse anti-human IgA1-AP | Southern Biotech | 9130-04 | 1:4000 |
| Mouse anti-human IgA2-AP | Southern Biotech | Sc-17803 | 1:4000 |
| Anti-Human IgG − Alkaline Phosphatase antibody produced in goat | SIGMA-Aldrich | A9544-.25ML | 1:4000 |
| Mouse Anti-Human IgG1 Fc-AP | Southern Biotech | 9054-04 | 1:4000 |
| Mouse Anti-Human IgG2 Secondary antibody, AP | Invitrogen | 05-3522 | 1:500 |
| Mouse Anti-Human IgG3 Secondary antibody, AP | Invitrogen | 05-3622 | 1:1000 |
| Mouse Anti-Human IgG4 Fc-AP | Southern Biotech | 9200-04 | 1:500 |

**Table S3. Differentially expressed proteins in psoriasis patients with low versus high anti-CA IgA levels.** Positive fold change values represent higher protein levels in the high IgA-CA group. Asterisks indicate proteins with >30% of values below the limit of detection.

| Protein | Gene | Uniprot ID | Fold Change | Raw P-value | FDR P-value |
|---|---|---|---|---|---|
| Eosinophil cationic protein | RNASE3 | P12724 | 1.718 | 0.037 | 0.978 |
| Azurocidin | AZU1 | P20160 | 1.507 | 0.035 | 0.978 |
| Chitinase-3-like protein 1 | CHI3L1 | P36222 | 1.401 | 0.045 | 0.978 |
| Protein delta homolog 1 | DLK1 | P80370 | 1.390 | 0.01 | 0.978 |
| C-C motif chemokine 18 | CCL18 | P55774 | 1.370 | 0.006 | 0.978 |
| Carboxypeptidase B | CPB1 | P15086 | 1.347 | 0.007 | 0.978 |
| Ras GTPase-activating protein 1 | RASA1* | P20936 | 1.318 | 0.032 | 0.978 |
| Fc receptor-like B | FCRLB | Q6BAA4 | 1.302 | 0.009 | 0.978 |
| NAD kinase 2, mitochondrial | NADK | O95544 | 1.299 | 0.041 | 0.978 |
| Immunoglobulin lambda constant 2 | IGLC2 | P0CG05 | 1.274 | 0.009 | 0.978 |
| Fc receptor-like protein 5 | FCRL5 | Q96RD9 | 1.268 | 0.011 | 0.978 |
| Alpha-(1,3)-fucosyltransferase 5 | FUT5 | Q11128 | 1.222 | 0.034 | 0.978 |
| Trypsin-2 | PRSS2 | P07478 | 1.220 | 0.028 | 0.978 |
| Procollagen C-endopeptidase enhancer 1 | PCOLCE | Q15113 | 1.217 | 0.047 | 0.978 |
| Secreted frizzled-related protein 3 | FRZB | Q92765 | 1.206 | 0.038 | 0.978 |
| Angiotensinogen | ANG | P03950 | 1.191 | 0.049 | 0.978 |
| Insulin-like growth factor-binding protein 7 | IGFBP7 | Q16270 | 1.167 | 0.042 | 0.978 |
| Intercellular adhesion molecule 3 | ICAM3 | P32942 | 1.144 | 0.045 | 0.978 |
| Beta-1,4-glucuronyltransferase 1 | B4GAT1 | O43505 | -1.117 | 0.025 | 0.978 |
| Brevican core protein | BCAN | Q96GW7 | -1.171 | 0.037 | 0.978 |
| Fas Ligand | FASLG | P48023 | -1.175 | 0.039 | 0.978 |
| Vascular Endothelial Growth Factor D | VEGFD | O43915 | -1.179 | 0.016 | 0.978 |
| Receptor for Advanced Glycation Endproducts | AGER | Q15109 | -1.192 | 0.005 | 0.978 |
| Follistatin | FST | P19883 | -1.271 | 0.011 | 0.978 |
| Growth differentiation factor 2 | GDF2 | Q9UK05 | -1.377 | 0.033 | 0.978 |
| Fibroblast growth factor 2 | FGF2* | P09038 | -1.430 | 0.034 | 0.978 |
| Insulin-like growth factor-binding protein 1 | IGFBP1 | P08833 | -1.601 | 0.041 | 0.978 |

## III. IL-15 and IL-23 synergize to trigger Th17 response by CLA$^+$ T cells in psoriasis

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ORIGINAL ARTICLE

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# IL-15 and IL-23 synergize to trigger Th17 response by CLA$^+$ T cells in psoriasis

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**Abstract**

IL-15 has emerged as a potentially relevant target in the IL-17 response in psoriasis. However, its mechanism is poorly characterized in humans. IL-15 and IL-23 are constitutively expressed in the psoriatic lesion. Also, *IL-15* is considered a susceptibility-associated gene in psoriasis, as are *IL-23R*, and *HLACW6*. Here, we studied the effect of IL-15 and IL-23 stimulation on the cytokine response of CLA+/CLA- T cells from 9 psoriasis patients and 3 healthy control subjects. To this end, CLA + and CLA- T cells from blood samples were cultured with epidermal cells from skin biopsies and treated with IL-15 and IL-23. After five days of culture, cytokines in supernatant were measured by ELISA or fluorescent bead-based immunoassay. There was a statistically significant increase in IL-17F and IL-17A production ($P < .001$) in cocultures of psoriasis skin-homing CLA + T cells with epidermal cells when stimulated with IL-15 and IL-23, but this effect was not observed in the cells of healthy controls. Interestingly, this response was reduced by around 50 to 80% by blocking HLA class I and II molecules. Our results point to the synergic action of IL-15 and IL-23 selectively for CLA + cells in psoriasis, leading to the induction of Th17 cell-related cytokines.

KEYWORDS
CLA, IL-15, IL-23, psoriasis, Th17

## 1 | INTRODUCTION

Psoriasis is the most prevalent T cell–mediated skin disease, affecting about 2%-3% of the Caucasian population. It is characterized by predominantly Th17 and Th1 immune responses, mostly through memory CD3 + CD45RO+ CLA + T cells. Circulating skin-tropic T cells, expressing cutaneous lymphocyte-associated antigen (CLA), are related to immunological mechanisms in the skin. These cells are considered important biomarkers of T cell–mediated skin diseases as they recirculate between skin and blood.[1,2] Unravelling the

main cytokine drivers of psoriasis by means of selective therapeutic treatments has shown that the IL-23/Th17 pathway is a key target for psoriasis and therefore an important axis in its development. Although the study of the molecular mechanisms in efficacious therapies for psoriasis suggests that complex cytokine circuits are maintained solely through Th17 cell responses,[3] many other cytokines may participate in this complex immune-based disease, especially those affecting the activation and expansion of pathogenic memory Th17 cells.[4] In this regard, IL-15 has emerged as a potentially relevant target in psoriasis that could be important in the IL-17 response.

IL-15 and its receptor are overexpressed in psoriasis,[5] and many IL-15-induced effects could play an important role in the immunopathogenesis of this condition, as reported for other inflammatory autoimmune diseases, such as rheumatoid arthritis.[6,7] Relevant functional activity of IL-15 in disease includes the induction of angiogenesis, the proliferation of T cells, the production of other proinflammatory cytokines and the inhibition of apoptosis in keratinocytes.[8-10] In addition, IL-15 promotes autoimmunity through the expansion of self-reactive memory T cells and by lowering the TCR activation threshold for low-avidity antigens on class I MHC.[11] In fact, IL-15 may play an early role in the pathogenesis of psoriasis, as suggested by the almost complete resolution of a psoriasis xenograft mouse model upon blockade of this interleukin.[5] In this model, injection of a monoclonal antibody against IL-15 gave rise to a reduction in epidermal hyperplasia, parakeratosis and inflammatory cell infiltration. Interestingly, an association between psoriasis and *IL-15* single-nucleotide polymorphisms (SNPs) within the PSORS9 locus has been reported.[12] Therefore, genetic and functional data support the involvement of IL-15 in the pathogenesis of psoriasis, and a new link to Th17 cells has been suggested, as previously described for other cytokines such as IL-23.[13] A better understanding of the effects of Th17-associated cytokines in psoriasis might help to elucidate the underlying molecular events underlying psoriasis, a disease characterized by high amounts of IL-15 and IL-23. On the one hand, IL-15 is produced by a wide range of cells,[14] such as dendritic cells, macrophages, fibroblasts and keratinocytes, and importantly, it is induced in response to innate microbial triggers [15] and other danger signals. On the other hand, IL-23 production has long been associated with dendritic cells in locally driving Th17 cell expansion and survival. Increased IL-23 levels in psoriatic skin have also been reported.[16] However, upstream IL-23 production by keratinocytes in mechanically injured skin has been proposed to precede dendritic cell polarization to drive T cell–specific responses in the skin.[17]

IL-15 belongs to the four α-helix bundle family of cytokines and has functional similarities with IL-2 and IL-7 in regulating T-cell turnover. However, IL-15 activity is generally associated with memory T cells,[18] preferentially pathogenic memory Th17 cells. Given these observations, the study of the cooperation between IL-15 and IL-23 could provide new insights into the altered Th17 cell response in psoriasis.

Here, we examined the responses of psoriatic ex vivo cocultures containing peripheral memory skin-homing CLA + or CLA- T cells in the presence of autologous lesional epidermal cells, under activation with IL-15 and IL-23. We found that these two cytokines acted synergically in psoriasis, leading CLA + T cells to produce higher amounts of IL-17F and IL-17A.

## 2 | MATERIALS AND METHODS

### 2.1 | Patients

This study was performed with human samples and in accordance with the Declaration of Helsinki. A total of 19 psoriasis patients and 4 healthy individuals were included in the study. All participants contributed voluntarily and provided written informed consent. Patients who had received any systemic treatment during the 4 weeks prior to the study or any topic treatment during the last 2 weeks were excluded in order not to obtain underestimated cellular activation. Patients and healthy subjects underwent two skin biopsies and a blood extraction.

### 2.2 | Circulating memory T cell and epidermal cell isolation

Memory CD45RA$^-$ CLA$^+$ and CLA$^-$ T cells were purified from blood samples after isolation of peripheral blood mononuclear cells by Ficoll (GE Healthcare, Princeton, NJ) gradient, and subsequent immunomagnetic separation, as described previously.[19] Around 90% of purity was obtained in both CLA + and CLA- T-cell subpopulations Figure S1. Skin biopsies were incubated overnight in dispase (Corning, Bedford, Mass) at 4°C, and the epidermal sheet was then peeled off the dermis. The epidermis was cut into small pieces, which were incubated in a trypsin solution (Biological Industries, Kibbutz Beit Haemek, Israel) for 15 minutes at 37°C. An equal volume of RPMI media (Sigma-Aldrich) containing 10% of FBS (Gibco) was added to inhibit trypsin action. Epidermal tissue was then mechanically disaggregated by gently up and down pipetting. The epidermal cell suspension (Epi) was transferred to fresh media [RPMI, 10% FBS, 1% penicillin-streptomycin (Sigma-Aldrich)], and the remaining tissue scaffold was discarded.

### 2.3 | Culture activation

Ex vivo cocultures consisted of the culture of 1.6x10$^4$ CLA$^+$ or CLA$^-$ T cells with 1x10$^4$ autologous epidermal cells (CLA$^+$/Epi or CLA$^-$/Epi, respectively), seeded together simultaneously in 96-well round-bottom plates (FALCON) in the culture media described above. Cocultures were left untreated or were activated for 5 days with 10 ng/mL of human recombinant IL-15 and/or IL-23 (Miltenyi Biotec, Bergisch Gladbach, Germany). Under a permanent concentration of IL-23 (10 ng/mL), the optimal concentration (10 ng/mL) of IL-15 was determined among a concentration range (see Figure S2. In cultures containing T cells or epidermal cells alone, the above-mentioned

amounts of each cell type were used, and activation with recombinant cytokines was performed in the same way. For the time course experiments, supernatants from activated cocultures were collected each day from replicated wells. For blocking assays, azide-free HLA-A/B/C (class I), HLA-DR (class II) neutralizing antibodies, or respective IgG2ak isotype controls (BioLegend), as well as IL-23-p19 (eBioscience, San Diego, CA) and IL-15 (BioLegend) neutralizing antibodies, or respective IgG1k isotype (BioLegend), were added to the cocultures at day 0, prior to activation, at a final concentration of 10 µg/mL.

### 2.4 | Cytokine quantification

Supernatants were kept frozen prior to cytokine quantification. IL-17A and IFN-γ were measured by ELISA on pre-coated plates (BioLegend), and IL-17F was measured by CBA fluorescent bead-based immunoassay (BD Biosciences).

### 2.5 | Flow cytometry assays

Cocultured cells were collected at the end of each experiment and then washed with PBS prior to staining. Cells were incubated with FcR Binding Inhibitor (eBioscience), anti-CD4-BV711, anti-CD8-AF700 and anti-HLADR-APC/Cy7 (BioLegend).

### 2.6 | Statistical analysis

Data are generally represented as the median and standard error media (SEM). For multiple comparison purposes, a Friedman test with Dunn's post-test was used. The differences between two groups were analysed by the Wilcoxon test. Differences were considered significant at a P value of less than 0.05 and represented by the following symbols: (*) or (#): $P < .05$; (**) or (##): $P < .01$; and (***) or (###): $P < .001$.

## 3 | RESULTS

### 3.1 | IL-23 synergizes with IL-15 to induce the production of IL-17F, IL-17A and IFN-γ by CLA + T cells, and this production is enhanced by the presence of autologous epidermal cells

To determine the participation of IL-15 and IL-23 in the production of Th17- and Th1-associated cytokines, namely IL-17F/A and IFN-γ, respectively, as well as the role of epidermal cells in the culture, epidermal cells and T cells were cultured together or separately for 5 days (n = 3-4). Importantly, the presence of epidermal cells was crucial for the selective production of IL-17F/A and IFN-γ by CLA + T cells upon activation. IL-17F, IL-17A and IFN-γ levels were synergistically increased by the simultaneous activation of cells with IL-15 and IL-23 Figure 1. Although the levels of IFN-γ were higher than those of IL-17F or IL-17A, a more selective production of Th17-associated cytokines was observed in CLA + T/Epi compared to CLA-/Epi cocultures. This finding indicates a greater involvement of epidermal cells in the IL-15/IL-23 synergistic effect on IL-17A and IL-17F production. In addition, a kinetic study of IL-15/IL-23 synergy showed a progressive increase in IL-17F and IL-17A secretion by CLA + T cells from day 3 to day 7 Figure S3A,B), while IFN-γ induction peaked at day 3 and slightly decreased thereafter Figure S3C). We also studied the activity of resident memory T cells present in the epidermal cell suspension (n = 2). IL-17F was barely detectable when 10,000 epidermal cells were activated with either Il-15 or IL-15/IL-23, or with anti-CD3/anti-CD28 or PMA/ION at 5 days. Conversely, IL-17F was clearly detected when the number of epidermal cells activated was increased to 100 000 or 300 000 Figure S4A,B), although no synergistic effect was observed. Similar results are observed for IL-17A and IFN-γ induction Figure S4A, B). The epidermal lymphocytic infiltrate of these samples was assessed by FACS staining with anti-CD45-APC and anti-CLA-PE antibodies. CD45 + cells, mostly CLA + T cells (70%-80%), accounted for only around 4% of the total epidermal cell suspension in these two patients Figure S4C). However, given the very low number present in our culture condition (10 000 cells), they are ruled out as the main source of the cytokines detected.

### 3.2 | IL-15- and IL-23-stimulated production of IL-17F and IL-17A by CLA + T cells and epidermal cells is specifically induced in psoriasis

Given that the presence of epidermal cells was crucial for optimal cytokine production, we next sought to evaluate responses of T/Epi cell cocultures in psoriasis (n = 8) and healthy controls (n = 3). IL-15 and IL-23 synergically induced IL-17F and IL-17A production by cocultures containing CLA + T cells Figure 2A,B. However, this synergic effect was not found in healthy control cocultures, thereby indicating that it is associated with the disease. Conversely, the induction of IFN-γ by IL-15 and IL-23 in psoriasis and healthy controls was similar Figure 2C, giving no clinical relevance to this cytokine.

### 3.3 | CLA + CD4+ T-cell activation induced by IL-15 and IL-23 is higher in psoriatic samples than in samples from healthy controls

T-cell activation was assessed by measuring the expression of HLA-DR by flow cytometry 5 days after stimulation. While 70% of CD8 + T cells were activated by IL-15 and IL-23, and no substantial differences were detected between the CLA + and CLA- subsets Figure 3B, A smaller proportion of CD4 + T cells expressed HLADR Figure 3A. However, the latter showed a preferential activation in CLA + memory T cells ($P < .05$). A similar pattern of activation was observed in cocultures from healthy donors, but CLA + CD4+ T cells showed less activation than those in the psoriasis cocultures

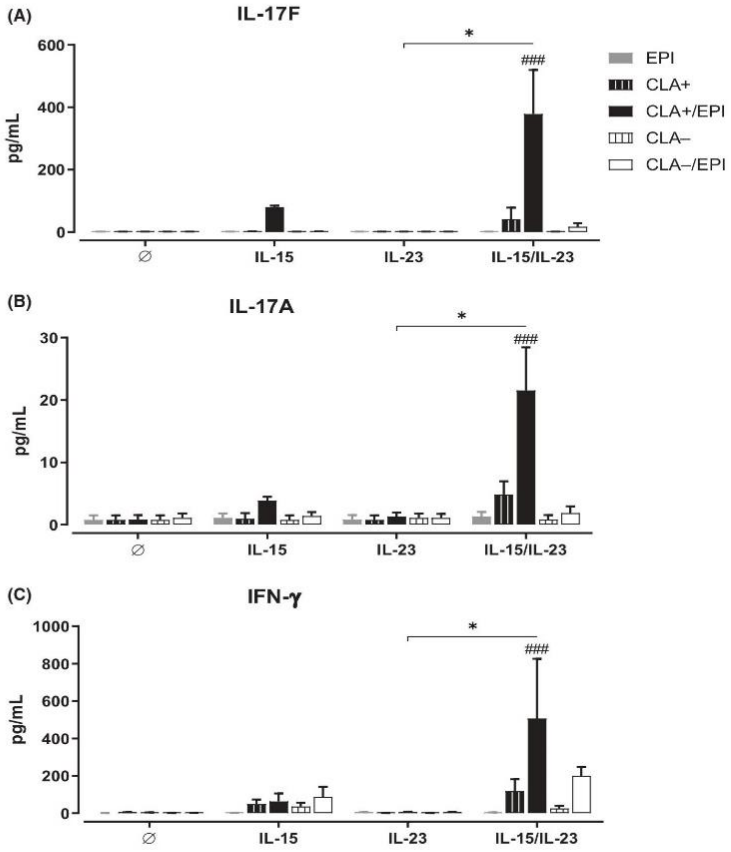


**FIGURE 1** IL-23 synergizes with IL-15 to produce IL-17F, IL-17A and IFN-γ. IL-17 production was especially enhanced by the presence of epidermal cells. Epidermal cells, $CLA^{+}$T or $CLA^{-}$ T cells from psoriasis patients were cultured alone or in T/epidermal cell coculture combinations, which were then activated by IL-15, IL-23 or both, or were left untreated. Supernatants were collected (n = 3) at day 5, and IL-17 (A), IL-17A (B) and IFN-γ (C) were measured. Data are represented as mean ± SEM. Two-way ANOVA and Dunnett's post-test were used to assess differences with basal untreated conditions (#: $P < .05$; ##: $P < .01$; ###: $P < .001$). Friedman test with Dunnett's post-test was used to compared different stimuli within the same coculture condition (*:$P < .05$; **:$P < .01$; ***:$P < .001$)

($P = .01$). Furthermore, we performed a time course experiment to determine the dynamics of CLA + CD4+ and CD8 + T-cell activation. The results showed that activation occurred earlier in CLA + CD4+ T cells and increased progressively till day 5 of culture. In contrast, no clear induction was observed in CLA- T-cell cultures Figure 3C. However, the increased number of activated CD8 + T cells was more pronounced at day 5 but was present in both CLA + and CLA- T-cell populations Figure 3D. Flow cytometry plots from representative patients are included as Figures S5 and S6.

### 3.4 | IL-17F synergistic production by IL-15 and IL-23 in CLA + T cells in psoriasis is strongly reduced after IL-15 or IL-23p19 blockade

We next studied the effect of IL-15 or IL-23p19 neutralization in co-cultures previously activated with both IL-15 and IL-23 on the production of Th17- and Th1-associated cytokines. Interestingly, the levels of IL-17F (n = 6) showed 80.1 and 30.5%inhibition by IL-15 and IL-23p19 blockade, respectively Figure 4A. As seen in previous experiments, IL-17A (n = 6) was mildly induced upon activation and its level was slightly decreased after IL-15 or IL-23p19 blockade Figure 4B. Finally, IFN-γ (n = 6) levels were measured as a Th1-associated cytokine and, in this case, while IL-15 neutralization resulted in 54.5% of inhibition, IL-23p19 blockade reduced IFN-γ levels by only 34,1% Figure 4C. All together, these results indicate that the synergistic effect of IL-15 and IL-23 is involved in IL-17F production by CLA + T cells.

### 3.5 | IL-17F, IL-17A and IFN-γ production induced by IL-15 and IL-23 is dependent on HLA-class I and II molecules

One of the mechanisms through which IL-15 is associated with autoimmunity might be via its capacity to reduce the TCR activation threshold, which results in increased responsiveness of CD4 and CD8 T-cell subsets and facilitates the activation and expression of autoreactive T cells.[3] Therefore, we sought to determine the effect of inhibiting antigen presentation through HLA-class I and II molecules on Th1- and Th17-associated cytokine production Figure 4D-F. Both neutralizing antibodies reduced cytokine production around 30%-80%, although HLA-class II blockade showed a greater reduction on Th17 products, 66,7% inhibition of IL-17F and 80% of IL-17A production compared to 26,7% inhibition of IFN-γ production. This

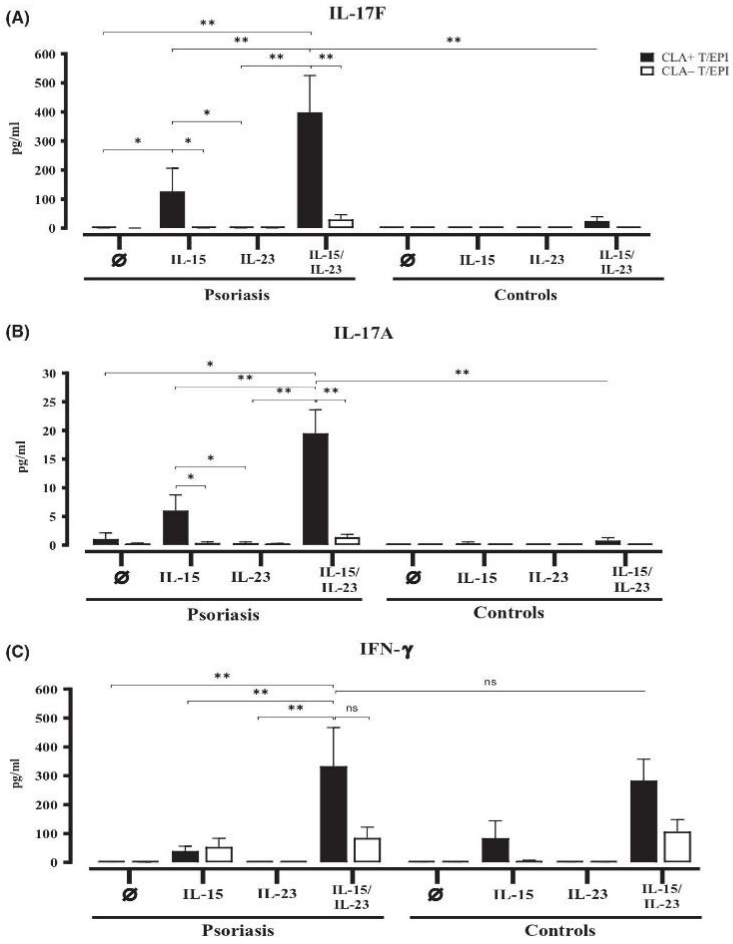


**FIGURE 2** Synergistic production of IL-17A and IL-17F by CLA + T cells and epidermal cells is induced in psoriasis by IL-15 and IL-23. Cytokine responses to IL-15 and/or IL-23 stimuli were compared between cocultures derived from psoriasis patients (n = 8) and healthy controls (n = 4). IL-17A (A), IL-17F (B) and IFN-γ (C) were measured in 5-day supernatants. Wilcoxon test was used to compared different stimuli within the same coculture condition in psoriasis or controls, whereas Mann-Whitney test was used to compared psoriasis and healthy control subjects (*:$P < .05$; **: $P < .01$; ***:$P < .001$)

observation suggests the involvement of both CD4 + and CD8 + T cells and potentially through self-antigen presentation.

## 4 | DISCUSSION

IL-15 and IL-23 are present in psoriatic lesions. However, their possible functional synergic activity on human memory T cells has not been addressed to date. Our results demonstrate how IL-15 and IL-23 cooperate to induce CLA + T cell–dependent production of IL-17F and IL-17A in psoriasis and in the absence of any T-cell activation, such as polyclonal activation or exogenous antigen addition.

