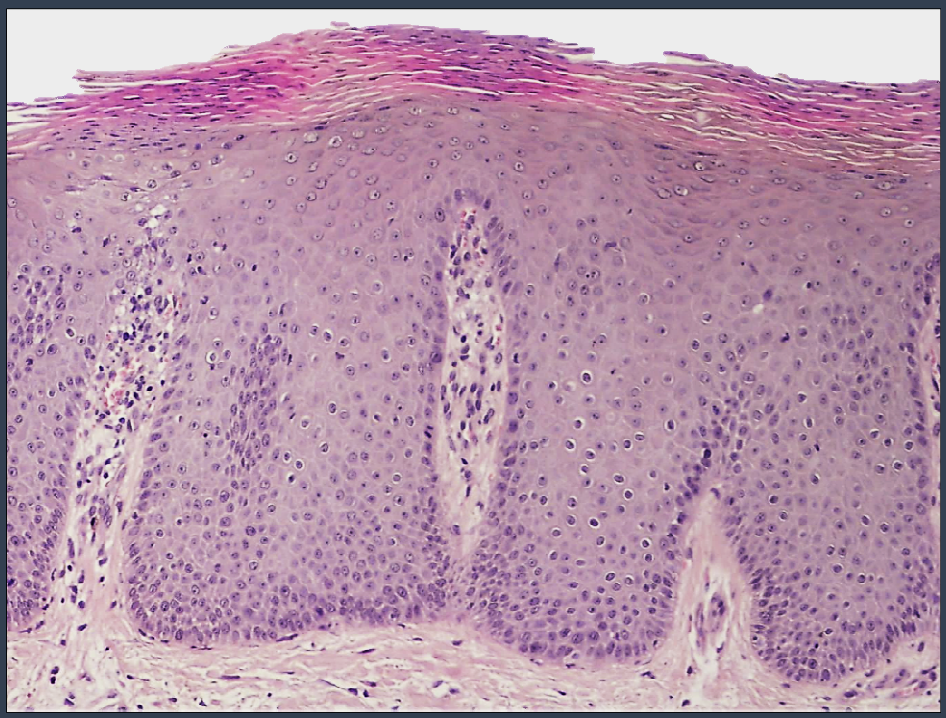
UNIVERSITAT DE BARCELONA

Characterization of a New Ex Vivo Model for the Study of Psoriasis Immunopathology

Ester Ruiz Romeu

Characterization of a New *Ex Vivo* Model for the Study of Psoriasis Immunopathology

Doctoral Thesis



Ester Ruiz Romeu | 2017

UNIVERSITAT DE BARCELONA

PhD Programme in Biomedicine

Characterization of a New *Ex Vivo* Model for the Study of Psoriasis Immunopathology

Thesis submitted by

Ester Ruiz Romeu

To qualify for the Doctorate degree by

University of Barcelona

Thesis Director and Tutor

Dr. Luis Francisco Santamaria Babí

Head of Translational Immunology Group

-

Associate Professor of Immunology

Department of Cellular Biology, Physiology, and Immunology

Faculty of Biology, University of Barcelona

-

External Consultant (Hospital del Mar)

" [...] es preciso saber dirigir la casualidad, así es como se obtienen los más difíciles resultados. "

El Conde de Montecristo (Alexandre Dumas,1845)

ACKNOWLEDGEMENTS

Achieving a doctorate is a long journey that goes beyond acquiring specific scientific knowledge, but entails many other valuable skills and competencies. One way or another, many individuals and institutions have contributed to overcoming this challenge, and to whom I am most grateful.

In the first place, to my director and mentor, Dr. Luis Francisco Santamaria, who has ecnouraged me to be a better scientist, and who has transferred me the "know-how" foundations for my future career. His perseverance and patience during the past years have been key to successfully completing this stage throughout my scientific life. I thank extensively his advice, comprehension and kindness, and I am honored to be part of his scientific projects.

I would also like to express my most sincere gratitude to Antonio Celada for his support and collaboration, and, not less important, for making me feel welcomed in his laboratory. Especially, it has been a great honor to dispose of an outer scientific view of my work from such an eminence.

I want to thank to our closest external collaborators, without whom it would have been impossible to carry out this work. To the dermatologists from Hospital del Mar (Barcelona), Hospital de Calella and Hospital Arnau de Vilanova (Lleida), who recruited the participants and provided the samples that have been used in this thesis.

Also, a very especial mention to our most distant collaborators, J. Jura, B. Bugara and B. Lipert from Krakow, and E. P. Prens and E. F. Florencia from Rotterdam, who were active participants in part of the research work.

Special thanks to the Immunology Unit at Hospital Clínic (Barcelona), the CCiTUB services, Microscopy facilities and the Pathological Anatomy service at Barcelona Science Park, for their technical support.

Thanks to my lab mates, friends, and more especially to my beloved family, that have made this process more bearable.

Finally, I am profoundly grateful to a few institutions who have supported and funded my work through their respective PhD fellowship programs:

- FI-DGR 2013-2014 program from Generalitat de Catalunya
- AAD-2013 program from Generalitat de Catalunya
- FPU-2013 program from Ministerio de Educación, Cultura y Deporte

Generalitat de Catalunya

GOBIERNO DE ESPAÑA
MINISTERIO DE EDUCACIÓN, CULTURA Y DEPORTE

INDEX

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

AMP	Antimicrobial peptide
APC	Antigen-presenting cell
CCL	CC-chemokine ligand
CCR	CC-chemokine receptor
CLA	Cutaneous lymphocyte-associated antigen
$CLA^{+/-}$/Epi	Cocultures of either CLA^{+} or CLA^{-} T cells and autologous epidermal cells
CTACK	Cutaneous T-cell–attracting chemokine (also known as CCL27)
CTLA	Cytotoxic T lymphocyte-associated antigen
CXCL	CXC-chemokine ligand
CXCR	CXC-chemokine receptor
DC	Dendritic cell
DETC	Dendritic epidermal T cell
EAE	Experimental autoimmune encephalomyelitis
E-selectin	Endothelial-cell selectin
GWAS	Genome-wide association study
ICAM	Intercellular adhesion molecule
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
ILC	Innate lymphoid cell
IMQ	Imiquimod
K17	Keratin 17
LC	Langerhans cell

LFA	Lymphocyte function-associated antigen
mAb	Monoclonal antibody
MCPIP1	Monocyte chemotactic protein-1–induced protein
MHC	Major histocompatiblity complex, also known as HLA (human leucocyte antigen) in humans
NF- κB	Nuclear factor-kappa B
NK	Natural killer
PASI	Psoriasis area severity index
pDC	Plasmacytoid dendritic cell
PDGF	Platelet-derived growth factor
PSGL	P-selectin glycoprotein ligand
P-selectin	Platelet selectin
PSORS	Psoriasis susceptiblity region
SAg	Superantigen
SE	*Streptococcus pyogenes* extract
SPE	Streptococcal pyrogenic exotoxin
S. pyogenes	*Streptococcus pyogenes*
STAT	Signal transducer and activator
Tc	T-cytotoxic
TCR	T-cell receptor
Th	T-helper
TLR	Toll-like receptor
TNF	Tumor necrosis factor
VCAM	Vascular cell adhesion molecule
VEGF	Vascular endothelial growth factor
VLA	Very late antigen

INTRODUCTION

1. T-cell skin tropism and immune surveillance

1.1 T-cell homing and skin tropism

Leukocytes in the blood migrate to tissues not randomly, but through specific molecular mechanisms. Therefore, peripheral blood leukocytes include a mixture of populations of circulating effector T lymphocytes, that result from clonal expansion and differentiation from naive T cells in secondary lymphoid tissues (spleen, peripheral lymph nodes, etc.), with different individual tissue tropism capabilities. This selective tissue tropism is primed by a combination of specific molecular interactions comprising important roles of several adhesion molecules and chemokines (Marelli-Berg et al., 2007), a process which is determined by the lymph node environment and tissue-derived dendritic cells (DCs) (Figure 1).

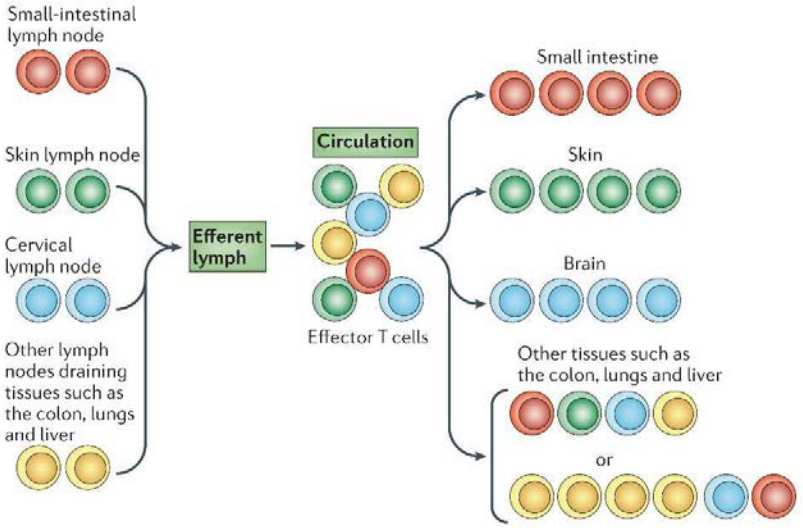


Figure 1. Generation of tissue-tropic effector T-cell subsets and their localization to extralymphoid tissues. Following their activation in secondary lymphoid tissues, effector T cells gain the ability to enter a wide range of tissue locations. Generally, which homing receptors are induced on T cells depends on the lymph node in which activation takes place. Then, effector T-cell populations drain through efferent lymph vessels and reach blood circulation through the thoracic duct. Skin, small intestine and brain seem to selectively recruit those effector populations generated in the relevant draining lymph nodes. However other tissues such as lungs, colon, liver and kidney also allow the entrance of effector T-cell populations generated in non-draining lymph nodes (Agace, 2006).

Within these populations, there is a subset consisting of T lymphocytes that are specially localized in cutaneous sites under inflammatory conditions. The surface expression of the cutaneous lymphocyte-associated antigen (CLA) identifies this pool of memory

effector skin-homing T cells. In fact, it is expressed by more than 90% of infiltrating T cells present in cutaneous inflammatory sites, but less than 20% of T cells in other non-cutaneous sites. This molecule is also present in about 15% of peripheral T cells (Picker et al., 1993), what allows easy isolation from blood extractions. The CLA antigen consists of a carbohydrate structure, similar to the sialyl Lewis X antigen, whose expression occurs as an epitope after a post-translational modification of a single cell-surface receptor, the platelet selectin (P-selectin) glycprotein ligand (PSGL)-1 (Fuhlbrigge et al., 1997), and is induced on T cells undergoing naive, expressing CD45RA, to memory, expressing CD45RO, transition in lymph nodes that drain the skin (Picker et al., 1993). The CLA antigen is a ligand for the endothelial-cell selectin (E-selectin) and P-selectin, adhesion molecules that are induced in endothelial cells under inflammatory conditions. Recently, CLA was also reported to be expressed on CD43 molecule, which selectively binds E-selectin (Fuhlbrigge et al., 2006). In addition, it is known that extravasation of CLA^+ T cells into the skin involves several other molecular interactions, including very late antigen (VLA)-4/vascular cell adhesion molecule (VCAM)-1 and lymphocyte function-associated antigen (LFA)-1/intercellular adhesion modelcule (ICAM)-1 (Babi et al., 1995), together with chemokines such as CC-chemokine ligand (CCL)-17 (Kupper and Fuhlbrigge, 2004). Other chemokines are also essential for the transendothelial migration, for example the cutaneous T-cell–attracting chemokine (CTACK), also known as CCL27, that is mainly produced in the skin by basal keratinocytes and is up-regulated in cutaneous inflammation (Homey et al., 2002) (Figure 2). Moreover, the expression of its cognate receptor CC-chemokine receptor (CCR)-10 on T cells is restricted to the CLA^+ $CD4^+$ subset (Hudak et al., 2002).

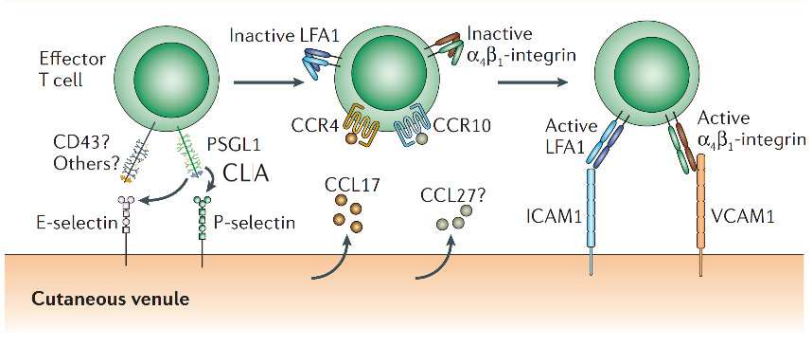


Figure 2. Multistep process of T-cell entrance into the skin. T-cell migration to cutaneous sites is regulated by the coordinated interaction of various cell-surface molecules on the T cell with their respective ligands on the surface of dermal venules (Agace, 2006). $\alpha_4\beta_1$: VLA-4.

1.2 Skin immune surveillance

The skin constitutes the body's largest and more exposed interface with the environment and thus plays an active defense role against external hostiles agents. Aside of its properties as a physical barrier, a robust system of immune surveillance participates either in maintaining homeostasis, or in cutaneous malignancies and infections (Kupper and Fuhlbrigge, 2004). The initiation and maintenance of a local effective immune response are crucially dependent on the orchestrated migration of T-cell subsets to distinct tissue locations, that is effector memory CLA^+ T cells to the skin. In non-inflamed skin, E-selectin and P-selectin are constitutively expressed at sufficient low level to support CLA^+ T cell rolling (Weninger et al., 2000). Additionally, resident immune cells include dermal DCs and epidermal Langerhans cells (LCs). During cutaneous inflammation, E-selectin and P-selectin expression is increased, promoting an influx of circulating effector memory CLA^+ T cells (Figure 3).

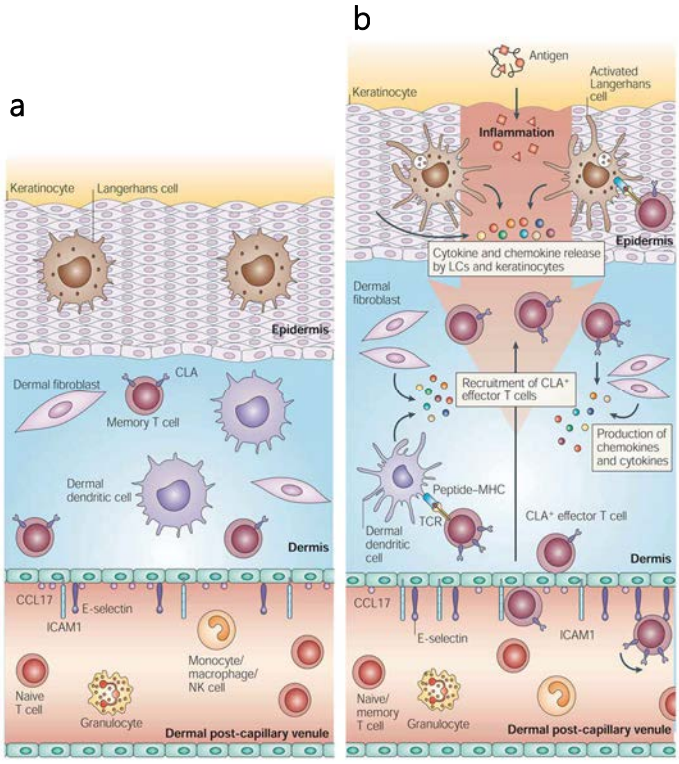


Figure 3. Non-inflamed skin and adaptive response in skin inflammation. a) In normal human skin, the immune surveillance is crucial in maintaining homeostasis, and it is especially mediated by several immune cells that are resident in the epidermis, basically LCs, and a dermal infiltrate composed of mast cells, DCs and a small number of CLA^+ memory T cells, whose migration into the non-inflamed skin is sustained by baseline levels of expression of E-selectin, CCL17 and ICAM-1. b) Upon epithelial injury and memory antigen-specific T cell generation, circulating CLA^+ T cells are recruited by the up-regulation of endothelial adhesion molecules and chemokines, encounter the cognate antigen carried by activated LCs and DCs, and are activated to proliferate and carry out specific functions (Kupper and Fuhlbrigge, 2004).

Overall, the fact that skin-homing T cells are actively involved in cutaneous inflammation through antigen recognition, and the possibility of selective isolation from blood through the expression of a surface marker, the CLA antigen, makes CLA^+ T cells an interesting tool for translational studies in skin immune-mediated diseases.

2. Circulating CLA^+ T lymphocytes as peripheral cell biomarkers in T-cell-mediated skin diseases

A biological marker, namely biomarker, would be defined as a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacological responses to a therapeutic intervention (Naylor, 2003). In practice, biomarkers include tools and technologies that improves understanding the prediction, cause, diagnosis, progression, regression or outcome of treatment of diseases. Then, either biochemical, molecular or cellular alterations that are measurable in tissues, cells, or fluids could be used as a potential biomarker (Mayeux, 2004). CLA^+ T cells have been proposed as cellular biomarkers for immune-mediated skin diseases that may provide translational information (Ferran et al., 2013a, supporting publication in page 153, in Appendix I), since they can be easily isolated from peripheral blood, and somehow solve the limitation represented by the need of a large number of skin biopsies for the study of those cells. However, if it is intended to get, for instance, severity- and clinical-associated outcomes from peripheral CLA^+ T cells, first it would be necessary to demonstrate the recirculation of those cells between affected skin and blood during cutaneous inflammation.

As previously described, CLA^+ T-cell rolling depends on the interaction of several cell-surface molecules on T cells and endothelial cells. Trafficking blockade through LFA-1/ICAM-1 interaction between T/endothelial cell, respectively, was indeed explored for potential therapeutic targeting (Gottlieb et al., 2002). The action of anti–LFA-1 treatments has shed new light regarding the recirculatory capacity of effector memory CLA^+ T cells in psoriasis and atopic dermatitis (Harper et al., 2008; Johnson-Huang et al., 2012; Vugmeyster et al., 2004), thus providing translational evidence in favor of effector memory CLA^+ T-cell recirculation between skin and blood during cutaneous inflammation. Actually, in both diseases, a rise in circulating CLA^+ T cells is observed in parallel to the clinical improvement of inflammation due to treatment (Harper et al.,

2008; Vugmeyster et al., 2004), either by inhibiting the LFA-1/ICAM-1–dependent transendothelial migration of CLA^+ T cells, and/or by the accumulation in the blood of those CLA^+ T cells that infiltrated the skin lesions and followed lymphatic drainage through dermal lymph nodes (Ferran et al., 2013a). Such lymphocyte accumulation in the blood, namely lymphocytosis, may develop a skin relapse after stopping the treatment (Johnson-Huang et al., 2012).

This phenomenon indicates that in T-cell mediated skin diseases implying chronicity, in which skin flares take place, recirculating CLA^+ T cells might carry substantial information about their antigen-specificity, thus acting as peripheral cell biomarkers (Ferran et al., 2013a). For instance, in atopic dermatitis or contact dermatitis, those CLA^+ T cells, but not CLA^- T cells, isolated from peripheral blood, respond to house dust mites or nickel, respectively (Santamaria-Babí et al., 1995). Other CLA^+ T-cell–associated selectivity for cutaneous antigens or allergens that belong to T-cell mediated skin pathologies, including cognate microbial antigens that match the pathogenesis, are shown in Table 1.

Disease	Antigen
Atopic dermatitis	*Dermatophagoides pteronyssinus* (Santamaria-Babí et al., 1995); Casein (Abernathy-Carver et al., 1995)
Contact dermatitis	Nickel (Santamaria-Babí et al., 1995)
Vitiligo	Melan-A (Ogg et al., 1998)
Herpex simplex	HSV-2 (Koelle et al., 2002)
Psoriasis	*Streptococcus pyogenes* (Ferran et al., 2013b)

Table 1. Compartmentalization of skin-specific antigenic immune response in CLA^+ T cells. (adapted from Santamaria-Babí, 2004).

Also, several studies reported that, in different T-cell–mediated cutaneous diseases, the clinical status can induce changes in phenotype, cell numbers or related mechanisms of CLA^+ T cells, as exposed in 2013a by Ferran et al. (summarized in Table 2). This view has been recently reported for atopic dermatitis (Czarnowicki et al., 2016). In summary, results obtained from different T-cell–mediated skin diseases highlight the relevance of circulating CLA^+ T cells. These cells may be a source of information as peripheral cell biomarkers, both in a predictive or retrospective way, as their number and phenotype parallel local cutaneous events and correlate with clinical activity and treatments.

Disease	CLA⁺ T cell phenotype	Associated clinical/function event	Key outcome
Atopic dermatitis	Pathological Th2 cells	In active form, circulating CLA⁺ T cells express activation markers and produce IL-4 and IL-13 (Akdis et al., 1997; Santamaria-Babí et al., 1995).	Memory CLA⁺ T cells freshly isolated express features and functional properties of *in vivo* activity.
		CGRP selectively activates circulating CLA⁺ T cells from atopic dermatitis patients and induce IL-13 (Antúnez et al., 2009).	Stress-associated CGRP contributes to the cross-talk between immune and nervous systems through CLA⁺ T cell function.
Skin tumors	Anti-tumoral T cells	Down-regulation of E-selectin in metastatic malignant melanoma leads to decreased tumor-infiltrating capacity of circulating CLA⁺ T lymphocytes (Weishaupt et al., 2007).	Blocked recruitment of activated tumor-specific T cells limits the effectiveness of immunotherapy. Enhancing migration of CLA⁺ T cells could be beneficial.
	Malignant T cells	In SS, a strong correlation is observed between % of circulating lymphoma CLA⁺ T cells and the extent of skin symptoms (Borowitz et al., 1993; Heald et al., 1993).	Targeted inhibition of tumor-specific CLA⁺ T cells might be therapeutically important.
Acute GvHD	Alloimmune suppressive CD4⁺ T cells.	Increased amount of CLA⁺ Tregs at engraftment is associated with the prevention of skin acute GvHD (Engelhardt et al., 2011, 2012).	Frequencies of CLA⁺ Tregs could be used to predict acute GvHD and to improve transplant survival.
Scleroderma	Profibrotic CD8⁺ T cells	Circulating CD8⁺ CLA⁺ T cells in early stages of diseases produce IL-13, accumulate in the skin and produce cytotoxic granules (Fuschiotti et al., 2013).	Early CD8⁺ T cells homing to the skin may represent a potential target for therapeutic intervention.
Psoriasis	Pathological T cells	% of circulating CLA⁺ T cells inversely correlates with PASI and BSA in acute psoriasis, but not in chronic form (Ferran et al., 2008). Number of circulating CLA⁺ Vγ9δ2⁺ T cells inversely correlated with PASI score and increased with successful treatment (Laggner et al., 2011).	CLA⁺ T cells are sequestered into inflamed skin and may recirculate between blood and skin.
		Number of circulating CD4⁺ CLA⁺ and CD3⁺ CLA⁺ HLA-DR⁺ is increased in active disease compared with chronic form (Pont-Giralt et al., 2006).	Activated CLA⁺ CD3⁺ cells in peripheral blood are preferentially involved in acute stages of psoriasis.

Table 2. CLA⁺ T cells clinical activity and relations with T-cell–mediated inflammatory cutaneous diseases. CGRP: calcitonin gene-related peptide; SS: Sézary syndrome; GvHD: graft versus host disease; Treg: T regulatory; PASI: psoriasis area severity index; BSA: body surface area. Summary of Ferran et al., 2013a.

3. Psoriasis: from epidermal to immunological skin disorder through translational research

Psoriasis, in its most common form, is a chronic, inflammatory, immune-mediated skin disease that affects about 2–3% of the Caucasian population. Psoriasis is now known to be the most prevalent T-cell–mediated skin disease, mostly through $CD3^+$ $CD45RO^+$ CLA^+ T cells, which occurs in individuals with genetic susceptibility, in conjunction with instigating environmental stimuli. However, this concept was not widely considered until the 1990s, but it has been the result of a continuous evolution of psoriasis pathogenic concepts, mainly through translational research. This process uses scientific investigation to advance the understanding of disease and to improve health by translating observations of bench-derived experimental discoveries to bedside clinical research, and vice versa, that is, the use of clinical observations that leads to scientific investigation (Guttman-Yassky and Krueger, 2007). In fact, psoriasis confers uniqueness for a disease studied through translational science, in which many rounds of bidirectional translational research have occurred over the years.

3.1 Histological features and early translational research in psoriasis

Affected skin is classically characterized by a focal formation of sharply demarcated, red and slightly raised lesions with constantly shedding silvery-white scales. These raised plaques derive from an excessive growth of keratinocytes, the specialized epithelial cells from the skin, which proliferate and mature rapidly. In fully developed lesions, there are a massive thickening of the epidermis (acanthosis), elongated epidermal rete ridges, and consequently elongated dermal papillae containing dilated capillaries. Terminal differentiation, normally occurring in upper granular keratinocytes, is disturbed in psoriasis, and that results in a diminished thickness or loss of the stratum granulosum and in an irregular and thickened stratum corneum (hyperkeratosis) with retention of the nuclei in squamous keratinocytes, namely corneocytes, (parakeratosis) (Schön and Boehncke, 2005; Wagner et al., 2010). Poorly adherent stratum corneum is formed due to low lipid cementation by corneocytes themselves, resulting in the characteristic scales and subsequent break in the protective barrier (Krueger, 2005; Lowes et al., 2007) (Figure 4).

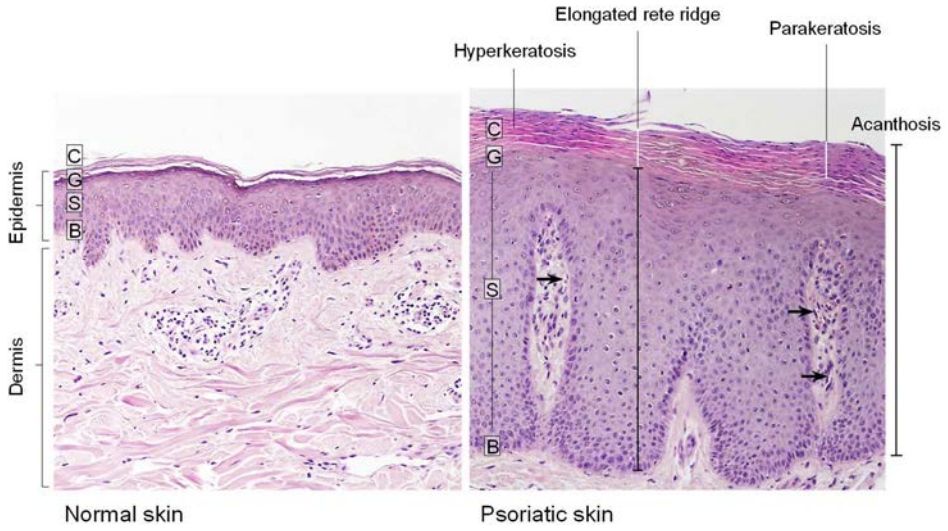


Figure 4. Pathological tissue alterations in psoriatic skin. As compared with normal skin, the epidermis in psoriatic skin suffer several histopathological alterations, including profound acanthosis (thickening of the viable cell layers) with elongated rete ridges, hyperkeratosis (thickening of the cornified layer), loss or reduction of the granular layer, and parakeratosis (persistence of the nuclei in the cornified layer). In addition, dermal blood vessels are increased in numbers and reach up to locations underneath the epidermis (arrows). B: basal layer; S: spinous layer; G: granular layer; C: cornified layer.

These histopathological features of the epidermis led to the idea that psoriasis was widely considered to be a disease caused by altered proliferation and differentiation of keratinocytes. It was not until 1983, when it was first proposed a potential role of the cellular immune system, due to the detection of a marked infiltration of leucocyte subsets in psoriasis lesions (Bos et al., 1983), which are now more accurately defined by later histological studies, and thus defining clear cellular changes in the skin (Krueger, 2005). Neutrophils have long been identified in active lesions within small foci in the stratum corneum, known as Munro microabscesses, since they have characteristic nuclei and cytoplasmic staining properties. T lymphocytes are found interspersed between keratinocytes throughout the epidermis and in somewhat larger quantities in the dermis. In fact, their subsets are not uniformly distributed in psoriasis lesions, as epidermal T cells are predominantly $CD8^+$ T cells, whereas dermal T lymphocytes are a mixture of $CD4^+$ and $CD8^+$ cells, with a $CD4^+$ predominance similar to that seen in peripheral blood. LCs are resident in normal epidermis and also can be found in psoriasis lesions, sometimes in increased abundance, and in rough terms, the number of myeloid $CD11c^+$ DCs present in psoriasis lesions equals or exceeds the number of T

cells (Krueger, 2005). Plasmacytoid CD11c$^-$ BDCA-2$^+$ DCs (pDCs) have also been detected in the dermis of psoriatic lesions (Nestle et al., 2005) (Figure 5).

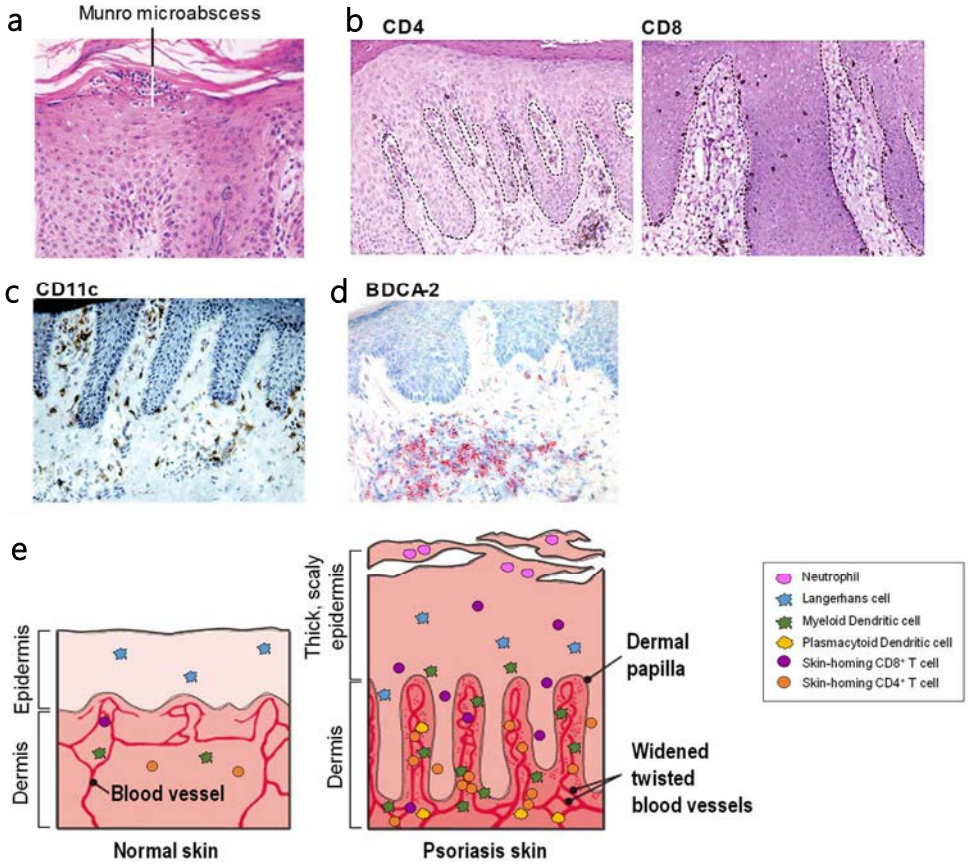


Figure 5. Infiltrating cells in psoriatic lesions. a) Neutrophils transmigrate through the epidermis and form the so-called Munro microabscesses (Schön and Boehncke, 2005). b) Upper dermis is infiltrated mostly by CD4$^+$ T cells, whereas most of the CD8$^+$ T cells are located within or close to the epidermis (Valdimarsson et al., 2009). c) CD11c$^+$ DCs are detected mainly within the upper dermis (Nestle et al., 2009). d) High numbers of BDCA-2$^+$ pDCs are present in the dermis of psoriatic lesions (Nestle et al., 2005). e) Cellular components of mature psoriatic plaque compared with normal skin. Normal skin contains an important number of resident and trafficking immune cells, as it is an immune-competent organ. However, in psoriasis, the leucocyte number is significantly increased, either by local expansion or high influx of immune cells that acquire migrating capacities through the dermis and the epidermis (adapted figure from Lowes et al., 2007). Positive staining: brown precipitate in b) and c); magenta dye in d).

3.2 Refining pathogenic concepts by the testing of first targeted therapeutics

The refinement of monoclonal antibody technologies and associated molecular biology, which was not yet available until the mid-80s, allowed the development of

targeted therapies for critical testing of the hypothesis on the primary defect in psoriasis: keratinocytes vs. cellular immunity. While inhibitors of keratinocyte proliferation resulted in a modest improvement, the marked improvement with the immunosuppression exerted by cyclosporine or tacrolimus suggested that activated T cells were pathogenic in psoriasis patients (Guttman-Yassky and Krueger, 2007). This hypothesis was further confirmed in 1995, when direct T-cell targeting with the fusion toxin DAB_{389}IL-2 (Gottlieb et al., 1995), and, a few years later, the costimulation blockade therapy with cytotoxic T lymphocyte-associated antigen (CTLA)4-Immunoglobulin (Ig) fusion protein, showed a reversal of clinical activity. Additionally, CTLA4-Ig not only reflected a reduction in activated T cells in lesions, but also in infiltrating DCs (Abrams et al., 2000), which might have an important role for ongoing disease activity, as it was later determined by especially activating and polarizing specific T-cell subsets. Therefore, outcomes in targeted therapeutics studies have contributed to a change in the pathogenic model from endogenous keratinocyte effects to one T-cell–mediated pathogenesis. However, early immune-targeted treatments were still not selective for polar T-cell subsets, that is for specific molecular pathways or cellular products.

3.3 Translational revolution in psoriasis: cytokine-targeted therapies and the discovery of the interleukin (IL)-23/T-helper (Th)17 pathway

With general acceptance of a T-cell–mediated pathogenesis in psoriasis, and based on more selective therapeutic treatments, molecular analyses started revealing the main cytokine drivers of psoriasis. Concurrent with the initial characterization of Th1/Th2 functions, and prior to the discovery of other Th lineages, psoriasis was believed to be mediated by the IL-12/Th1 pathway, as IL-12 and interferon (IFN)-γ were reported to be elevated in psoriasis skin lesions (Yawalkar et al., 1998). The generation of ustekinumab, a monoclonal antibody (mAb) directed against the p40 subunit of IL-12 had shown clinical efficacy (Kauffman et al., 2004). Later, p40 subunit was reported to also associate with p19 subunit to form IL-23 (Oppmann et al., 2000). In 2003, Th17 lineage, characterized by the production of IL-17, was reported (Aggarwal et al., 2003). It used the lineage-specific RORγt transcription factor and required, among others, IL-23, which promoted expansion and activation of Th17 cells which express several pro-inflammatory cytokines, including IL-17A, IL-17F, IL-21 and IL-22 (see timeline in Figure 6). Ustekinumab resulted to also interact with IL-23p40. IL-23R and IL-23 are elevated in psoriasis skin lesions (Tonel et al., 2010), and polymorphisms of genes that encode either IL-12/23p40 or the IL-23R are linked to psoriasis (Cargill et al., 2007, and see Table

4 on page 36). Subsequent studies then demonstrated that ablation of, or antibody treatment against, the p40 subunit was either protective or attenuating in experimental autoimmune encephalomyelitis (EAE) and collagen-induced arthritis murine models. In contrast, genetic ablation of IL-12p35–specific subunit was not protective (Benson et al., 2011). In addition, an early study suggested that IL-23 might be the dominant p40 cytokine in psoriasis, since mRNAs of IL-12/23p40 and IL-23p19–specific subunit were 10-fold up-regulated, whereas IL-12p35 levels were not overexpressed (Lee et al., 2004). Therefore, IL-23 mediates many disease pathologies previously attributed to IL-12, including psoriasis. This discovery led to a refocusing of anti-psoriatic therapies. For instance, the SMART anti–IL-12 antibody was discontinued in 2003 owing to its inability to target IL-23 (Teng et al., 2015).

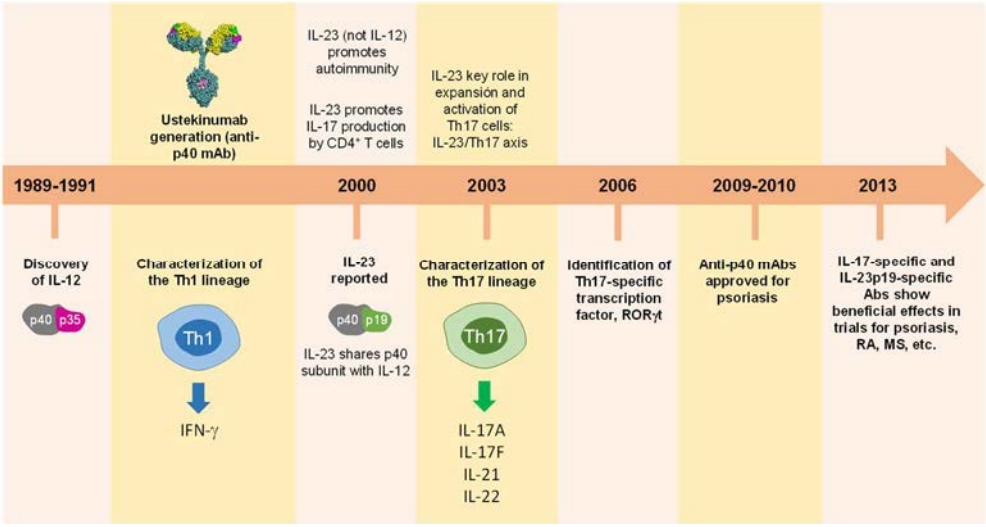


Figure 6. Timeline of IL-23/Th17 axis discovery and elucidation of key targets for psoriasis. Adapted from Benson et al., 2011, and Gaffen et al., 2014.

The extent and severity of lesions are quantified according to the Psoriasis Area and Severity Index (PASI), that integrates combined measures of the involved area, erythema, induration and desquamation. Anti-psoriatic biological therapies are evaluated on the basis of the frequency of patients with moderate to severe disease, generally with PASI>10, who show 75% reduction in baseline PASI score (PASI-75). The discovery of the IL-23/Th17 pathway led to fundamental changes in finding new targets for psoriasis treatments. After anti-p40 mAbs approval, many other mAbs specific for IL-17s and IL-17 receptor, as well as against IL-23p19, have shown promising results in

psoriasis, with 70–90% of patients reaching PASI-75, as well as in other chronic diseases (Kopp et al., 2015) (Table 3 and Figure 7). Particularly, the anti–IL-17s mAbs may induce a more specific response. For instance, ixekizumab (anti–IL-17A) and brodalumab (anti–IL-17RA), not only have shown impressive clinical outcomes, but they have also revealed a rapid normalization, within 2 weeks, of the expression of hundreds of disease-related genes prior to disease reversal, which usually takes place within 6 weeks (Krueger et al., 2012; Russell et al., 2014).

Targeting cytokines and their receptors with mAbs has become a mainstream therapeutic approach for autoimmune disorders, as they are generally well tolerated and safer than traditional immunosuppressive medications (Carretero et al., 2015). However, it is difficult to pinpoint which cytokine should be targeted because of the oversimplification as to how individual cytokines work in the network in psoriasis or other complex immune-based diseases. In fact, some biologics targeting potentially involved cytokines have been however discontinued for psoriasis. For instance, anti–IL-22 mAb fezakinumab, anti–IFN-γ mAb fontolizumab, anti–IFN-α mAb MEDI-545, or anti–IL-8 mAb ABX-IL-8, have all been withdrawn because of the lack of enough clinical efficacy achieved in early phases of clinical trials (Bissonnette et al., 2010; Harden et al., 2015; Mahil et al., 2016; Pinto-Almeida and Torres, 2014). Others like tumor necrosis factor (TNF)-α antagonists, which had demonstrated a high efficacy, remain important therapeutic agents for psoriasis since approval in 2006. Despite they represented a counterargument for the main role of T cells in the development of skin lesions, subsequent work demonstrated that TNF-α–blockade effectiveness was associated to suppression of IL-23 and IL-17 production, either by TNF-α–induced IL-23 production by DCs, and/or by synergizing with IL-17 to induce altered protein expression in keratinocytes (Lowes et al., 2005; Zaba et al., 2007). Therefore, discontinued drugs, the study of the specific mechanisms in efficacious therapies, and the fact that almost all disease-defining genes in patients with psoriasis are reversed through IL-17–signaling blockade, it is suggested that complex cytokine circuits are maintained solely through expression of IL-17A or IL-17F (Noda et al., 2015).

For these reasons, psoriasis is probably the best example of skin disease where rapid advances in pathogenic understanding and development of new therapeutics have occurred through translational science approach (Guttman-Yassky and Krueger, 2007).