The observation that the synergic effect of IL-15 and IL-23 on IL-17F/A response is restricted to CLA + T cells could be particularly relevant for our understanding of the cutaneous immune response in psoriasis. CLA + memory T cells are peripheral cellular biomarkers for many T cell–mediated cutaneous diseases in humans, specially psoriasis.[20,21] CLA + T cells recirculate between blood and psoriatic lesions, and CLA + T cells isolated from blood seem to be more sensitive to mediators such as IL-15 and IL-23, which are present in the lesions. In addition, this synergic effect is directly linked to the presence of autologous epidermal cells in culture, thereby indicating that cooperation between T cells and epidermal cells occurs together with the synergic action of IL-15 and IL-23. In our model, a general homogenate from the epidermal layer was obtained. We studied the putative role of skin resident memory T cells ($T_{RM}$), which are part of this epidermal cell suspension, in the synergic effect observed. Almost null IL-17F induction was found when epidermal cells were stimulated on their own at the same numbers used for cocultures with CLA + and CLA- T cells (10,000 EPI cells). This observation could be attributed to the low lymphocytic infiltrate, which hardly reached 4% of the total cell populations. However, when the number of epidermal cells in culture was increased, IL-17F/A and IFN-γ induction is noted by the presence of IL-15, but no synergistic effect with IL-23 was found. Future efforts should be made to identify the cell type and intermediate mediators within the epidermal cell population that participates in this IL-15/IL-23 synergy.

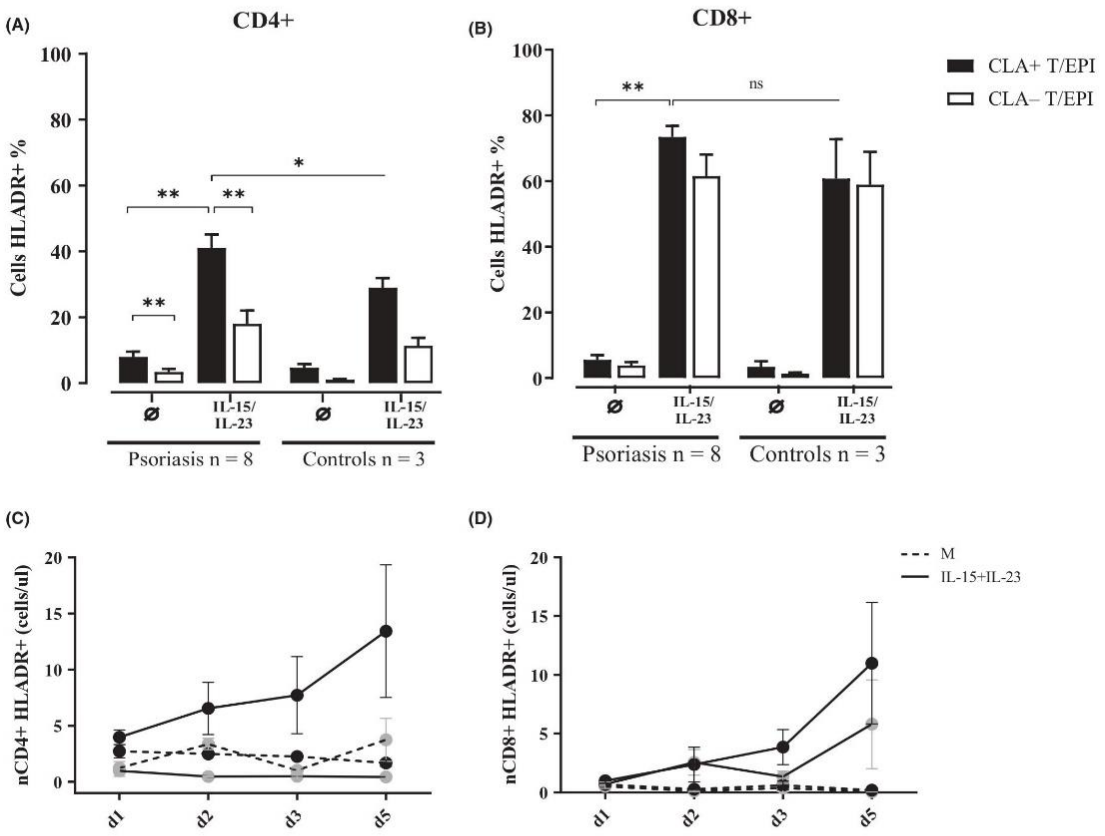


**FIGURE 3** IL-15 and IL-23 enhance the activation of CD4 + T cells in psoriasis patients compared to control subjects. Percentages of activated HLA-DR$^+$ CD4 (A) and CD8 (B) T cells after IL-15 and IL-23 stimulation are shown in psoriasis patients (n = 6) and healthy controls (n = 3). Wilcoxon test was used to compared different stimuli within the same coculture condition in psoriasis or controls, whereas Mann-Whitney test was used to compared psoriasis and healthy control subjects (*:$P < .05$; **: $P < .01$; ***:$P < .001$). Lines represent net increase (cell/ul) of HLA-DR + CD4+ (C) or CD8+ (B) T cells at days 1, 2, 3 and 5 after IL-15 and IL-23 stimulation, at a basal level (dashed line, M), and after cytokine activation (continuous line, IL-15/23). CLA + T cells are shown as black squares and CLA- T cells as grey circles. Data are presented as mean and SEM (n = 4)

In a hypothesized model for this mechanism in psoriasis, we propose that extravasated CLA + T cells, when exposed to lesional IL-15 and IL-23 and in close contact with epidermal cells, produce IL-17F and IL-17A, both clinically relevant mediators in psoriasis, and IFN-γ, a cytokine that contributes to inflammation in this disease but whose therapeutic neutralization has minimal clinical efficacy.[22] Intermediate mediators of this cytokine cascade may be involved. However, further experiments are needed to confirm this notion.

Little attention has been paid to the functional relevance of IL-17F in psoriasis. Our results indicate that IL-15 and IL-23 induce a higher production of IL-17F than IL-17A. Interestingly, this feature has been reported in previous studies by our group,[21] when CLA + T cells were used in ex vivo models of psoriasis. However, independent studies have also reported higher IL-17F than IL-17A concentration in serum and skin samples from patients with psoriasis.[23,24] The observation that CLA + T cells from psoriasis patients appear to produce more IL-17F in our ex vivo assays endorses the idea that this cytokine plays a relevant role in the development of the disease. Recently, the importance of IL-17F in psoriasis patients has been supported by the observation of the clinical efficacy of bimekizumab, an antibody that neutralizes IL-17A and IL-17F.[25]

Several antibodies that specifically neutralize IL-23, through its subunit p19, are currently being tested for clinical use. The first results of phase III clinical trials have shown how these biologics reduce skin area affected by psoriasis and the severity index (PASI), thereby highlighting the involvement of IL-23 in the development of the disease.[26] In the present study, we demonstrate how anti-p19 treatment reduced IL-17F production by CLA + T cells in cocultures. This effect was limited to this cytokine and was not observed for IL-17A or IFN-γ. Therefore, our data also contribute to our current understanding of the role of IL-23 in psoriasis. On the basis of our results, we propose that a bifunctional antibody neutralizing IL-15

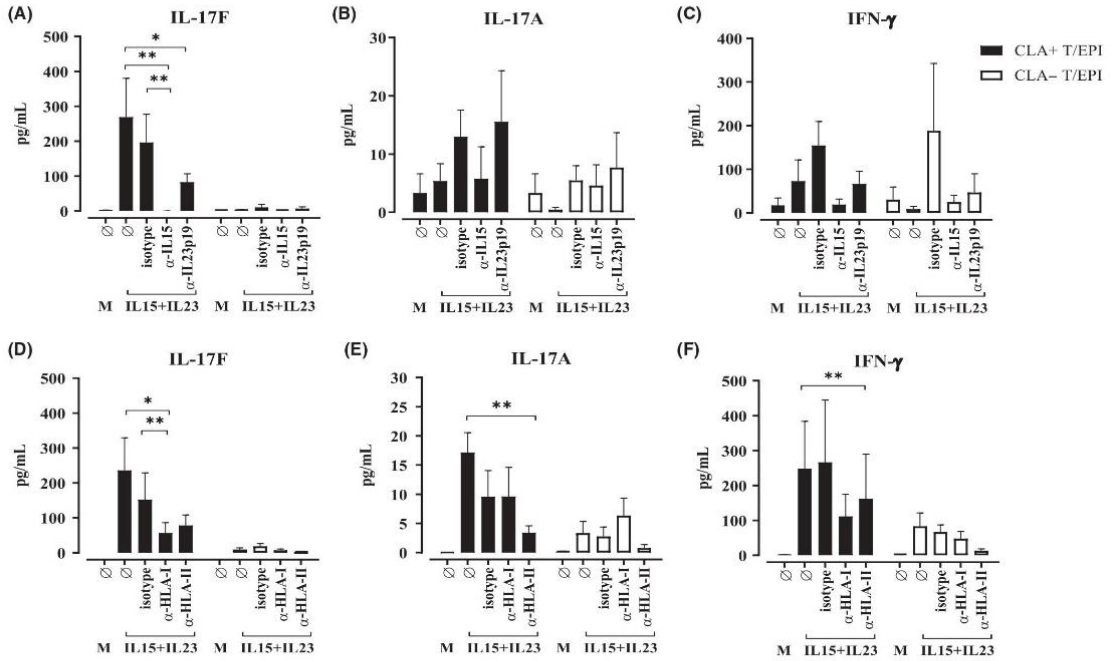


**FIGURE 4** Th17- and Th1-associated cytokine production is reduced upon either IL-15 or IL-23 neutralization and is dependent on HLA class I and II. Blocking antibodies against IL-15 or IL-23p19 were added to IL-15- and IL-23-activated cocultures. After 5 days, supernatants (n = 6) were collected and IL-17F (A), IL-17A (B) and IFN-γ (C) were measured. Also, blocking antibodies against HLA-A/B/C (class I) or HLA-DR (class II) were added to cocultures of psoriatic samples prior to activation with IL-15 and IL-23. After 5 days, supernatants (n = 9) were collected and IL-17F (D), IL-17A (E) and IFN-γ (F) were quantified. Basal cytokine levels without any stimuli and stimulated with IL-15 and IL-23 but without neutralizing antibodies are also shown. Wilcoxon test was used to compare two different groups (*$P < .05$; **$P < .01$; ***$P < .001$)

and IL-23 in psoriasis patients may significantly reduce the signs and symptoms of the disease.

However, despite several in vivo studies in mice, the functional role of IL-15 in psoriasis in humans is poorly characterized. In this context, the present study is the first to demonstrate that blocking IL-15 in an ex vivo model of psoriasis considerably reduces IL-17F and to a lesser extent IL-17A and IFN-γ production by CLA + T cells and not by CLA- T cells. Our results are consistent with the findings of previous studies in which IL-15 blockade induced the resolution of psoriasis in a xenograft mouse model,[5] or in which treatment of xenograft of AGR129 mice with IL-15Ra, an endogenous antagonist of IL-15, inhibited the development of psoriasis-like inflammation.[27] Indeed, our results support the notion that IL-15 stimulates the production of IL-17, mainly IL-17F, more effectively than IL-23, as previously reported.[28]

At the genetic level, an association analysis described a link between SNPs in *IL-15*, within the psoriasis susceptibility 9 (PSORS9) locus, and psoriasis in Chinese families,.[12] This finding, together with the strong genetic association between psoriasis and the p40 subunit of IL-23 and its receptor (IL-23R),[29] may lead us to consider whether these two cytokines cooperatively play a functional role in the development of this disease. Such a synergic effect has already been reported for other cytokines within the same families, such as IL-2 and IL-12, corresponding to the IL-15 and IL-23 families, respectively.[30,31] Conversely, the analysis of cytokine production induced by IL-15 and IL-23 in cultures in which HLA class I or class II molecules were neutralized indicated that both CD4 + and CD8 + T cells are involved, although HLA class II blockade had a greater effect on Th17 cytokine levels. The observation that HLA class I and class II neutralizing antibodies block IL-15/IL-23-induced cytokine production by CLA + T cells in the presence of autologous epidermal cells indicates the recognition of autoantigens presented by epidermal cells. We cannot discard the involvement of melanocyte-related autoantigen (ADAMTSL5) or cathelicidin (LL37) in this process. Most importantly, we found that the synergic action of IL-15 and IL-23 induced greater activation of CD4 + T cells in cultures from psoriasis patients than in those from healthy controls, while the levels of CD8 + T-cell activation were similar. Overall, these findings indicate that CD4 + T cells play a determinant role in IL-17 secretion in the context of psoriasis. Nonetheless, we believe that results from direct T cell–derived cytokines are more functional readouts of T-cell activity.

In summary, this study demonstrates the novel synergic action of IL-23 and IL-15, two cytokines present in the psoriatic lesion. The relevance of this finding is that this synergy in IL-17F and IL-17A production is restricted to skin-related memory T cells of psoriasis patients and is not found in healthy controls. Altogether, our results

contribute to clarifying the role of IL-23 in psoriasis and support the relevance of IL-15 as a potential therapeutic target.

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## CONFLICT OF INTEREST

The authors state no conflict of interest.

## AUTHORS' CONTRIBUTIONS

CJG, ERR, LSB, MF and RMP conceived the project, with the contribution of AC. CJG and ERR performed the experiments and analysed the data. MF, MS, PG and AC provided the clinical material and contributed to data curation. CJG, ERR and LSB wrote the manuscript. All the authors have read and approved the final manuscript.

## DATA AVAILABILITY STATEMENT

No data sets were generated or analysed during the current study.

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## SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

**Figure S1**. Purity of CLA + and CLA- subpopulations after magnetic beads separation. CLA + and CLA- T cell subpopulations were separated using column-based MACS manual separators and the anti-CLA microbead kit for human in two steps: anti-CLA-PE and anti-mouse IgG-beads. Stained memory T cells, pre-separation, and purified subpopulations were analyzed in FACS. Around 90% of purity is obtained for both CLA + and CLA- T cells after separation following the standard protocol.

**Figure S2**. IL-15 and IL-23 synergistic effect appears when added at 10ng/ml to cell culture. To determine the optimal concentration of IL-15 and IL-23 in this study epidermal cells and $CLA^+T$ or $CLA^-$ T cells from psoriasis patients were cocultured and then activated by IL-15 (10ng/ml), IL-23 (10ng/ml), increasing concentration of IL-15 in combination with IL-23 (10ng/ml), or were left untreated. Supernatants were collected at day 5 (n = 1) and IL-17A (a) and IFN-γ (b) were measured. Although there is a slight induction of IL-17A and IFN-γ at 5ng/ml of IL-15; the synergistic effect between IL-15 and IL-23 is clearly present when both are added at 10ng/ml to the culture, as shown by the increase of IL-17A and IFN-γ levels.

**Figure S3**. Kinetics of IL-17F, IL-17A and IFN-γ induction after IL-15, IL-23 or both stimulation of CLA + or CLA- T cell coculture with autologous epidermal cells. (a, b) IL17F and IL17A secretion progressively increased at day 3, 5 and 7 only when both IL15 and IL23 were added. (c) IFN-γ secretion mean showed a pick at day 3 and then a decrease at days 5 and 7 only with IL15 + IL23 activation. Data are shown as mean plus standard error of the mean (SEM).

**Figure S4**. IL-15 and IL-23 synergistic effect is not observed in skin resident memory T cells. Functionality of resident memory T cells present in patients epidermal cell suspensions was tested in vitro (n = 2). (a) Cultures of increasing number of epidermal cells (10.000, 100.000 and 300.000) were left untreated or activated with either IL-15, IL-23 or both. (b) PMA/ION and αCD3/αCD28 stimuli were also included as positive control of T cell activation. After 5 days of culture, IL-17F, IL-17A and IFN-γ levels were measured in supernatants. Data are represented as mean and standard deviation (SD). (c) Epidermal cell suspensions were stained with anti-CD45-APC and anti-CLA-PE antibodies to determine lymphocyte infiltration. Flow cytometry plots show results from the two patients whose cultures were analyzed in a and b. PMA: phorbol 12-myristate 13-acetate, ION: ionomycin.

**Figure S5**. IL-15 and IL-23 enhace activation of CD4 + T cells in psoriasis compared to controls. After 5 days of CLA ± T cell/EPI cultures untreated or activated with IL-15 plus IL-23, cells were haversted and stained with anti-CD4-BV711, anti-CD8-AF700 and anti-HLA-DR-APC/Cy7. Flow cytometry plots show results from one representative patient and control.

**Figure S6**. IL-15/IL-23 induced increase in the number of activated CLA + CD4+ or CD8 + T cells is time dependent. After 1, 2, 3 and 5 days of CLA ± T cell/EPI cultures activated with IL-15 plus IL-23, cells were haversted and stained with anti-CD4-BV711, anti-CD8-AF700 and anti-HLA-DR-APC/Cy7. Flow cytometry plots show results from one representative patient.

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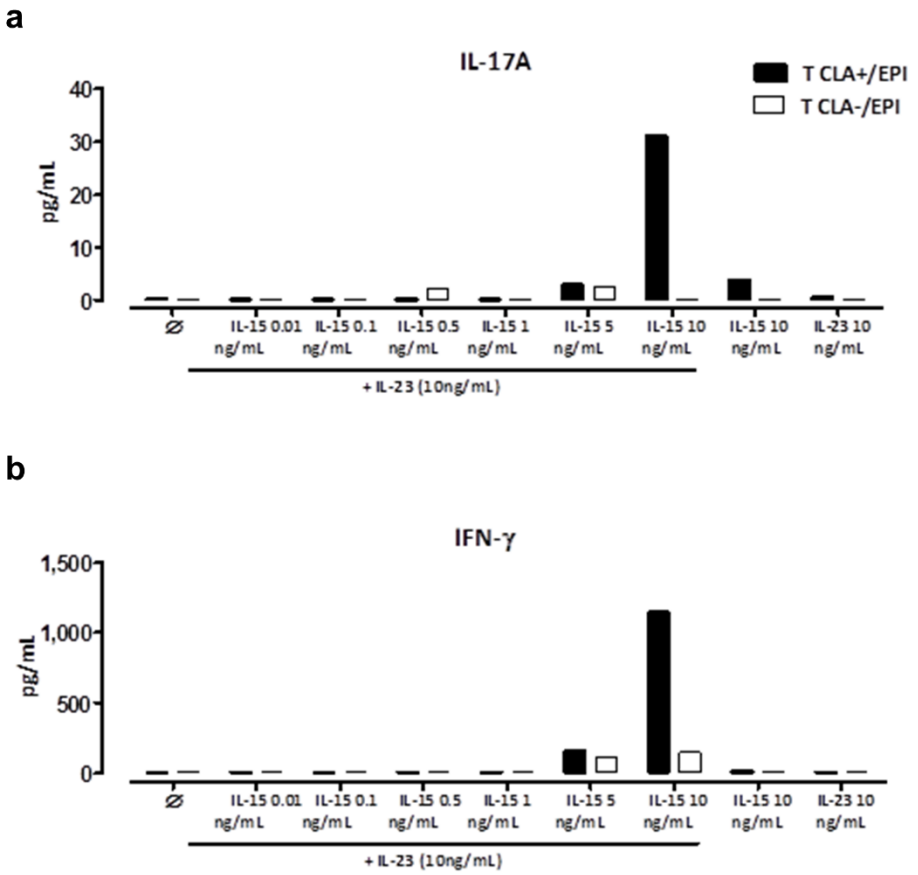


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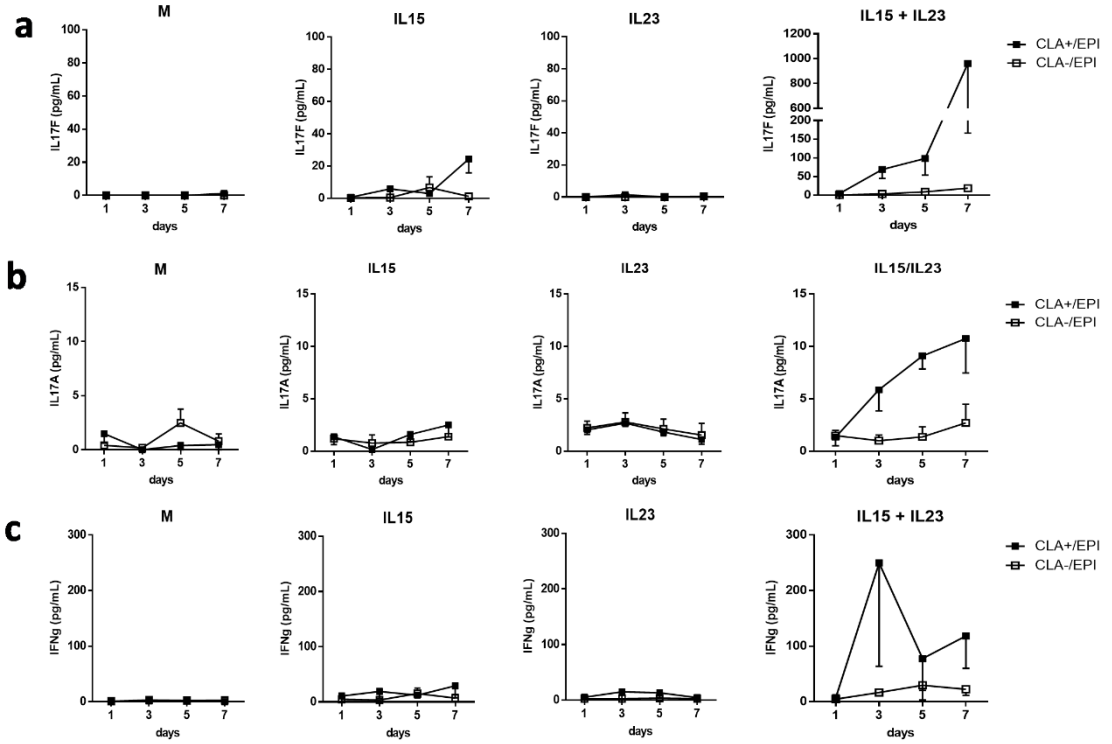


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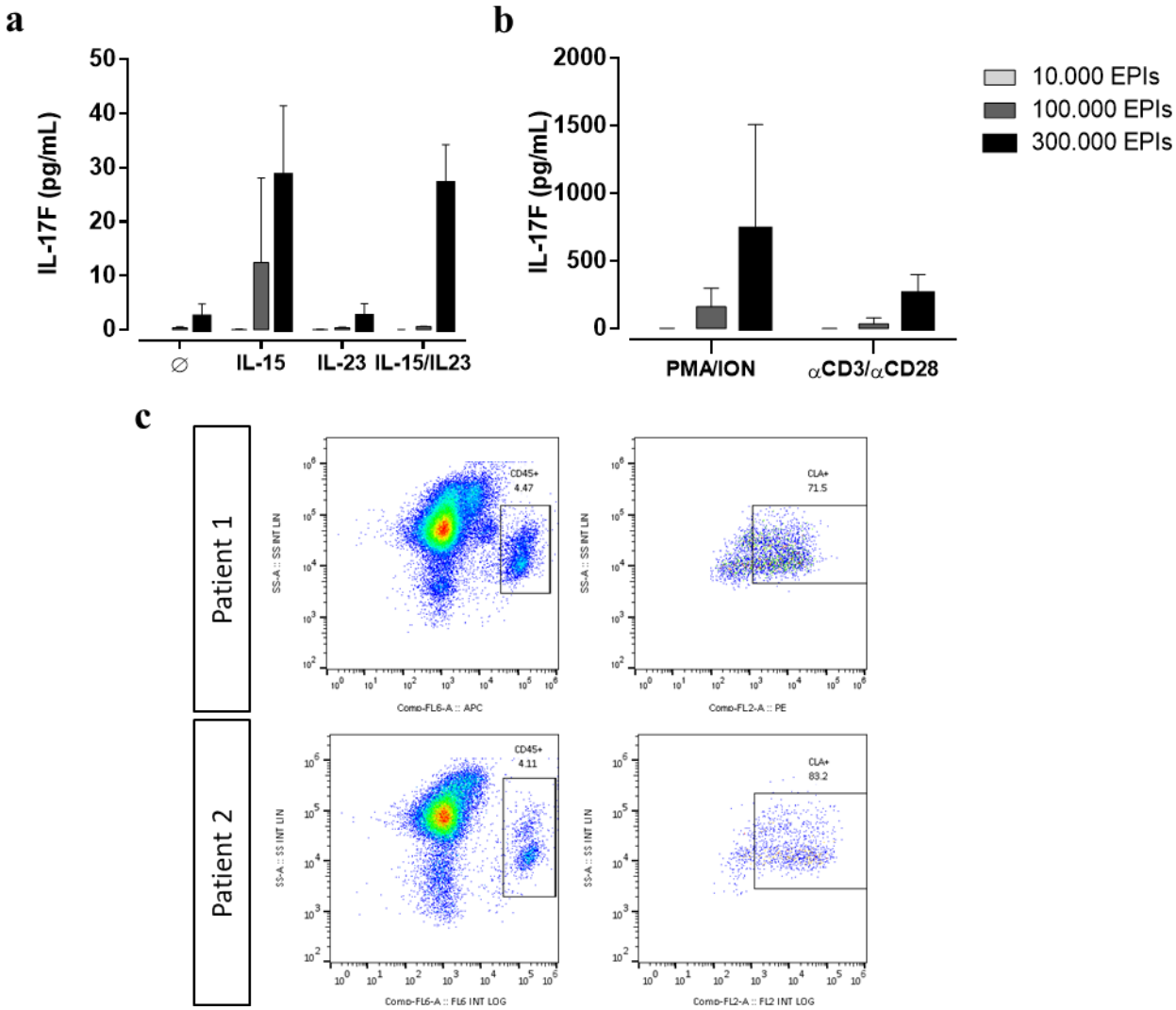