Name	Manufacturer	Target	Formulation	Psoriasis	Psoriatic arthritis	Rheumatoid arthritis	Other inflammatory diseases
Ustekinumab	Janssen	IL-12–p40 and IL-23–p40	Fully human mAb	Marketed	Marketed	Phase II	Crohn's disease (Phase III)
Secukinumab	Novartis	IL-17A	Fully human mAb	Pre-registration	Phase III	Phase III	Ankylosing spondylitis (Phase III), asthma (Phase II) and multiple sclerosis (Phase II)
Ixekizumab	Eli Lilly	IL-17A	Humanized mAb	Phase III	Phase III	Phase II	–
Brodalumab	Amgen/ AstraZeneca	IL-17RA	Fully human mAb	Phase III	Phase III	–	Asthma (Phase II)
Tildrakizumab	Merck/ Sun Pharma	IL-23–p19	Fully human mAb	Phase III	–	–	–
Guselkumab	Janssen	IL-23–p19	Fully human mAb	Phase III	–	Phase II	–
ABT-122	AbbVie	IL-17A and TNF-α	Dual-variable-domain immunoglobulin	–	–	Phase II	–
AMG 139	AstraZeneca/ Amgen	IL-23–p19	Fully human mAb	Phase I	–	–	Crohn's disease (Phase II)
BI 655066	Boehringer Ingelheim	IL-23–p19	Humanized mAb	Phase II	–	–	Ankylosing spondylitis (Phase II) and Crohn's disease (Phase II)
CNTO 6785	Janssen	IL-17A	Fully human mAb	–	–	Phase II	–
CJM112	Novartis	IL-17A	mAb	Phase I/II	–	–	–
COVA322	Janssen/ Covagen	IL-17A and TNF-α	Bispecific antibody fusion protein	Phase I/II	Preclinical	Preclinical	Ankylosing spondylitis (preclinical)
ALX-0761	Merck Serono/ Ablynx	IL-17A and IL-17F	Bispecific half-life-extended nanobody	Phase I	–	–	–
Bimekizumab	UCB	IL-17A and IL-17F	Humanized mAb	Phase I	Phase I	–	–
LY3074828	Eli Lilly	IL-23–p19	Humanized mAb	Phase I	–	–	–
SCH-900117	Merck	IL-17A	Fully human mAb	–	–	Phase I	–

*Other early stage products in development include LY3114062 (Phase I in inflammatory arthritis) and RG7624 (Phase I for autoimmune diseases). IL-12, interleukin-12; IL-12–p40, p40 subunit of interleukin-12; IL-17A, interleukin-17A; IL-17F, interleukin-17F; IL-17RA, IL-17 receptor A; IL-23, interleukin-23; IL-23–p19, p19 subunit of interleukin-23; IL-23–p40, p40 subunit of interleukin-23; mAb, monoclonal antibody; TNF-α, tumour necrosis factor-α.

Table 3. Drug candidates targeting the IL-23/Th17 pathway (Bartlett and Million, 2015).

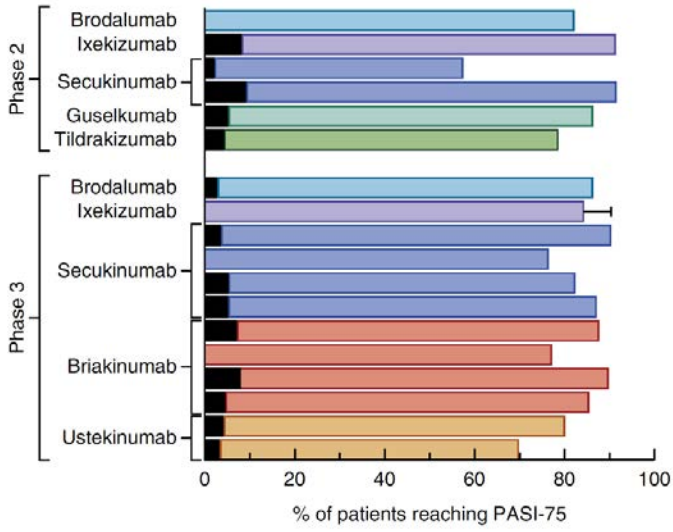


Figure 7. Efficacy of IL-12/23p40, IL-23p19 and IL-17A or IL-17RA antagonists in treating patients with moderate to severe psoriasis. Colored bars indicate the highest reported efficacy for any dose groups within studies of 12–16 weeks treatment period. Black bar indicates the associated placebo group (Teng et al., 2015).

4. Psoriasis immunopathology

Translational research has helped defining the immune cellular components in psoriasis lesions and how those cells may interact to form the lesion.

4.1 Lesions development

The plaque formation in uninvolved skin of predisposed individuals can be triggered by physical injury (the Koebner phenomenon), stress, drugs and infections with streptococcus or other microbes (Liu et al., 2006). Although these are diverse stimuli, all might activate cellular immunity, and likely involving some antigen-based trigger or innate stimulation that leads to up-regulation of chemokines and adhesion molecules (Liu et al., 2006). In general terms, T cells and DCs, and the cytokines and chemokines that they secrete, collaborate to create the pro-inflammatory milieu that stimulates the proliferation of resident keratinocytes and endothelial cells, finally deriving into the tissue growth pattern of psoriasis lesions (Bowcock and Krueger, 2005).

During the onset of psoriasis, accumulation of mononuclear cells (skin-homing T cells, monocytes and DCs) are found around dermal vessels, without any involvement in the epidermis (left panel in Figure 8). In fact, it has been suggested that CLA^+ T cells are present in non-lesional skin before epidermal hyperplasia takes place (Davison et al., 2001a), and evidence of early apparition of CLA^+ T cells in the margin zone of spreading lesions has been reported (Vissers et al., 2004). Then, this perivascular infiltrate deepens into the tissue, where CTACK is synthesized by keratinocytes, a chemokine that attracts CLA^+ T cells and thus might initiate the entry of those T cells in the skin (Homey et al., 2002). In acute lesion, large numbers of leucocytes migrate into the skin and the presentation of antigens by antigen-presenting cells (APCs) (e.g. DCs or LCs) to $CD4^+$ T cells leads to the synthesis of cytokines, which in turn stimulate keratinocytes that become activated to proliferate (central panel in Figure 8). ICAM-1, CD40 and HLA-DR are expressed on the surface of some basal and suprabasal keratinocytes. Activation after binding cognate receptors of these molecules during leucocyte trafficking through the epidermis, including T cells, $CD11c^+$ DCs and neutrophils, also provides stimulus for keratinocyte hyperplasia. Then, keratinocytes themselves are stimulated to secrete their own cytokines, which can act in an autocrine and/or paracrine manner on immune cells and promote angiogenesis through vascular endothelial growth factor (VEGF), platelet-derived growth factor (PDGF), and other endothelial cell mitogens (Liu et al., 2006), thus maintaining the psoriatic process, in which proliferative rate of

keratinocytes and leucocyte infiltrates reach an equilibrium that persists over the years (Bowcock and Krueger, 2005). This means that new expression of adhesion molecules by endothelial cells will allow the extravasation of leukocytes into the lesion, including more skin-homing CLA^+ T cells that can also be activated in an antigen-specific manner, leading to the secretion of more cytokines to perpetuate the process (Smith and Barker, 2006) (right panel in Figure 8, and also see Figure 9).

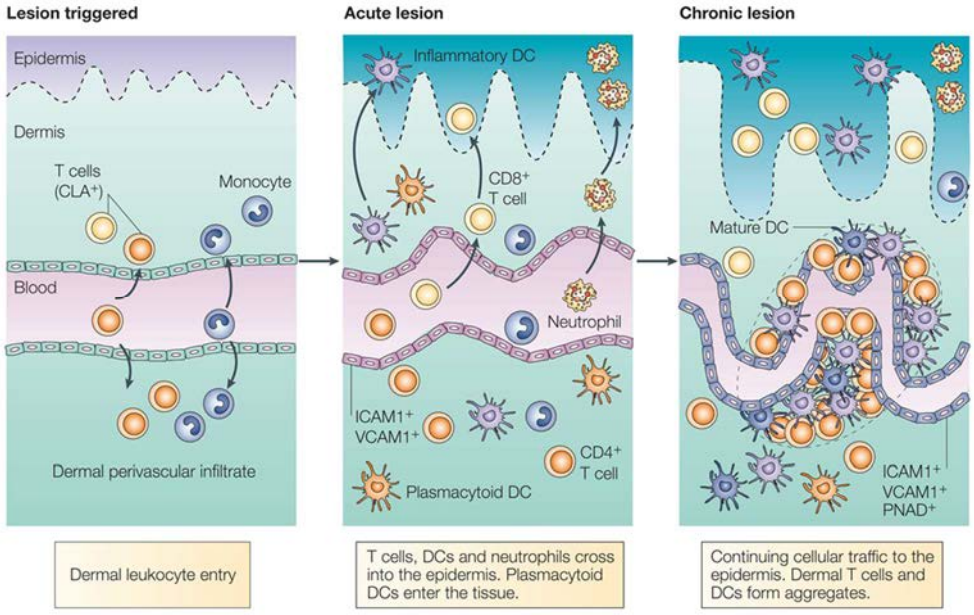


Figure 8. Development of psoriatic plaque. The interval between the triggering of a new lesion and the formation of chronic plaque is characterized by the progressive accumulations of leucocytes and epidermal hyperplasia. When the lesion is settled, perivascular accumulations of T cells and mature DCs might function as a secondary lymphoid tissue. Whereas normal endothelial cells that line the blood vessels in the skin do not express ICAM-1 or VCAM-1, the expression of these molecules is up-regulated in acute and chronic psoriatic lesions (Bowcock and Krueger, 2005).

4.2 Innate and adaptive immunity in psoriasis

Two fundamentally different cell types interact in the formation of a psoriatic lesion: epidermal keratinocytes and mononuclear leukocytes. Most of the T cells involved in psoriasis lesions are memory cells ($CD45RO^+$ or $CD45RA^-$) that mainly belong to the Th1/T-cytotoxic (Tc)1 and Th17/Tc17 cell subsets defined by the production of interferon (IFN)-γ, IL-17A and IL-17F, respectively. The release of these cytokines induces and acts synergistically to produce several products by keratinocytes, which in turn acquire

activating and recruiting effects. For instance, chemokines such as CXC-chemokine ligand (CXCL)-8, also known as IL-8, and CXCL1, also known as GRO-α, recruit neutrophils; CCL20 has been shown to attract CCR6-expressing Th/Tc17 cells; and CXCL9–11 allow the migration of activated CXC-chemokine receptor (CXCR)-3–expressing T cells, which are increased in psoriasis lesions (Rottman et al., 2001). Interestingly, upper keratinocytes increase the production of antimicrobial peptides (AMPs), such as those belonging to S100 calcium binding proteins, including the S100A7–9 and S100A12, and β-defensins, which at certain concentration gradient can act as leucocyte chemoattractants as well (Bowcock and Krueger, 2005; Ghannam et al., 2011; Oppenheim et al., 2003; Weinberg et al., 2012) (Figure 9).

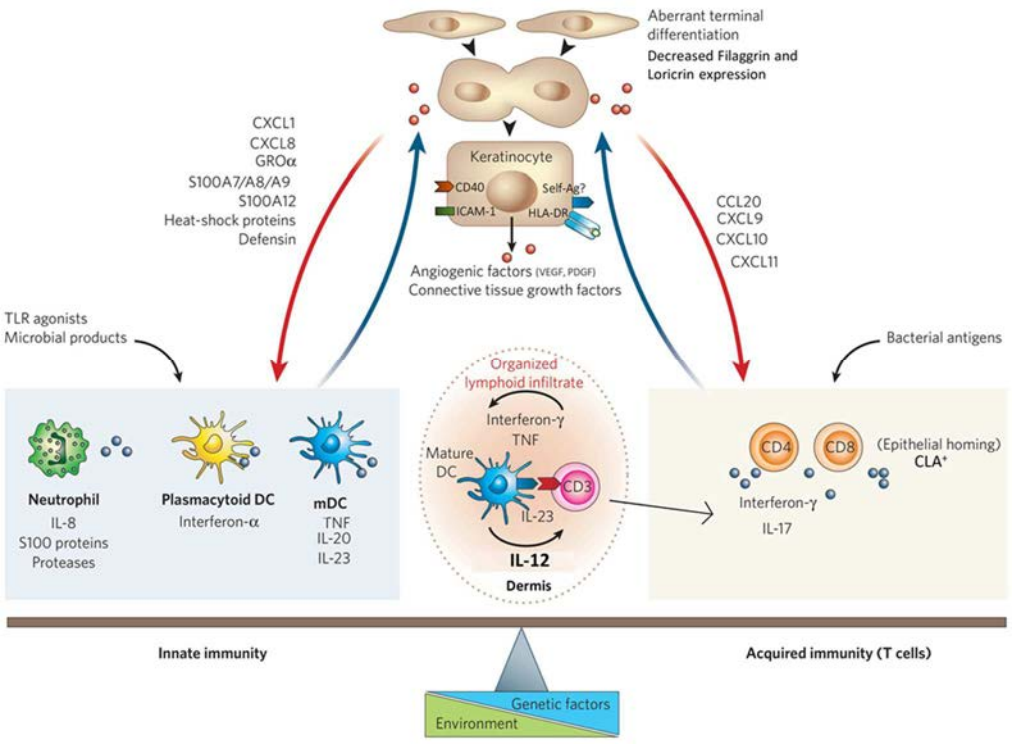


Figure 9. In established lesions, there is a balance between the innate and the adaptive immune systems. Environmental triggers can activate innate cells or T cells, which might chronically respond to a persistent self-antigen. Immune-system–derived cytokines act on keratinocytes which in turn release chemokines acting on innate/adaptive immune cells, together with cytokines and growth factors, express adhesion molecules, and perpetuate the hyperplasia and aberrant differentiation of the epidermis (adapted figure from Lowes et al., 2007).

Activated pDCs produce IFN-α, and their presence could be important in the innate initiation of the disease. Conversely, myeloid $CD11c^+$ DCs produce IL-12 and IL-23, that stimulates and help differentiation *in situ* of Th1 and Th17 cells, respectively. Interestingly, it has been demonstrated so far that IL-23/Th17 constitutes the crucial axis in the development and maintenance of psoriasis, as previously described. Finally, in addition to the increased expression levels of AMPs, altered expression of differentiation-associated proteins has been described. For instance, loricrin and filaggrin, both proteins involved in the proper formation of the cornified envelope, are reduced in psoriatic lesions (Bergboer et al., 2012). Such decreased expression can also be triggered by the inflammatory environment and contributes to the impairment of the skin barrier (Gutowska-Owsiak et al., 2012; Kim et al., 2011) (Figure 9). This phenomenon has also supported the hypothesis of a potential aggravation of the disease and homeostasis loss through external infectious agents or dysregulation of microbial components in the skin (Di Meglio et al., 2011).

4.3 Additional Th cell lineages in psoriasis: Th9 and IL-9

Our understanding of human Th cell lineages continues to evolve as additional Th profiles are described. Thus, the exploration of their T-cell products could help understanding psoriasis disease, as is the case of Th9 and its cytokine-signature, IL-9. IL-9 contributes to several inflammatory diseases, ranging from allergic to autoimmune pathologies. Despite some discrepancies, cellular sources of IL-9 may vary in different type and location of inflammatory diseases. Therefore, either in immune models for airway allergic, or lung and gut parasitic infections, mast cells, Th2 cells, natural killer (NK)-T cells and innate lymphoid cells (ILC) group 2, might produce IL-9 promoting allergic inflammation or parasite clearance, respectively (Noelle and Nowak, 2010; Spits and Di Santo, 2011; Wilhelm et al., 2012). However, Th9 cells may additionally contribute to these pathologies. Th9 cells lack expression of other Th cell lineage-specific transcription factors and cytokines (Noelle and Nowak, 2010), and, despite transcription factors PU.1 and IRF4 are required for its development (Chang et al., 2010; Staudt et al., 2010), there is still unclear evidence for Th9 lineage uniqueness.

IL-9R is expressed on immune cells, in which IL-9 could be promoting an autocrine and paracrine cell expansion, and on non-hematopoietic cells, such as lung epithelia and keratinocytes, in which it could induce the production of chemotactic factors (Hong et

al., 2015; Little et al., 2001). Studies in EAE models revealed that IL-9 may also contribute to autoimmune disease development, especially by facilitating Th17 cells expansion, which express high levels of IL-9R (Elyaman et al., 2009; Nowak et al., 2009). Recent studies in a murine model of ulcerative colitis suggest that IL-9 promotes gut inflammation through epithelial barrier impairment (Gerlach et al., 2014). However, as most of the existing reports are based on *in vitro* studies or murine models, a mounting interest in humans has led to new characterizations about Th9 biology and function in human disease.

In the last years, mounting evidence of clinical significance of IL-9 and Th9 cells in the human has been suggested, especially regarding to chronic autoimmune and inflammatory systemic diseases. For instance, in ulcerative colitis, IL-9 and IL-9R are predominantly involved in its pathogenesis (Nalleweg et al., 2015). Also, IL-9 is increased in sera and synovial fluids of rheumatoid arthritis patients (Ciccia et al., 2015), acting as a T-cell growth factor, and *IL9R* polymorphism has been linked to this disease (Burkhardt et al., 2009). In psoriatic arthritis, circulating numbers of Th9 cells correlates with disease activity, and interestingly they decrease after anti–TNF-α and ustekinumab treatments, suggesting a clinical involvement (Ciccia et al., 2016).

Recently, IL-9 was demonstrated to have a putative involvement in psoriasis. Aside from the evidence of *IL9* gene presence in the psoriatic susceptibility region (5q31.1) (Friberg et al., 2006; Modi et al., 1991), IL-9 production was found in supernatants from activated $CD4^+$ T cells from psoriasis patients, and higher numbers of $IL\text{-}9R^+$ and $IL\text{-}9^+$ cells were found in psoriatic skin lesions than in healthy samples (Schlapbach et al., 2014; Singh et al., 2013). In addition, those studies revealed a relevant link between Th9 and skin by showing that human healthy blood-derived Th9 cells primarily belonged to the CLA^+ skin-homing effector T-cell population (Schlapbach et al., 2014). Therefore, tissue localization of Th9 cells in the skin may constitute a key factor for psoriasis disease that should be longer studied, especially in relation to Th17 cells activity.

5. Clynical types of psoriasis: plaque and guttate psoriasis

Despite the distinctive appearance of the skin lesions, several clinical phenotypes of psoriasis are recognized that share identical histopathological changes in the skin but with highly variable morphology, distribution, severity, course and patient's age at

disease. Chronic plaque psoriasis, commonly named as "psoriasis vulgaris", accounts for 90% of the cases. Plaque-type patients generally develop the first psoriasis presentation at any age, starting as <1 cm round-oval plaques that peripherally expand to form plaques of one or several centimeters in diameter (Langley et al., 2005), a process which leads to moderate–severe form of disease in more than a third of plaque psoriasis patients, represented by more than 10% of body surface affection. In addition, the lesions are typically symmetrically distributed on the knees, scalp, elbows and body folds (Figure 10a).

Other clinical subtypes of psoriasis include the guttate psoriasis, an acute form with a 2% prevalence of total cases of psoriasis, in which this thesis has been mainly focused. Lesions are small drop-shaped papules of less than 1 cm in diameter, ranging from 5–10 to over 100 lesions centripetally distributed (Figure 10b). In contrast with the chronic plaques, guttate psoriasis is especially common in adolescents and early adulthood, an age distribution that was also observed in our recruited participants (Figure 10c). Also, after the onset of symptoms, guttate psoriasis is usually self-limiting, resolving within a few months (Griffiths and Barker, 2007). Interestingly, 33–40% of those guttate patients develop chronic plaque disease in the future, and guttate flares are seen in already existing plaque-affected patients (Ko et al., 2010; Langley et al., 2005).

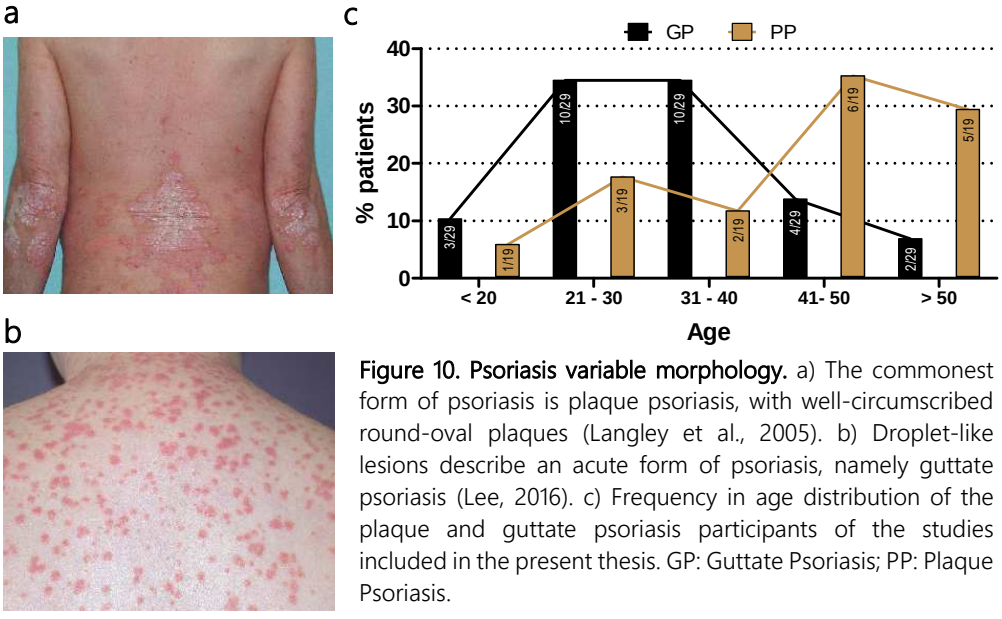


Figure 10. Psoriasis variable morphology. a) The commonest form of psoriasis is plaque psoriasis, with well-circumscribed round-oval plaques (Langley et al., 2005). b) Droplet-like lesions describe an acute form of psoriasis, namely guttate psoriasis (Lee, 2016). c) Frequency in age distribution of the plaque and guttate psoriasis participants of the studies included in the present thesis. GP: Guttate Psoriasis; PP: Plaque Psoriasis.

6. Genetic basis of psoriasis

Population studies show that about 30% of the individuals with psoriasis vulgaris have an affected first degree relative, which increases up to a 50% chance of developing the disease if both parents and a sibling are affected (Griffiths and Barker, 2007). Psoriasis has an acknowledged but complex genetic basis, but functional relationships between the predisposing genetic factors and the cellular elements of the pathogenic immunity are not well worked out (Liu et al., 2006). At least 12 psoriasis susceptibility loci, termed PSORS, have been identified (Table 4), and several additional loci have been assigned by linkage analysis and genome-wide association studies (GWAS) (Duffin and Krueger, 2009; Wagner et al., 2010).

Psoriasis susceptibility region (OMIM reference number)	Locus	Molecules differentially regulated in psoriasis
PSORS1 (#177900)	6p21.3	HLACw6, TNF, cornodesmosin, MHC class I
PSORS2 (#602723)	17q25	TIMP-2, SLC9A3R1, NAT9, RAPTOR
PSORS3 (#601454)	4q	VDBP, RRH
PSORS4 (#603935)	1q21	EDC, S100A7, S100A8, S100A9, LCE3B, LCE3C, loricrin
PSORS5 (#604316)	3q21	Transferrin, SLC12A8
PSORS6 (#605364)	19p13	JunB, SGTA
PSORS7 (#605606)	1p	IL-23R
PSORS8 (#610707)	16q	NOD2
PSORS9 (#607857)	4q31–4q34	ND
PSORS10 (#612410)	18p11.23	ND
PSORS11 (#612599)	5q31.1–5q33.1	IL-12p40
PSORS12 (#612950)	20q13	ZNF313 (also termed RNF114)

Abbreviations: EDC, epidermal differentiation complex; IL, interleukin; LCE, late cornified envelope protein; NAT, N-acetyltransferase; ND, not described; NOD, nucleotide-binding oligomerization domain; OMIM, Online Mendelian Inheritance in Man; PSORS, psoriasis susceptibility region; IL-23R, IL-23 receptor; RAPTOR, regulatory associated protein of mammalian target of rapamycin; RNF, RING finger protein; RRH, retinal pigment epithelium-derived rhodopsin homolog; SGTA, small glutamine-rich tetratricopeptide repeat-containing protein alpha; SLC, solute carrier; TIMP, tissue inhibitor of metalloproteinases; TNF, tumor necrosis factor; VDBP, vitamin D-binding protein; ZNF, zinc finger protein.

Table 4. Psoriasis susceptibility loci (Wagner et al., 2010).

The strongest association was found for the PSORS1 locus, which is located within the major histocompatibility complex (MHC) class I region on chromosome 6p. This association has been virtually replicated in all linkage studies of psoriasis, and accounts for 35–50% of the heritability of the disease, (Capon et al., 2002; Trembath et al., 1997). Although several genes within this locus encode proteins that are expressed in the skin, its most relevant allele is the HLA-Cw6. Indeed, in a northern European study, this allele was found in 60–65% of patients and only in 15% of healthy controls. HLA-Cw6 is a strong marker for early-onset psoriasis. In one study, 85% of patients with onset before the age of 40 years had at least one HLA-Cw6 allele, compared with 15% of those with onset after this age (Griffiths and Barker, 2007). Actually, guttate psoriasis, an acute onset form previously described, is strongly associated with PSORS1 (Asumalahti et al., 2003). Furthermore, HLA-Cw6–positive psoriasis patients differ from the HLA-Cw6–negative in several clinical features, involving a higher risk of developing the disease (Gudjonsson et al., 2006), a more sever profile, and show a better clinical response to biological treatments like ustekinumab (Talamonti et al., 2013). Despite HLA-Cw6 is still an interesting candidate gene as the susceptibility factor at PSORS1, since it might be involved in immune responses at the level of antigen presentation, no disease-specific mutations have been identified. And as the penetrance of PSORS1 is proposed to be about 10–15%, other genetic variants and/or environmental factors are required for disease development (Bowcock and Krueger, 2005).

7. Psoriasis and streptococcal infection

7.1 Streptococcal throat infections impact on psoriasis course

As a multifactorial disease, genetics partly explains the onset of psoriasis in predisposed individuals. Therefore, a clear additional environmental influence is needed as a trigger factor. Although infections by various microorganisms have been implicated in the disease process, including fungi (e.g. *Candida albicans*), several studies agree with the crucial link between throat infections with β-hemolytic streptococci and the initiation and exacerbation of psoriasis. Actually, it has been reported that among HLA-Cw6–positive psoriasis patients, pharyngitis caused by *Streptococcus pyogenes* (*S. pyogenes*) –a group A β-hemolytic streptococci– may contribute to the onset or exacerbation of the inflammatory process of skin lesions. Interestingly, the close relation between the HLA-Cw6 allele and guttate-type psoriasis is also reflected by the fact that

streptococcal pharyngitis often precedes the onset of this particular phenotype of the disease in 56–97% of cases (Prinz, 2001), and raised anti-streptolysin O (ASO) titers are usually found, likely indicating an infection with group A streptococci. For these reasons, it is suggested that guttate psoriasis patients may display a particular genetically determined sensitivity to streptococcal infection (Weisenseel et al., 2002). Besides these observations, the streptococcal influence is not limited to this acute psoriasis subtype, but there is evidence demonstrating that infection by this microbe in psoriatic plaque patients results in an exacerbation of the disease symptoms (Gudjonsson et al., 2003). Also, such infections are approximately 10 times more frequent in chronic plaque psoriasis patients compared with control population (Gudjonsson et al., 2003), their tonsils are more frequently infected by β-hemolytic streptococci than are recurrently infected tonsils from patients without psoriasis (Sigurdardottir et al., 2013), and, finally, their serum IgG titers against *S. pyogenes* are increased compared with controls (El-Rachkidy et al., 2007). Remarkably, tonsillectomy has been described to be effective in ameliorating psoriasis evolution (Rachakonda et al., 2015; Thorleifsdottir et al., 2012). Therefore, streptococcal infection is clinically relevant for psoriasis development, either in guttate or plaque forms (Thorleifsdottir et al., 2016).

Other entry routes for streptococcal antigens could be considered as instigators of the disease. For instance, *S. pyogenes* has been detected in the skin but not in the throats of some patients with guttate psoriasis (Fahlén et al., 2012), and some authors have proposed that its transitory intracutaneous presence during streptococcal pharyngitis would be enough to generate an antibacterial immune response that would lead to autoimmunity against local skin proteins by molecular mimicry (Mallbris et al., 2005), a concept discussed in the next section. Overall, the relationship between streptococcal antigens and subsequent psoriatic clinical events has to be further studied.

7.2 Functional association between *S. pyogenes* and CLA$^+$ T cells in psoriasis

7.2.1 Streptococcal tonsilitis and T cell sensitization.

DCs may induce the expression of tissue-specific homing receptors on T cells consistent with the organ from which those DCs come. However, DCs from the upper respiratory truck and the tonsils may constitute an exception, since they seem to generate some skin-homing CLA$^+$ T cells (Sabat et al., 2007). Actually, 5–10% of tonsillar T cells express

CLA, and it has been reported that streptococcal superantigens (SAgs) can induce expression of CLA via induction of IL-12 (Berg et al., 1991; Leung et al., 1995a). SAgs are microbial toxins that bind to the variable region of the T-cell receptor (TCR)Vβ chain and to MHC class II molecules. Such binding is not restricted by polymorphic determinants of MHC class II molecules or antigen presentation, resulting in extensive heterogeneity in T cell clonal activation. However, each SAg is associated with a characteristic TCRVβ 'fingerprint', for example, streptococcal pyrogenic exotoxin (SPE)-C triggers the activation and expansion of T cells carrying Vβ2.1, Vβ3.2, Vβ12.5 and Vβ15.1 with a strong preference for Vβ2.1 (Proft and Fraser, 2016). Therefore, initially researchers had proposed a role for SAgs as triggers of psoriasis disease. Actually, skin biopsies from patients with acute guttate psoriasis, but not from healthy controls, demonstrated expansion of $V\beta2^+$ T cells containing an extensive junctional diversity compatible with a SAg rather than a conventional antigen-driven T-cell response (Leung et al., 1995b). Such relation between CLA^+ T cells and the response to streptococcal SAgs in psoriasis patients was also supported by the increased TCRVβ2 chain expression in those skin-homing CLA^+ T lymphocytes in psoriasis vulgaris (Davison et al., 1999). However, later evidence determined that streptococcal SAgs only induced higher proliferative rates in CLA^+ $V\beta2^+$ T cells from active guttate psoriasis, but not from chronic plaque psoriasis (Davison et al., 2001b).

Further analysis of TCR usage in psoriasis lesions provided evidence of a conventional antigen driven T-cell activation (Menssen et al., 1995). The proposed SAg-dependent expansion of T cells sharing particular TCRVβ regions would not explain the continuous presence of the same clonal TCR rearrangements in chronic psoriatic plaques that persist in relapsing psoriasis, thus suggesting that a stable antigen-specific pathogenic T-cell response is involved in psoriatic inflammation over prolonged periods of time (Prinz et al., 1999; Vollmer et al., 2001). Also, identical TCRβ chain rearrangements in CLA^+ T cells, but not in CLA^- T cells, in streptococcal angina and in psoriasis vulgaris skin lesions were identified (Diluvio et al., 2006). Furthermore, previous data showed that T-cell lines from psoriatic lesions may show a strong cross-reactivity to streptococcal antigens (Valdimarsson et al., 1997). Altogether, these findings suggest that SAg might be involved in the development of guttate flares and may generally facilitate at least, the migration of tonsillar T cells to the skin through the induction of CLA expression, but the key step affecting the effector memory skin-homing T cell generation would be likely subjected to an antigen-specific selection of naive T cells in the tonsils (Diluvio et

al., 2006; Elder et al., 2010) (Figure 11, steps 1–3). This first priming step would be considered as a sensitization phase, similar to the sequence of events that take place in an immune reaction (Sabat et al., 2007).

7.2.2 Cross-reacting T cells in psoriasis progression: an autoimmune disorder?

Following the first priming step, there might be a silent phase without clinically skin-associated symptoms as long as a precipitating factor, or several, such as trauma, infections, genetic predisposition, and stress episodes, triggers an effector phase that is initiated by the skin infiltration of various immune cells. Such infiltrate includes oligoclonal T cells driven by conventional antigens, which would contain dominant clones that can persist for a long time (Valdimarsson et al., 2009). It has been proposed that dermal $CD4^+$ T cells respond to streptococcal peptidoglycans, and, interestingly, dermal macrophages containing such peptidoglycans have been detected (Baker et al., 2006). Those streptococcal antigens could reach the skin through the bloodstream, where they are taken up by monocytes or macrophages that migrate as APCs into developing psoriatic lesions during streptococcal pharyngitis (Valdimarsson et al., 2009; Weisenseel and Prinz, 2005), or through compromised skin (Bergboer et al., 2012) (Figure 11, steps 4–6). However, those intracutaneous streptococcal antigens do not seem to persist long enough in psoriatic lesions to continue activating the persistent oligoclonal expansion of antigen-specific T cells. Then, it has been suggested that psoriasis maintenance could be explained by an autoimmune reaction, in which those infiltrating T cells might recognize and proliferate clonally in response to auto-antigens exposed or activated by the inflammatory process (Valdimarsson et al., 2009) (Figure 11, step 7). Therefore, some authors support the hypothesis of a T-cell specificity, especially from $CD8^+$ T cells, that could be cross-reacting to auto-epitopes presented by HLA-Cw6 or other HLA class I molecules on keratinocytes surface and/or cross-presenting DCs (Gudjonsson et al., 2004; Valdimarsson et al., 2009). In this regard, it was reported the existence of common determinants between human keratins, structural filamentous proteins of the epithelia, and streptococcal M-protein, a virulence factor that prevents opsonization. Interestingly, throat infections preceding the onset or worsening of psoriatic lesions are only associated with the three groups of β-hemolytic streptococci (A, C and G) that express M-protein on their surface (Gudjonsson et al., 2003).

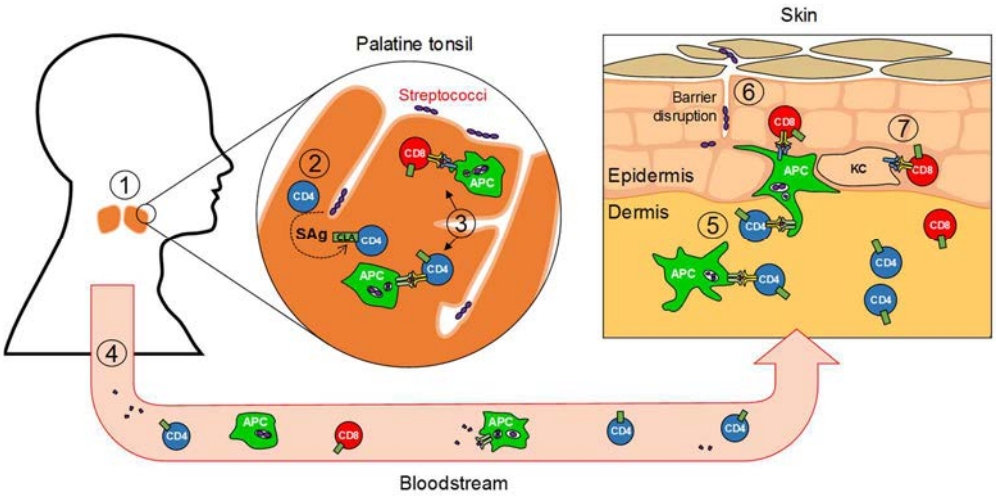


Figure 11. Proposed pathogenic concept of streptococci-induced psoriasis. 1) Primary or worsening psoriasis manifestations are often triggered by tonsillar infection, especially by β-hemolytic *S. pyogenes*. 2) SAgs induce on T cells the expression of the skin-homing receptor CLA. 3) Repetitive TCR rearrangements reflects a clonal expansion of potentially cross-reactive $CD4^+$ and $CD8^+$ T cells that recognize streptococcal components that were internalized by APCs. 4) Effector memory T cells expressing CLA enter the bloodstream, together with some streptococcal components that are taken up by migrating APCs. 5) Psoriatic immune response involves an increased frequency in lesions of T cells primed against streptococcal antigens that become re-activated by the presence of streptococcal antigens, carried by migratory APCs or entering through the disrupted skin (6), or by molecular mimicry through cutaneous self-peptides (7). Scheme adapted from Prinz, 2001.

Those studies have been carried out by using peptides from keratin 17 (K17), which is up-regulated in psoriatic lesions, and M-protein from *S. pyogenes*, that showed sequence similarities, as well as motifs predicted to bind to HLA-Cw6 (Johnston et al., 2004). Subsequently, a markedly increased frequency of T cells that recognize such homologous determinants were detected in the blood of psoriasis patients, and >90% were positive for CLA (Gudmundsdottir et al., 1999; Johnston et al., 2004). Consequently, M-protein–primed T cells may recognize up-regulated keratin epitopes via molecular mimicry. Similar studies with predicted K17 sequences to bind to HLA class II molecule HLA-DRB1*04, that is significantly associated with psoriasis, although not as strongly as HLA-Cw6, showed a CD4-dependent response as well (Shen et al., 2005). However, $CD4^+$ T cells showed little response to M-protein determinants (Baker et al., 2001), but still responded to streptococcal peptidoglycans, as previously mentioned.

In addition to skin keratins, other putative autoantigens in psoriasis have been described over the years, such as heat shock proteins, the AMP LL37, the melanocytic antigen ADAMTSL5, and, more recently, enzymatically processed lipids by phospholipase A2 and presented through CD1a to CD1a-autoreactive T cells (Arakawa et al., 2015; Besgen et al., 2010; Cheung et al., 2016; Lande et al., 2014). Ddespite they might be not associated with common streptococcal determinants, still support an autoimmune reaction in psoriasis.

In spite of all this evidence, long-term treatments with antibiotics have not been effective for psoriasis (Owen et al., 2001), probably because streptococci can exist in both extracellular and intracellular spaces, thus forming intracellular reservoirs in the tonsillar epithelia and macrophages, which can contain a quiescent bacterial load after antibiotic therapies. These streptococci can re-activate, re-colonize and cause symptoms again, whereas tonsillectomy might remove this pool of streptococci (Österlund et al., 1997; Thorleifsdottir et al., 2016), a chirurgic treatment that has indeed been associated with psoriasis improvement in either plaque and guttate psoriasis patients, in 49% and 75% of the cases, respectively (Thorleifsdottir et al., 2016). In fact, frequency in circulating CLA^+ T cells that recognize shared M-protein and human keratins determinants decrease after tonsillectomy and correlate with the degree of clinical improvement (Thorleifsdottir et al., 2012).

Overall, skin-homing CLA^+ T cells might constitute the functional link between genetic predisposition, microbial infection in psoriasis (Prinz, 2001), and the appearance or worsening of the psoriatic lesions. Therefore, the study of the phenotypical outcome from isolated circulating CLA^+ T cells in terms of their immune response displayed against *S. pyogenes*, could bring valuable information about disease status and course and would support the translational value of CLA^+ T cells as cellular biomarkers.

8. Mouse models for the study of psoriasis and limitations

Psoriasis is only observed in humans, but numerous psoriasis-like murine models have been developed in order to constitute an approach for the study of this disease. Skin stratification is similar between mouse and human, although the number of cellular layers for each compartment are thicker in human. Also, murine immune system

contains particular subtypes of cells, including abundant γδ T cells and dendritic epidermal T cells (DETC), that are highly reduced or absent, respectively, in humans (Figure 12). Despite these differences, some models are still useful that share histological features and molecular pathways (Pasparakis et al., 2014; Wagner et al., 2010).

A mouse model that resembles all aspects of psoriasis has not yet been established, although one of the more informative models is the xenograft model, using symptomless pre-psoriatic human skin (Boyman et al., 2004; Flutter and Nestle, 2013; Wagner et al., 2010), but it entails a high technical difficulty. This kind of model has shown a relevant role of IFN-α of the innate immune system in driving adaptive response, since blocking this molecule prevented T-cell–dependent development of psoriasis in the grafted skin (Nestle et al., 2005). However, mAbs against IFN-α in humans have shown no clinical activity (Bissonnette et al., 2010).

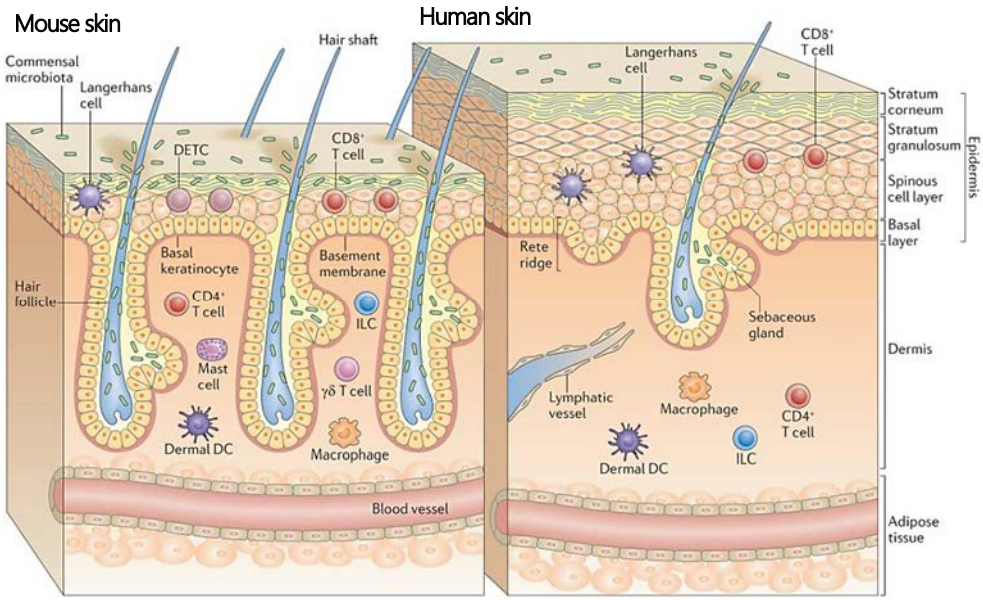


Figure 12. Structure and cellular components of the skin in mice and humans. Aside from the densely presence of hair follicles, the most characteristic differences of murine skin are its less number of epidermal layers and its content of immune cell types that do not currently exist in human skin, such as DETCs, or that are more representative in the skin inflammatory response in mice than in humans, such as γδ T cells (Pasparakis et al., 2014).