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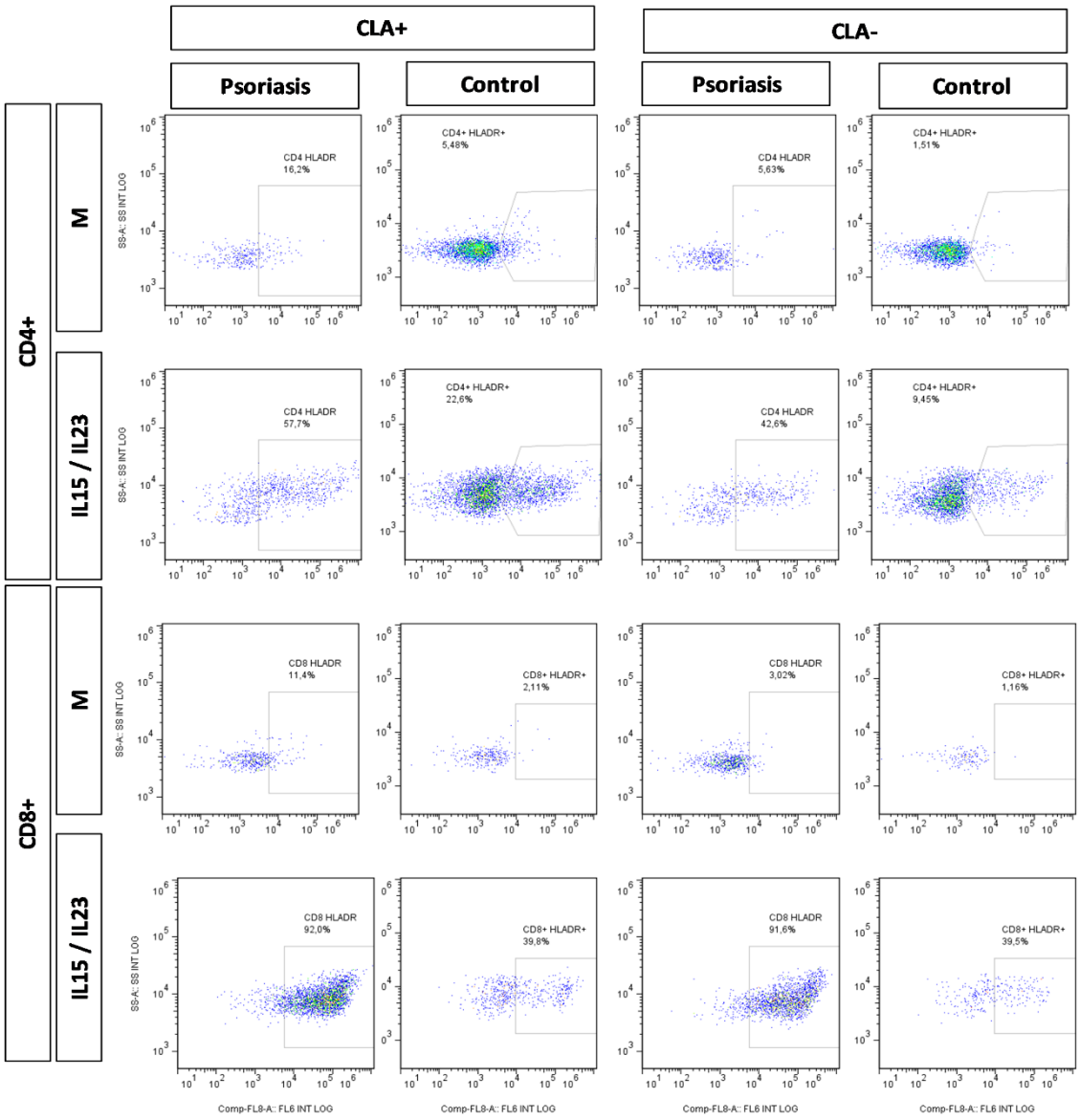


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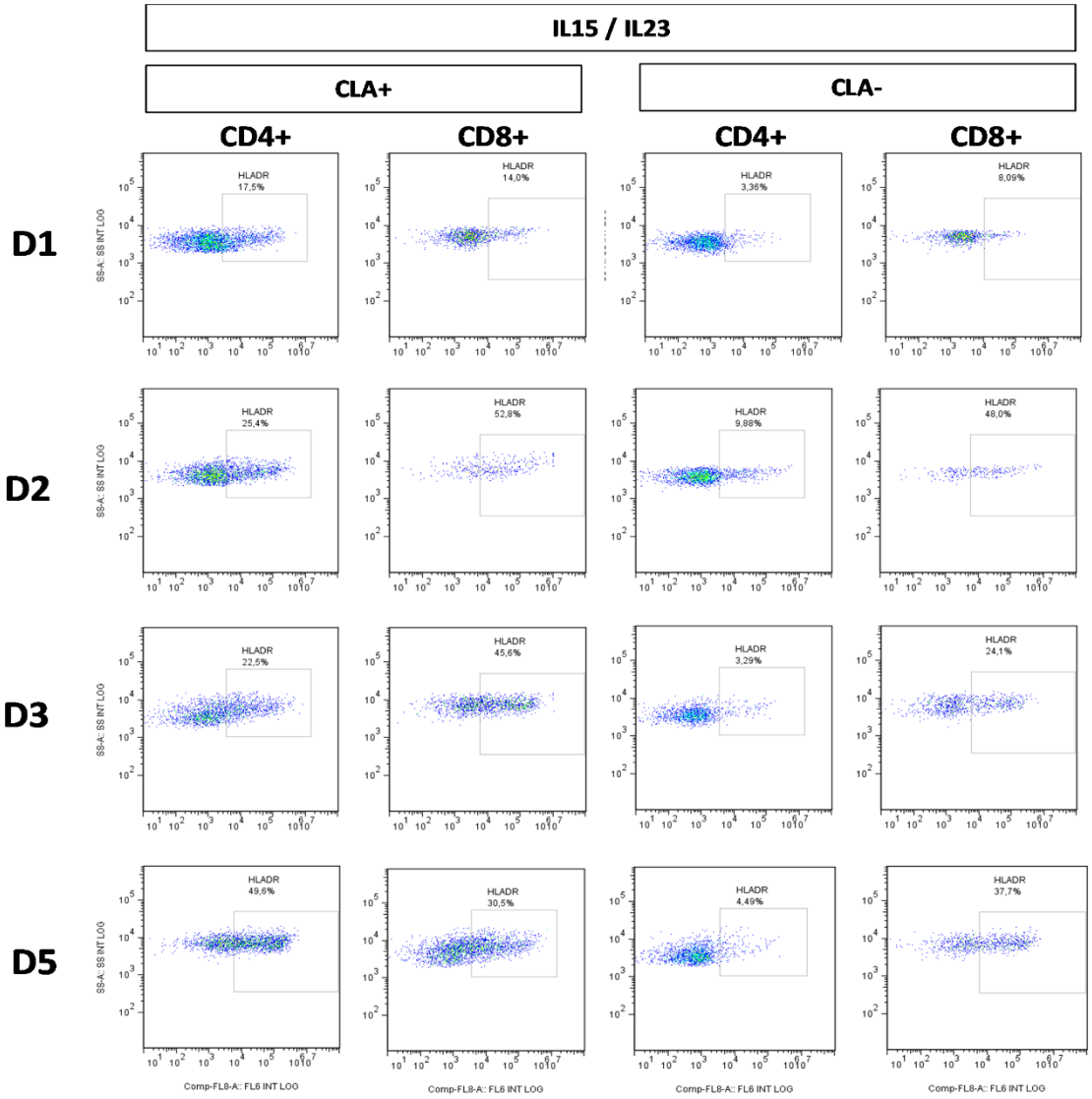


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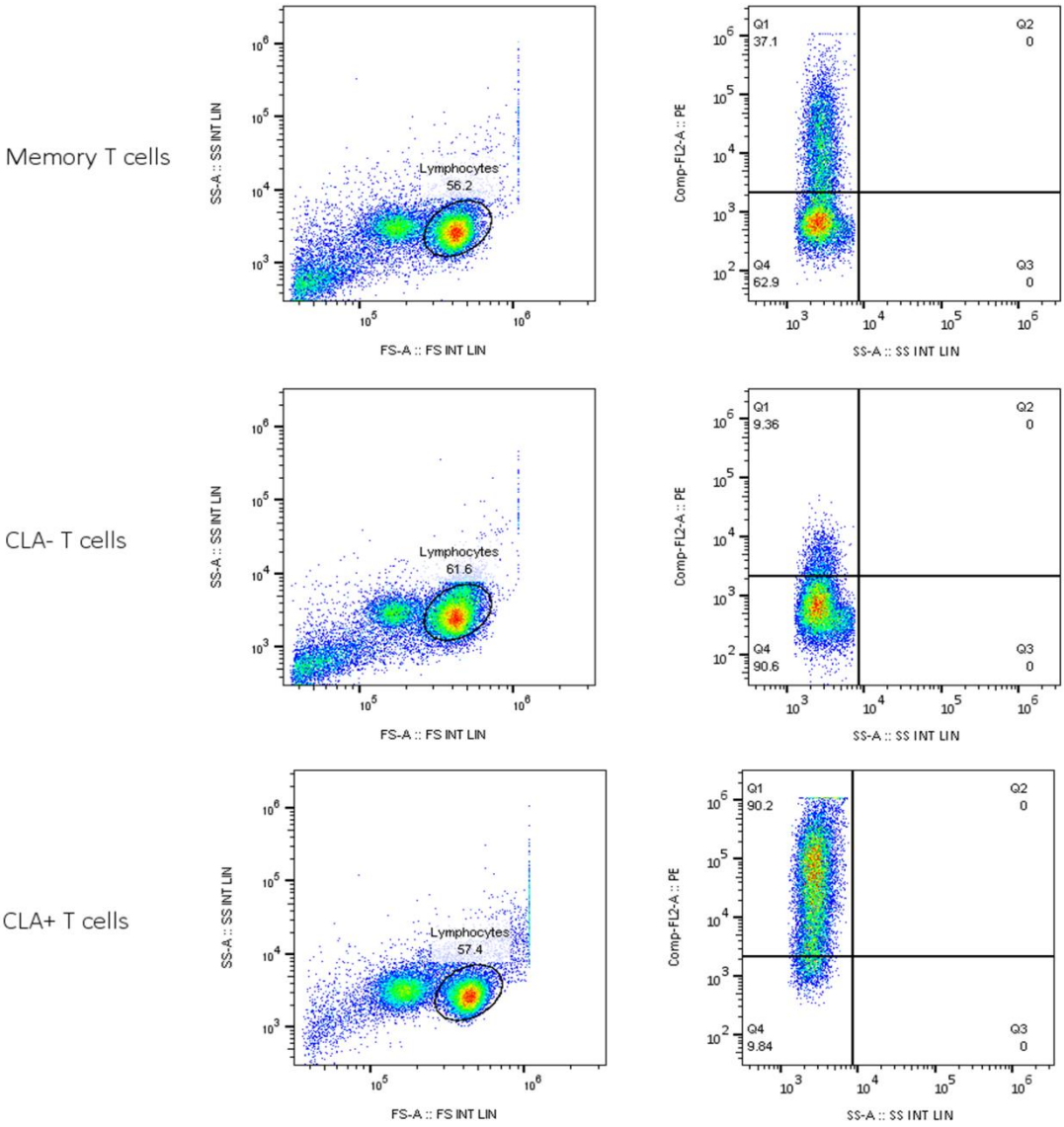


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# DIRECTOR'S REPORT

As supervisor of the present Doctoral Thesis, Dr. Luis F. Santamaria Babí hereby state that the student Carmen de Jesús Gil has actively participated in the overall process conducting to a considerable amount of work done during the last four years as part of the group of Translational Immunology (UB). This includes the peer-reviewed papers that conform this thesis, as reflected by their author order and composition. The candidate, Carmen de Jesús Gil, had an essential role in the development and execution of the experimental design, data acquisition and treatment, and in the discussion and publication of the main results and conclusions.

What follows below is a concise overview of the peer-reviewed papers included in the current Doctoral Thesis:

**PAPER I**

Title: Specific IgA and CLA+ T-Cell IL-17 Response to *Streptococcus pyogenes* in Psoriasis

Authors: **Carmen de Jesús-Gil**, Lídia Sans-de San Nicolàs, Ester Ruiz-Romeu, Marta Ferran, Laura Soria-Martínez, Anca Chiriac, Antonio Celada, Ramon M Pujol, Luis F Santamaria-Babí

Journal: Journal of Investigative Dermatology

Year of publication: 2020

DOI: 10.1016/j.jid.2019.12.022

JCR Impact Factor (2019): 7.143 JCR 5 Years Impact Factor: 6.867

Rank: Q1 Dermatology

Doctoral student participation: the candidate carried out most of the experiments, analyzed the data and performed the graphs and tables, discussed the results, and wrote the manuscript.

**PAPER II**

Title: Interplay between Humoral and CLA+ T Cell Response against *Candida albicans* in Psoriasis

Authors: **Carmen de Jesús-Gil**, Lídia Sans-de San Nicolàs, Ester Ruiz-Romeu, Marta Ferran, Laura Soria-Martínez, Irene García-Jiménez, Anca Chiriac, Josep M Casanova-Seuma, Josep M Fernández-Armenteros, Sherry Owens, Antonio Celada, Michael D Howell, Ramon M Pujol, Luis F Santamaria-Babí

Journal: International Journal of Molecular Sciences

Year of publication: 2021

DOI: 10.3390/ijms22041519

JCR Impact Factor (2019): 4.556 JCR 5 Years Impact Factor: 4.653

Rank: Q1 Biochemistry & Molecular Biology

Doctoral student participation: the candidate carried out most of the experiments, analyzed the data and performed the graphs and tables, discussed the results, and wrote the manuscript.

**PAPER III**

Title: IL-15 and IL-23 synergize to trigger Th17 response by CLA+ T cells in psoriasis

Authors: **Carmen de Jesús-Gil**, Ester Ruiz-Romeu, Marta Ferran, Marc Sagistrà, Anca Chiriac, Pablo García, Antonio Celada, Ramon M Pujol, Luis F Santamaria-Babí

Journal: Experimental Dermatology

Year of publication: 2020

DOI: 10.1111/exd.14113

JCR Impact Factor (2019): 3.368 JCR 5 Years Impact Factor: 2.786

Rank: Q1 Dermatology

Doctoral student participation: the candidate carried out part of the experiments, analyzed the data and performed most of the graphs and tables, discussed the results, participated in the writing of the manuscript and actively performed the experiments and script changes required during the revision.

# SUMMARY OF RESULTS

The main goal of this thesis was to explore novel mechanisms on how environmental microbes and psoriatic inflammatory microenvironment interact with $CLA^+$ memory T cells from psoriasis patients to trigger IL-17 response, which is essential in psoriasis immunopathology. We used a translational *ex vivo* model of psoriasis based on the coculture of circulating skin homing $CLA^+$ T cells with autologous epidermal cells from non-treated psoriasis patients. Challenging their response against relevant microbial agents, here *S. pyogenes* and *C. albicans*, and lesional pro-inflammatory cytokines, as IL-15 and IL-23, revealed significant IL-17A and IL-17F responses, sustaining psoriasis pathogenesis. We studied the exposure of patients to microbes through specific humoral response against *S. pyogenes* and *C. albicans* in plasma, revealing increased but heterogeneous antibody levels in psoriasis subtypes compared with controls.

Regarding the first objective, we characterized the humoral immune response against *Streptococcus pyogenes* and *Candida albicans* in psoriasis patients from either plaque or guttate subtypes. We found increased *S. pyogenes*-specific IgA (anti-SE), but not IgG, both in plaque as in guttate psoriasis when compared to healthy individuals but also to patients with atopic dermatitis, a different inflammatory skin condition. These results further support the unique association of β-hemolytic *Streptococci* and psoriasis disease. Of note, presence of anti-SE IgA was reported in plaque psoriasis patients with negative ASO and whose flares were not associated with *Streptococcal* infection. In this regard, to identify the potential site of encounter with this pathogen, IgA subtypes were analyzed and revealed preferential anti-SE IgA1 response, which is regularly secreted in the upper respiratory tract. Previous studies from our group showed preferential induction of Th17, Th1 and Th9 cytokines by *S. pyogenes* in $CLA^+$ T/EPI cocultures from psoriasis patients (Ruiz-Romeu et al. 2018; Ruiz-Romeu et al. 2016b). Finally, we assessed whether these cytokine responses were associated with *S. pyogenes* exposure in patients. Interestingly, only anti-SE IgA levels positively correlated with IL-17F and IL-17A induction in SE-activated $CLA^+$ T/EPI cocultures from plaque and guttate psoriasis respectively.

On the contrary, generally increased IgA and IgG against *C. albicans* (anti-CA) were detected exclusively in plaque psoriasis patients, whereas similar antibody responses were observed in guttate psoriasis and healthy individuals. Of note, *C. albicans* fresh infection was discarded in both psoriasis and healthy individuals, by using a commercial diagnostic kit. Next, T cell response against *C. albicans* was fully characterized in our *ex vivo* model of coculture, comparing between different forms of disease. Preferred IL-17 response in CA-activated $CLA^+$ T/EPI condition was similarly observed in cocultures from plaque and guttate psoriasis just as healthy controls, in which IL-17F prevailed over IL-17A, whilst significantly increased CA-induced IL-9 was only reported in $CLA^+$ T/EPI cocultures from psoriasis patients. In general, higher cytokine responses were observed in guttate psoriasis compared to chronic plaque form of disease. As done for *S. pyogenes*, the linkage between specific cellular and humoral immune responses was studied. This

analysis revealed that anti-CA IgA levels, but not IgG, were directly associated with CA-induced IL-17 response *in vitro* by both $CLA^+$ and $CLA^-$ T/EPI cocultures in plaque psoriasis particularly. Interestingly, for plaque psoriasis patients, disease duration positively correlated with levels of anti-CA IgA in plasma and $CLA^+$ T cell-induced IL-17F/A response *in vitro*. Based on these premises, we hypothesized that specific characteristics of patients with increased anti-CA IgA levels could be accountable for their clinical course of disease. To address this matter, a broad proteomic profiling of plasma samples was assessed in a different cohort of plaque psoriasis patients. Generally increased proteins in patients with higher anti-CA IgA plasma levels were implicated in antimicrobial humoral immune response, cell chemotaxis and inflammation. Subsequent validation of differentially expressed proteins confirmed increased levels of CCL18, CHI3L1 and AZU1 in plasma from plaque psoriasis patients compared to guttate psoriasis and healthy individuals. Further association with disease severity, age of onset or disease duration supported the translational value of these proteins linked to exposure to *C. albicans* in psoriasis.

With respect to the second objective, the influence of the lesional pro-inflammatory cytokines IL-15 and IL-23 was assessed in our *ex vivo* model of psoriasis. Synergistic induction of IL-17F and IL-17A was observed in $CLA^+$ T/EPI cocultures from psoriasis patients stimulated with IL-15 and IL-23, but not in healthy controls. This effect was directly dependent on the presence of autologous epidermal cells and associated to the activation of $CD4^+$ T cells in psoriasis patients, whilst expansion of activated ($HLA\text{-}DR^+$) $CD8^+$ T cells was similar in $CLA^+$ and $CLA^-$ T cell subsets, as well as in psoriasis and controls. As part of the epidermal cell suspension, the eventual implication of resident memory T cells was evaluated but discarded based on the lack of IL-17 response in single EPI and $CLA^-$T/EPI cocultures, as well as the low rate of $T_{RM}$ ($CD45^+$) in our coculture, representing only around 4% of the total epidermal cell suspension. Because the epidermal vicinity was needed for the synergistic effect, their potential action as antigen presenting cells was assessed. HLA class II blockade significantly reduced Th17 products, whilst HLA-I neutralization partially impacted Th17 and Th1 cytokines induction. Altogether, these data reinforced the role of circulating $CLA^+$ $CD4^+$ T cells as key effector cells under the IL-15 and IL-23 synergy. Finally, the specific contribution of IL-15 and IL-23 in the induction of $CLA^+$ Th17 response was evaluated. Neutralization of IL-15, and to a lesser extent IL-23p19, in previously IL-15 and IL-23 activated cocultures showed significant reduction of induced IL-17F, whereas minor effects over IL-17A and IFN-γ secretion, emphasizing the role of IL-15 in this synergy.

In summary, this work has characterized novel mechanisms on the skin-tropic memory T cells related to triggers of psoriasis. We proved the heterogeneous exposure to the environmental microbes *S. pyogenes* and *C. albicans*, which directly promote $CLA^+$ T memory-dependent IL-17 response *in vitro*. And we showed how the lesional cytokines IL-23 and IL-15 synergistically induce IL-17 response by $CLA^+$ T cell in the presence of autologous epidermal cells in psoriasis.

# GENERAL DISCUSION

Psoriatic skin inflammation is caused by a complex interplay between the immune system, genetic susceptibility, and environmental factors with a leading role for the IL-17/IL-23 axis in the disease pathogenesis, demonstrated by biological therapies targeting this pathway. Circulating $CLA^+$ memory T cells, representing the subset of effector memory T lymphocytes that are functionally associated to the skin, constitute a profitable tool with translational value to understand the mechanisms underlying T cell-mediated skin conditions, such as psoriasis (Ferran et al. 2013b). Given this premise, we studied how relevant disease triggers, like microorganisms (*S. pyogenes* and *C. albicans*) and pro-inflammatory cytokines (IL-15 and IL-23), affect $CLA^+$ T cell responses in our *ex vivo* model of coculture. For those microbial environmental factors, we also studied the connection between *in vitro* cytokine responses and patients' exposure to the same microbe.

Currently, the study of microbiota composition in association to chronic diseases mainly rely on metagenomic profiling of different body regions, that identifies the presence of bacterial strains based on their DNA, but enough attention should be paid to the extent of contact between the host and that microbe in particular (Sharma and Gilbert 2018). Indeed, some authors have found discrepancies between molecular (DNA-based) and serological (antibody-based) studies (Zhang et al. 2018). Microbe-specific antibodies can be easily measured in blood and provide information not only of the exposure status to this microorganism but also about the possible site of encounter, regarding the diverse antibodies isotypes (IgG, IgA, IgE). Activated B cells rearrange and change the type of antibody they produce through the mechanism called isotype class switch, which generally occurs at secondary lymphoid organs and requires the help of T cells recognizing the same antigen and expressing co-stimulatory molecules. Based on the information that antibodies could give, we chose this straightforward approach for our study to relate adaptive $CLA^+$ memory T cell response with humoral responses to the same microorganism.

Streptococcal throat infections are strongly related to triggering or exacerbating skin lesions in both guttate and plaque forms of psoriasis, but specially in patients with early disease onset and who carry the HLA-Cw*06 allele (Wisenseel et al. 2002). Remarkably, regardless of the subtype of psoriasis, we observed increased anti-SE IgA and IgG levels in patients with positive HLA-Cw*06 (see APPENDIX I, Figure S1). This finding goes along with previous studies from our group showing increased cytokine response against *S. pyogenes* in guttate psoriasis patients positive for HLA-Cw*06 (Ruiz-Romeu et al. 2016b). Conversely, such association is not observed for *C. albicans*-specific antibodies levels in psoriasis (see Figure S1 in the APPENDIX I), which are indeed raised in patients negative for the HLA-Cw*06 allele.

Our study revealed raised levels of anti-SE IgA type 1 in plasma from plaque and guttate psoriasis patients, compared to healthy and atopic dermatitis subjects, even in patients with no history of recent streptococcal-mediated tonsillitis and negative anti-streptolysin O (ASO) antibody titer.