Other easier approaches include the use of the imiquimod (IMQ)-induced model of psoriasis, which has increased in the past few years. The topical application of this

component constitutes a simple, rapid and effective method of inducing psoriasis in mice compared to other psoriasis models, and, importantly, there are clinical observations indicating that IMQ can exacerbate psoriasis in patients (Fanti et al., 2006). Moreover, IMQ is a potent immune activator and a Toll-like receptor (TLR)-7 (TLR7) ligand, and thus provides a model which highlights the pathogenic involvement of cytokines and the innate immune system in psoriasis (Wagner et al., 2010). Keratinocytes do not express TLR7, therefore the effect of the IMQ application implies other targets as LCs, which are induced to migrate to the skin lymph nodes (Suzuki et al., 2000), or pDCs, which become mature (Gibson et al., 2002). In this model, psoriatic-associated transcripts are increased as soon as 24 hours after the first topical application (Walter et al., 2013), and resembles most of the human lesion features: acanthosis, parakeratosis, abnormal epidermis differentiation and immune cell infiltration, including mononuclear cells, neutrophils, DCs and T cells. However, despite the IL-23/Th17 axis is crucial for the IMQ psoriasiform model development (van der Fits et al., 2009), the skin hyperplasia and inflammation are primarily mediated by IL-22, which is produced by Th17 cells in mice, whereas IL-17 seems to have a lesser role (Krueger, 2012) (Figure 13). Conversely, abolition of IL-22 activity in humans induces no consistent improvements in a large fraction of psoriasis patients (Antoniu, 2012), while blocking IL-17A or IL-17RA leads to a rapid strong clinical improvement in more than 80% of treated subjects.

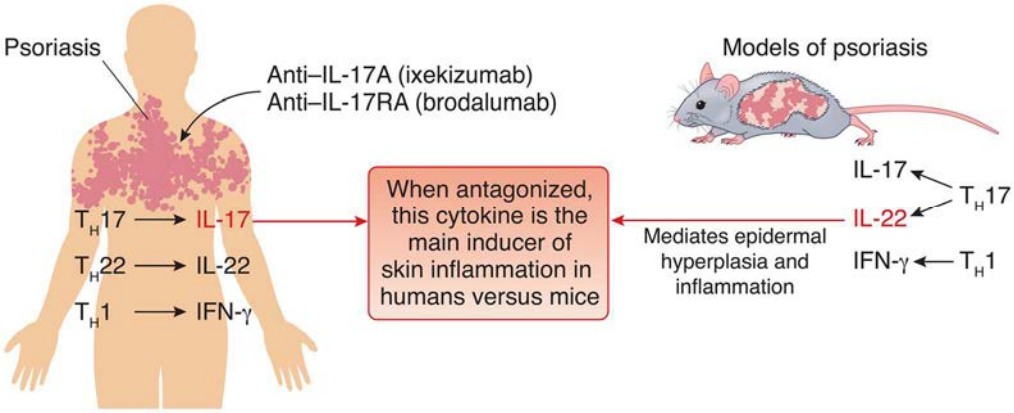


Figure 13. Skin inflammation in patients with psoriasis and mouse models of disease. Different cytokines play key roles as main inducers of skin inflammation and histological features in humans and mice. While in humans, biological therapies point to a clear contribution of IL-17 signaling pathways, in mice those mirrored effects seem to be orchestrated by IL-22 (Krueger, 2012).

Thus, key cytokine divergence between psoriasis mouse models and real disease in humans argues for a need of further translational studies to better understand cytokine mechanisms in psoriasis skin inflammation, especially focusing on IL-17 effects. Also, murine models are far to represent the multifactorial background of this disease that humans do, as exposed in previous sections.

9. A new *ex vivo* model

9.1 Background and rationale

- Circulating skin-homing CLA^+ T cells constitute peripheral cellular biomarkers for human T-cell–mediated skin diseases, thus providing a tool for translational evaluation of effector responses upon their interaction with cognate pathogens that match the pathogenesis.

- The clinical association between *S. pyogenes* throat infections and guttate or plaque psoriasis flares is well established, being especially strong in guttate psoriasis.

- The need of new translational tools due to: 1) limitations of the existing non-human models to evaluate psoriasis as a human complex disease; and 2) assumptions in disease mechanisms requires demonstration in human immune system

9.2 *Ex vivo* coculture with memory T cells and autologous epidermal cells

On the one hand, in order to reproduce the initial interaction between T lymphocytes and epidermal cells in psoriasis, a new *ex vivo* model has been established. It consists of cocultures that contain psoriatic circulating effector memory CLA^+ or CLA^- T cells, together with autologous epidermal cells obtained from the skin lesions (abbreviated as CLA^+/Epi or CLA^-/Epi). The coculture is then activated with a *S. pyogenes* extract (SE), that consists of a sonicated preparation containing isolated strains from infected tonsils of psoriasis patients. Then, this system allows the evaluation of this clinically relevant trigger in the generation of the immune response in the context of the cellular components in psoriatic lesions (Figure 14).

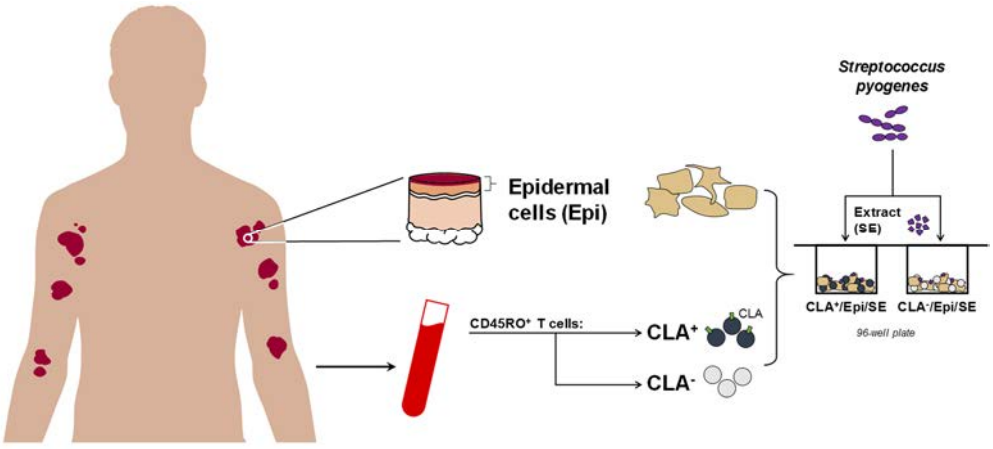


Figure 14. *Ex vivo* human model for the study of psoriasis. Epidermal cells are isolated from lesional skin punches, and are cocultured with autologous skin-homing CLA^+ effector memory T cells isolated from peripheral blood. CLA^- T cells are used as a control for other restricted-tissue tropisms. Simultaneously, cocultures are challenged with SE.

The activation of the CLA^+/Epi coculture with SE leads to the production of cytokines from Th1/Th17/Th22 profiles and chemokines associated with psoriatic lesions, as well as to the activation and proliferation of epidermal cells *in vitro* and *in vivo* (Ferran et al., 2013b, see full article in page 157, in Appendix I). Furthermore, the use of human samples in this model allows the evaluation of the effector function triggered by the *ex vivo* stimulation with SE together with other patient's associated features, such as the genetic (HLA-Cw6) and environmental triggering factors (previous streptococcal pharyngitis), duration of disease, the severity of disease and/or the psoriasis form (see left panel in Figure 15, page 50).

On the other hand, as this model is trying to reproduce the initial inflammatory focus of psoriatic lesions, the use of coculture supernatants on *in vitro* cultured normal cutaneous resident cells, such as keratinocytes. This approach provides a tool to evaluate the effector function capability of such conditioned supernatants to switch keratinocyte gene expression from a healthy profile to a psoriatic-like one, especially allowing for the exploration of IL-17A downstream regulated targets (see right panel in Figure 15, see page 50).

AIM AND OBJECTIVES

The general aim of the present dissertation is the use of the *ex vivo* approach to further characterize diverse aspects of psoriatic disease, which are exposed in the following objectives:

- **Objective 1:** To characterize the CLA^+ T-cell–dependent responses profiles induced by SE, and distinguish clinical profiles according to genetics (HLA-Cw6 allele), environmental-associated factors/parameters (pharyngitis, ASO), and/or severity of disease (PASI).

- **Objective 2:** To identify and characterize new genes potentially involved in psoriasis through the evaluation of the action of enriched supernatants from SE-activated CLA^+/Epi cocultures on cutaneous resident cells (e.g. keratinocytes).

The diagram shown in Figure 15 outlines the integrative approaches and steps that were followed to achieve the objectives.

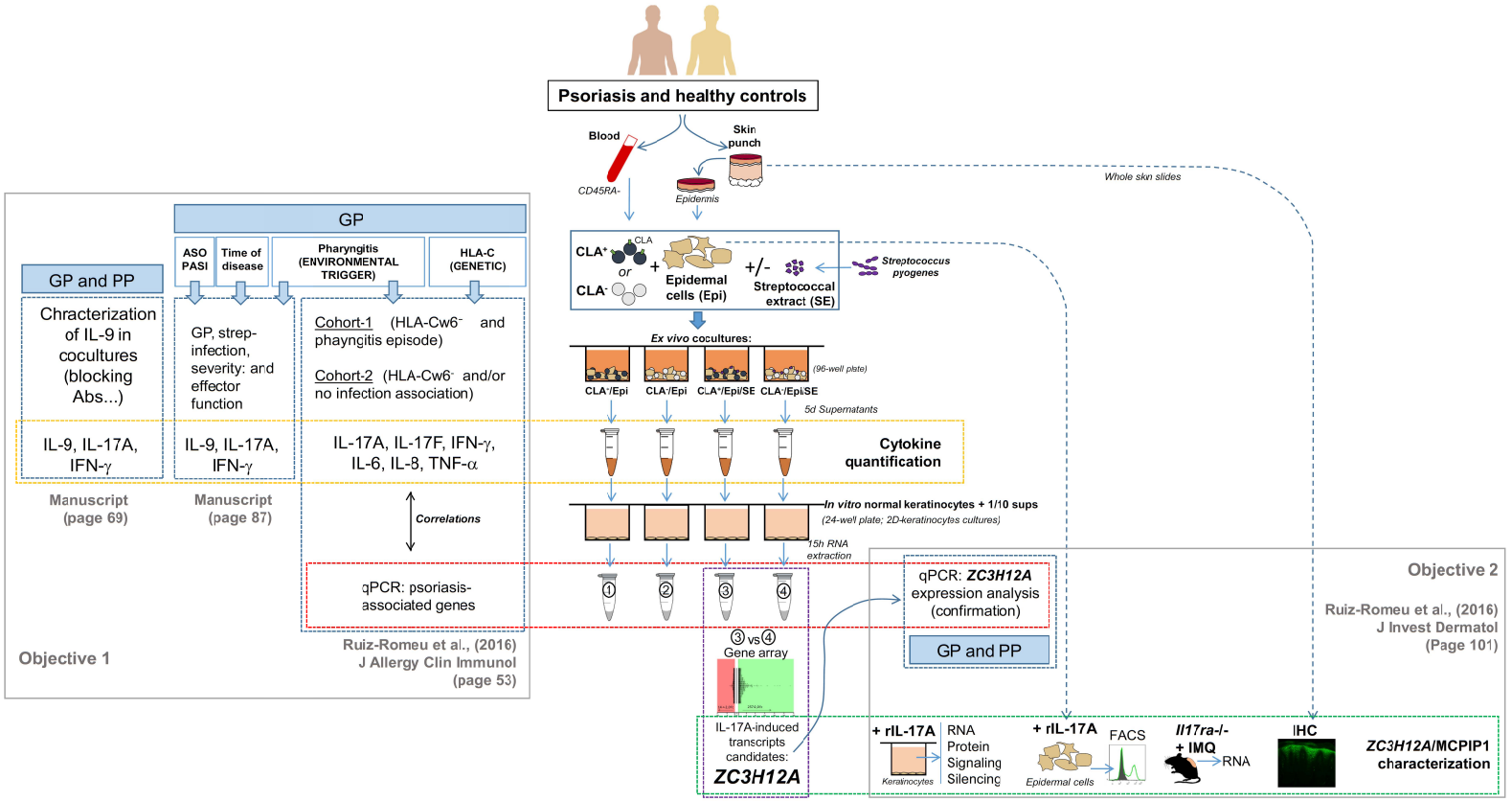


Figure 15. Diagram illustrating the integrated approaches used in this thesis. All procedures are subjected to the isolation of cellular components (memory CLA^+/CLA^- T cells and epidermal cells) from psoriasis or healthy control subjects. Left side of the figure represents the studies carried out with patients with guttate (GP) and/or plaque psoriasis (PP) patients, and that were focused on the characterization of the effector responses exerted by the *ex vivo* cocultures. The lower right side of the figure shows the identification and characterization process of a selected IL-17–induced target in keratinocytes, the ribonuclease MCPIP1, which is encoded by *ZC3H12A* gene.

PUBLICATIONS

Streptococcus pyogenes–induced cutaneous lymphocyte antigen–positive T cell–dependent epidermal cell activation triggers T_H17 responses in patients with guttate psoriasis

Ester Ruiz-Romeu, BSc,[a] Marta Ferran, MD,[b] Marc Sagristà, MD,[c] Julià Gómez, PhD,[d] Ana Giménez-Arnau, MD, PhD,[b] Krisztina Herszenyi, MD,[e] Péter Hóllo, MD, PhD,[e] Antonio Celada, MD, PhD,[f] Ramon Pujol, MD, PhD,[b] and Luis F. Santamaria-Babí, PhD[a] *Barcelona, Spain, and Budapest, Hungary*

Background: Guttate psoriasis (GP) is characterized by acute onset of small, rounded psoriatic lesions. Although this particular phenotype of psoriasis is usually associated with streptococcal throat infections and mainly occurs in HLA-Cw6$^+$ patients, the specific immunologic response to this innate stimulus that causes these skin lesions is poorly understood.
Objective: This study aims to elucidate how key cellular elements of patients with GP respond to *Streptococcus pyogenes* and whether this initial immune response is favored by the genetic and environmental background of these patients.
Methods: Circulating memory T cells and autologous epidermal cells from samples from either patients with GP (n = 14) or healthy control subjects (n = 6) were cocultured *ex vivo* in the presence of an *S pyogenes* extract. Levels of the psoriasis-associated cytokines IL-17A, IL-17F, IFN-γ, TNF-α, IL-6, and IL-8 were determined. The expression of several genes with increased (*DEFB4*, *S100A7*, *LCN2*, *IL36G*, *IL8*, *CXCL9*, *CXCL10*, and *CXCL11*) or decreased (*FLG* and *LOR*) transcripts in psoriatic lesions was examined in keratinocytes treated with coculture supernatants.
Results: When skin-homing effector memory cutaneous lymphocyte antigen–positive T cells were used in cocultures, a T_H17-dominant response was observed, as reflected by the higher amounts of IL-17A and IL-17F than IFN-γ. Moreover, a higher T_H17 response was observed in cells isolated from patients with flares associated with a streptococcal tonsillitis and with the HLA-Cw6 allele (cohort 1). In addition, in normal keratinocytes the supernatants from these cocultures induced an increase in IL-17–associated genes, such as *DEFB4*, *S100A7*, *LCN2*, *IL36G*, and *IL8* but a decrease in *FLG* and *LOR*, thereby confirming the role of activated T_H17 cells.
Conclusion: This study reveals a dominant T_H17 response of cutaneous lymphocyte antigen–positive T cells activated by epidermal cells and *S pyogenes* in patients with GP. (J Allergy Clin Immunol 2016;138:491-9.)

Key words: *T_H17, streptococcal pharyngitis, guttate psoriasis, cutaneous lymphocyte antigen, HLA-Cw*0602*

Psoriasis is a phenotypically and genetically heterogeneous disease associated with various environmental triggers, and as such, it is commonly observed in patients with multifactorial diseases. One of the most compelling susceptibility factors for psoriasis is the presence of the HLA-Cw*0602 allele, which is found in approximately 50% of European patients and confers a higher risk of having the disease.[1] The clinical features of HLA-Cw6$^+$ patients with psoriasis differ from those of HLA-Cw6$^-$ patients. In this regard the former show earlier onset of psoriasis, a more severe profile, and a better clinical response to ustekinumab.[2,3]

Streptococcal infection is a recognized trigger of psoriasis,[4,5] especially guttate psoriasis (GP), in which *Streptococcus pyogenes* throat infection precedes lesion onset in 56% to 97% of cases.[6] In addition, patients with chronic plaque psoriasis tend to be more susceptible to *S pyogenes* throat infection than control subjects, and this infection can exacerbate psoriatic disease.[7] Furthermore, these patients frequently have increased serum IgG levels against *S pyogenes*.[8] Finally, tonsillectomy has been described to be effective in ameliorating psoriasis evolution.[9,10]

In HLA-Cw6$^+$ patients with psoriasis, *S pyogenes* infections can contribute to the onset or exacerbation of psoriatic lesions.[11] Moreover, guttate-type onset is confined mostly to those carrying the HLA-Cw6 allele, and because streptococcal pharyngitis often precedes the onset of this particular psoriatic phenotype,[6] it has been proposed that patients with GP display a particular genetically determined sensitivity to streptococcal infection.[12] This microbial trigger in patients with GP provides a useful tool to study a clear starting point of the disease and to examine early events in its development.[13]

From [a]Translational Immunology and [f]Macrophage Biology, Department of Cellular Biology, Physiology and Immunology, Universitat de Barcelona; [b]the Department of Dermatology, Hospital del Mar, Research Group in Inflammatory Dermatologic Diseases, IMIM, Universitat Autònoma de Barcelona; [c]Hospital Sant Jaume de Calella, Barcelona; [d]Laboratori de Referència de Catalunya, El Prat de Llobregat, Barcelona; and [e]the Department of Dermatovenerology and Oncodermatolgy, Semmelweis Egyetem, Budapest.

Supported by ERDF grants from the Instituto de Salud Carlos III (PI09/2222 and PI13/01845 to L.F.S.-B., PI09/07811 to A.G.-A., and PI13/01716 to R.P.). L.F.S.-B. coordinated projects and grants. E.R.-R. was the recipient of a PhD fellowship from the Ministerio de Educación, Cultura y Deporte.

Disclosure of potential conflict of interest: A. Giménez-Arnau serves on the board of Novartis, Genentech, URIACH Pharma, and Almirall; receives grant funding from Novartis and URIACH; and receives speakers' fees from Novartis, GlaxoSmithKline, and Leo Pharma. A. Celada receives research funding from the Spanish Ministry of Economy. L. F. Santamaria-Babí receives research funding from RDF grants from the Instituto de Salud Carlos III Ester Ruiz Romeu receives research funding from Ministerio de Educación, Cultura y Deporte. The rest of the authors declare that they have no relevant conflicts of interest.

Received for publication July 29, 2015; revised January 22, 2016; accepted for publication February 5, 2016.

Available online April 4, 2016.

Corresponding author: Luis F. Santamaria-Babí, PhD, University of Barcelona, Translational Immunology, Baldiri i Reixac, 10 Barcelona, Barcelona 08028, Spain. E-mail: luis.santamaria@ub.edu.

The CrossMark symbol notifies online readers when updates have been made to the article such as errata or minor corrections

0091-6749/$36.00

http://dx.doi.org/10.1016/j.jaci.2016.02.008

Abbreviations used

ASO: Anti-streptolysin O
CLA: Cutaneous lymphocyte antigen
CLA^+/Epi or CLA^-/Epi: Cocultures of T cells and epidermal cells
Ct: Cycle threshold
GP: Guttate psoriasis
SE: *Streptococcus pyogenes* extract

Circulating cutaneous lymphocyte antigen $(CLA)^+$ T lymphocytes might constitute peripheral cell biomarkers that reflect many skin-related mechanisms that take place in cutaneous lesions of T cell–mediated skin diseases.[14] CLA^+ T cells are present in psoriatic lesions[15] and might be involved in their initiation because they are present in the marginal zone of spreading lesions before the appearance of epidermal hyperplasia.[16,17] In acute stages of psoriasis, circulating CLA^+ T cells present an activated phenotype, and their number inversely correlates with the severity of the disease, thereby supporting the notion that these cells are sequestered and subsequently recirculated.[14,18] In addition, *S pyogenes* induces CLA expression in T cells,[19] and CLA^+ T cells are related to streptococcal response in patients with psoriasis,[4] as reflected by the observation that patients with this skin disease present an increased CLA^+ T-cell population expressing T-cell receptor Vβ2, a receptor for streptococcal superantigens. Interestingly, CLA^+ T cells present in the tonsils and psoriatic lesions of the same patient show an identical T-cell receptor β rearrangement, thereby suggesting migration of these cells from the tonsils to the skin.[20] The immunologic mechanisms involved in initiation of psoriatic lesions are poorly characterized and constitute an important topic for research in patients with this disease.[21]

We recently developed an *ex vivo* model in which the *S pyogenes*–induced activation of circulating effector memory CLA^+ T cells, but not CLA^- T cells, and of autologous epidermal cells from patients with psoriasis reproduces most of the key immune-inflammatory mediators described in patients with psoriasis.[22] The clinical association of *S pyogenes* throat infection and GP flare is well established; however, neither the cytokine profile nor the effector function of *S pyogenes*–induced CLA^+ T cell–mediated epidermal cell activation in HLA-$Cw6^+$ patients is well understood. Given these genetic and environmental backgrounds, it was hypothesized that such skin-homing T cells from HLA-$Cw6^+$ patients with GP respond to exposure to *S pyogenes* determinants, especially in patients challenged after recent reported streptococcal tonsillitis.

Our results indicate that *S pyogenes* induces a preferential T_H17 response only in cultures of CLA^+ T cells and epidermal cells from patients with GP. These cultures produced inflammatory mediators that led to an IL-17–dependent psoriatic effector function in keratinocytes. This response was much higher in HLA-$Cw6^+$ patients with increased anti-streptolysin O (ASO) blood levels whose GP flares were associated with streptococcal throat infection.

METHODS

Patients

The study included 14 patients with GP and 6 healthy control subjects. Detailed characteristics of the patients can be found in Table E1 in this article's Online Repository at www.jacionline.org. All participants participated voluntarily and provided written informed consent. Patients and healthy subjects underwent 1 or 2 skin biopsies and a blood extraction. The samples were collected after a minimum of 6 weeks without any systemic treatment. The study was approved by the Medical Ethics Committee of Hospital del Mar (Barcelona, Spain) and conducted according to the principles established in the Declaration of Helsinki. Patients were classified into 2 groups: cohort 1 patients were carriers of the HLA-Cw*0602 allele with a flare associated with pharyngeal infection, whereas cohort 2 patients had no evident previous episode of pharyngitis and/or did not carry the HLA-Cw*0602 allele.

Isolation of circulating memory CLA^+/CLA^- T-cell and epidermal cell suspensions

Circulating memory CLA^+ or CLA^- T cells were purified from PBMCs obtained by using Ficoll (GE Healthcare, Princeton, NJ) gradient separation with 40 to 60 mL of blood. Three consecutive immunomagnetic separations were then carried, as described previously.[22] Punch skin biopsy specimens were incubated in Dispase solution (Corning, Bedford, Mass) overnight at 4°C before peeling off the epidermis from the dermis. The epidermal sheet was cut into small pieces and incubated with trypsin (Biological Industries, Kibbutz Beit Haemek, Israel) for 30 minutes at 37°C and then mechanically disaggregated by gently pipetting against the tissue. Remaining tissue was discarded, and epidermal cell suspension was obtained by means of centrifugation.

Cocultures of T cells and epidermal cells and activation with *S pyogenes* extract

The coculture system was performed by seeding 50,000 circulating memory CLA^+ or CLA^- T cells with 30,000 autologous epidermal cells in a 96-well flat-bottom microwell plate (Nunc, Rosklide, Denmark) with culture medium (RPMI, 1% penicillin-streptomycin [Sigma-Aldrich, St Louis, Mo], and 10% FBS [Gibco BRL-Life Technologies, Grand Island, NY]) for 5 days. *Streptococcus pyogenes* extract (SE) was obtained from sonicated *S pyogenes* isolated from throat swabs of patients with psoriasis and was used at a final concentration of 5 μg/mL. Designations CLA^+/Epi or CLA^-/Epi are used in reference to cocultures of T cells and epidermal cells.

Human keratinocyte cultures

Pooled primary human epidermal keratinocytes were cultured in 24-well plates (Corning, Corning, NY) in CnT-07 medium (CELLnTEC, Bern, Switzerland). After reaching confluence, cells were washed with PBS (Lonza, Verviers, Belgium), and new medium was added with 1:10 diluted coculture supernatants and left for 15 hours before RNA extraction.

RNA isolation and quantitative real-time PCR

RNA from cell cultures was isolated with the PureLink RNA Mini Kit (Ambion, Carlsbad, Calif). cDNA was synthesized by using oligo(dT) (Biotools, Madrid, Spain), dNTPs (GeneCraft, Lüdinghausen, Germany), M-MLV RT 5x Buffer, and M-MLV RT RNase (H-) Point Mutant (Promega, Madison, Wis). Subsequent analysis with SYBR Green (Applied Biosystems, Warrington, United Kingdom)–based quantitative real-time PCR was performed with an ABI Prism 7900HT instrument (Applied Biosystems, Foster City, Calif). Gene expression was calculated by using the $\Delta-\Delta$ cycle threshold (Ct) method (with the mean cycle threshold value for β-actin [*ACTB*] and the gene of interest for each sample). The equation 1.8 e(CtACTB − Ctgene of interest) $\times 10^4$ was used to obtain normalized values.[23] Primer sequences (Sigma-Aldrich) are shown in Table E2 in this article's Online Repository at www.jacionline.org. Fold increase of mRNA levels was obtained by using expression of keratinocytes under stimulation with supernatants of CLA^+ or CLA^- T cells with epidermal cells without SE for each patient as an individual baseline, thus reducing the noise and increasing the analytic power.

Cytokine quantification

After 5 days of culture, supernatants were taken. IL-17A, IFN-γ, IL-6, TNF-α, and IL-8 levels were then measured with a multiplex fluorescent bead–based immunoassay with the Diaclone DIAplex kit (Gen-Probe,

Besançon, France). Human IL-17F levels were quantified with the CBA kit (BD Biosciences, San Diego, Calif). A Gallios flow cytometer (Beckman-Coulter, Brea, Calif) was used to run the samples, and FCAP Array software was used for analysis.

HLA-Cw*0602 typing

DNA was obtained from T-cell blasts. Human PBMCs (1×10^6) were activated *in vitro* with PHA (10 μg/mL, Sigma-Aldrich). At days 4 to 5, IL-2 was added to generate T-cell blasts. Growing cells were transferred from 48- to 24-well plates, reaching at least 2 confluent wells at the end of culture (creating a T-cell line). At days 7 to 8, cells were harvested, and DNA typing for HLA-C*0602 was performed with the protocol described below.

DNA purification and PCR

Purification of total DNA from cultured cells was done with the DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany) according to a Spin-Column Protocol. DNA was quantified with a NanoDrop Spectrophotometer (Thermo Scientific, Waltham, Mass) and made up to concentrations of 100 or 50 ng/μL. HLA-C polymorphisms were examined with sequence-specific oligonucleotide primed PCR (PCR-SSO) technology. Briefly, target DNA was amplified by using PCR with biotinylated primers specifically designed for amplified exons 2 and 3 of HLA-C alleles. The PCR amplicon was then denatured and hybridized to complementary oligonucleotide probes immobilized on fluorescence-coated microsphere beads. At the same time, the biotinylated PCR product was labeled with phycoerythrin-conjugated streptavidin and immediately examined with a Luminex 200 (Luminex, Austin, Tex) by using the Quicktype software (LIFECODES; Gen-Probe). We followed the guidelines of the 2010 World Health Organization nomenclature Committee for factors regarding the HLA system.

Statistical analysis

To further characterize the pathogenesis of GP, we used the model established in our previous work, in which we defined the role of SE-induced CLA^+ T cells and epidermal cell activation in an *ex vivo* coculture.[22] Data were assumed to be not normally distributed. Nonparametric tests (Mann-Whitney *U* and Kruskal-Wallis tests with the Dunn posttest) were used to compare the medians of cytokine concentrations produced by cocultures and their effect on keratinocytes in terms of mRNA expression. Data were represented as medians ± interquartile ranges either by box plots (minimum to maximum) or solid lines. Broken axes were used for outlier values. The Wilcoxon matched pairs test was used to compare T_H17/T_H1 cytokine production by the same samples. For linear regression analysis, log transformation of non-Gaussian outcomes was applied to make their distribution approximately normal, and the Pearson coefficient correlation was used. For this purpose, nondetectable cytokine concentrations were assigned to 0.1 pg/mL for inclusion in the analysis (1 cohort 2 value for IL-17A and 1 cohort 1 value and 2 cohort 2 values for IFN-γ). Differences were considered significant at a *P* value of less than .05.

RESULTS

S pyogenes induces production of IL-17A/F in cultures of CLA^+ T cells and epidermal cells from patients with GP

Cultures containing CLA^+ T cells and autologous epidermal cells (CLA^+/Epi) from patients with GP (n = 14) in the presence of SE displayed the highest activation (Fig 1). This SE-induced activation led to production of the T cell–derived cytokines IL-17A, IL-17F, and IFN-γ ($P < .05$; Fig 1, *A-C*), thus indicating the capacity of SE to promote both a T_H17 and T_H1 response. This observation is consistent with current knowledge about the mixed T_H17/T_H1 responses in patients with this disease.[24] However, a clear T_H17 profile was observed because both medians and pairwise comparison analysis for individual values showed significantly higher production of IL-17A (336 ± 23.2/1,860 pg/mL) and IL-17F (3,885 ± 254.2/33,264 pg/mL) compared with IFN-γ (21.6 ± 0/113.8 pg/mL; $P < .05$; Table I and see Fig E1, *A*, in this article's Online Repository at www.jacionline.org). Also, these patients showed a higher production of IL-17F than IL-17A ($P = .02$, Table I). Interestingly, SE led to a significant increase in levels of the T_H17 differentiation inducer IL-6 (76.2 ± 8.8/139.2 pg/mL), as well as TNF-α (11 ± 0/108.1 pg/mL) and IL-8 (12,902 ± 2,388/26,290 pg/mL; $P < .05$; Fig 1, *D-F*, respectively).

Activation of cocultures containing non–skin-homing CLA^- T cells (n = 13) did not cause any significant difference in the production of these cytokines, clearly presenting a trend toward higher IFN-γ production in 77% of psoriatic CLA^- T-cell cocultures compared with IL-17A, although without significance ($P = .81$; see Fig E1, *B* and *C*). Similarly, cocultures with healthy samples showed no significant IL-17A, IL-17F, or IFN-γ production (Fig 1, *A-C*), and there was no preferential T_H17 or T_H1 response (see Fig E1, *A* and *B*). Significant production of TNF-α was observed only in SE-activated CLA^+/Epi cocultures from control samples (16.3 ± 3.7/51.3 pg/mL; Fig 1, *E*).

$HLA\text{-}Cw6^+$ patients with GP and previous pharyngitis display a strong T_H17 response to SE

Patients with GP were classified into 2 cohorts to further characterize clinical profiles on the basis of differential responses to SE. Those carrying the HLA-Cw6 allele and with flares associated with previous pharyngeal infection were grouped in cohort 1 (n = 6). Note that the HLA-Cw6 allele was not present in any of the healthy control subjects (Table II). The remaining patients not satisfying either of these 2 parameters were grouped into cohort 2 (n = 8, Table II). No significant difference was found between median ASO levels from 2 cohorts (cohort 1: 331 vs cohort 2: 387 IU/mL), although most cohort 1 patients had positive blood titers (ASO, ≥200 IU/mL; 83.3%), as well as earlier onset of lesions, than cohort 2 patients (83.3% vs 37.5%, respectively; Table II and see Table E1). In addition, median Psoriasis Area Severity Index scores were not significantly different between cohorts (cohort 1: 4.3 vs cohort 2: 6.6, Table II).

SE-activated CLA^+/Epi cocultures from cohort 1 patients showed higher cytokine production than those from cohort 2 patients, reaching significance for IL-17A, IL-17F, IL-6, and IL-8 ($P < .05$; Fig 2, *A*, *B*, *D*, and *F*, respectively). No statistical difference was found for IFN-γ or TNF-α production between cohorts. Within each cohort, only IL-17A, IL-17F, and IL-6 showed a significantly higher production by coculture with CLA^+ T cells than by those with CLA^- T cells from the same subjects (Fig 2, *A*, *B*, and *D*, respectively). The same comparison was significant in cohort 2 samples only for IL-8 production (Fig 2, *F*). Furthermore, in cohort 1 IL-17A (1,861 ± 533.5/4,730 pg/mL) and IL-17F (24,053 ± 4,972/71,783 pg/mL) production by SE-activated CLA^+/Epi cocultures was significantly higher than IFN-γ production (47.38 ± 13.86/305.5 pg/mL). Additionally, a significant paired difference was observed for IL-17A compared with IFN-γ values within these same supernatants ($P < .05$, Table I and Fig E2 in this article's Online Repository at www.jacionline.org). Conversely, only significantly higher production of IL-17F than IFN-γ was found in cohort

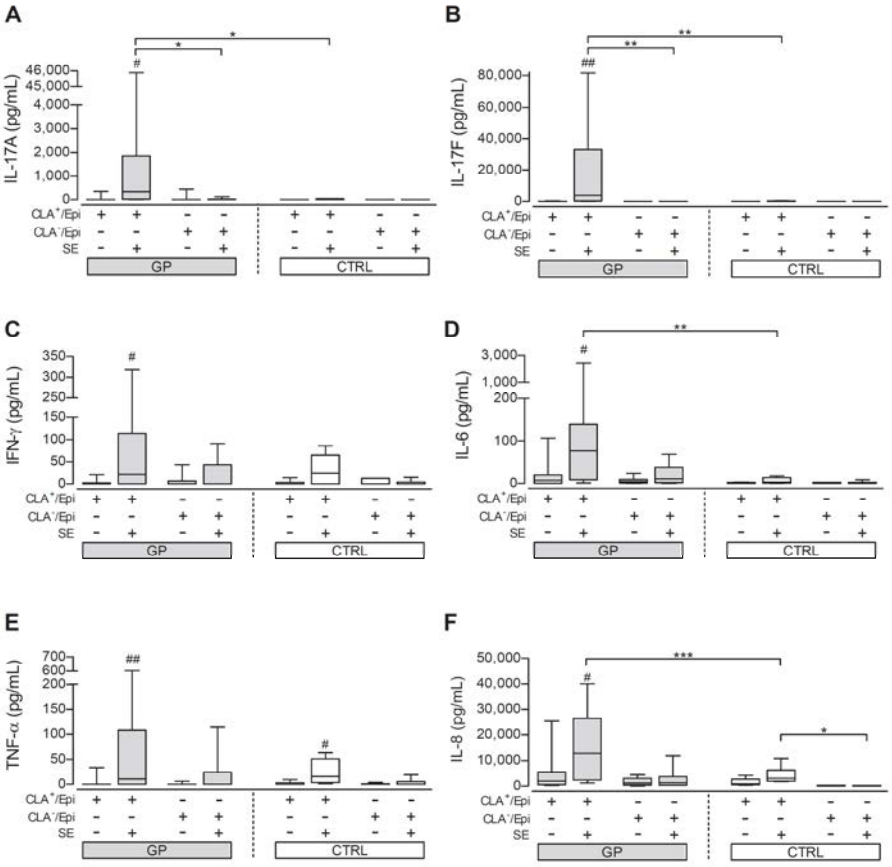


FIG 1. SE-activated cocultures of CLA^+ T cells and epidermal cells from patients with GP produce high levels of psoriasis-associated cytokines. Cocultures of supernatants from 14 patients with GP and 6 healthy control (*CTRL*) samples were collected at day 5, and IL-17A **(A)**, IL-17F **(B)**, IFN-γ **(C)**, IL-6 **(D)**, TNF-α **(E)**, and IL-8 **(F)** concentrations were measured. Data are represented by box plots (minimum to maximum and median ± interquartile range). Cytokine production by cocultures stimulated with SE were compared with basal cytokine values by using the Mann-Whitney *U* test: #$P < .05$ and ##$P < .01$. SE-stimulated conditions were compared by using the Kruskal-Wallis ANOVA test, followed by Dunn multiple comparison: *$P < .05$, **$P < .01$, and ***$P < .001$. Cell types and SE presence (+) for each coculture condition are defined below the *x-axis*.

TABLE I. T cell–derived T_H17 and T_H1 cytokine production by SE-activated CLA^+/Epi cocultures from GP samples

	Median ± IQR (pg/mL)			IL-17A vs IL-17F		IL-17A vs IFN-γ		IL-17F vs IFN-γ	
	IL-17A	IL-17F	IFN-γ	*P* value*	*P* value†	*P* value*	*P* value†	*P* value*	*P* value†
All patients (n = 14)	336 (23.2/1,860)	3,885 (254.2/33,264)	21.6 (0/113.8)	.09	**.02**	**.03**	**.005**	**.002**	**.008**
Cohort 1 (n = 6)	1,861 (533.5/14,730)	24,053 (4,972/71,783)	47.38 (13.86/305.5)	.07	.13	**.009**	**.03**	**.009**	.13
Cohort 2 (n = 8)	32.83 (0.1/322.4)	303.5 (61,35/2364)	2.8 (0/89.52)	.27	.38	.48	.31	**.03**	.32

Values in boldface indicate statistical significance.
IQR, Interquartile range.
*Mann-Whitney *U* test for median comparison.
†Wilcoxon test for paired samples.

2–derived supernatants (Table I). Interestingly, amounts of IL-17A from cohort 1 showed a significant inverse correlation with time elapsed since symptom onset ($r = -0.91$, $P = .01$; Fig 2, *G*), whereas no such correlation occurred with IFN-γ (Fig 2, *H*).

Conditioned cohort 1 supernatants induce a higher upregulation of psoriasis-associated genes in normal human keratinocytes

Given the significant differences observed in cytokine production between the 2 cohorts, their effector functions were

TABLE II. Characteristics of the study population

Parameter	Whole population	
	Patients with GP (n = 14)	Healthy control subjects (n = 6)
HLA-Cw0602 positive (%)	11 (78.6)	0 (0)

Parameter	GP population		
	Cohort 1 (n = 6)	Cohort 2 (n = 8)	*P* value*
HLA-Cw0602 positive (%)	6 (100)	5 (62.5)	
Confirmed *S pyogenes* pharyngeal infection (%)	6 (100)	0 (0)	
ASO titer (IU/mL), median (±IQR)	331 (226.3/452.3)	387 (67.3/387.3)	.2
ASO ≥200 (%)	5 (83.3)	4 (50)	
Lesion flare <2 mo (%)	5 (83.3)	3 (37.5)	
Age (y), median (±IQR)	39.5 (28.25/47)	33.0 (23.5/40)	.44
PASI score, median (±IQR)	4.3 (3.7/7.7)	6.6 (3.6/14.5)	.4

Parameters in boldface indicate the 2 indispensable conditions for cohort 1 inclusion.
IQR, Interquartile range; *PASI*, Psoriasis Area Severity Index.
*Mann-Whitney *U* test between cohorts 1 and 2.

examined in keratinocytes. Supernatants from cohort 1–derived CLA^+/Epi cocultures (n = 6) showed a higher gene induction capacity than that elicited by supernatants from their respective CLA^-/Epi cocultures, as well as compared with that from cohort 2 or control supernatants (n = 5; Fig 3, *A*). This capacity was only significant for the IL-17–induced transcripts *DEFB4*, *S100A7*, *LCN2*, *IL36G*, and *IL-8* but not for the IFN-γ–associated chemokines *CXCL9*, *CXCL10*, and *CXCL11* (Fig 3, *A*). Supernatants from cohort 2 patients and control subjects had no effect on keratinocyte mRNA expression.

In addition, amounts of IL-17A produced by SE-activated CLA^+/Epi cells showed a significant correlation with IL-17–related genes (Fig 3, *B*, and see Table E3 in this article's Online Repository at www.jacionline.org), whereas no correlation was observed when compared with amounts of IFN-γ (see Fig E3 and Table E3 in this article's Online Repository at www.jacionline.org).