Nowadays, ASO titer is universally used for the clinical diagnosis of β-hemolytic streptococcal infections, whose serological levels peak around 3-6 weeks after infection but fall back to normal after 6 months. Because streptococcal sore throat often occurs close to guttate lesions flare-ups, increased ASO titers are commonly observed in these patients linking the onset of disease with the infection. Actually, ASO titers positively correlated with Th17, Th1 and Th9 responses *in vitro* by $CLA^+$ T/EPI cocultures from guttate psoriasis patients (Ruiz-Romeu et al. 2018) (also in APPENDIX II). However, the sustained course of plaque psoriasis masks the detection of upraised ASO titer, leading to a misleading disassociation with *S. pyogenes* infection in these patients. In the chronic form of psoriasis, streptococcal biofilm formation within the tonsils may constitute a reservoir of bacteria responsible for recurrent relapses, which would also explain the success of tonsillectomy in treating certain of these cases (Allen et al. 2018). In fact, despite negative ASO, our group has previously reported higher $CLA^+$ T cells cytokine responses against *S. pyogenes* in plaque psoriasis cocultures when compared with healthy controls (Ruiz-Romeu et al. 2018) (see APPENDIX II page 139). As the first defensive organ in the orogastric entry, the palatine tonsils are key part of the mucosal immune system and because they are extensively colonized by *S. pyogenes* in psoriasis, we suggest that anti-SE IgA blood levels may be an useful new parameter to shape exposure to *S. pyogenes* in psoriasis patients, particularly in those with chronic course of disease. Likewise, although anti-SE IgA levels were not significantly different between psoriasis subtypes, most of the patients with anti-SE IgA above the patients' average correspond to the chronic plaque rather than guttate type. Importantly, despite guttate psoriasis often clears spontaneously, in some patients the disease eventually evolves into chronic plaque psoriasis. Still no clear parameters have been established to identify guttate psoriasis patients who are more likely to progress towards the chronic form of the disease (Ko et al. 2010; Martin et al. 1996; Pfingstler et al. 2016). Hence, we propose the monitoring of anti-SE IgA levels in non-treated guttate psoriasis patients in future long-term follow-up studies for tracking guttate to plaque evolution.

*Candida albicans* has been associated to exacerbation of psoriatic lesions in humans(Fry and Baker 2007)) and mice models (Hurabielle et al. 2020), and increased mucosal colonization of *Candida* spp. is observed in psoriasis patients (Pietrzak et al. 2018), but insights on specific cellular and humoral response against this fungus in different types of psoriasis remained unexplored. Our study reported that psoriasis patients exposure to *Candida albicans* exerts a different pattern in plaque and guttate forms of psoriasis, showing significantly increased anti-CA IgA and IgG only in the former compared to healthy individuals. Despite candidiasis was not evaluated at the time of samples collection, fresh *Candida* infection was discarded later in both psoriasis and controls using a diagnostic kit based on specific IgA quantification. The fact that upraised antibodies against *C. albicans* appear in the chronic form of psoriasis could be explained

since these patients may have faced repeated cycles of immunosuppressive treatments over the course of disease. Effective psoriasis treatment reduces IL-17 responses in patients, which are essential for immunity against fungi (Okada et al. 2016; Puel 2020), therefore facilitating *C. albicans* colonization and induction of anti-CA IgA. The increased presence of this fungus will subsequently induce IL-17F/A responses by $CLA^+$ memory T cells, thus fueling the pro-inflammatory loop underlying psoriasis pathogenesis. A scheme of this hypothetical explanation is illustrated in the Figure S2 from the APPENDIX I (page 130). Indeed, positive correlation of disease duration with anti-CA IgA and IgA2 plasma levels is exclusively observed in plaque psoriasis patients. Despite B-cells are dispensable and antibodies may not be pathogenic per se in psoriasis (Thomas et al. 2019), increased IgA would rather indicate abnormal mucosal immune response, which may be related to patients heterogeneity and possible comorbidities. With this premise, a high throughput proteomic profile of plasma samples was assessed using Olink technology, which revealed increased proteins involved in antimicrobial humoral response, cell chemotaxis and inflammatory response in patients with higher anti-CA IgA levels. Although we could not confirm their association with *Candida*-specific IgA in an independent cohort of patients, possibly due to the lower number of patients, several proteins with anti-candida activity, such as RNASE3, CHI3L1 and AZU1, were significantly increased in plasma from plaque psoriasis patients compared to healthy controls. Likewise, CCL18, which mediates $CLA^+$ T cell homing to skin, was significantly increased in plaque versus guttate psoriasis and associated with disease severity. Of note, AZU1 and CCL18 have been related to periodontal disease (Davanian et al. 2012; Nalmpantis et al. 2020), which has been also described as another comorbidity of psoriasis (Zhang et al. 2020). Similarly, inflammatory bowel disease (IBD), which is related to increased presence of *C. albicans* in the gut mucosa (Gerard et al. 2015), is also considered a classical comorbidity of psoriasis. Interestingly, we observed similar anti-CA IgA levels in plasma from plaque psoriasis and IBD patients, both above the levels detected in guttate psoriasis (see Figure S3 in the APPENDIX I, page 131). Consequently, we suggest that future studies assessing anti-CA IgA levels may be valuable for plaque psoriasis patients stratification and evaluation of comorbidities.

Additionally, cellular immune response to *C. albicans* showed preferential induction of Th17 cytokines in $CLA^+$ T/EPI cocultures in psoriasis and healthy individuals. It is not uncommon that skin-tropic effector memory T cells actively recognize *Candida* spp. as they are part of our normal mycobiota, but this is of special interest in the context of psoriasis since *Candida*-induced IL-17 cytokines may fuel the pro-inflammatory loop present at cutaneous lesions in both plaque and guttate forms of disease. Selective IL-9 production by skin-tropic $CLA^+$ $CD4^+$ T cells after stimulation with *C. albicans* has been previously reported in healthy subjects (Schlapbach et al. 2014), however, we did not found such increased IL-9 response by $CLA^+$ T cells in healthy

controls, which is probably due to differences in the experimental design (they cocultured antigen-pulsed $CD14^+$ monocytes with purified $CD4^+$ memory T cells, whilst our coculture comprises epidermal cells as source of APCs and a mixture of $CD4^+$ and $CD8^+$ effector memory $CD45RA^-$ T cells). This same study also described increased presence of IL-9-positive cells in psoriatic lesional skin suggesting they may participate in cutaneous inflammation. Our data sustains this finding since significantly increased IL-9 induction by skin-tropic $CLA^+$ T cells against *Candida* was observed in plaque and guttate psoriasis patients compared with healthy individuals. Importantly, previous works from our group demonstrated how IL-9 directly supported IL-17A response and promoted $CLA^+$ T cell survival *in vitro* (Ruiz-Romeu et al. 2018) (see APPENDIX II, Figure 3 on page 138). Altogether, *Candida albicans* may contribute to cutaneous lesions because of its direct and indirect heightening of Th17 responses in both acute and chronic forms of psoriasis.

Of note, we reported a direct association between antigen-specific IgA and Th17 responses *in vitro*, which lead us to hypothesize about a T cell-B cell cooperation in psoriasis. Proinflammatory Th17 cells are effective B-cell helper and intestinal Th17 cells have been proved to contribute to high-affinity IgA production (Hirota et al. 2013); in fact, mice lacking the IL-17R showed impaired intestinal IgA response (Cao TA et al 2012). Despite the exact mechanism is not fully understood, unlike the direct contribution of IL-21 to IgA class switch, the role of IL-17 in IgA responses is more likely to be indirectly mediated by upregulating pIgR receptor then contributing to IgA secretion into the intestinal lumen (Cao et al. 2012; Dann et al. 2015), enhancing B-cell activators (Mitsdoerffer et al. 2010; Shibui et al. 2012) and modulating B-cells migration within the germinal centers (Ferretti et al. 2016). Besides their major role at the gastrointestinal mucosa, the presence of IgA secreting cells has been proved in healthy skin, where they uphold tissue homeostasis (Wilson et al. 2019). Importantly, altered B-cell subset, with generally increased IgA producing cells, and elevated serum IgA have been reported in psoriasis (Kahlert et al. 2019; Thomas et al. 2019). Therefore, we hypothesized that the cooperation of antigen-specific Th17 cells and B-cells may result in raised IgA response in psoriasis patients (see Figure S4 in the APPENDIX I, page 132). In the case of *S. pyogenes*, we demonstrated a direct correlation between anti-SE IgA and $CLA^+$ T cell-induced IL-17F and IL-17A responses in our *ex vivo* model of disease for plaque and guttate psoriasis respectively. Coexistence of *S. pyogenes* and $CLA^+$ T cells in the tonsils lead to the hypothesis that cutaneous lesions are mediated by tonsillar T cells that migrate towards the skin, where they are activated by proinflammatory environment inducing IL-17 secretion (Valdimarsson et al. 2009). Our observation reinforces this link between palatine tonsils (IgA production), group A streptococci (*S. pyogenes*) and skin immune response ($CLA^+$ T cells) in psoriasis. Similarly, levels of anti-*C. albicans* IgA in plasma positively correlated to IL-17F and IL-17A induction by this fungus in $CLA^+$ T and $CLA^-$ T/EPI cocultures from plaque

psoriasis patients exclusively. This would involve that *Candida*-specific Th17 cells cooperate with B-cells both in cutaneous tissue, linked to the presence of $CLA^+$ T cells, such as the skin or oral mucosa, and extracutaneous sites, such as gastrointestinal tract, in which $CLA^-$ T cells are mainly found.

Finally, exploring the influence of the lesional pro-inflammatory cytokines IL-15 and IL-23 in our *ex vivo* model of psoriasis unveiled their synergistic effect over IL-17F and IL-17A induction by $CLA^+$ T cells in cocultures from psoriasis but not healthy controls. Hoeve and colleges showed that IL-23 and IL-15 independently enhanced IL-17 secretion only in mitogenically stimulated T cells, but not in the resting ones (Hoeve et al. 2006). Particularly, we observed the synergy between IL-15 and IL-23 in the absence of direct T-cell activation (polyclonal or antigenic) but exclusively in the presence of autologous epidermal cells. This demand of epidermal cells may be related to the requirement of previous T cell activation reported by Hoeve, which could involve the presentation of autoantigens (ADAMTSL5 or LL37) by epidermal cells *in vitro* in our model. Actually, the finding that HLA-I and HLA-II neutralization reduced IL-15/IL-23-induced Th17 and Th1 cytokines secreted by $CLA^+$ T cells further support this theory.

Alternatively, the requisite presence of epidermal cells could implicate tissue resident memory T cells ($T_{RM}$) as part of the epidermal cellular mixture, which are increased and have been implicated in the maintenance and recurrence of psoriatic lesions (Gallais Sérézal et al. 2019; Rachael A. Clark 2015). Nonetheless their contribution to the synergic effect of IL-15 and IL-23 was discarded by various observations. On the one hand, the induction of IL-17F/A was not observed neither in $CLA^-$ T/EPI cocultures nor in single cultures of epidermal cells activated with the IL-15/IL-23 cocktail. Furthermore, lymphocytic infiltration of the epidermal compartment barely reached 4% of the total cell suspension, implying a limited number of $T_{RM}$ cells in our coculture condition. Last but not least, when increasing the number of epidermal cells cultured alone, IL-17F/A and IFN-γ levels increased after IL-15 stimulation but no synergistic effect with IL-23 was found. Altogether, the observation that this cooperative effect is restricted to $CLA^+$ memory T cells could be particularly relevant for our understanding of the cutaneous immune response in psoriasis.

*In vivo* studies suggested a functional role for IL-15 in psoriasis, since its neutralization resolved or abrogated the development of psoriasis-like skin inflammation in different xenograft models (Bouchaud et al. 2013; Villadsen et al. 2003). In humans, SNPs in the IL-15 gene have been associated with psoriasis in Caucasians and Chinese populations (Weger et al. 2008; Zhang et al. 2007). In addition to its pro-inflammatory nature, IL-15 is known to inhibit differentiation and apoptosis of keratinocytes (Luo et al. 2016; Ruckert et al. 2000), which are both hallmarks of

psoriasis pathogenesis. However, its functional role over Th17 cells and IL-17 cytokines induction in psoriasis is poorly characterized.

In our model, IL-15 alone is even more effective than IL-23 in enhancing IL-17F and IL-17A production by psoriatic $CLA^+$ T cells, as previously reported for healthy human T cells (Hoeve et al. 2006). And what is more, we reported a considerable reduction of IL-17F, and to a lesser extent IL-17A and IFN-γ, production by $CLA^+$ T cells after IL-15 neutralization. This effect was partially observed with the IL-23p19 blockade solely. On this basis, we proposed the bifunctional neutralization of IL-15 and IL-23 as an alternative therapeutic approach to improve psoriasis management. Interestingly, a recent work modeling different cytokines networks involved in psoriasis pathogenesis suggested that targeting IL-15 and IL-23/IL-17 at the same time would also be more effective (Pandey et al. 2021).

Additionally, special attention should be paid to the relevance of IL-17F in psoriasis. Our results indicate that IL-15 and IL-23 induced a higher production of IL-17F than IL-17A by $CLA^+$ T cells. This preferred IL-17F response was observed after *C. albicans* stimulation as well, and has been previously reported by our group for *S. pyogenes* (Ruiz-Romeu et al. 2018; Ruiz-Romeu et al. 2016b). Besides, higher IL-17F than IL-17A concentration has been shown in serum (Kolbinger et al. 2017) and skin (Soderstrom et al. 2017) samples from psoriasis patients. Most importantly, the clinical relevance of IL-17F in psoriasis has been recently supported by the increased efficacy of bimekizumab, a neutralizing antibody targeting IL-17A, IL-17F and IL-17A/F heterodimer, when compared to ustekinumab, a selective IL-17A inhibitor (Reich et al. 2021).

In summary, this work established how environmental triggers such as microbes or lesional pro-inflammatory cytokines interact specifically with circulating $CLA^+$ T cells, the subset of memory T cells representing the cutaneous adaptive immune system in psoriasis. First, plasma levels of *S. pyogenes* and *C. albicans*-specific IgA indicated that non-treated psoriasis patients display heterogenic exposition to these microbes that directly induce IL-17F and IL-17A responses, thus probably fueling psoriatic immunopathogenesis. This is a matter that we believe should be further explored in the clinic. And finally, this study demonstrated the synergic action of IL-15 and IL-23 in inducing $CLA^+$ Th cell-dependent IL-17F/A responses in psoriasis, supporting the still scarcely characterized role of IL-15 in psoriasis inflammation.

# CONCLUSIONS

During the elaboration of this thesis, we have reached the following conclusions on the study of $CLA^+$ T cell response to:

*Streptococcus pyogenes*

- Plaque and guttate psoriasis patients present increased *Streptococcus pyogenes*-specific IgA compared with healthy and atopic dermatitis subjects, indicating higher exposure to this microorganism despite no history of throat infection and negative ASO titer.
- Levels of *S. pyogenes*-IgA are directly associated to IL-17F and IL-17A responses by $CLA^+$ memory T cells against this bacterium in plaque and guttate psoriasis respectively, supporting the link between *Streptococcal* presence in tonsils and cutaneous lesions in psoriasis.

*Candida albicans*

- Plaque, but not guttate, psoriasis patients display increased plasma levels of IgA and IgG against *Candida albicans*, which is probably related to disease chronicity.
- Cytokine response to *C. albicans* is restricted to $CLA^+$ T cells and dominated by IL-17F in psoriasis and healthy controls, whereas *Candida*-induced IL-9 is significantly higher in psoriasis.
- Levels of *C. albicans*-specific IgA, but not IgG, are directly associated with IL-17F and IL-17A responses by $CLA^+$ and $CLA^-$ memory T cells in plaque psoriasis, suggesting cutaneous and extra-cutaneous implications of this fungus.
- Plaque psoriasis patients present raised plasma levels of CCL18, CHI3L1 and AZU1 proteins compared with guttate and controls, which have anti-candida properties and are likely related to periodontitis.

Lesional pro-inflammatory cytokines: IL-15 and IL-23

- IL-15 and IL-23 synergistically induce IL-17F and IL-17A responses particularly by skin-tropic $CLA^+$ $CD4^+$ T cells in psoriasis, but not in psoriatic $CLA^-$ T cells or in healthy controls.
- The synergy between IL-15 and IL-23 on Th17 cells requires the presence of autologous epidermal cells but does not depend on skin resident memory T cells.

Of particular interest, our results support IL-17F as a fundamental mediator in psoriasis, which is secreted to a greater extent than IL-17A by $CLA^+$ memory T cells after stimulation with both microbial and lesional pro-inflammatory cytokines.

# IMPLICATIONS AND FUTURE PERSPECTIVES

This thesis confirms that circulating $CLA^+$ effector memory T cells are key responders to relevant psoriasis triggers, preferentially inducing IL-17 cytokines. Therefore, upon being actively recruited to lesional skin, these cells may contribute to the feedforward inflammatory loop and perpetuate disease pathogenesis. Certainly, new questions arise from the results of this thesis some of which are detailed below:

- Is antigen-specific IgA better than ASO to evaluate long term exposure to *S. pyogenes* in chronic psoriasis?
- Could *S. pyogenes*-specific IgA levels be used to monitor patients with guttate psoriasis that are more likely to progress towards the chronic form of disease?
- Concerning the appearance of candidiasis in patients treated with IL-17 inhibitors, could measuring *C. albicans*-specific IgA levels prior treatment be useful to classify psoriasis patients candidates for the most suitable treatment approach?
- Is *C. albicans* exposure a reliable link to IL-17-associated comorbidities in psoriasis?
- Are antigen-specific Th17 and B cells cooperating to induce microbe-specific IgA in psoriasis? If so, where does this collaboration take place?
- Regarding the IL-15 and IL-23 synergy, which are the antigens presented by MHC-I and MHC-II on epidermal cells that contribute to T cell activation? Are they presented by keratinocytes, Langerhans cells or both?
- Are there some other intermediate mediators, probably released from epidermal cells, involved in the IL-15 and IL-23 synergistic effect?

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# APPENDIX I: Supplementary figures

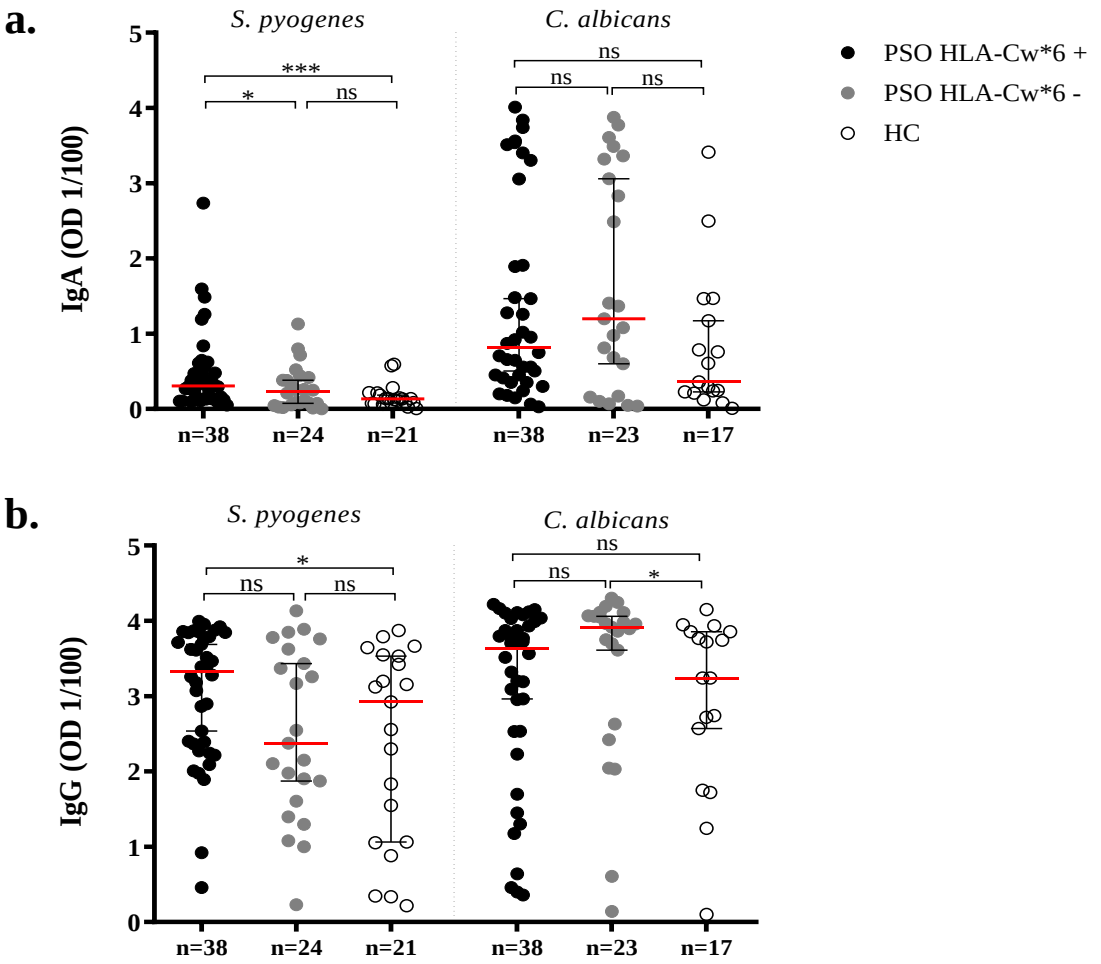


**Figure S1. Figure S1. Analysis of *S. pyogenes* and *C. albicans* specific humoral response regarding presence of HLA-C*6 allele in psoriasis patients.** Microbe-specific **(a)** IgA and **(b)** IgG levels are shown, measured as optical density (OD) of 1/100 plasma dilutions. PSO: psoriasis; HC: healthy controls. Mann-Whitney tests was used to compare between groups. P values are indicated as: ns: p >0.05; *: p <0.05; ***: p<0.001

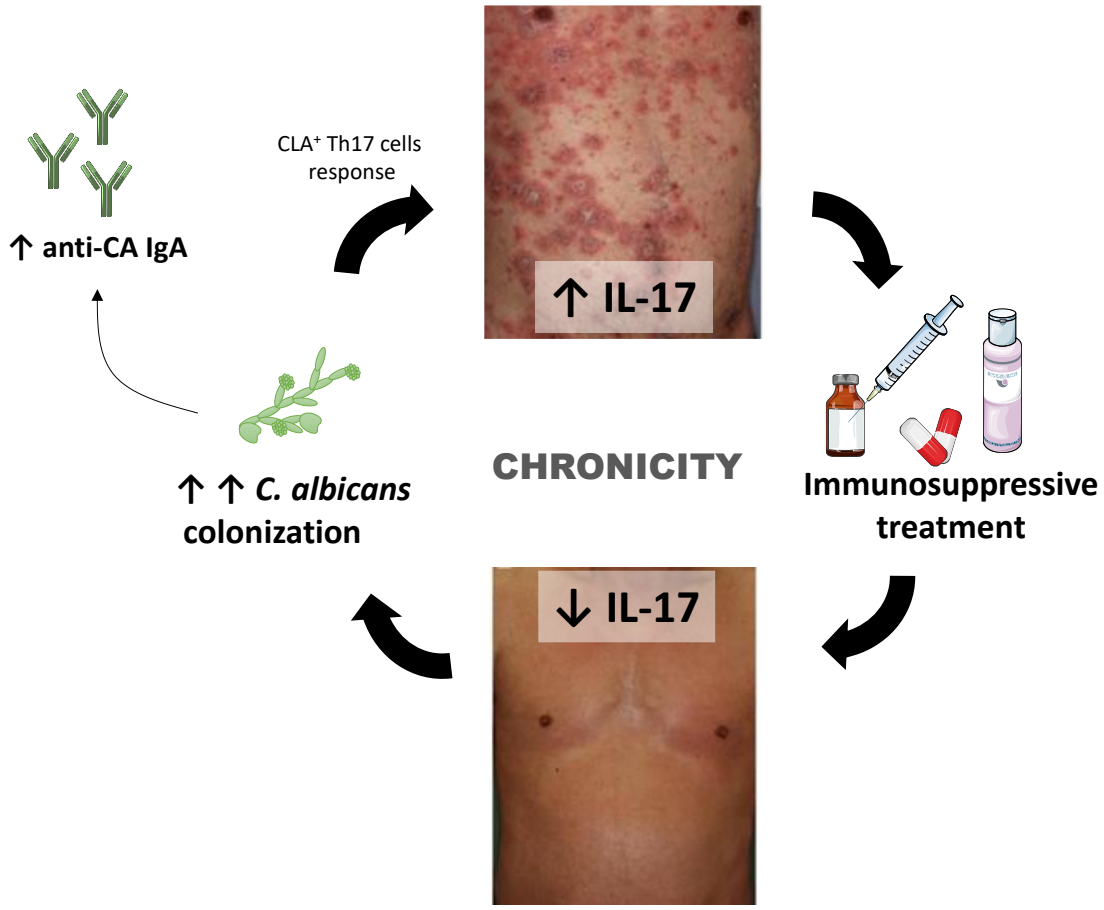


**Figure S2. Hypothetic model of *C. albicans* increased exposure in plaque psoriasis.** Plaque psoriasis patients may face repeated cycles of immunosuppressive treatments over the course of disease. Effective psoriasis treatments reduce IL-17 responses in patients, which are essential for anti-fungal immunity. This leads to increase *C. albicans* colonization, as revealed by the presence of anti-CA IgA, and specific induction of IL-17F/A by $CLA^+$ T cells, contributing to the pro-inflammatory loop underlying psoriasis pathogenesis and responsible for recurrent flare-ups. Patient images obtained from Ochi M, et al CEN Case Rep. 2019.

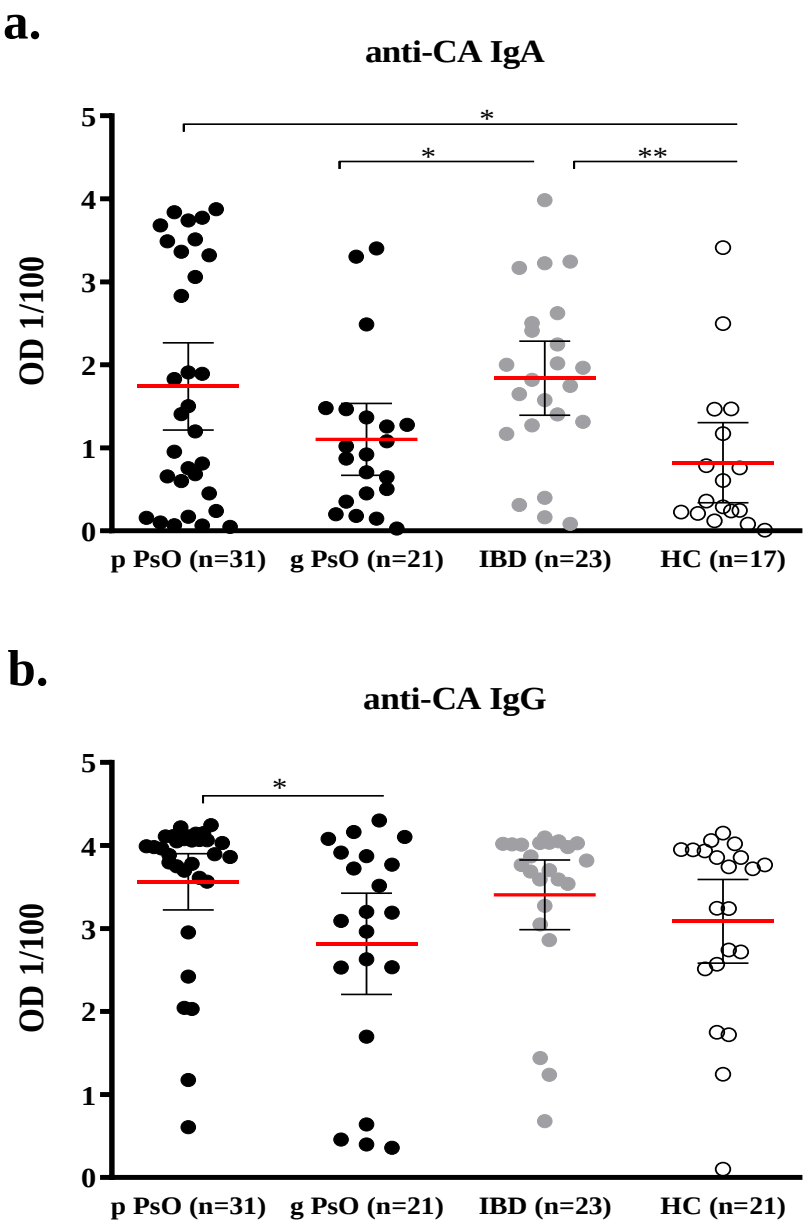


**Figure S3. Similar *Candida*-specific IgA and IgG in plaque psoriasis and inflammatory bowel disease patients.** Levels of (a) IgA and (b) IgG specific for *C. albicans* are shown, measured as optical density (OD) of 1/100 plasma dilutions. pPSO: plaque psoriasis; gPSO: guttate psoriasis; IBD: inflammatory bowel disease; HC: healthy controls. Mann-Whitney tests was used to compare between groups; p values are indicated as: *: $p < 0.05$; **: $p < 0.01$

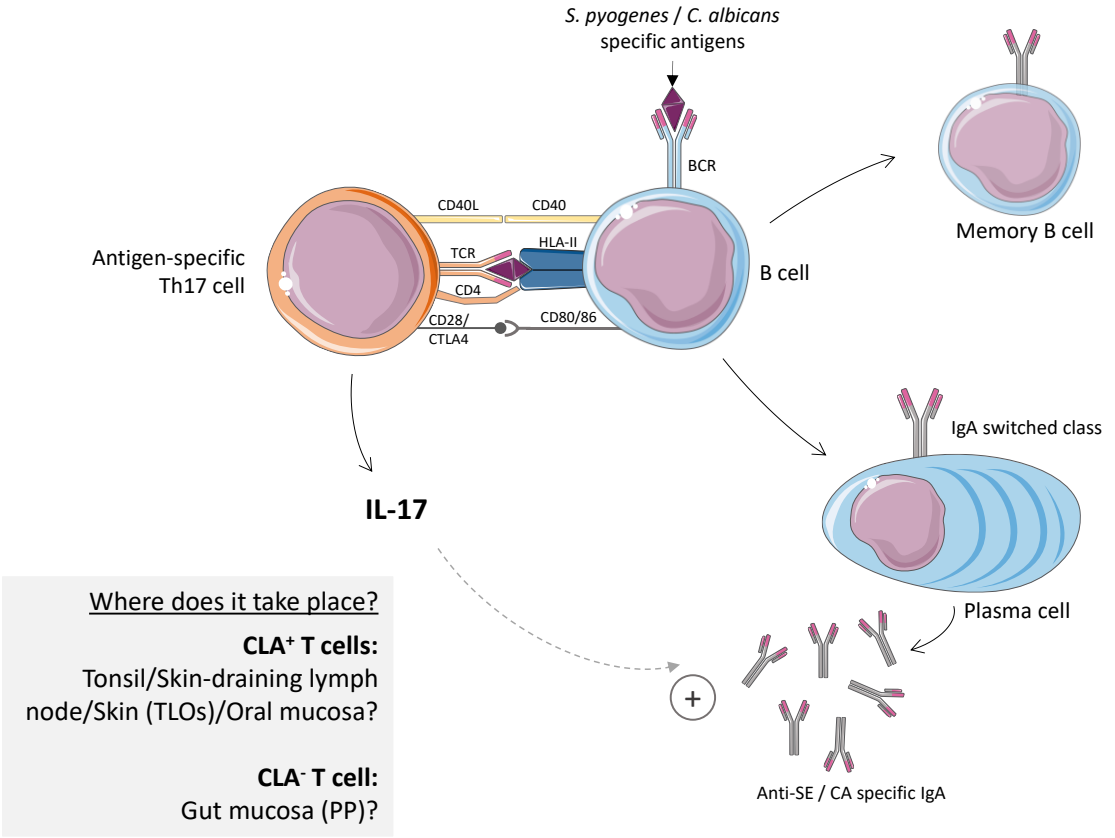


**Figure S2. Hypothetic model of *S. pyogenes* and *C. albicans*-specific Th17 cell-B cell cooperation in psoriasis.** In psoriasis patients, Th17 cells recognizing specific antigens from *Streptococcus pyogenes* or *Candida albicans* may cooperate with B cells specific to the same antigen, that will be internalized and presented within their HLA-II to the specific TCR on Th17 cells, and together with the interaction of co-stimulatory molecules (CD40L-CD40, CD8/CTLA4-CD80/CD86) will lead to: 1) activation of Th17 cells and induction of IL-17 and other cytokines and 2) activation and clonal expansion of B cells, which will differentiate into memory B cells and plasma cells that will undergo IgA class switch, indirectly favored by IL-17, and secrete high-affinity IgA antibodies against these microbes. This phenomenon of T and B cell collaboration occurs in specialized follicular environments, therefore we propose tonsils, skin-draining lymph nodes, tertiary lymphoid organs (TLOs) in the skin or oral mucosa for *S. pyogenes* or *C. albicans*-specific $CLA^+$ Th17 cells and Peyer's patches (PP) at the gut mucosa for C. albicans-specific $CLA^-$ Th17 cells. BCR: B cell receptor, CA: C. albicans, SE: S. pyogenes, TCR: T cell receptor.

# APPENDIX II: Supporting publications

ORIGINAL ARTICLE

# Microbe-Dependent Induction of IL-9 by CLA$^+$ T Cells in Psoriasis and Relationship with IL-17A

Ester Ruiz-Romeu[1], Marta Ferran[2], Carmen de Jesús-Gil[1], Pablo García[2], Marc Sagristà[3], J.M. Casanova[4], J.M. Fernández[4], Anca Chiriac[5], Péter Hóllo[6], Antonio Celada[7], Ramon M. Pujol[2] and Luis F. Santamaria-Babí[1]

IL-9 is present in psoriatic lesions and is produced by lymphocytes. However, it is not known whether this cytokine is induced by relevant pathogenic triggers of psoriasis, such as *Streptococcus pyogenes*. Here we addressed the production of IL-9 in response to various pathogens in a psoriatic ex vivo model. Extracts of *S. pyogenes* and *Candida albicans* triggered the production of IL-9 and also IL-17A and IFN-γ. This induction was dependent on the interaction between CLA$^+$ T cells and epidermal cells. Neutralization of IL-9 reduced *S. pyogenes*-induced IL-17A production by CLA$^+$ T cells but had no effect on IFN-γ production. Also, IL-9 increased the survival of circulating psoriatic CLA$^+$ T cells. Co-cultures from patients with guttate or plaque psoriasis with *S. pyogenes* produced similar amounts of IL-9. High cytokine responses in streptococcal-driven guttate patients paralleled peaks in Psoriasis Area Severity Index and anti-streptolysin O levels. Our results confirm that IL-9 promotes inflammation in psoriasis by up-regulating IL-17A production and support the clinical association of the immune response by streptococcal-sensitized CLA$^+$ T cells with this cytokine, especially in guttate psoriasis.

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## INTRODUCTION

The onset of guttate psoriasis is preceded by tonsil infection by *Streptococcus pyogenes* in 56–97% of patients (Prinz, 2001), and raised anti-streptolysin O (ASO) titers are commonly detected. Furthermore, the streptococcal influence is not limited to this acute subtype of psoriasis. Indeed, there is evidence that throat infection by this microbe in patients with plaque psoriasis exacerbates the symptoms of the disease (Gudjonsson et al., 2003). Also, such infections are approximately 10 times more frequent in individuals with chronic plaque psoriasis than in a control population (Gudjonsson et al., 2003). The co-culture of circulating skin-homing cutaneous lymphocyte-associated antigen (CLA)$^+$ T cells with autologous epidermal cells and activation by an extract of *S. pyogenes* (SE) provides an ex vivo model through which to study psoriasis. Such co-cultures have shown a high production of psoriasis-associated cytokines, including the main signature products of T cytotoxic (Tc) 17/T helper type (Th) 17 and Tc1/Th1 cells, namely IL-17A and IFN-γ, respectively (Ferran et al., 2013b; Ruiz-Romeu et al., 2016). Therefore, the components of this ex vivo approach provide an optimal scenario in which to address other less known inflammatory mediators in psoriasis.

For instance, there is mounting evidence that the cytokine IL-9 and Th9 cells are clinically relevant in humans, especially regarding chronic autoimmune and inflammatory systemic diseases (Burkhardt et al., 2009; Ciccia et al., 2015, 2016). Recently, an association between Th9 and skin in humans has been described, because healthy blood-derived Th9 cells are mainly an effector skin-homing CLA$^+$ T-cell population (Schlapbach et al., 2014). In addition, it has been proposed that IL-9 has a putative role in psoriasis; however, little is known about its functional role in patients with psoriasis. Notably, *IL9* is present in the psoriatic susceptibility region (5q31.1) (Friberg et al., 2006; Modi et al., 1991), it is found in supernatants from CD4$^+$ T cells from psoriasis patients after polyclonal activation, and psoriatic skin lesions have higher numbers of IL-9R$^+$ and IL-9$^+$ cells than samples from healthy subjects (Schlapbach et al., 2014; Singh et al., 2013). IL-9 may be involved in Th17 inflammation and angiogenesis in a murine model of psoriasis (Singh et al., 2013).

Here, we report that IL-9 was produced mostly by CLA$^+$ T cells in SE-activated psoriatic co-cultures and that this

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*Abbreviations: ASO, anti-streptolysin O; CA, Candida albicans; CLA, cutaneous lymphocyte-associated antigen; CLA$^-$/Epi, co-cultures of CLA$^-$ T cells and autologous epidermal cells; CLA$^+$/Epi, co-cultures of CLA$^+$ T cells and autologous epidermal cells; DC, dendritic cell; HLA, human leukocyte antigen; PASI, Psoriasis Area Severity Index; SE, Streptococcus pyogenes extract; SEB, superantigen staphylococcal enterotoxin B; Th, T helper type*

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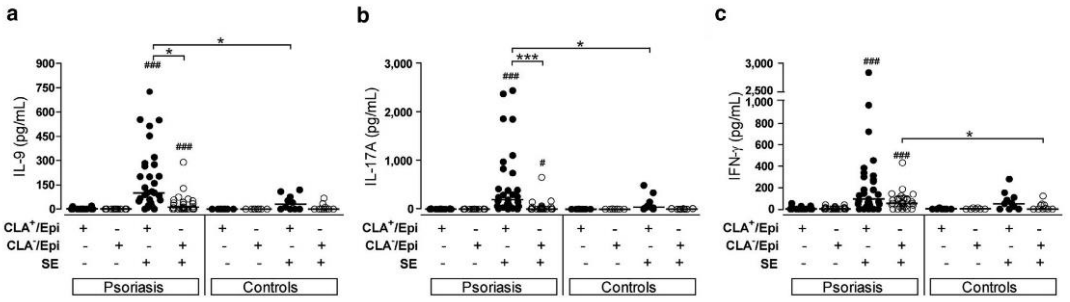


**Figure 1. Circulating psoriatic memory CLA$^+$ T cells produce IL-9, together with IL-17A and IFN-γ, upon activation with *Streptococcus pyogenes*.** Peripheral circulating psoriatic (n = 31) or healthy control (n = 10) CD45RA$^-$CLA$^+$ or CLA$^-$ T cells were co-cultured with autologous epidermal cells (Epi), and (**a**) IL-9, (**b**) IL-17A, and **(c)** IFN-γ were measured in supernatants after 5 days of stimulation with SE. Cell types and presence (+) or absence (−) of pathogens for each co-culture condition are shown below the x-axis. Data are represented by dot plots, each representing the values of a supernatant from a patient-derived co-culture, and by the median. *P*-value symbols (#) indicate differences with basal untreated conditions. $^*P < 0.05$, $^{\#}P < 0.05$, $^{\#\#\#}P < 0.001$. CLA, cutaneous lymphocyte antigen; CLA$^-$/Epi, co-cultures of CLA$^-$ T cells and autologous epidermal cells; CLA$^+$/Epi, co-cultures of CLA$^+$ T cells and autologous epidermal cells; SE, *S. pyogenes* extract.

production was dependent on the interaction of epidermal cells and human leukocyte antigen (HLA) class I and II presentation. This response showed a similar pattern to that of IL-17A, the production of which was in turn enhanced by IL-9. Furthermore, during guttate psoriasis flares derived from streptococcal infection, such cytokine production peaked after 1−2 months of disease duration and was associated with a high psoriasis area severity index (PASI) score and ASO titer results.

## RESULTS

### SE induces the production of IL-9, together with IL-17A and IFN-γ, by circulating effector memory CLA$^+$ T cells and autologous epidermal cells in psoriasis

Supernatants generated from 31 co-cultures performed with clinical samples from patients with psoriasis and 10 healthy control subjects were collected at day 5 for IL-9 quantification. IL-17A and IFN-γ were determined simultaneously within the same well (Figure 1). IL-9 was preferentially induced in psoriasis-derived co-cultures containing CLA$^+$ T cells (median = 101.3 ± interquartile range (IQR) = 40.3/275.7 pg/ml) over non−skin-homing CLA$^-$ T cells (median = 11.88 ± IQR = 0/42.51 pg/ml) and over co-cultures of CLA$^+$ T cells and autologous epidermal cells (CLA$^+$/Epi) from healthy control samples (median = 27.03 ± IQR = 0/83.7 pg/ml) (Figure 1a). A similar response was observed for the production of IL-17A (Figure 1b) and IFN-γ (Figure 1c), the latter also showing a higher production in activated co-cultures of CLA$^-$ T cells and autologous epidermal cells (CLA$^-$/Epi) than in those from healthy control subject samples ($P < 0.05$) (Figure 1c).

Other pathogens, such as *Candida albicans* (CA) and staphylococcal enterotoxin B (SEB), may activate this co-culture system (Ferran et al., 2013b). However, when CLA$^+$/Epi or CLA$^-$/Epi co-cultures were directly challenged with CA, IL-9 production was not observed in healthy donors. In this regard, such production was specific to psoriasis-derived CLA$^+$/Epi co-cultures (see Supplementary Figure S1a online), as observed for SE. These selective responses of CLA$^+$/Epi co-cultures to SE and CA in psoriatic samples were not due to a poor response from the co-cultures of healthy control samples, because SEB activation exerted the same IL-9 induction capacity in both types of donor (see Supplementary Figure S1b).

CLA$^+$ and CLA$^-$ T cells were co-cultured with autologous dendritic cells (DCs) or epidermal cells. Activation with SE, CA, and SEB (see Supplementary Figure S2 online) preferentially induced the production of IL-9 (see Supplementary Figure S2a−c), IL-17A (see Supplementary Figure S2d−f), and IFN-γ (see Supplementary Figure S2g−i) by CLA$^+$ T cells only in the presence of epidermal cells (n = 3). However, such a selective response by CLA$^+$ T cells was not observed when using DCs (n = 3). Thus, CLA$^+$ and CLA$^-$ T cells produced IL-9 (see Supplementary Figure S2a−c), whereas CLA− T cells produced more IFN-γ than CLA$^+$ T cells (see Supplementary Figure S2g−i). Neither DCs nor epidermal cells alone showed cytokine production in the presence of SE (see Supplementary Figure S2d−f).

### SE-induced IL-9 production by circulating CLA$^+$ T cells requires autologous epidermal cells and is dependent on HLA-mediated presentation

To understand the contribution of epidermal cells to IL-9 production in the co-culture, the IL-9 content, along with that of IL-17A and IFN-γ, was quantified in cultures with either T cells alone or with lesional epidermal cells (Figure 2a−c). SE activity was minimal in purified memory T-cell cultures, and the presence of autologous lesional epidermal cells led to the production of IL-9, IL-17A, and IFN-γ, greatly enhancing IL-9 and IL-17A production by CLA$^+$ T compared with CLA$^-$ T cells ($P < 0.05$, Figure 2a and b). Such effector responses were dependent on HLA class I and II molecules, because when these molecules were neutralized in the co-culture, cytokine production was inhibited by about 50% and 90−100%, respectively (Figure 2d−f, and see Supplementary Figure S3 online). Thus, potential interactions with SE-derived antigen(s), through HLA class I or II molecules, may be involved in the production of IL-9 (Figure 2d) and in that of IL-17A and

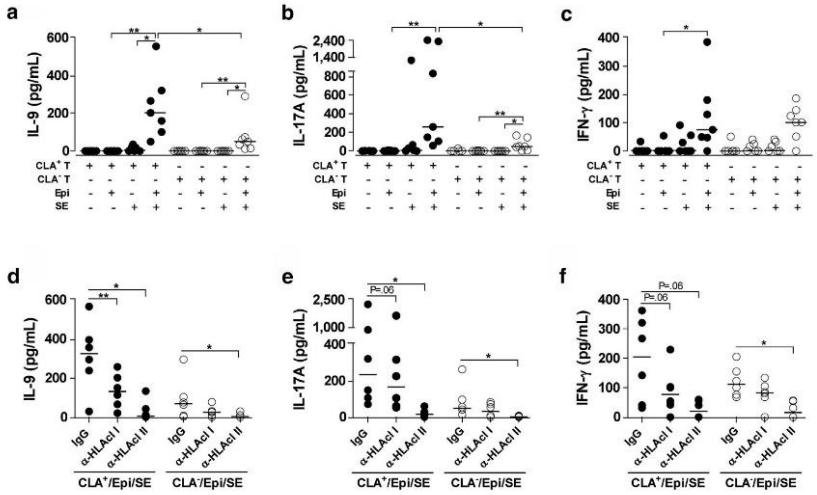


**Figure 2. *Streptococcus pyogenes*-associated IL-9, IL-17A, and IFN-γ production by psoriatic co-cultures is dependent on the presence of epidermal cells and on HLA class I and II molecules.** $CLA^+$ or $CLA^-$ T cells alone or co-cultured with autologous epidermal cells were activated with SE or left untreated. (**a**) IL-9, (**b**) IL-17A, and (**c**) IFN-γ were measured in each cell culture supernatant after 5 days (n = 7). Cell types and presence (+) or absence (−) of SE for each culture condition are shown below the x-axis. HLA class I and class II molecules were neutralized at day 0 in the SE-activated co-cultures, or co-cultures were treated with control IgG isotype. (**d**) IL-9, (**e**) IL-17A, and (**f**) IFN-γ measured at day 5 are shown (n = 6), and pairwise compared with respect to isotype values. Data are represented by dot plots, each representing the values of a supernatant from a patient-derived co-culture, and by the median. CLA, cutaneous lymphocyte-associated antigen; $CLA^-$/Epi, co-cultures of $CLA^-$ T cells and autologous epidermal cells; $CLA^+$/Epi, co-cultures of $CLA^+$ T cells and autologous epidermal cells; HLA, human leukocyte antigen; SE, *S. pyogenes* extract. $^*P < 0.05$, $^{**}P < 0.01$.

IFN-γ (Figure 2e and f, respectively), This notion is consistent with previously reported results (Ferran et al., 2013b). Activation of CD4-depleted psoriatic co-cultures with SE or CA did not lead to the detectable production of IL-9, IL-17A, or IFN-γ (see Supplementary Figure S4a–c online). However, IL-17F, which is usually produced in higher amounts than IL-17A and is also known to be generated by the co-cultures (Xue et al., 2016), was detected in two of four experiments with SE (see Supplementary Figure S4d).

### Kinetics of SE-dependent production of IL-9 by $CLA^+$ T cells and contribution to IL-17A production in psoriasis

The kinetics of IL-9 production upon SE stimulation of psoriasis-derived $CLA^+$/Epi co-cultures were examined at different time points during 5 days of culture. IL-9, IL-17A, and IFN-γ followed the same increasing tendency along the 5 days (Figure 3a). Although IL-9 displayed no peak preceding IL-17A and IFN-γ production, blocking assays showed that IL-17A production showed a 50% dependence on IL-9, whereas no such clear effect was observed for IFN-γ production (Figure 3b, and see Supplementary Figure S5 online). Because T-cell survival is increased by IL-9 (Parrot et al., 2016) and Th17 cells express high levels of IL-9R (Elyaman et al., 2009; Nowak et al., 2009), we assessed T-cell survival in psoriatic $CLA^+$ and $CLA^-$ T cells. IL-9 reduced the percentage of T cells undergoing early apoptosis and increased the percentage of living cells within the $CLA^+$ subset (see Supplementary Figure S6a, S6b online).

### SE-induced production of IL-9 by in $CLA^+$/Epi co-cultures in guttate and plaque psoriasis-derived samples

The cytokine profile was examined in guttate and plaque psoriasis (Figure 4). In guttate psoriasis-derived co-cultures activated by SE, a significant paired difference was found for IL-17A (median = 293.6 ± IQR = 48.5/979.4 pg/ml) compared with IL-9 (median = 101.3 ± IQR = 22.6/275.7 pg/ml) or IFN-γ (75.5 ± 35.2/300.9 pg/ml) values within the same supernatants, thereby confirming a clear predominant Th17 profile. Conversely, no clear preferential response toward IL-9, IL-17A, or IFN-γ production was found in plaque psoriasis, because the respective amounts measured were similar (median = 110 ± IQR = 42.6/406.3, median = 139 ± IQR = 56.35/352.1, and median = 111.4 ± IQR = 24.9/304.2 pg/ml, respectively).