Filaggrin and loricrin expression is downregulated in normal keratinocytes by supernatants from SE-stimulated CLA^+/Epi cocultures from psoriatic cohort 1

Altered expression of cornification proteins results in barrier dysfunction.[23] It has also been described that gene expression of proteins involved in the proper formation of the cornified envelope, such as loricrin and filaggrin, are reduced in psoriatic lesions.[26] This observation might explain the impairment and weakening of the skin barrier and abnormal differentiation pattern characteristic of this disease. However, the mechanism underlying reduced skin barrier function in patients with psoriasis is unknown.[27] Interestingly, only keratinocytes exposed to supernatants from SE-activated CLA^+/Epi cocultures from cohort 1 showed a higher reduction of both *FLG* and *LOR* transcripts (Fig 4, *A*). In contrast, supernatants from cohort 2 patients and control subjects generally maintained baseline levels. Additionally, IL-17A in supernatants from SE-activated CLA^+/Epi cocultures inversely correlated with *FLG* mRNA expression ($r = -0.64$, $P = .04$; Fig 4, *B*). However, this correlation was not statistically significant for *LOR* expression (Fig 4, *B*). None of these expressions correlated with IFN-γ levels (see Fig E3 in this article's Online Repository at www.jacionline.org).

DISCUSSION

The study of induction of antigen-specific immune responses with clinically relevant stimuli in responding patients allows identification of pathologic translational mechanisms of several immunologic diseases, including psoriasis. Indeed, although psoriasis is considered to be triggered by innate stimuli[21,28] and GP is related to streptococcal throat infections, this clinically relevant microbe association within its natural history has received little attention.[29] In addition, although the T_H17 response is clinically validated in patients with this disease,[30] no studies have focused on a possible T_H17 response induced by *S pyogenes* in patients with GP.

The present study indicates that SE induces a preferential T_H17 response in patients with psoriasis, specifically through interaction of circulating effector memory CLA^+ T cells and lesional epidermal cells, with effector functions in normal keratinocytes comparable with those found in psoriatic epidermal cells. Such a T_H17 response and effector function were even higher in patients who were HLA-$Cw6^+$ and whose skin flare was related to *S pyogenes* throat infection.

Increased expression of IL-23 receptor by CLA^+ T cells, either $CD4^+$ or $CD8^+$, from the tonsils of patients with psoriasis has been shown. These $CD4^+$ T cells also coexpress high CCR6 levels. Both receptors are surface markers of T_H17 cells.[31,32] In addition, tonsillar CLA^+ T cells share identical Vβ expression with T cells present in psoriatic lesions of the same patient,[20] thereby indicating their migration from tonsils to skin. Thus circulating CLA^+ T cells from patients with GP induced by streptococcal infection should preferentially respond to SE and produce IL-17. In fact, the results of this work support this notion and extend this concept. The innate stimulus that induces an adaptive T_H17 response is poorly understood in patients with GP. These findings are in line with the observation of high serum IL-17 levels in patients with psoriasis with early spreading guttate morphology.[33] In addition, a bimodal immune activation has recently been described in patients with psoriasis, in which the onset of new lesions shows a predominantly IL-1/T_H17 rather than T_H1 phenotype, with the latter associated with a late-phase reaction.[34] Interestingly, patients from this study who had a GP flare induced by *S pyogenes* and were HLA-$Cw6^+$ (cohort 1) presented, on average, more than 20 times more IL-17A after SE activation than patients without those clinical features (cohort 2). These results suggest that a patient whose flare is associated with *S pyogenes* throat infection would have a greater number of T_H17 circulating CLA^+ T cells responding to SE. Given the inverse correlation of IL-17A levels with the time elapsed from throat infection and the consecutive lesion onset, numbers of T_H17 circulating CLA^+ T cells would probably increase shortly after infection and would progressively reduce over time.

We have previously shown that the memory CLA^+ T cell–dependent *S pyogenes* production of IL-17A and IFN-γ can be reduced in this coculture model by blocking antibodies for HLA class I and class II, which caused 50% and almost 100% inhibition, respectively.[22] Given this observation, both $CD4^+$ and $CD8^+$ T cells might be involved in this activation. In fact, *S pyogenes* superantigens activate T cells in patients with

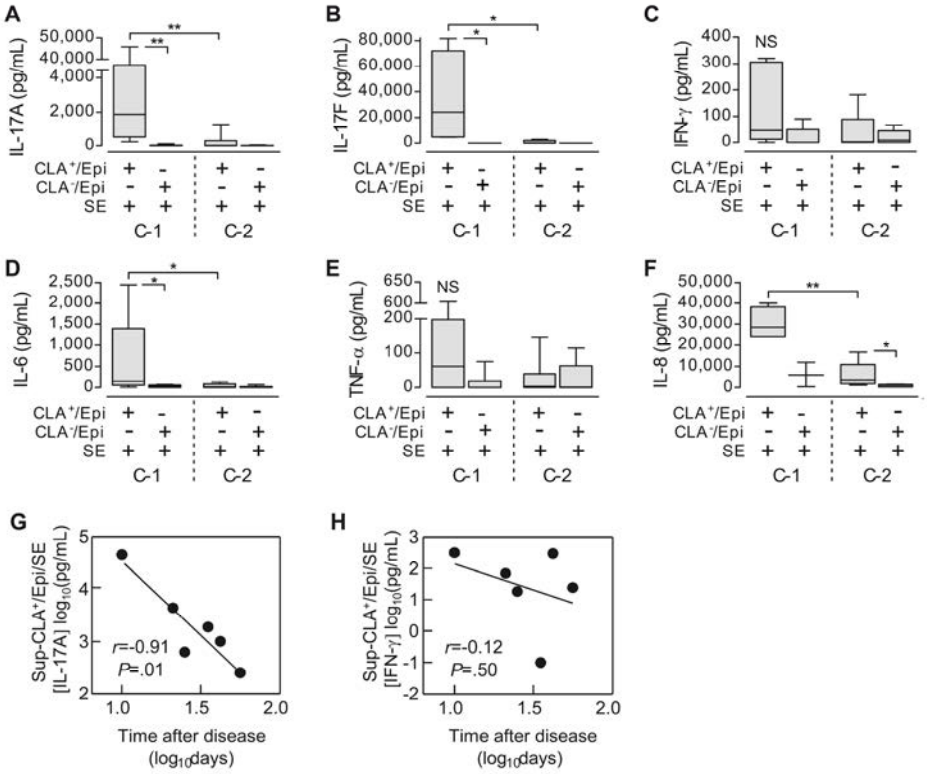


FIG 2. T_H17 response is increased in HLA-Cw6$^+$ patients with GP with previous pharyngitis. **A-F,** Cytokine production by cohort 1 (C-1, n = 6; except for IL-8 CLA$^-$/Epi/SE, n = 3) and cohort 2 (C-2, n = 5). Data are expressed as box plots (minimum to maximum and median ± interquartile range). The Mann-Whitney *U* test was used to compare cytokine production: **P* < .05 and ***P* < .01. *NS,* Not significant. Cell types and SE presence (+) for each coculture condition are defined below the *x-axis.* Linear correlation between cohort 1 SE-activated CLA$^+$/Epi production of IL-17A **(G)** or IFN-γ **(H)** with time elapsed since disease onset.

acute GP,[19] and antigen-specific T-cell activation can also take place because the cross-reaction of CD8$^+$ T cells with human keratin and streptococcal antigens has been reported.[4] A limitation of the present study is that it did not address the mode of T-cell activation in the *ex vivo* model in detail. The main goal was first to clarify the *S pyogenes*–specific immune response in a population of patients with GP by using this model.

The observation that the response to *S pyogenes* is preferentially associated with circulating CLA$^+$ T cells in patients with GP is of relevance with respect to understanding the initial events of the lesions in early stages of the disease. Among other cell types, CLA$^+$ T cells are present at the edges of psoriatic lesions before epidermal hyperplasia occurs.[16,17] In patients with GP, circulating CLA$^+$ T cells express activation markers, such as HLA-DR, which are not present in control subjects,[18] and these cells recirculate during cutaneous inflammation.[14] Thus this preferential response of circulating CLA$^+$ T cells to *S pyogenes* and their T_H17 cytokine production seems to be related to the early events of lesion formation, during which a molecular mimicry mechanism[4] or microbial activation events take place.[19]

Acute lesions in patients with GP spread rapidly. To better understand this process, we explored the effector capacity of supernatants from SE-specific CLA$^+$ T cells on normal keratinocytes. With this aim, we studied how the inflammatory milieu induced by SE-CLA$^+$/Epi activation in patients with GP affects nonlesional epidermal cells of the surrounding tissue, a mechanism likely driven by immune mediators produced by adaptive and innate cross-talk between epidermal cells and immunocyte infiltrates, as represented by the cocultures. The supernatants of SE-activated CLA$^+$/Epi cocultures from psoriatic cohort 1 patients induced the highest mRNA expression of genes found to be increased in the psoriatic epidermis. Expressions of chemokines recruiting both neutrophils *(IL8)* and T cells (*CXCL9*, *CXCL10*, and *CXCL11*) were upregulated in keratinocytes. The latter T-cell chemoattractants can enhance the chemotaxis of skin-homing CLA$^+$ T cells through their CXCR3 receptor.[35] Other upregulated genes included the antimicrobial peptides *DEFB4*, *S100A7*, and *LCN2*, which are highly expressed in psoriatic lesions and might modulate immune responses in patients with this disease,[36,37] and *IL36G*, an important cytokine that might regulate the IL-23/T_H17 pathway

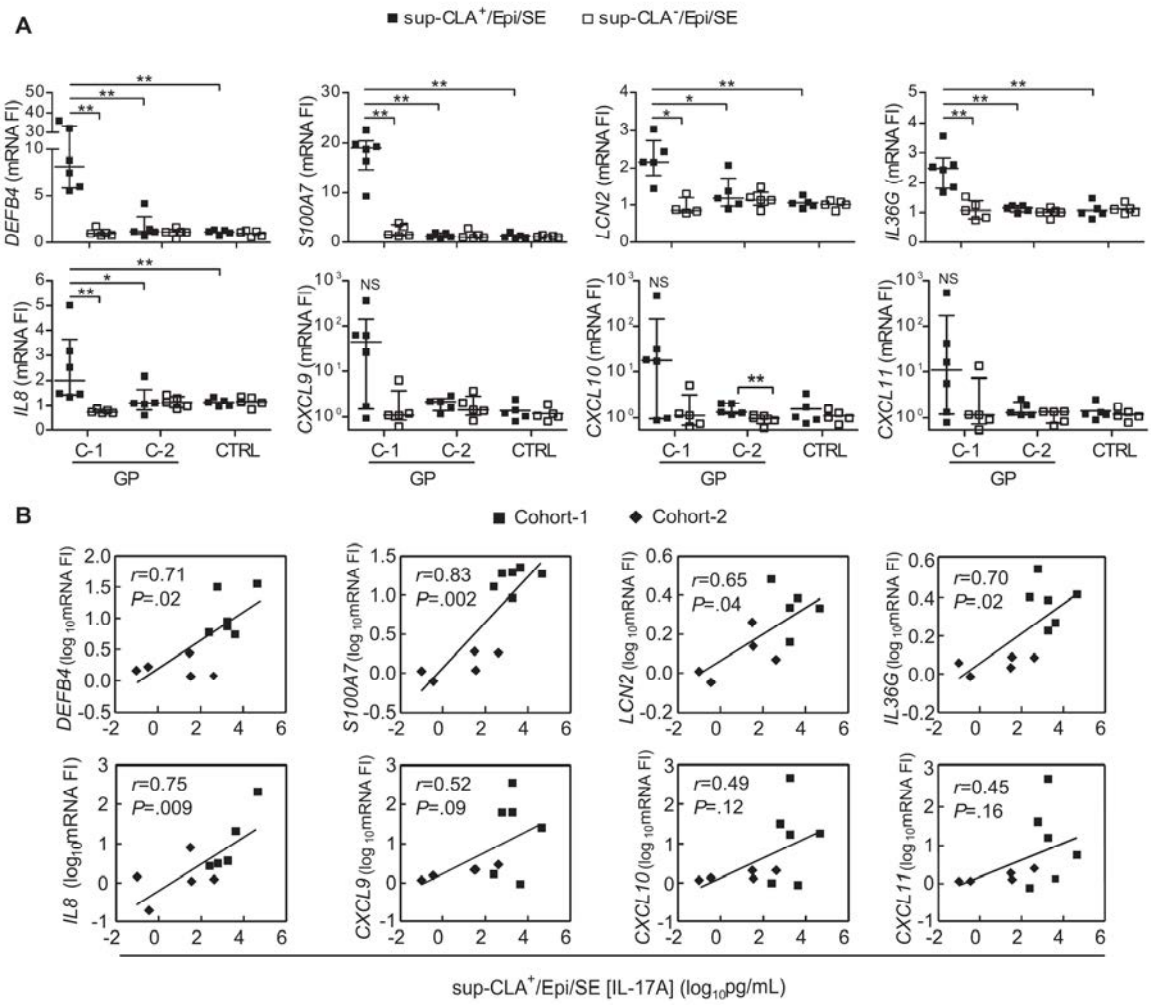


FIG 3. Supernatants from psoriatic cohort 1–derived CLA^+/Epi cocultures trigger a higher expression of psoriasis-associated transcripts in keratinocytes. **A,** Gene induction capacity of SE-activated CLA^+/CLA^- coculture supernatants from cohort 1 (*GP C-1*; n = 6) is compared with that of cohort 2 (*GP C-2*; n = 5) and control subjects (*CTRL*; n = 5). The Mann-Whitney *U* test was used for median comparisons: $*P < .05$ and $**P < .01$. **B,** Correlation between IL-17A concentrations of 5-day supernatants from SE-activated CLA^+/Epi cocultures and the mRNA levels induced in keratinocytes. *FI,* Fold increase.

in patients with psoriasis.[38,39] In addition, *IL36G* has recently been described as a biomarker for psoriatic skin lesions and is also induced by other innate stimuli.[40,41] Interestingly, the expression of these genes is related to initial events in the psoriatic lesion, and *DEFB4*, *S100A7*, *LCN2*, *IL36G*, and *IL8* are known to be induced by IL-17 in keratinocytes.[42] It is of relevance that the upregulation of these genes in supernatant-stimulated keratinocytes correlated with their respective contents of IL-17A but not IFN-γ. Thus in nonlesional surrounding keratinocytes SE-induced CLA^+ T and lesional epidermal cell activation would favor the early recruitment of T cells migrating to skin and neutrophils in a T_H17 environment.

Conversely, keratinocytes treated with the same supernatants of cohort 1–derived CLA^+/Epi/SE samples showed decreased mRNA levels for *FLG* and *LOR*. The products of these 2 genes are important skin barrier proteins that show an abnormal low expression in psoriatic lesions, which could be modulated by the underlying inflammatory milieu.[25,27,43] Similarly, it has been reported that IL-17 decreases *FLG* expression in keratinocytes,[44] thereby supporting the observed correlation between *FLG* expression and IL-17A content in the supernatants used for keratinocyte stimulation. In patients with atopic dermatitis, *FLG* mutations confer strong genetic determinants for cases with long-term duration.[45,46] However, patients with atopic dermatitis who are not carriers of such mutations might also have an acquired defect in *FLG* expression through the T_H2-derived IL-4/IL-13 atopic inflammatory response.[47] Thus our data further reinforce the key contribution of the immune

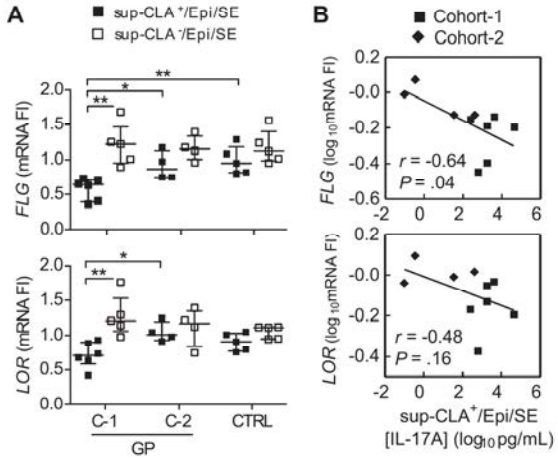


FIG 4. Expression of the skin barrier–associated genes *FLG* and *LOR* is downregulated by SE-activated CLA^+/Epi coculture supernatants from GP cohort 1 samples. **A,** mRNA fold increase (*FI*) expression levels in keratinocytes treated with SE-activated CLA^+/CLA^- coculture supernatants from GP cohort 1 (*GP C-1*; n = 6), GP cohort 2 (*GP C-2*; n = 4), or healthy control subjects (*CTRL*; n = 5) samples. The Mann-Whitney *U* test was used for median comparisons: $*P < .05$ and $**P < .01$. **B,** Linear correlation between IL-17A concentrations within SE-activated CLA^+/Epi coculture supernatants and respective mRNA expression in keratinocytes.

response to skin barrier defects, which contribute to the development of these diseases.

Interestingly, genes of the late cornified envelop 3B and 3C, which are expressed in epithelial cells, are risk factors for psoriasis. A pathogenic model of psoriasis has recently been put forward in which skin barrier abnormalities in patients with psoriasis facilitate infection, innate immunity activation, and T_H17 response induction, similar to allergen sensitization through the skin in patients with atopic dermatitis.[26] Because *Streptococcus* species have been shown to be present in psoriatic lesions,[48] our data support the notion of a link between SE-induced activation of CLA^+ T cells and skin barrier disruption within a T_H17 context, especially in patients belonging to cohort 1 (HLA-Cw6 patients and patients with GP with a flare caused by *S pyogenes* throat infection). *S pyogenes* has been detected in the skin but not the throats of patients with GP,[49] and some authors have proposed that a transitory intracutaneous presence of *S pyogenes* during streptococcal pharyngitis would be enough to generate an antibacterial immune response that would lead to autoimmunity against local skin proteins by molecular mimicry.[50] In fact, our model might reproduce the molecular events that occur when *S pyogenes* reaches the lower layers of the skin and interacts with CLA^+ T cells in patients with psoriasis, thus revealing a secondary portal of entrance for streptococcal antigens.

In summary, these data shed new light on the innate induced adaptive immune response in patients with GP. The preferential T_H17 response induced by a clinically relevant trigger, such as *S pyogenes*, in skin-associated circulating effector memory CLA^+ T cells has a significant effect on keratinocytes. This effect seems to be even greater in patients with type I psoriasis[51] carrying the HLA-Cw6 allele and presenting with GP triggered by *S pyogenes* throat infection. These new mechanisms might help unravel the acute course and severe presentation of the disease.

Clinical implications: The environmental innate stimulus *S pyogenes* induces a preferential T_H17 response through key cellular elements of HLA-$Cw6^+$ patients with GP. This response produces psoriatic features in keratinocytes, including decreased expression of filaggrin and loricrin.