### Peripheral $CLA^+$ T-cell effector response to *S. pyogenes* in guttate psoriasis parallels ASO levels and PASI score with a common peak after disease onset

Guttate flares with positive ASO titers (≥200 IU/ml) and preceded by throat infections (n = 13) showed variable ASO levels and PASI scores when they were distributed on the basis of duration of disease, from 15 days to 4 months, in which still there was evidence of a prior pharyngitis (Figure 5a). The highest ASO blood levels and PASI scores were found among guttate flares between 1 and 2 months after disease onset. Lower ASO titer results (mean < 200 IU/ml) were found in two of the three patients with long-duration (>1 year) guttate psoriasis with no clear history of streptococcal infection. Despite incomplete resolution of their lesions, these patients presented milder severity in

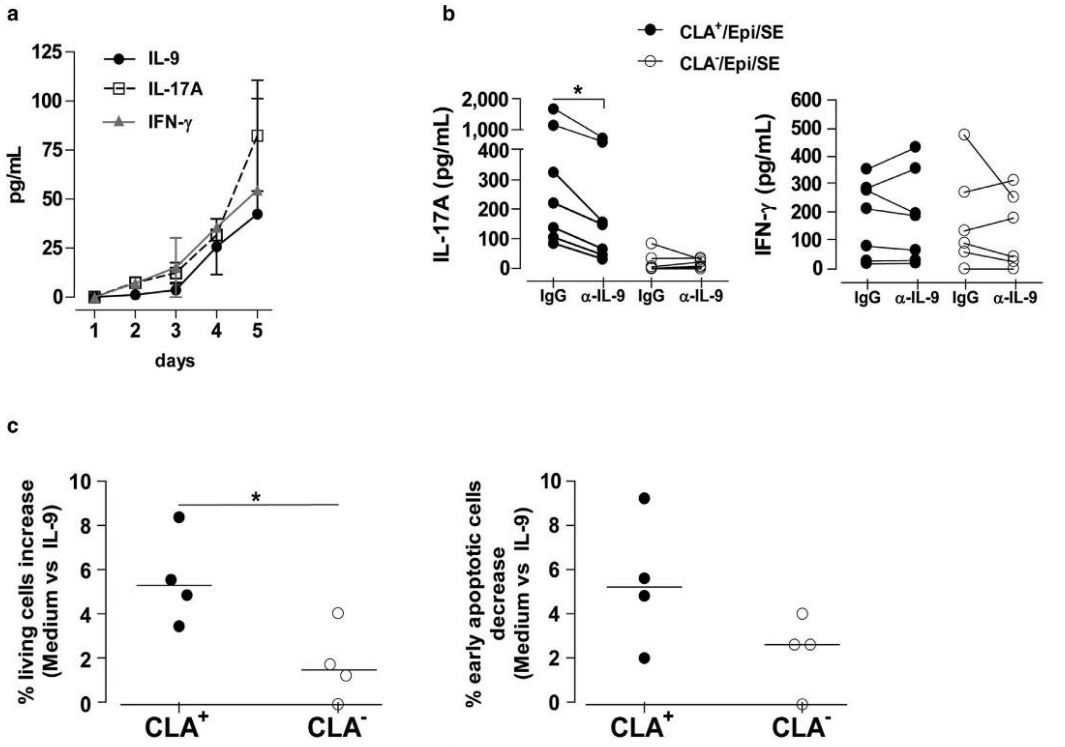


**Figure 3. IL-9 production induced by *Streptococcus pyogenes* follows similar kinetics to the production of IL-17A and IFN-γ and enhances IL-17A production.** (**a**) IL-9, IL-17A, and IFN-γ were measured in supernatants from psoriatic $CLA^+$/Epi co-cultures that were activated for 1 (n = 2−3), 2 (n = 1−2), 3 (n = 2−3), 4 (n = 1−2), and 5 (n = 2−3) days with SE. Each symbol represents the median ± interquartile range. (**b**) Neutralizing antibodies against IL-9 or IgG isotype control were added at day 0 of culture and were activated by SE. Next, IL-17A and IFN-γ levels were measured in 5-day supernatants from psoriatic $CLA^+$/Epi and $CLA^-$/Epi co-cultures (n = 7), and paired results were compared with respect to isotype values. (**c**) $CLA^+$ and $CLA^-$ T cells were cultured with or without IL-9 for 5 days. An increase or decrease in living or early apoptotic cells, respectively, is shown (n = 4). Each dot represents the values of a supernatant from a patient-derived co-culture. *$P < 0.05$. CLA, cutaneous lymphocyte-associated antigen; $CLA^-$/Epi, co-cultures of $CLA^-$ T cells and autologous epidermal cells; $CLA^+$/Epi, co-cultures of $CLA^+$ T cells and autologous epidermal cells; SE, *S. pyogenes* extract.

terms of PASI score. In contrast, patients with plaque psoriasis, who had long-duration chronic disease, presented high PASI scores but had negative ASO titer results, thus confirming the reported lack of prior throat infections in this group (n = 10) (Figure 5a). Such clinical temporal variability observed in guttate psoriasis samples was also reproduced in terms of cytokine responses, in the $CLA^+$/Epi/SE condition for IL-9, IL-17A, and IFN-γ (Figure 5b), and the highest values were found again in $CLA^+$/Epi/SE co-cultures derived from guttate samples from patients with 1−2 months of disease duration. Consequently, ASO and cytokine levels finely correlated (Figure 5c, upper panels), showing significance in the case of IL-9 ($r = 0.68$, $P = .003$) and IL-17A ($r = 0.55$, $P = .02$) production and near significance for that of IFN-γ ($r = 0.45$, $P = .07$). Similarly, the PASI score significantly correlated with IL-9 ($r = 0.61$, $P = .01$) and IFN-γ ($r = 0.55$, $P = .02$) production and near significance was found with IL-17A levels ($r = 0.48$, $P = .06$) (Figure 5c, lower panels).

## DISCUSSION

The role of IL-9 in human psoriasis is poorly characterized, and it is not known whether clinically associated triggers of psoriasis can induce the production of this cytokine. Using an ex vivo model of psoriasis, we show CLA-dependent production of IL-9 upon activation with *S. pyogenes* and *C. albicans*, a supporting role in IL-17A response, and an association with clinical features in guttate flares.

Given the biomarker capacity of peripheral $CLA^+$ T cells, they may provide evidence of relevant disease-associated inflammatory mediators (Czarnowicki et al., 2017; Ferran et al., 2013a). In our model, IL-9, together with IL-17A and IFN-γ, was preferentially produced by psoriatic memory $CLA^+$ T cells upon activation with SE and in the presence of autologous lesional epidermal cells. This model more accurately matches a cutaneous-like context, because a highly specific immune response by skin-homing $CLA^+$ T cells was observed. Conversely, the use of DCs did not induce such a clear CLA-selective response. In fact, the amount of IFN-γ

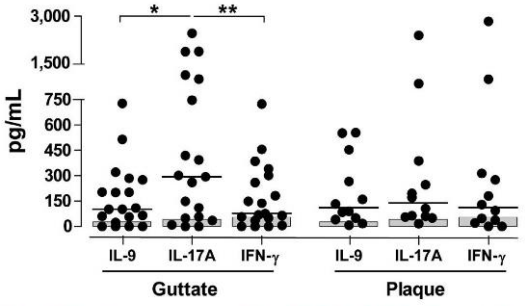


**Figure 4. *Streptococcus pyogenes*-induced IL-9 by CLA$^+$ T cells occurs in guttate and plaque psoriasis-derived co-cultures.** Paired IL-9, IL-17A, and IFN-γ production in SE-activated CLA$^+$/Epi co-cultures were compared within samples obtained from guttate psoriasis (n = 19) and plaque psoriasis (n = 12) patients. Gray chart bars indicate median cytokine levels produced by healthy control co-cultures (n = 10). Data are represented by dot plots, each representing the values of a supernatant from a patient-derived co-culture, and by the median. *$P < 0.05$, **$P < 0.01$. CLA, cutaneous lymphocyte-associated antigen; CLA$^+$/Epi, co-cultures of CLA$^+$ T cells and autologous epidermal cells; SE, *S. pyogenes* extract.

produced by CLA$^-$ T cells was higher than that by CLA$^+$ T cells. Also, IL-17A, which is highly associated with cutaneous defense, was produced in higher amounts by epidermal cells than by DCs when co-cultures were activated by SE and SEB, even though the former are a more heterogeneous population of cells. Thus, SE-induced IL-9 production by psoriatic CLA$^+$/Epi co-cultures occurred in a specific manner, because activation with SEB equally induced IL-9 in both psoriasis and healthy control subject samples. Activation with CA was also tested. In contrast to recent studies performed with healthy CLA$^+$ T cells activated by autologous pulsed monocytes with CA (Schlapbach et al., 2014), we found that IL-9 production was associated mainly with the CLA$^+$/Epi from patients with psoriasis but not in healthy control subjects. This difference may be attributed to the use of unpulsed autologous epidermal cells.

The production of IL-9, IL-17A, and IFN-γ was dependent on HLA class I and class II presentation and on the presence of epidermal cells as a possible source of antigen-presenting cells. Cytokine production was completely reduced by the addition of blocking antibodies against HLA class II molecules. This observation supports the proposed role of CD4$^+$ T cells as source of IL-9 (Schlapbach et al., 2014). Indeed, cytokine production induced by SE and CA in the co-culture was sustained mainly by the action of CD4$^+$ T cells, as observed in CD4-depleted co-cultures. However, SE induced detectable amounts of IL-17F in half of these co-cultures, thereby indicating that CD8$^+$ T cells alone may also be relevant in responding to SE. About 50% of IL-9, IL-17A, and IFN-γ production was dependent on HLA class I presentation. Given that this subset accounts for a minor fraction of the lymphocyte population in the co-culture, this finding indicates a noteworthy secondary role of CD8$^+$ T cells, even in activated conditions (data not shown). These results are in the line with our previous observations (Ruiz-Romeu et al., 2016) and deserve further attention.

IL-9 is associated with increased IL-17 production. In the K5.hTGF-β1 transgenic mouse, IL-9 injection increases IL-17A mRNA expression but has no effect on IFN-γ (Singh et al., 2013). In purified human CD4$^+$ T cells from patients with psoriasis, IL-9 enhances IL-17A production, and IL-9 is required in healthy CLA$^+$ T cells for a maximal activation-induced increase of IL-17$^+$ CD4$^+$ T cells (Schlapbach et al., 2014; Singh et al., 2013). However, it is not known whether IL-9 is involved in the innate induced production of IL-17A by antigen-specific responding T cells in psoriasis. Also, a transient increase in IL-9$^+$ cells with a peak at day 2 has been reported in polyclonal-activated CLA$^+$ T cells from healthy individuals, and this peak preceded the increase in other effector cells, including IL-17 and IFN-γ effector cells (Schlapbach et al., 2014). Despite the lack of an apparent preceding IL-9 peak, IL-9 neutralization resulted in a 50% reduction in SE-induced IL-17A production in CLA$^+$/Epi co-cultures, whereas the amount of IFN-γ was not altered. Indeed, given that IL-9R is highly expressed by Th17 cells (Elyaman et al., 2009; Nowak et al., 2009) and by activated healthy CLA$^+$ T cells (Schlapbach et al., 2014), the increased survival observed in psoriatic CLA$^+$ T cells treated with IL-9 supports the link between IL-9 and the IL-17 production by these cells.

Psoriasis is frequently described as being characterized by predominantly Th17 and Th1 immune responses (Lowes et al., 2008). Here, we propose that Th9 cells additionally contribute to psoriatic inflammation. Furthermore, in two of the existing forms of disease, namely guttate and plaque psoriasis, SE activation of CLA$^+$/Epi co-cultures resulted in a similar response regarding IL-9 production. However, a predominant Th17 response in guttate psoriasis was observed, thus confirming our previous report (Ruiz-Romeu et al., 2016). However, no predominant response was observed in plaque psoriasis, because levels of IL-17A were slightly diminished, but without significance, compared with the levels detected in guttate psoriasis, and thus the plaque form of the disease presented a mixed Th9/Th17/Th1 response to SE.

The functional association between *S. pyogenes* and psoriatic skin lesions has been determined to be through effector memory skin-tropic CLA$^+$ T cells. These cells may be generated in the tonsils and migrate to the skin, because identical CLA$^+$ T-cell clones have been found in both tonsils and cutaneous lesions in psoriasis patients (Diluvio et al., 2006). In guttate psoriasis developed after an episode of pharyngotonsillitis, the immune response exerted by *Streptococcus* pyogenes-sensitized CLA$^+$ T cells to re-stimulation with SE may be associated with some of their clinical features, including disease severity. The common pattern of PASI and ASO and cytokine levels in CLA$^+$/Epi/SE co-cultures suggests that, within a particular period of time, the ex vivo behavior against re-stimulation with *S. pyogenes* of circulating CLA$^+$ T cells supports this view. Conversely, co-cultures from patients with plaque psoriasis without any recent history of throat infection still showed a greater response to SE than healthy control individuals. However, the possibility that these patients also presented a streptococcal burden cannot be ruled out (Sigurdardottir et al., 2013; Thorleifsdottir et al., 2016). Also, the long interval since

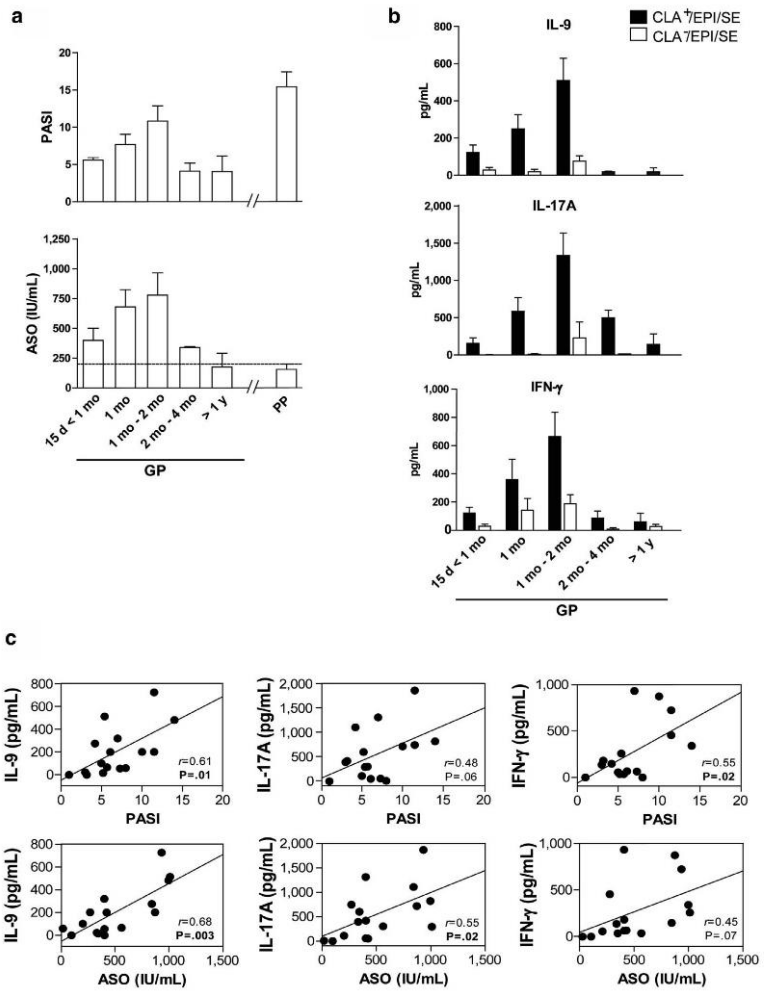


**Figure 5. Clinical parameters correlate with cytokine production in *Streptococcus pyogenes*-activated guttate CLA$^+$/Epi co-cultures.** (**a**) PASI, ASO, and (**b**) cytokine levels from guttate psoriasis samples (GP) (n = 16) were distributed on the basis of disease duration of each of the patients (minimum of two per group). The PASI score and ASO titer results are also shown in **a** for patients with plaque psoriasis (PP) (n = 11). Dashed line indicates the cutoff sensitivity for positive ASO result (200 IU/ml). (**c**) Cytokine values produced by SE-activated CLA$^+$/Epi co-cultures from guttate psoriasis patients showed a linear correlation with their respective PASI and ASO levels. ASO, anti-streptolysin O; CLA, cutaneous lymphocyte-associated antigen; CLA$^-$/Epi, co-cultures of CLA$^-$ T cells and autologous epidermal cells; CLA$^+$/Epi, co-cultures of CLA$^+$ T cells and autologous epidermal cells; mo, month; PASI, Psoriasis Area Severity Index; SE, *S. pyogenes* extract; y, year.

disease onset and the lack of acute activity of disease stage make it difficult to establish a link between past infections, disease symptoms, and circulating CLA$^+$ T-cell activity against SE in chronic plaque psoriasis.

Limitations of this study include the indeterminacy of a real uniqueness of IL-9 T-cell source in the co-cultures. Also, although we report data from patients with guttate psoriasis with varying duration of disease, it was not possible to perform a follow-up study in these patients. Although we report that patients with either guttate and plaque psoriasis carry skin-tropic memory CLA$^+$ T cells with IL-9 effector properties, the impact on lesion development is not clear.

In summary, IL-9 effector response, in addition to IL-17A and IFN-γ—which are already known associated cytokines in psoriasis—is exerted preferentially by circulating psoriatic CLA$^+$ T cells cultured with autologous lesional epidermal cells via activation with *S. pyogenes*. Furthermore, IL-9 might be functionally significant by supporting IL-17A levels, thereby suggesting a potential contribution to psoriasis immunopathology. The IL-9 response in guttate psoriasis samples followed a similar production pattern to that of IL-17A and IFN-γ, according to time of flare. Given the translational information provided in this study, the results support the notion that IL-9 participates in the cytokine network in psoriasis.

## MATERIALS AND METHODS

### Patients

This study was performed with human samples and in accordance with the Declaration of Helsinki. A total of 39 nontreated psoriasis patients and 10 healthy individuals were recruited from three hospitals (see Supplementary Table S1 online). All participants contributed

voluntarily and provided written informed consent. Psoriatic samples were from patients with guttate (n = 19) and plaque (n = 12) lesions, without any age or sex restriction. Patients who had received systemic treatment in the 6 weeks before sample collected were excluded to avoid the underestimation of cell activation. Patients and healthy subjects underwent two skin biopsies, which were punched in lesions in psoriatic patients, and a blood extraction.

### Circulating memory T-cell and epidermal cell isolation

Memory $CD45RA^{-}CLA^{+}$ and $CLA^{-}$ T cells were purified from blood as described previously (Ferran et al., 2013b). Occasionally, an intermediate depletion of $CD4^{+}$ T cells was performed. Skin biopsy samples were incubated overnight in Dispase (Corning, Bedford, MA) at 4°C, then the epidermal sheet was peeled from the dermis. The epidermis was cut into smaller pieces, which were then incubated in trypsin solution (Biological Industries, Kibbutz Beit Haemek, Israel) for 30 minutes at 37°C.

### Generation of DCs from peripheral blood monocytes

$CD14^{+}$ cells purified by immunomagnetic separation were cultured at a final density of $10^{6}$/ml with 50 ng/ml of IL-4 and GM-CSF (Miltenyi Biotec, Bergisch Gladbach, Germany). Half the medium was replaced by new supplemented medium at day 4. At day 7, cells were washed and counted for proper culture conditions.

### Cultures and pathogen activation

Ex vivo co-cultures involved the culture of total or CD4-depleted $5 \times 10^{4}$ $CLA^{+}$ or $CLA^{-}$ T cells with $3 \times 10^{4}$ autologous epidermal cells ($CLA^{+}$/Epi or $CLA^{-}$/Epi, respectively) in 96-well flat-bottom plates (Nunc, Roskilde, Denmark) in the medium described. Also, co-cultures were performed with $5 \times 10^{4}$ autologously generated DCs, which were seeded with thawed $5 \times 10^{4}$ $CLA^{+}$ or $CLA^{-}$ T cells. Co-cultures were left untreated or activated for 5 days with the following pathogen preparations: an extract of *S. pyogenes* obtained from sonicated *S. pyogenes* isolated from throat swabs from patients with psoriasis, used at 1 μg/ml; commercial CA preparation (Greer Labs, Lenoir, NC) at 20 μg/ml; and SEB (Sigma-Aldrich) at 100 ng/ml. In cultures containing only T cells, the mentioned amounts of each cell type were used, and activation with SE was performed in the same way. For the time course experiments, supernatants from SE-activated co-cultures were collected each day from replicated wells. For blocking assays, azide-free HLA-A/B/C (class I), HLA-DR (class II), or IL-9 neutralizing antibodies, or respective isotype IgG controls (Biolegend, San Diego, CA), were added at day 0 to co-cultures from psoriatic patient samples before activation at a final concentration of 10 μg/ml.

### Apoptosis assay

Isolated $10^{5}$ $CLA^{+}$ and $CLA^{-}$ T cells from patients with psoriasis were cultured in the medium described supplemented or not with 20 ng/ml of IL-9 (Miltenyi Biotec) for 5 days. Cells were then washed with cold phosphate buffered saline and stained with Annexin-V-APC (BD Bioscience, San Jose, CA) and DAPI and analyzed by flow cytometry.

### Cytokine quantification

Multiplex fluorescent bead-based immunoassay with DiacloneDIAplex kit (Gen-Probe, Besançon, France) was used for IL-17A and IFN-γ measurements. IL-9 concentration was measured by ELISA using precoated plates (Biolegend).

### Statistical analysis

Data are generally represented as individual dots and the median. For multiple comparison purposes, one-way analysis of variance Kruskal-Wallis with Dunn posttest was used. Differences between two groups were analyzed by the Mann-Whitney test, and for paired comparisons Wilcoxon match pairs test was used. Pearson correlation coefficient was used to assess cytokine similarity. Differences were considered significant at a *P*-value of less than 0.05.

## CONFLICT OF INTEREST

The authors state no conflict of interest.

## ACKNOWLEDGMENTS

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## SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at www.jidonline.org, and at https://doi.org/10.1016/j.jid.2017.08.048.

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**SUPPLEMENTARY MATERIAL**

**Table S1. List of participants in the study.**

| Participants | Age | Diagnostic | PASI |
|---|---|---|---|
| 26/02/2013 | 38 | Guttate | 2.9 |
| 09/10/2013 | 23 | Guttate | 3.2 |
| 10/10/2013 | 20 | Guttate | 1 |
| 17/02/2014 | 34 | Guttate | 7.2 |
| 08/04/2014 | 34 | Guttate | 4.7 |
| 09/04/2014 | 30 | Guttate | 7.3 |
| 07/05/2014 | 23 | Guttate | 5.4 |
| 03/07/2014 | 26 | Guttate | 3 |
| 25/09/2014 | 32 | Guttate | 5 |
| 14/01/2015 | 30 | Guttate | 5.7 |
| 15/01/2015 | 34 | Guttate | 5.4 |
| 19/01/2015 | 25 | Guttate | 11.5 |
| 26/02/2015 | -- | Guttate | 11.5 |
| 27/05/2015 | 19 | Guttate | 6.1 |
| 25/06/2015 | 21 | Guttate | 4.2 |
| 09/11/2015 | 38 | Guttate | 7.8 |
| 04/02/2016 | 30 | Guttate | 8 |
| 11/05/2016 | 20 | Guttate | 10 |
| 23/05/2016 | 33 | Guttate | 7 |
| 28/03/2017 | 50 | Guttate | 4.8 |
| 13/06/2017 | 39 | Guttate | 2 |
| 15/06/2017 | 17 | Guttate | 1 |
| 19/12/2013 | 21 | Plaque | 18 |
| 12/02/2014 | 28 | Plaque | 8.4 |
| 02/12/2014 | 43 | Plaque | 13 |
| 10/12/2014 | 59 | Plaque | 15 |
| 15/10/2015 | 32 | Plaque | 10 |
| 27/10/2015 | 42 | Plaque | 10 |
| 03/11/2015 | 65 | Plaque | 12 |
| 02/03/2016 | 43 | Plaque | 30 |
| 26/04/2016 | 58 | Plaque | 14.1 |
| 03/05/2016 | 39 | Plaque | 12 |
| 10/05/2016 | 59 | Plaque | 15 |
| 19/05/2016 | 45 | Plaque | 28 |
| 02/03/2017 | 37 | Plaque | 24 |
| 09/03/2017 | 67 | Plaque | 7 |
| 16/03/2017 | 56 | Plaque | 25 |
| 21/03/2017 | 20 | Plaque | 8 |
| 20/10/2011 | 30 | Control | -- |
| 19/12/2012 | 32 | Control | -- |
| 16/10/2013 | 47 | Control | -- |
| 22/10/2013 | 40 | Control | -- |
| 14/01/2014 | 51 | Control | -- |
| 20/01/2014 | 49 | Control | -- |
| 07/11/2014 | 50 | Control | -- |
| 21/11/2014 | 20 | Control | -- |
| 16/06/2015 | 47 | Control | -- |
| 14/07/2015 | 50 | Control | -- |

**FIGURE S1**

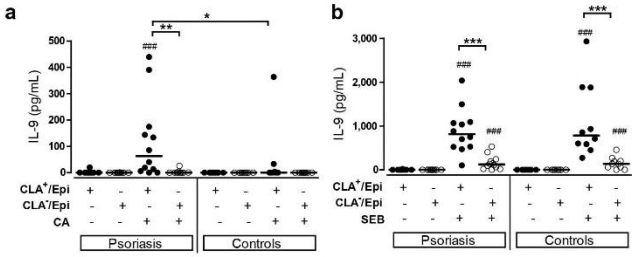


**Figure S1. *Candida albicans* induces selective IL-9 production by psoriatic memory CLA$^+$ T cells, and SEB also indistinctly exerts IL-9 induction in healthy donors.** IL-9 levels were measured in 5-day supernatants from psoriatic (n=12) or healthy control (n=10) cocultures activated by CA (d) or SEB (e). Cell types and presence (+) or absence (-) of pathogens for each coculture condition are shown below the x-axis. Data are represented by dot plots, each representing the values of a supernatant from aa patient-derived coculture, and by the median. P values symbols (#) indicate differences with basal CLA$^+$/Epi or CLA$^-$/Epi untreated conditions.

**FIGURE S2**

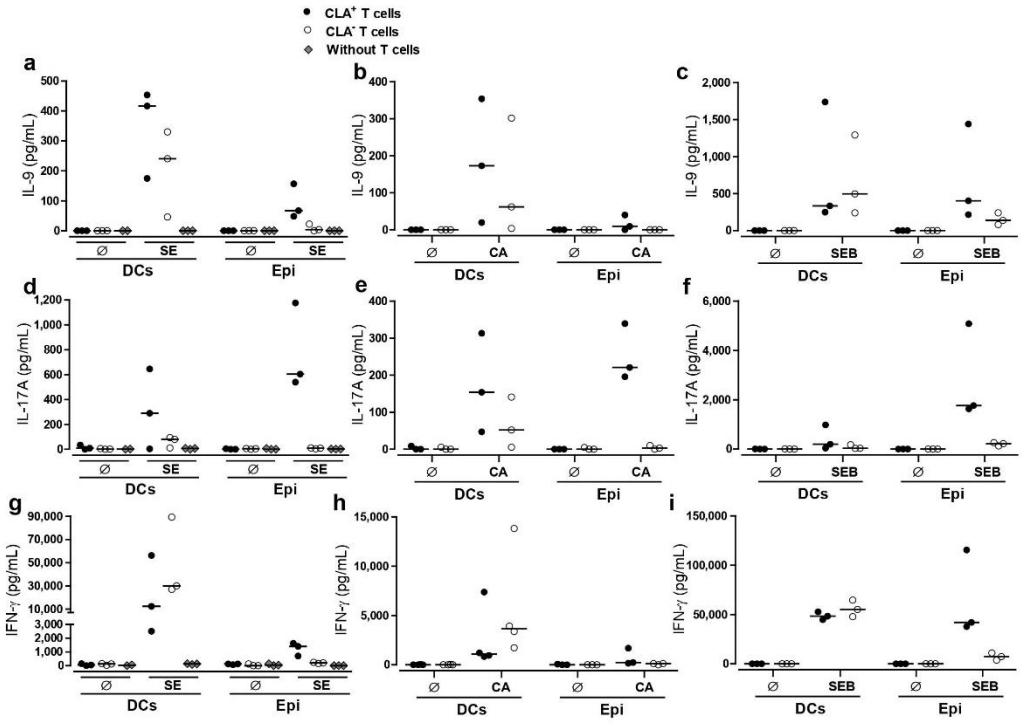


**Figure S2. Autologous epidermal cells provide a selective CLA$^+$T-cell–dependent response in a psoriatic coculture system.** Circulating CLA$^+$ and CLA$^-$ T cells were cocultured with autologous dendritic cells derived from peripheral monocytes (DC; n=3) or autologous epidermal cells (Epi; n=3). DCs and Epi were also cultured without T cells. Cultures were then activated with SE, CA and SEB, and IL-9, IL-17A and IFN-γ were measured at day 5. Data are represented by dot plots, each representing the values of a supernatant from a patient-derived coculture, and by the median.

**FIGURE S3**

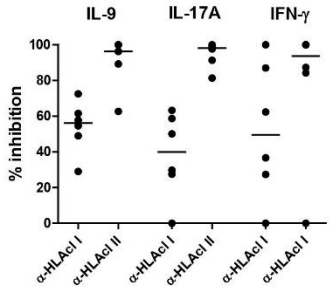


**Figure S3. IL-9, IL-17A and IFN-γ production by SE is HLA class I and II-dependent.** Percentage of inhibition of cytokine production achieved by HLA class I or II blockade after 5 days of SE activation of $CLA^+$/Epi cocultures with respect to isotype control (n=6). Data are represented by dot plots, each representing the values of a patient-derived coculture, and by the median.

**FIGURE S4**

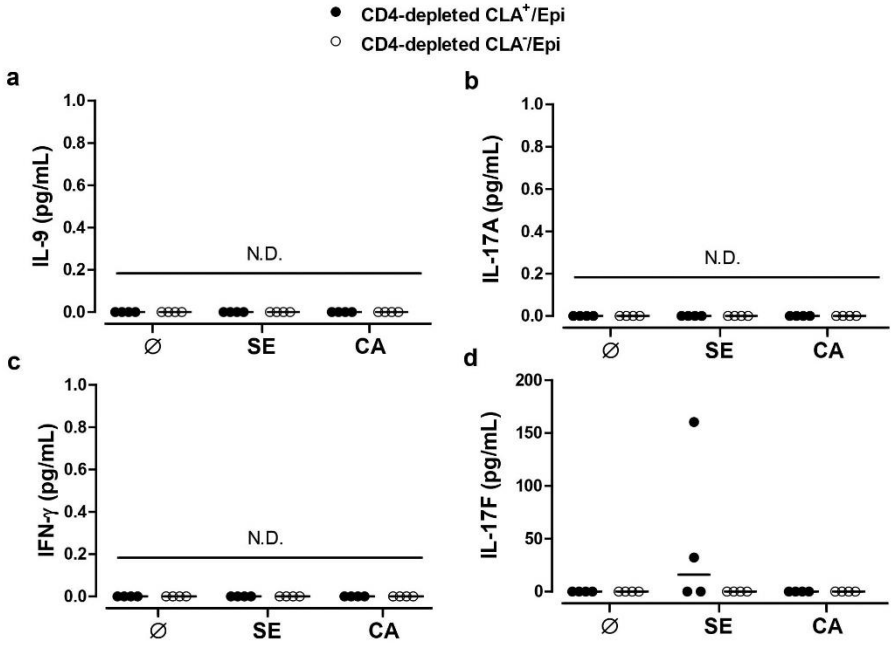


**Figure S4. Depletion of $CD4^+$ T cells impairs cytokine production by the cocutlures.** $CD4^-$-depleted $CLA^+$ and $CLA^-$ T cells were cocultured with autologous epidermal cells from psoriatic donors and activated with SE or CA(n=4). IL-9 (a), IL-17A (b) IFN-γ (c) and IL-17F (d) were measured at day 5. Data are represented by dot plots, each representing the values of a patient-derived coculture, and by the median. N.D.: not-detectable.

**FIGURE S5**

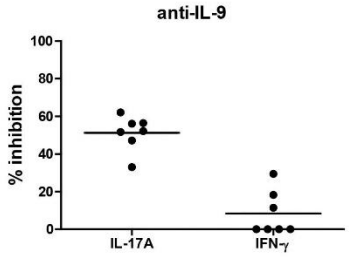


**Figure S5. SE-induced IL-17A production on CLA$^+$/Epi coculture is partially dependent on IL-9.** Percentage of IL-17A and IFN-γ inhibition produced by IL-9 blockade in CLA$^+$/Epi cocultures after 5 days of SE activation with respect to isotype control (n=7). Data are represented by dot plots, each representing the values of a patient-derived coculture, and by the median.

**FIGURE S6**

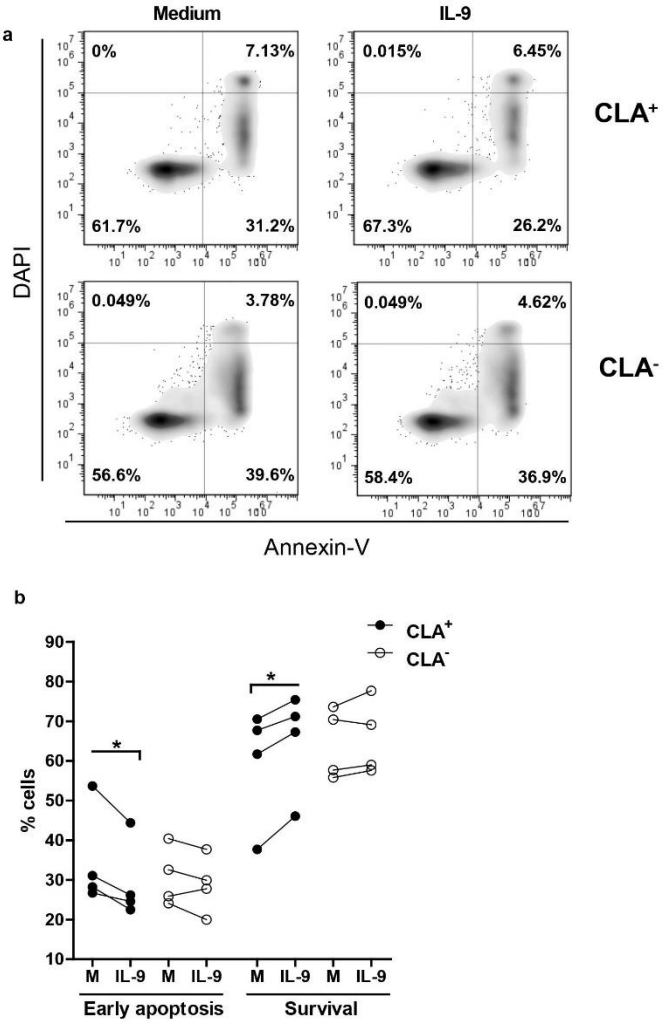


**Figure S6. IL-9 preferentially enhances CLA$^+$ T cell-survival.** $10^5$ peripheral CLA$^+$ or CLA$^-$ T cells isolated from the blood of patients with psoriasis (n=4) were cultured with medium (M) or with medium supplemented with IL-9 (20 ng/mL). At day 5, cells were stained with Annexin-V-APC and DAPI, and the percentage of living cells (Annexin-V$^-$/DAPI$^-$) and early apoptotic cells (Annexin-V$^+$/DAPI$^{-/low}$) was determined by flow cytometry (a,b). b) Data were pairwise compared between IL-9-treated and untreated cells. Data are represented by dot plots, each representing the values of the T cells of one patient, and by the median.

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# CLA+ T Cell Response to Microbes in Psoriasis

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*Streptococcus pyogenes* throat infection is a clinically relevant trigger of both guttate and chronic plaque psoriasis, and it provides an ideal context in which to study the pathogenesis of these diseases using an antigen-dependent approach. Circulating cutaneous lymphocyte-associated antigen (CLA) positive (+) memory T cells are a subset of peripheral lymphocytes whose phenotype and function are related to immunological mechanisms in the skin. These cells are considered peripheral biomarkers of T-cell-mediated skin diseases. The coculture of autologous epidermal cells with CLA+ T cells from psoriasis patients activated by *S. pyogenes* allows the reproduction of the *ex vivo* initial molecular events that occur during psoriatic lesion formation. With cooperation of autologous epidermal cells, *S. pyogenes* selectively activates CLA+ T cells both in guttate and plaque psoriasis, inducing key mediators, including an IL-17 response. Here, we explore potential new mechanisms of psoriasis development including the influence of HLA-Cw6 on *S. pyogenes* CLA+ T cell activation in guttate psoriasis, the relevance of IL-9 on microbe induced IL-17 response in guttate and plaque psoriasis, and novel effector functions of *Candida albicans*. This review will summarize recent knowledge of psoriatic mechanisms elicited by microbes that have been studied through an innovative translational perspective based on CLA+ T cell-mediated cutaneous immune response.

**Keywords: psoriasis, cutaneous lymphocyte-associated antigen, homing, *Candida albicans*, *Streptococcus pyogenes***

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## INTRODUCTION

Molecular studies of psoriasis lesions and patients have allowed translational research to generate potent and novel therapies (1). However, our understanding of the influence of environmental factors on the psoriatic cutaneous immune response is still limited (1). Several microorganisms, including bacteria, fungi, and viruses, have been postulated to be potential triggers and/or exacerbating factors of psoriasis (2). Bacterial genome DNA sequencing in psoriasis is an area of great interest, some microorganisms have been identified but their functional relevance for psoriasis is still to be determined. Psoriasis can be classified as early or late onset (3). The former is associated with the HLA-Cw6 allele, streptococcal throat infection, and a higher tendency to be generalized

(4, 5). Interestingly, patients with this type often present a more intense inflammatory lymphocytic infiltrate and are more likely to receive biological therapy (6). All these observations suggest that the presentation of psoriasis is associated with the present bacterial infection. *Streptococcus pyogenes* throat infection is a well-characterized infectious trigger of gutatte psoriasis (GP) and chronic plaque psoriasis (CPP). More than 60 years ago, it was reported that two-third of GP patients present an acute sore throat 12 weeks before the skin eruption (7). Similarly, CPP can also be triggered by *S. pyogenes* throat infections (8), and interestingly, CPP patients have a higher incidence of recurrent sore throats compared with controls (9, 10). The presence of *S. pyogenes* has been detected in the blood of both GP and CPP patients (11). In addition, tonsillectomy can be a useful therapeutic intervention in CPP patients with a history of streptococcal-associated exacerbations (12). It has been proposed that psoriasis tonsillar $CLA^+$ T cells (13) activated by streptococcal antigens migrate to the skin where they react to antigens that share sequence homology with the streptococcal proteins (14). However, other microbes may also participate in psoriasis. Fungal cutaneous infections caused by *Candida albicans* have been associated with exacerbation of the disease and a higher frequency of intestinal *C. albicans* isolation in psoriasis patients than controls has been reported (2), although the mechanisms involved in *C. albicans*-induced psoriasis remain poorly characterized.

Interestingly, microbes such as *C. albicans* induce type I interferon response and, type I interferon production by plasmacytoid dendritic cells in skin has been stated to be an important trigger for psoriasis development (15). However, clinical efficacy blocking antibodies against IFN-α have not shown clinical efficacy in psoriasis (16), rising questions about the translational relevance of this mechanism.

In this review, we cover the current state of the art in psoriasis immunopathogenic mechanisms brought out by disease-related microorganisms, such as *S. pyogenes* or *C. albicans*. We focus on cutaneous immune response mediated by $CLA^+$ T cells and how these microbes affect T cell activation and production of clinically relevant cytokines.

## CIRCULATING $CLA^+$ T CELLS AND THE STUDY OF THE CUTANEOUS IMMUNE SYSTEM

The immune responses of T cells during cutaneous chronic inflammation in psoriasis involve a subset of memory T lymphocytes that can be distinguished from other memory T cells by the surface expression of the cutaneous lymphocyte-associated antigen (CLA) antigen. This antigen is a cell surface carbohydrate that allows the identification of memory T cells that belong to the cutaneous immune system. CLA is an adhesion molecule expressed by 15% of circulating T cells in humans, and by most (>90%) skin-infiltrating T cells, contrary to other inflamed organs (17). In addition to several ligands for chemokine receptors (CCR10, CCR4, CCR6, and CCR8), CLA binds to E-selectin and together with the interaction between the very late antigen-4/vascular cell adhesion protein-1 and lymphocyte function-associated antigen-1/intercellular adhesion molecule-1, forms a code bar system enabling skin lymphoid infiltration (18). The relevance of circulating $CLA^+$ T cells in the cutaneous immune response lies not only in the skin-seeking capacity of these cells but also in their functional relation to the immune response that occurs in inflamed cutaneous lesions. This feature is derived from the recirculating capacity of these cells between skin lesion and blood during cutaneous inflammation (18, 19). The antigen-specific response and phenotype of circulating $CLA^+$ T cells has been studied in many human skin conditions. $CLA^+$ T cells respond to antigens, allergens, or superantigens involved in disease by triggering T cell-mediated skin diseases, such as psoriasis, atopic dermatitis, and contact dermatitis (18). Furthermore, the phenotype and function of these cells are related to the clinical status of the patient, thereby explaining why circulating $CLA^+$ T cells are considered peripheral cell biomarkers of T cell-mediated cutaneous disease in humans (18). Using $CLA^+$ T cells from psoriasis patients and healthy controls, our group explores the influence of microbes on cutaneous immune response in psoriasis.

## $CLA^+$ T CELL ACTIVATION BY *S. pyogenes* IN PSORIASIS INDUCES IL-17 AND IL-9 RESPONSES

Studying the antigen-specific immune response of $CLA^+$ T cells induced by clinically relevant triggers of psoriasis may allow the identification of the translational mechanisms involved in psoriasis. The stimulation of autologous coculture $CLA^+$ T cells and epidermal cells with *S. pyogenes* leads to an inflammatory immune response that shows the hallmarks of psoriasis. By contrast, the same stimulation of $CLA^-$ cells from the same patient or cultures using $CLA^+/CLA^-$ T cells from healthy controls does not have this effect (20). The $CLA^+$ T cell response in this model is related to the clinical response of patients in terms of anti-streptolysin O levels, PASI, and duration of flare in GP (21), and to anti-streptolysin O in CPP (20). This activation is determined by the presence of autologous epidermal cells (lesional/non-lesional) and MHC class I and class II presentation. Supernatants of *S. pyogenes*-activated cocultures of $CLA^+$ T cells and epidermal cells induce epidermal hyperplasia upon intradermal injection in mouse skin (20). IL-17A and IL-17F production is probably the most relevant effect of *S. pyogenes* on $CLA^+$ T cells in psoriasis. The influence of *S. pyogenes* through the response of these cells and the relevance of IL-17 production in GP have been extensively studied (21). In $HLA\text{-}Cw6^+$ patients whose GP flare is associated with a pharyngitis episode, the Th17-associated response is greater than that exerted by samples from GP patients not associated with pharyngitis. In fact, significantly higher levels of IL-17A, IL-17F, and even IL-6, which participates in Th17-differentiation, were found (21). Thus, the observed response of psoriasis memory T cells to *S. pyogenes* seems to be restricted to $CLA^+$ T cells, leading to IL-17 production. This cytokine is a key driver of psoriasis, and its

neutralization in patients, or receptor blockade improves the skin condition (1).

The cytokine IL-9 is involved in chronic inflammation and has recently been associated with psoriasis (22, 23). We have demonstrated how *S. pyogenes* preferentially induces IL-9 production during the coculture of autologous CLA$^+$ T cells and epidermal cells in psoriasis but not in healthy controls. IL-9 is produced in the same culture conditions in which IL-17A and IL-17F are detected in a time-dependent manner. IL-9 production is dependent on MHC class I and class II presentation, and it preferentially prolongs CLA$^+$ T cell survival. Higher amounts of IL-9 were detected in psoriasis patients than in healthy controls, but no differences were observed between GP and CPP patients (24). IL-9 has been associated with increased IL-17A production in an animal model of psoriasis (23). Since *S. pyogenes* induces both IL-9 and IL-17A, we examined the interaction between these two cytokines in CLA$^+$ T cells. To this end, we blocked IL-9 function using a neutralizing. A 50% reduction in IL-17A production, but not IFN-γ, was found when was IL-9 was neutralized in CLA$^+$ cells activated by *S. pyogenes*.

Our studies have shown that, in CPP patients without clinical evidence of *S. pyogenes* infection, only CLA$^+$ T cells respond to this microbe in comparison to healthy controls. This observation indicates that psoriasis patients present an adaptive immune response to *S. pyogenes* through IL-17A, IL-17F, IL-9, and IFN-γ production (20, 21, 24) and suggests that *S. pyogenes* modulates the response of the CLA$^+$ T cells that maintain psoriatic lesions, i.e., pyogenes infection has been describe to participate in CPP infection, since higher levels of IgG against *S. pyogenes* proteins are detected in psoriasis patients in comparison to healthy controls (25). Some studies have reported the presence of the genera *Streptococcus* in normal and psoriatic skin (26) and the isolation of *S. pyogenes* in the skin of GP patients (4), probably leading to cutaneous immunization and a CLA$^+$ T cell-restricted response in psoriasis.

## CLA$^+$ T CELL RESPONSE TO *C. albicans* IN PSORIASIS

The cutaneous adaptive immune response to *C. albicans* infection is mediated by a Th17 profile since Th17 cells are essential for anti-fungal barrier immunity (27). Patients with Th17 deficiencies have an increased susceptibility to candidiasis (28), and CD45RA$^+$ human T cells may lead to an increase in the number of IL-17 and IFN-γ-producing cells (29). Cutaneous candidal infections have been reported in association with psoriasis exacerbation (30); however, the mechanisms by which *C. albicans* induces psoriasis are poorly understood (2). In psoriasis, *C. albicans*-derived superantigens may induce an expansion of lymphocytes expressing the T-cell receptor variable region beta 5.1 (31). Like in the case of *S. pyogenes*, CLA$^+$ T cells, together with autologous epidermal cells, preferentially respond to *C. albicans* extract by inducing IL-9, IL-17A, and IFN-γ production in psoriasis. This response appears to be restricted to CLA$^+$CD4$^+$ memory T cells since CD4-depleted CLA$^+$ memory T cells do not respond to this microorganism in a coculture model with psoriasis cells (24). These results are in line with the expected immune response to *C. albicans* in the skin. However, the observed preferential response of CLA$^+$ T cells in psoriasis suggests an adaptive immune response to *C. albicans*, underlying its importance as a relevant antigen likely to be involved in triggering the disease.

## INFLUENCE OF *S. pyogenes* AND *C. albicans* ON IL-17 ADAPTIVE IMMUNE RESPONSE IN PSORIASIS

The precise mechanisms by which environmental factors trigger psoriasis are not well understood (1). Biological therapies have revealed the clinical relevance of the IL-23/IL-17 axis in this skin disease. Thus, environmental factors that contribute to fueling the IL-23/IL-17 response may induce the condition. The observation that CLA$^+$ T cells in psoriasis patients respond to skin *S. pyogenes* and *C. albicans* extracts indicates a relationship between memory T cells and environmental microbes. Such preferential sensitization to these microorganisms in psoriasis can be either at the tonsillar level in psoriasis through the abnormal generation of CLA$^+$ T cells or at the skin level, since the presence of both *S. pyogenes* and *C. albicans* in psoriatic lesions (13). The CLA$^+$ T cell response to these microbes is based on IL-17A, IL-17F, IL-9, and IFN-γ production. This response indicates that these skin-homing cells will migrate to psoriatic lesions and thus that they may be involved in the local inflammatory response. IL-17A and IL-17F are clinically validated mediators of psoriasis. *S. pyogenes*-driven IL-9 production through CLA$^+$ T cells supports IL-17A production in human lymphocytes, since *in vitro* neutralization of IL-9 reduces IL-17A production by 50% (24).

A current model of IL-17 production in psoriasis considers that some autoantigens, such as LL-37 and ADAMTS-like protein 5, would activate T17 cells (32), initiating the immune circuit of the psoriasis pathogenetic mechanism in the disease. Also, IL-23 production by inflammatory dendritic cells favors the generation and maintenance of the T17 phenotype in psoriasis. Interestingly, regarding the possible interplay between *C. albicans* and *S. pyogenes* and the IL-23/Th17 axis, it has been recently shown that *C. albicans* stimulates dendritic cells to release IL-23 (33). There is a complex interplay between these two microbes and CLA$^+$ T cells in psoriasis; however, the influence of microbes in psoriasis may be more complex that originally believed since microbiota studies demonstrate the presence of a range of microorganisms in psoriatic lesions (34). The functional relevance of these microorganisms for the disease has not been determined (35).

In summary, the observations made to date suggest that circulating CLA$^+$ T cells in psoriasis patients produce increased amounts of IL-17A, IL-17F, and IL-9, in comparison to healthy controls, when activated by *S. pyogenes* (**Figure 1**). Interestingly, the response to *C. albicans* is restricted mainly to CLA$^+$ T cells in cocultures with autologous epidermal cells in psoriasis with a similar cytokine profile. Psoriatic lesions produce several chemokines to attract skin-seeking CLA$^+$ T cells (21) with IL-17 capacity to the skin with potential to induce IL-17-dependent autoantigens and promote and maintain lesion activity. The study of the cutaneous immune response of CLA$^+$ T cells allows us to gain insight into how environmental factors, such microbes, shape psoriasis inflammation.

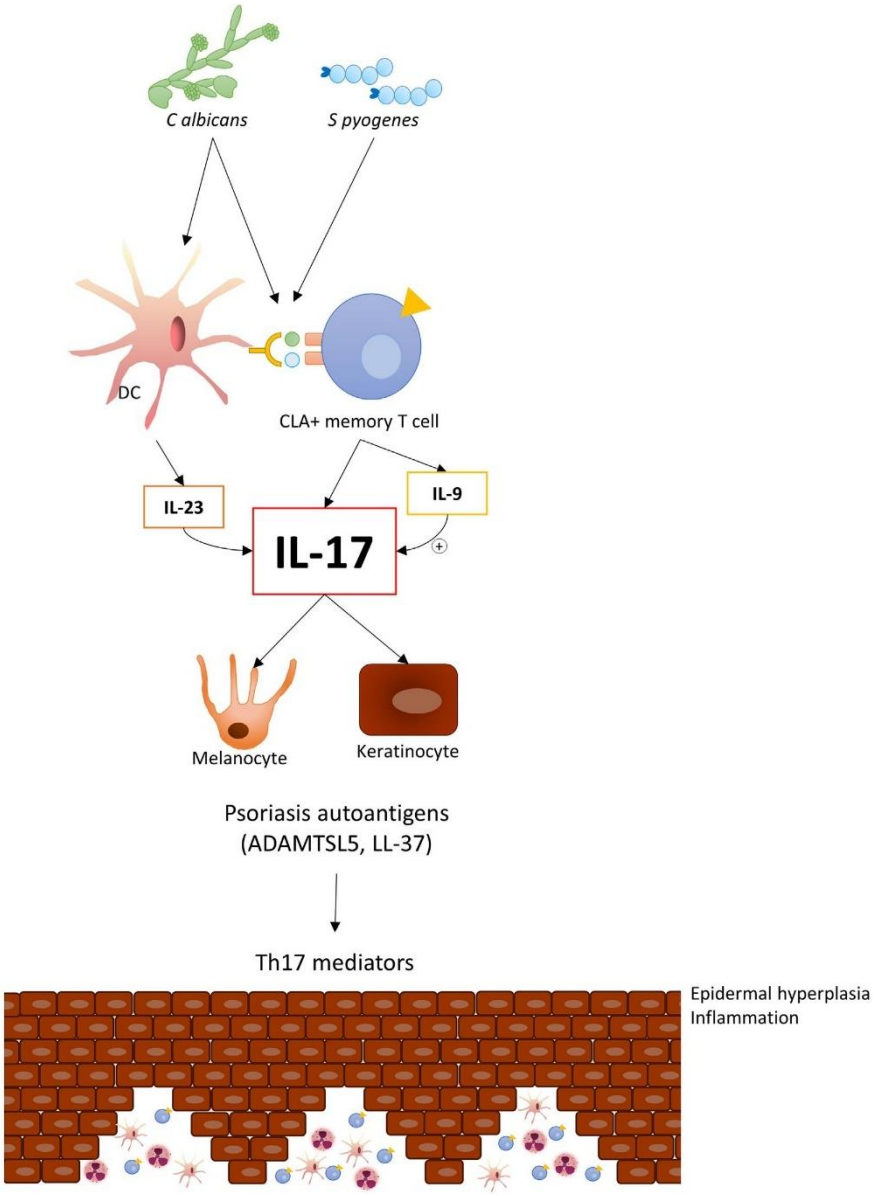


**FIGURE 1** | *Candida albicans* and *Streptococcus pyogenes* induce IL-17 response in circulating CLA$^+$ T cells in psoriasis, thereby indicating an established adaptive immune response to these microorganisms in this disease. Upon migration to cutaneous lesions, these cells react with those microbes and locally trigger IL-17 and IL-9 production, which will contribute to inducing psoriasis autoantigens.

## AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

LFSB conceived the ideas and together drafted the manuscript. All authors revised and approved the final version of the manuscript.

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# The Translational Relevance of Human Circulating Memory Cutaneous Lymphocyte-associated Antigen positive T Cells in Inflammatory Skin disorders

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## ABSTRACT

Circulating memory T cells are heterogeneous in their tissue tropism. The skin-seeking T cells subset express the cutaneous lymphocyte-associated antigen (CLA) on their surface. $CLA^+$ memory T cells not only migrate from blood to skin, but also recirculate between blood and skin. Studying $CLA^+$ memory T cells in cutaneous diseases has allowed a better understanding of immune-inflammatory mechanism that take place in the skin. The analysis of the phenotypical features of these cells, their antigen specificity, cytokine production profile and changes in relationship to clinical status and therapies among other characteristics, has led to the concept that they constitute peripheral cellular biomarkers in T cell-mediated cutaneous conditions. $CLA^+$ memory T cells are of relevance in the pathogenesis of several cutaneous diseases such as psoriasis, atopic dermatitis, vitiligo and drug-induced allergic reactions, to name a few. The interaction of circulating $CLA^+$ T cells with skin resident cells has been investigated in different *ex vivo* coculture models made out of clinical samples. Interestingly, microbes that are present in the skin or related with human skin diseases are preferentially recognized by $CLA^+$ T cells. Thus, the interaction of *Streptococcus pyogenes* with $CLA^+$ T cells in psoriasis is providing novel concepts that help to understand disease immunopathogenesis. The goal of this review is to present latest results in the field of $CLA^+$ T cells in T cell-mediated inflammatory skin diseases and their translational relevance for human immunodermatology.

**Keywords:** skin-homing lymphocytes, cutaneous inflammation, CLA+ T cell, human, translational, immunodermatology

# INTRODUCTION

The existence of a cutaneous immune system in humans was postulated almost 50 years ago (1) and recently reformulated (2). In order to understand the adaptive immune response regionally, for those human memory T cells that belong to the cutaneous immunity, a skin-specific cell marker would be of great help. The cutaneous lymphocyte-associated antigen (CLA) constitutes a relevant marker that identifies the subset of memory T lymphocytes functionally related to skin physiology. CLA was discovered in 1990 by serendipity as a cell surface carbohydrate preferentially expressed by T cells present in cutaneous inflamed tissues, but no other organs (3). Since then, a comprehensive number of scientific evidences described in humans support the role of CLA as a relevant marker to identify skin-associated memory T cells involved in T cells-mediated cutaneous inflammation, that provide translational information in numerous different human skin diseases. CLA is more than a mere cell surface carbohydrate (3), preferentially expressed on $CD45RO^+$ T cells, that binds to endothelial E-selectin and mediates cell adhesion and transendothelial migration together with other molecules such as LFA-1, VLA-4 and CCR10 (4). $CLA^+$ T lymphocytes are found in circulation and in inflamed and healthy skin but not infiltrating other non-cutaneous sites (3,5). The fact that some $CLA^+$ T cells are found in circulation deserves special attention due to the consequences of blocking LFA-1 in patients with atopic dermatitis and psoriasis (6,7). During treatment with efalizumab, patients present a circulating lymphocytosis of $CLA^+$ T cells. If the treatment is interrupted a flare in disease occurs. One explanation to this effect is that $CLA^+$ T cells recirculate between blood and skin during cutaneous inflammation. Blocking their extravasation through LFA-1 leads to accumulation of those cells in blood, consequently when the blockade is released the cells enter abruptly into lesions and make flare. This mechanism of $CLA^+$ T cell recirculation has important implications for the translational relevance of studying circulating $CLA^+$ T cells in human inflammatory skin disorders (Figure 1a). $CLA^+$ memory T cells participate in pathological mechanisms of inflammatory disorders by recognizing key triggers of disease and producing cytokines that affect cells of the skin. The phenotype of those cells can reflect clinical status of the patients. The goal of this review is to update this information on circulating $CLA^+$ T cells in different human skin inflammatory diseases.

# PSORIASIS

In psoriasis $CLA^+$ T cells are contributing to understand the pathological mechanisms from a translational point of view using *ex vivo* studies with clinical samples. Although psoriasis is considered to be triggered by LL-37 (8) and IFN-alpha (9,10), the translation of this mechanism into patients is complex since anti-

IFN-alpha (11), anti-IFN-gamma (12) and anti-IL-22 do not induce clinical improvement, to name a few clinically invalidated mechanisms. *Streptococcus pyogenes (S. pyogenes)* infection is the best characterized clinically relevant trigger of psoriasis in patients (13). The molecular mechanism that links *S. pyogenes* and IL-17 response are starting to be clarified in psoriasis (14). Only $CLA^+$ memory T cells, but not $CLA^-$, preferentially respond to *S. pyogenes* in an autologous coculture of T cells and cutaneous epidermal cells from psoriasis patients (15). Besides, psoriatic patients that are negative for anti-Streptolysin O antibody (ASO) present increases levels of immunoglobulin A, but not IgG, to *S. pyogenes*, which are directly associated with $CLA^+$ T cell dependent IL-17 response to *S. pyogenes in vitro* (16). These results suggest that increased exposure to *S. pyogenes*, as demonstrated by the presence of specific humoral immune response even in ASO negative patients, upon recognition by $CLA^+$ T cells can fuel pathogenic IL-17 production. Remarkably, similar association has been recently described for *C. albicans*, which is a potent IL-23/Th17 inducer too (17). Some other new mechanisms in psoriasis relating $CLA^+$ T cells and IL-17 have been recently revealed. The cytokine milieu present in psoriatic lesions can influence IL-17 response by $CLA^+$ T cells. IL-15 and IL-23, both present in psoriatic lesions, have been shown to synergize with $CLA^+$ T cells and autologous epidermal cells to produce IL-17A and IL-17F in psoriasis. This phenomenon occurs without the use of any exogenous stimulus, in a MHC-dependent way, and independently of resident T cells (18), but it does not take place using $CLA^-$ T cells or cells from healthy controls. This is an example of how psoriatic skin cytokine microenvironment interacts specifically with skin-related memory T cells generating the IL-17 response critical for psoriasis initiation and maintenance. IL-9 is another cytokine studied in the context of $CLA^+$ T cells function in psoriasis in the *ex vivo* coculture model with autologous epidermal cells (19). *S. pyogenes* preferentially triggers IL-9 in $CLA^+$ T cells together with other mediators such IL-17A. In contrast to previous studies where IL-9 it is not induced by natural stimulus, IL-9 is produced in a time-dependent way and not transiently. Interestingly, the neutralization of *S. pyogenes*-induced IL-9 by $CLA^+$ T cells decreased IL-17A production by 50%.

Furthermore, descriptive analysis of T cell subpopulations in psoriatic patients revealed differential role for $CD4^+$ and $CD8^+$ T lymphocytes. Whereas CLA expression is associated with skin recruitment of $CD4^+$ central memory T cells ($T_{CM}$), particularly those $CCR4^+$ and $CCR6^+$, suggesting a specific role for these cells in patrolling the skin compartment; $CD8^+$ T cells are more likely to accumulate in psoriatic skin and stay as resident memory T cells ($T_{RM}$) (20). Still, there is a need for psoriatic models, closely representing human disease, to be used as drug screening platforms. Recently, Shin JU *et al* developed psoriatic human

3D skin constructs (pHSCs) by incorporating T cells, over the classical approach based on the use of patient-derived keratinocytes or fibroblasts treated with psoriasis-related cytokines (21). As lymphocyte source, they tested *in vitro* polarized Th1/Th17 cells and $CCR6^+$ $CLA^+$ T cells from psoriasis patients, both of which showed psoriatic phenotype on epidermal cells along with disease-associated cytokine profile. Interestingly, when different psoriatic drugs were tested, those pHSCs with $CCR6^+$ $CLA^+$ T cells responded differently to the ones with *in vitro* polarized Th1/Th17 cells, highlighting the relevance of using patient-$CLA^+$ T cells to better address and even anticipate specific therapeutical responses *in vitro*, moving one step forward to personalized medicine in psoriasis field.

$CLA^+$ lymphocytes have been extensively studied in the context of psoriasis disease, as last reviewed here (22). The importance of $CLA^+$ T cells in psoriasis resides not only in their contribution to lesion formation but also in their role as peripheral biomarkers of successful treatment, as it has been confirmed by recent studies. For example, a significant reduction of circulating $CLA^+$ T cell, as well as IL-6 and IL-22, has been associated to clinical efficacy after anti-TNF-alpha treatment (23). Similarly, a reduction on the number of $CLA^+$ Th17/Tc17 and Th22/Tc22 cells was observed after 6 weeks of phototherapy and balneotherapy, which positively correlated with the reduction on PASI score (24).

## ATOPIC DERMATITIS

$CLA^+$ T cells are involved in initiation and perpetuation of atopic dermatitis (AD) (25), since they are functionally related to cutaneous inflammation (4,26). Recent blood phenotyping studies on peripheral blood mononuclear cells from AD patients and healthy controls (HC) have illustrated increased percentage of $CLA^+$ memory T cells in moderate-to-severe AD patients compared with age-matched HC, but a decrease with increasing age only in AD patients (27,28). Expansion of $CLA^+$ T cells is accompanied by predominance of $CLA^+$ Th2/Tc2 and Th22/Tc22 response in AD. Whereas $CLA^+$ Th2 cell counts are similarly increased across all ages and are significantly higher than HC, $CLA^+$ Th22 cell counts increase with age only in AD and its levels are also higher than in HC (27–29). $CLA^+$ Th22 levels correlate with severity parameters (SCORAD: Scoring Atopic Dermatitis, EASI: Eczema Area and Severity Index), pruritus and IL-17 producing cells (29,30). $CLA^+$ $IL\text{-}13^+$ T cells positively correlate with SCORAD, serum IgE levels and IL-22 frequencies (27). Regarding Th1/Tc1 cell subsets, AD is characterized by decreased $CLA^+$ Th1/Tc1 frequencies in conjunction with negative correlations between $CLA^+$ $IFN\text{-}gamma^+$ T cells and SCORAD, and $CLA^+$ $IL\text{-}13^+$ and $IL\text{-}22^+$ populations. $CLA^+$ Th1 frequencies increase with age both in AD and HC, but do not reach the HC levels, and they are associated with disease duration. All these data

support an imbalanced $CLA^+$ Th1:Th2 cell ratio that increases with age in both AD and HC, but remains decreased in AD (27–29).

Expression of the indicator of T cell mid-activation, inducible costimulatory molecule (ICOS), has been reported to be enhanced both in $CLA^+$ and $CLA^-$ memory T cell subsets in AD patients compared to HC and psoriasis (PSO) patients, predominantly in the skin-homing subset, and correlates with SCORAD. In contrast, expression of Human Leukocyte Antigen (HLA)-DR, the chronic T cell activation MHC class II antigen, is similar in infants regardless AD status, but increases with age reaching high levels in adult AD patients versus HC and PSO patients, particularly among $CLA^+$ T cell population, which correlates with SCORAD (28,29,31). Besides, $CLA^+$ regulatory T cells ($T_{regs}$) frequencies are higher in AD compared with HC and PSO patients, and they correlate with both ICOS and HLA-DR, and also with clinical parameters (SCORAD and IgE levels) (31). Intriguingly, OX40, another costimulatory molecule predominantly expressed on T cells and required for long-term memory responses, is mainly expressed by $CLA^+$ memory T cells in both AD patients and HC (32). A recent epigenetic report has extended the knowledge on the $CLA^+$ T cells of male adult patients with severe AD and allergen specific IgE sensitization (33). Peripheral blood mononuclear cells sorted into four different T cell populations ($CD8^+$, $CD4^+$, $CD4^+$ $CD45RA^+$ and $CD4^+$ $CLA^+$) have revealed differentially DNA methylations in 40 protein-coding genes in the $CD4^+$ $CLA^+$ subset in AD patients versus HC. Among them, IL-13 gene promoter shows decreased methylation levels, which negatively correlate with IL13 mRNA expression levels in this subset.

# VITILIGO

Melanocyte-specific circulating memory $CD8^+$ $CLA^+$ T cells induce melanocyte apoptosis, together with other mechanisms such as cell detachment triggered by E-cadherin disruption (34), contribute to melanocyte loss and the development of depigmented skin lesions in vitiligo. Several blood endotyping studies have depicted that vitiligo patients have low frequencies of circulating $CD4^+$/$CD8^+$ $CLA^+$ $T_{EM}$/$T_{CM}$ cells compared to PSO patients, being similar to HC, supporting that $CLA^+$ T cells migrate to the skin (35,36). Within T cell subsets, vitiligo patients have the highest frequency of $CD4^+$/$CD8^+$ $CLA^+$ T cells producing IFN-gamma compared with AD, PSO, and alopecia areata (AA) patients, and HC (35), supporting that vitiligo is caused by a type 1 T cell response (37,38). Consistent with the literature describing $CD8^+$ T cell role in melanocytes death under IFN-gamma stimulation, $CD8^+$ $CLA^+$ T cell population producing IFN-gamma predominate over $CD4^+$ $CLA^+$ T cell subset in vitiligo patients (35). Interestingly, the same study showed the highest frequency of $CD4^+$/$CD8^+$ $CLA^+$ T cells producing IL-9 in

vitiligo patients in comparison with AD, PSO and AA patients and HC, pointing out for the first time a possible role for IL-9 on the physiopathology of vitiligo. Furthermore, Th17 subset, either $CLA^+$ or $CLA^-$, was also increased in vitiligo compared to HC, AD and, surprisingly, PSO patients, a disease associated to be driven by IL-17 activation. Regarding $CLA^+$ Th22 subset, it was also augmented in vitiligo patients compared to PSO and HC (35). In accordance to this fact, IL-22 has been reported to participate in the pathogenesis of vitiligo, as it promotes IL-1β secretion from keratinocytes what cause the suppression of melanogenesis and melanocyte migration as well as the induction of melanocyte apoptosis (39). The active participation of autoreactive $CD8^+$ T cells in vitiligo indicates that immune tolerance has been disrupted. Several studies have shown that vitiligo patients have a reduced amount of infiltrating $T_{regs}$ in non-lesional, perilesional and lesional skin (40,41). Vitiligo patients also have fewer amounts of total circulating $T_{regs}$ compared to AD and PSO patients, the difference being not that obvious in the $CLA^+$ $T_{regs}$ subset (35).

## ALOPECIA AREATA

CLA relevance in alopecia areata in humans was first revealed by Yano et al., describing higher positivity in peripheral blood mononuclear cells, $CD4^+$ and $CD8^+$ lymphocytes from AA compared with HC, and that CLA positivity negatively correlated with clinical improvement (42). Later, phenotyping research on peripheral blood mononuclear cells described that $CLA^{+/-}$ Th2 cell frequencies are similar between AA and AD patients, both higher than HC, and they correlate with AA severity; whereas skin homing Tc2 and Th22 are significantly higher in AA versus HC (43). Also, positive correlations have been reported not only between $CLA^+$ Th2/Tc2 and Th22/Tc22, but also between $CLA^+$ Th17/Tc17 and Th22/Tc22, particularly in the $CD8^+$ subset. In addition to this, skin homing $CD4^+/CD8^+$ $T_{CM}$ cell counts are higher in AA patients, compared with AD patients and HC, unlike $CD4^+/CD8^+$ $T_{EM}$ frequencies, and $CD4^+$, but not $CD8^+$, subset shows HLA-DR activation in $T_{CM}$ cells. Regarding $T_{regs}$ cells, diminished frequencies of total and $CLA^+$ $T_{regs}$ have been described in AA patients compared to AD patients and HC, which correlate with skin homing Th9, Th2/Tc2, Tc17 and Tc22 counts.

## OTHER INFLAMMATORY SKIN DISEASES

In lichen planus there is accumulation of $CLA^+$ T cells in the epithelium of the buccal mucosa in oral lichen planus (OLP), as well as in the epidermis of skin biopsies in cutaneous lichen planus (CLP) (44). Increased E-selectin expression in lesional biopsies from OLP over perilesional tissue, together with a significantly higher proportion of $CD8^+$ $CLA^+$ T cells were observed by immunohistochemical analysis. It has been

shown cutaneous T-cell-attracting chemokine (CTACK, also CCL27) secretion by oral keratinocytes which increased in the presence of IFN-gamma and actively attracted $CLA^+$ memory T cells to the oral epithelium. Although both normal oral mucosa and lesions from chronic OLP showed low levels of CTACK expression, it may still play a role in early recruitment of T cells and immunopathogenesis of OLP (45).

Although the role of $CLA^+$ T cells in non-immediate drug-induced cutaneous reactions was first reported two decades ago (46), there is still a lot to learn about them in drug allergy. Recently, it has been shown that in drug rash with eosinophilia and systemic symptoms (DRESS) (47) IL-4 and IL-13 producing $CD4^+$ T cells are increased during active disease and decline with recovery, pointing at the relevance of $CLA^+$ Th2 cells in the pathogenesis of DRESS. In β-Lactam hypersensitivity a new T-cell subset has been proposed, blood- and skin-derived clones specific for piperacillin expressed high levels of skin-homing chemokine receptors and migrated in the presence of the ligands CCL27 (48).

# DISCUSSION

The involvement of $CLA^+$ T cells in different inflammatory skin disorders, with diverse pathological immunological mechanisms, makes them interesting for human dermatology (Figure 1b). Also, circulating memory $CLA^+$ T cells specific for disease relevant antigens/allergens have reported, for example *S. pyogenes* in psoriasis (22) or house dust mite and *Staphylococcus aureus* enterotoxin B in atopic dermatitis (4). Despite the functional relevance of memory $CLA^+$ T cells has been better described in psoriasis and atopic dermatitis (4,22,49); their potential role in other T cell-mediated skin conditions is still to be fully investigated. Various approaches that involve minimal manipulation of those cells are providing immunological information that relates to clinic in a translational way. Although animal models and complex *in vitro* models are providing important information to understand human inflammatory skin disorders, there is a need to use clinical human samples to gather patients' genetic background and real diseased skin cells (50). For example, animal models cannot reflect the immune response present in patients after several years of chronic cutaneous inflammation and numerous flares. Different aspects can influence the immune response in inflammatory skin diseases such as genetic background, disease endotype/patient heterogeneity, local antigen presentation by human epidermal cells, impact of environmental triggers of disease or neurogenic inflammation. This integrated view is the approach that $CLA^+$ T cells exploration follows.

Nonetheless, the interplay between circulating and tissue resident $CLA^+$ T cells should be further addressed. Particularly since the later have been described to persist in resolved skin after treatment and to be involved in the recurrence of cutaneous lesions in psoriasis, vitiligo, or fixed drug eruptions (51).

We are clearly still at the top of the iceberg unravelling the information that those lymphocytes can provide for human inflammatory cutaneous conditions. For all these translational capacities, circulating memory $CLA^+$ T cells can be proposed as peripheral cellular biomarkers in human inflammatory skin disorders.

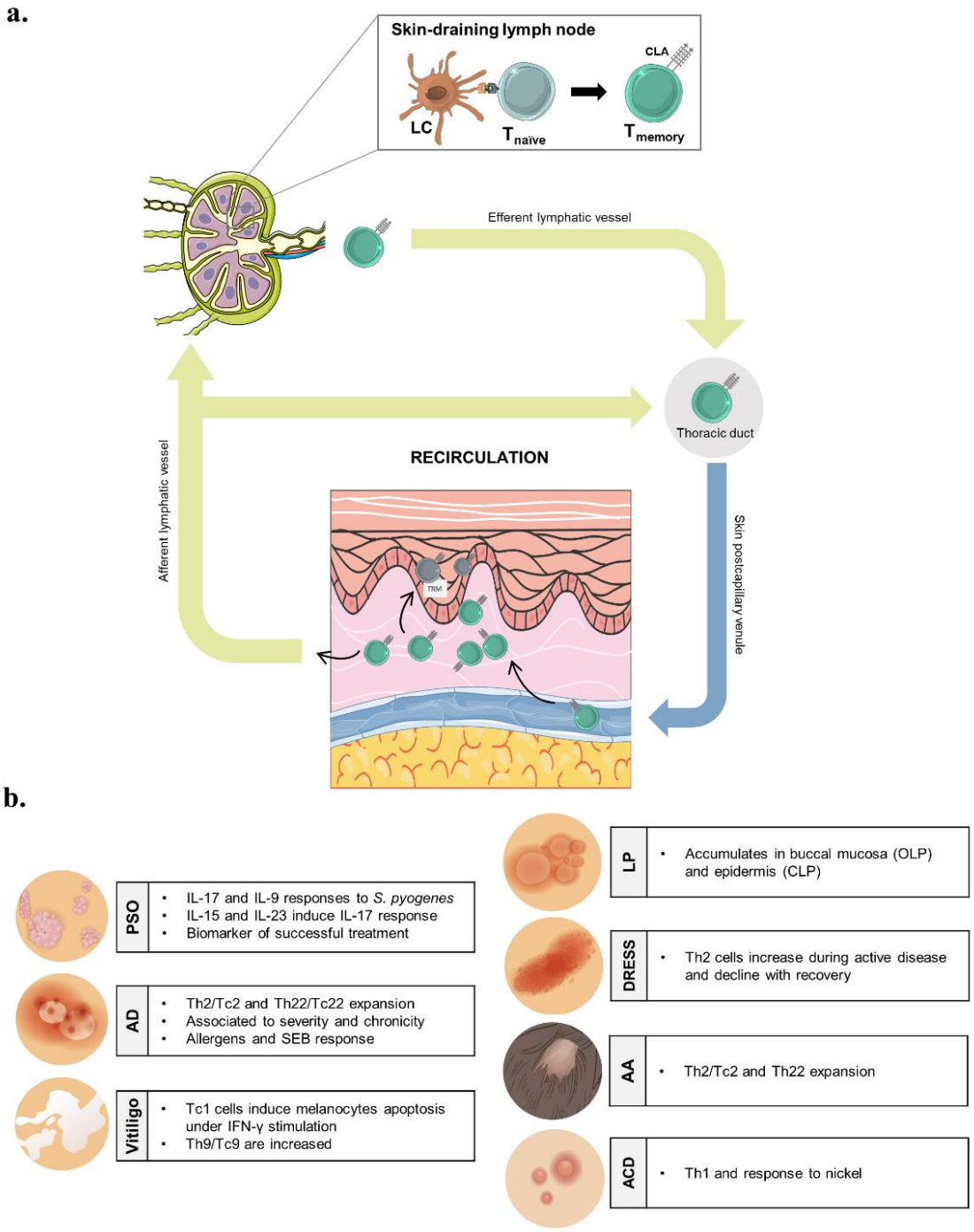


**Figure 1. Translational relevance of human circulating memory CLA$^+$ T cells in inflammatory skin disorders. (a)** In skin-draining lymph nodes, antigen-specific Langerhans cells encounter naïve T cells generating memory T cells that express the skin-homing receptor CLA. CLA$^+$ memory T cells enter the general circulation, through efferent lymphatic vessels, and will extravase to inflamed skin. Some CLA$^+$ memory T cells leave cutaneous tissue through afferent lymphatic vessels, recirculating back to the bloodstream. Whereas others are retained at the epidermal layer as resident memory T cells **(b)** Principal antigen/allergen/autoantigen-induced effector functions of CLA$^+$ memory T cells in human inflammatory cutaneous conditions. AA, Alopecia Areata; ACD, Allergic Contact Dermatitis; AD, Atopic Dermatitis; CLA, Cutaneous-associated Lymphoid Antigen; CLP, Cutaneous Lichen Planus; DRESS, Drug Rash with Eosinophilia and Systemic Symptoms; LC, Langerhans cells; OLP; Oral Lichen Planus; PSO, Psoriasis; TRM; T resident memory. This figure was created using images from SMART Servier (https://creativecommons.org/licenses/by/3.0/), WikiHow (https://creativecommons.org/licenses/by-nc-sa/3.0/) and Freepik.es (@brgf).

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