REFERENCES

1. Gudjonsson JE, Karason A, Runarsdottir EH, Antonsdottir AA, Hauksson VB, Jónsson HH, et al. Distinct clinical differences between HLA-Cw*0602 positive and negative psoriasis patients—an analysis of 1019 HLA-C- and HLA-B-typed patients. J Invest Dermatol 2006;126:740-5.
2. Talamonti M, Botti E, Galluzzo M, Teoli M, Spallone G, Bavetta M, et al. Pharmacogenetics of psoriasis: HLA-Cw6 but not LCE3B/3C deletion nor TNFAIP3 polymorphism predisposes to clinical response to interleukin 12/23 blocker ustekinumab. Br J Dermatol 2013;169:458-63.
3. Chiu HY, Wang TS, Chan CC, Cheng YP, Lin SJ, Tsai TF. Human leucocyte antigen-Cw6 as a predictor for clinical response to ustekinumab, an interleukin-12/23 blocker, in Chinese patients with psoriasis: a retrospective analysis. Br J Dermatol 2014;171:1181-8.
4. Valdimarsson H, Thorleifsdottir RH, Sigurdardottir SL, Gudjonsson JE, Johnston A. Psoriasis—as an autoimmune disease caused by molecular mimicry. Trends Immunol 2009;30:494-501.
5. McFadden JP, Baker BS, Powles AV, Fry L. Psoriasis and streptococci: the natural selection of psoriasis revisited. Br J Dermatol 2009;160:929-37.
6. Prinz JC. Psoriasis vulgaris—a sterile antibacterial skin reaction mediated by cross-reactive T cells? An immunological view of the pathophysiology of psoriasis. Clin Exp Dermatol 2001;26:326-32.
7. Gudjonsson JE, Thorarinsson AM, Sigurgeirsson B, Kristinsson KG, Valdimarsson H. Streptococcal throat infections and exacerbation of chronic plaque psoriasis: a prospective study. Br J Dermatol 2003;149:530-4.
8. El-Rachkidy RG, Hales JM, Freestone PP, Young HS, Griffiths CE, Camp RD. Increased blood levels of IgG reactive with secreted *Streptococcus pyogenes* proteins in chronic plaque psoriasis. J Invest Dermatol 2007;127:1337-42.
9. Thorleifsdottir RH, Sigurdardottir SL, Sigurgeirsson B, Olafsson JH, Sigurdsson MI, Petersen H, et al. Improvement of psoriasis after tonsillectomy is associated with a decrease in the frequency of circulating T cells that recognize streptococcal determinants and homologous skin determinants. J Immunol 2012;188:5160-5.
10. Rachakonda TD, Dhillon JS, Florek AG, Armstrong AW. Effect of tonsillectomy on psoriasis: a systematic review. J Am Acad Dermatol 2015;72:261-75.
11. Mallbris L, Wolk K, Sánchez F, Ståhle M. HLA-Cw*0602 associates with a twofold higher prevalence of positive streptococcal throat swab at the onset of psoriasis: a case control study. BMC Dermatol 2009;9:5.
12. Weisenseel P, Laumbacher B, Besgen P, Ludolph-Hauser D, Herzinger T, Roecken M, et al. Streptococcal infection distinguishes different types of psoriasis. J Med Genet 2002;39:767-8.
13. Leung DY, Gately M, Trumble A, Ferguson-Darnell B, Schlievert PM, Picker LJ. Bacterial superantigens induce T cell expression of the skin-selective homing receptor, the cutaneous lymphocyte-associated antigen, via stimulation of interleukin 12 production. J Exp Med 1995;181:747-53.
14. Ferran M, Romeu ER, Rincón C, Sagristà M, Giménez Arnau AM, Celada A, et al. Circulating CLA+ T lymphocytes as peripheral cell biomarkers in T-cell-mediated skin diseases. Exp Dermatol 2013;22:439-42.
15. Picker LJ, Treer JR, Ferguson-Darnell B, Collins PA, Bergstresser PR, Terstappen LW. Control of lymphocyte recirculation in man. II. Differential regulation of the cutaneous lymphocyte-associated antigen, a tissue-selective homing receptor for skin-homing T cells. J Immunol 1993;150:1122-36.
16. Davison SC, Ballsdon A, Allen MH, Barker JN. Early migration of cutaneous lymphocyte-associated antigen (CLA) positive T cells into evolving psoriatic plaques. Exp Dermatol 2001;10:280-5.
17. Vissers WH, Arndtz CH, Muys L, Van Erp PE, de Jong EM, van De Kerkhof PC. Memory effector (CD45RO+) and cytotoxic (CD8+) T cells appear early in the margin zone of spreading psoriatic lesions in contrast to cells expressing natural killer receptors, which appear late. Br J Dermatol 2004;150:852-9.
18. Pont-Giralt M, Giménez-Arnau AM, Pujol RM, Santamaria-Babi LF. Circulating CLA+ T cells from acute and chronic psoriasis patients manifest a different activation state and correlation with disease severity and extension. J Invest Dermatol 2006;126:227-8.
19. Leung DY, Travers JB, Giorno R, Norris DA, Skinner R, Aelion J, et al. Evidence for a streptococcal superantigen-driven process in acute guttate psoriasis. J Clin Invest 1995;96:2106-12.

20. Diluvio L, Vollmer S, Besgen P, Ellwart JW, Chimenti S, Prinz JC. Identical TCR B-chain rearrangements in streptococcal angina and skin lesions of patients with psoriasis vulgaris. J Immunol 2006;176:7104-11.
21. Nestle FO, Kaplan DH, Barker J. Psoriasis. N Engl J Med 2009;361:496-509.
22. Ferran M, Galván AB, Rincón C, Romeu ER, Sacrista M, Barboza E, et al. *Streptococcus* induces circulating CLA+ memory T-cell-dependent epidermal cell activation in psoriasis. J Invest Dermatol 2013;133:999-1007.
23. Chan JR, Blumenschein W, Murphy E, Diveu C, Wiekowski M, Abbondanzo S, et al. IL-23 stimulates epidermal hyperplasia via TNF and IL-20R2–dependent mechanisms with implications for psoriasis pathogenesis. J Exp Med 2006;203: 2577-87.
24. Lowes MA, Kikuchi T, Fuentes-Duculan J, Cardinale I, Zaba LC, Haider AS, et al. Psoriasis vulgaris lesions contain discrete populations of Th1 and Th17 T cells. J Invest Dermatol 2008;128:1207-11.
25. Henry J, Toulza E, Hsu CY, Pellerin L, Balica S, Mazereeuw-Hautier J, et al. Update on the epidermal differentiation complex. Front Biosci (Landmark Ed) 2012;17:1517-32.
26. Bergboer JG, Zeeuwen PL, Schalkwijk J. Genetics of psoriasis: evidence for epistatic interaction between skin barrier abnormalities and immune deviation. J Invest Dermatol 2012;132:2320-31.
27. Kim BE, Howell MD, Guttman-Yassky E, Gilleaudeau PM, Cardinale IR, Boguniewicz M, et al. TNF-α downregulates filaggrin and loricrin through c-Jun N-terminal kinase: role for TNF-α antagonists to improve skin barrier. J Invest Dermatol 2011;131:1272-9.
28. Nestle FO, Conrad C, Tun-Kyi A, Homey B, Gombert M, Boyman O, et al. Plasmacytoid predendritic cells initiate psoriasis through interferon-α production. J Exp Med 2005;202:135-43.
29. Ko HC, Jwa SW, Song M, Kim MB, Kwon KS. Clinical course of guttate psoriasis: Long-term follow-up study. J Dermatol 2010;37:894-9.
30. Krueger JG, Fretzin S, Suárez-Fariñas M, Haslett PA, Phipps KM, Cameron GS, et al. IL-17A is essential for cell activation and inflammatory gene circuits in subjects with psoriasis. J Allergy Clin Immunol 2012;130:145-54.
31. Annunziato F, Cosmi L, Santarlasci V, Maggi L, Liotta F, Mazzinghi B, et al. Phenotypic and functional features of human Th17 cells. J Exp Med 2007;204: 1849-61.
32. Sigurdardottir SL, Thorleifsdottir RH, Valdimarsson H, Johnston A. The association of sore throat and psoriasis might be explained by histologically distinctive tonsils and increased expression of skin-homing molecules by tonsil T cells. Clin Exp Immunol 2013;174:139-51.
33. Choe YB, Hwang YJ, Hahn HJ, Jung JW, Jung HJ, Lee YW, et al. A comparison of serum inflammatory cytokines according to phenotype in patients with psoriasis. Br J Dermatol 2012;167:762-7.
34. Christophers E, Metzler G, Röcken M. Bimodal immune activation in psoriasis. Br J Dermatol 2014;170:59-65.
35. Rottman JB, Smith TL, Ganley KG, Kikuchi T, Krueger JG. Potential role of the chemokine receptors CXCR3, CCR4, and the integrin alphaEbeta7 in the pathogenesis of psoriasis vulgaris. Lab Invest 2001;81:335-47.
36. Morizane S, Gallo RL. Antimicrobial peptides in the pathogenesis of psoriasis. J Dermatol 2012;39:225-30.
37. Gudjonsson JE, Ding J, Johnston A, Tejasvi T, Guzman AM, Nair RP, et al. Assessment of the psoriatic transcriptome in a large sample: additional regulated genes and comparisons with in vitro models. J Invest Dermatol 2010;130: 1829-40.
38. Lowes MA, Russell CB, Martin DA, Towne JE, Krueger JG. The IL-23/T17 pathogenic axis in psoriasis is amplified by keratinocyte responses. Trends Immunol 2013;34:174-81.
39. Tortola L, Rosenwald E, Abel B, Blumberg H, Schäfer M, Coyle AJ, et al. Psoriasiform dermatitis is driven by IL-36–mediated DC-keratinocyte crosstalk. J Clin Invest 2012;122:3965-76.
40. D'Erme AM, Wilsmann-Theis D, Wagenpfeil J, Hölzel M, Ferring-Schmitt S, Sternberg S, et al. IL-36γ (IL-1F9) is a biomarker for psoriasis skin lesions. J Invest Dermatol 2015;135:1025-32.
41. Li N, Yamasaki K, Saito R, Fukushi-Takahashi S, Shimada-Omori R, Asano M, et al. Alarmin function of cathelicidin antimicrobial peptide LL37 through IL-36γ induction in human epidermal keratinocytes. J Immunol 2014;193:5140-8.
42. Chiricozzi A, Guttman-Yassky E, Suárez-Fariñas M, Nograles KE, Tian S, Cardinale I, et al. Integrative responses to IL-17 and TNF-α in human keratinocytes account for key inflammatory pathogenic circuits in psoriasis. J Invest Dermatol 2011;131:677-87.
43. Roberson ED, Bowcock AM. Psoriasis genetics: breaking the barrier. Trends Genet 2010;26:415-23.
44. Gutowska-Owsiak D, Schaupp AL, Salimi M, Selvakumar TA, McPherson T, Taylor S, et al. IL-17 downregulates filaggrin and affects keratinocyte expression of genes associated with cellular adhesion. Exp Dermatol 2012;21: 104-10.
45. Barker JN, Palmer CN, Zhao Y, Liao H, Hull PR, Lee SP, et al. Null mutations in the filaggrin gene (FLG) determine major susceptibility to early-onset atopic dermatitis that persists into adulthood. J Invest Dermatol 2006;127:564-7.
46. Henderson J, Northstone K, Lee SP, Liao H, Zhao Y, Pembrey M, et al. The burden of disease associated with filaggrin mutations: a population-based, longitudinal birth cohort study. J Allergy Clin Immunol 2008;121:872-7.
47. Howell MD, Kim BE, Gao P, Grant AV, Boguniewicz M, DeBenedetto A, et al. Cytokine modulation of atopic dermatitis filaggrin skin expression. J Allergy Clin Immunol 2009;124(suppl 2):R7-12.
48. Fahlén A, Engstrand L, Baker BS, Powles A, Fry L. Comparison of bacterial microbiota in skin biopsies from normal and psoriatic skin. Arch Dermatol Res 2012;304:15-22.
49. Mallbris L, Larsson P, Bergqvist S, Vingård E, Granath F, Ståhle M. Psoriasis phenotype at disease onset: clinical characterization of 400 adult cases. J Invest Dermatol 2005;124:499-504.
50. Weisenseel P, Prinz JC. Incidental detection of S. pyogenes-DNA in psoriatic skin by PCR. Arch Dermatol Res 2005;296:573-6.
51. Henseler T, Christophers E. Psoriasis of early and late onset: characterization of two types of psoriasis vulgaris. J Am Acad Dermatol 1985;13:450-6.

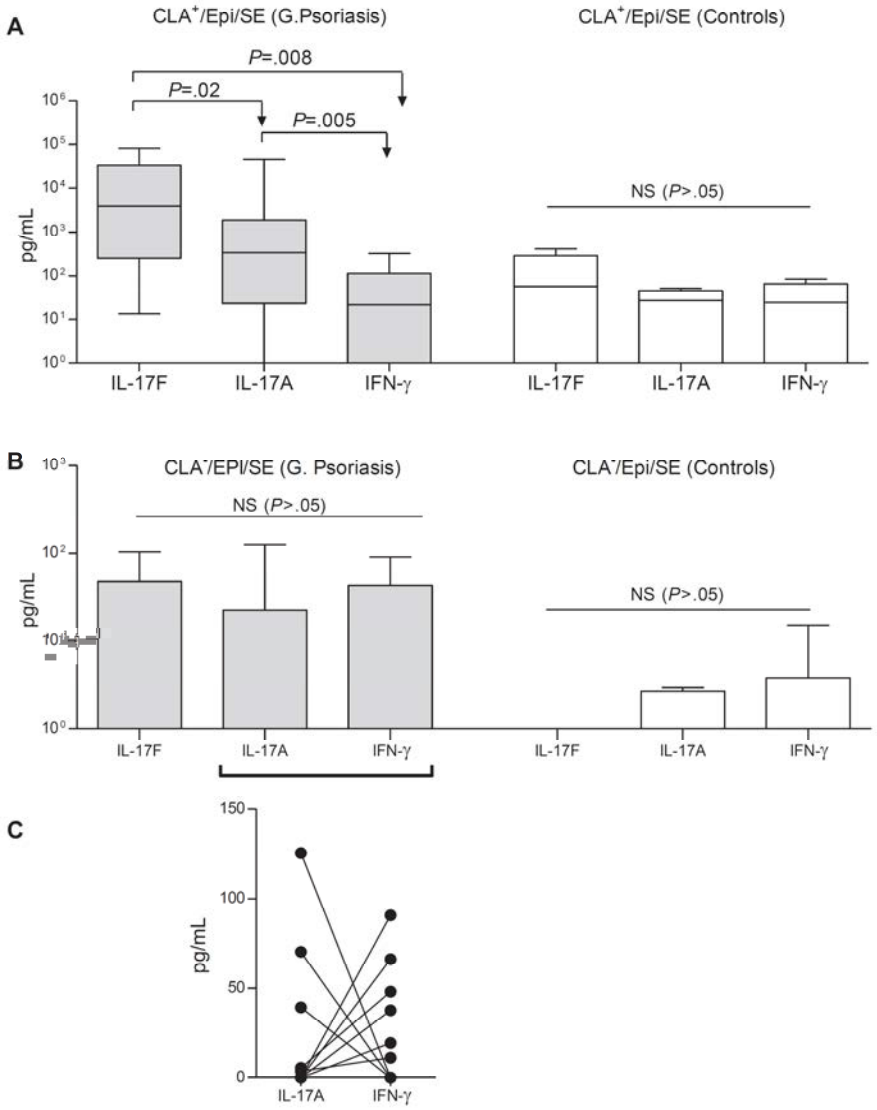


FIG E1. T_H17-predominant response in CLA^+ T cells from patients with GP cultured with autologous epidermal cells induced by SE. **A** and **B,** Wilcoxon paired test analysis (marked as *arrows*) comparing IL-17A, IL-17F, and IFN-γ production in culture supernatants. **C,** Detailed paired data of IL-17A and IFN-γ cytokine concentrations from SE-activated CLA^-/Epi cocultures (n = 13) from GP samples expressed as *dots* for each subject. *NS,* Not significant.

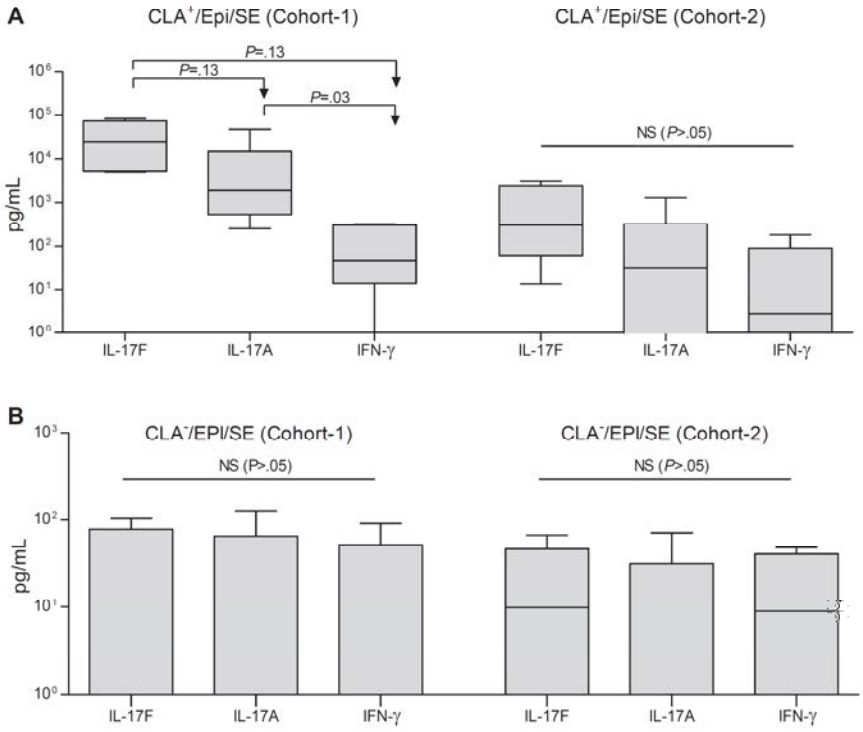


FIG E2. Pairwise comparison indicates higher production of IL-17A than IFN-γ in cohort 1 CLA^+/Epi/SE cocultures. Wilcoxon paired test analysis (marked as *arrows*) compared IL-17A, IL-17F, and IFN-γ production in supernatants from CLA^+/Epi/SE **(A)** and CLA^-/Epi/SE **(B)** cocultures. *NS,* Not significant.

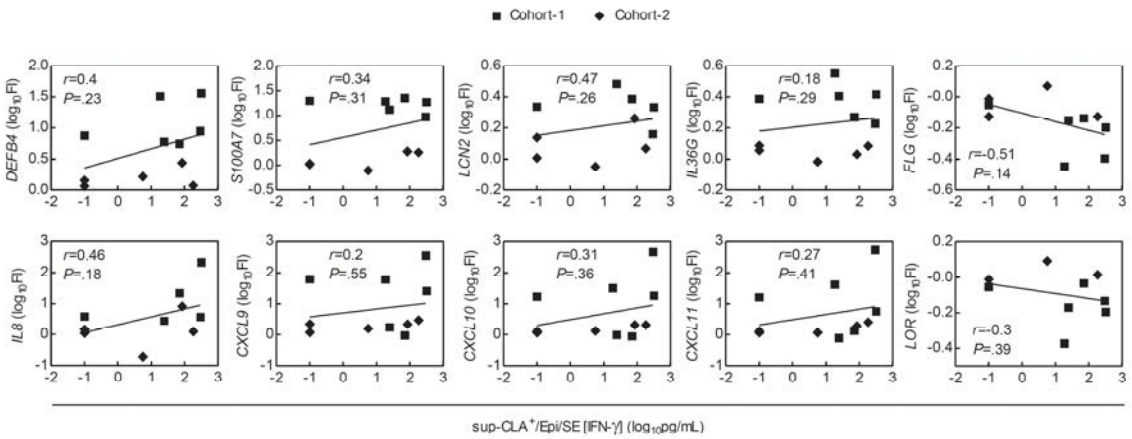


FIG E3. IFN-γ from SE-activated CLA^+/Epi cell cocultures does not present a significant correlation with upregulation of gene expression in keratinocytes. Correlation between the contents of IFN-γ in the GP-derived supernatants from CLA^+/Epi cocultures (cohort 1, n = 6; cohort 2, n = 5) and their respective mRNA levels of induction/reduction in keratinocytes.

TABLE E1. Clinical data of patients with GP

Patient	Age (y)	PASI score	Disease duration	HLA- C*0602	Onset associated with pharyngeal *S pyogenes* infection	ASO (IU/mL)	Cohort
26/10/2011	25	14.8	1 mo	Yes	Uncertain	278	2
02/11/2011	41	4	3 wk	Yes	Yes	337	1
07/02/2012	23	7.6	2 mo	Yes	Yes	443	1
21/02/2012	46	25.2	3 mo	No	Uncertain	58	2
10/04/2012	30	8	25 d	Yes	Yes	243	1
18/04/2012	33	13.4	20 d	Yes	Uncertain	414	2
02/05/2012	33	6	7 d	Yes	Uncertain	337	2
09/05/2012	41	4	5 wk	Yes	Yes	480	1
12/06/2012	65	4.6	10 d	Yes	Yes	176	1
26/02/2013	38	2.9	6 wk	Yes	Yes	325	1
09/10/2013	23	3.2	3 y	Yes	No	404	2
10/10/2013	20	1	Years	Yes	No	99	2
17/02/2014	42	7.2	2 mo	No	No	18	2
08/04/2014	34	4.7	5 wk	No	Uncertain	95	2

PASI, Psoriasis Area Severity Index.

TABLE E2. Sequences of human primers used in the study

	5′→3′ Forward	5′→3′ Reverse
ACTB	AGCCTCGCCTTTGCCGATCC	ACCATCACGCCCTGGTGCCT
DEFB4	CCAGCCATCAGCCATGAGGGT	GGAGCCCTTTCTGAATCCGCA
S100A7	AAGAAAGATGAGCAACAC	CCAGCAAGGACAGAAACT
LCN2	CCACCTCAGACCTGATCCCA	CCCCTGGAATTGGTTGTCCTG
IL36G	GGGCCGTCTATCAATCAATG	TGATAACAGCAACAGTGATG
IL8	ATGACTTCCAAGCTGGCCGTGG	TTATGAATTCTCAGCCCTCTTCAAAA
CXCL9	TGCAAGGAACCCCAGTAGTGA	GGTGGATAGTCCCTTGGTTGG
CXCL10	GAACTGTACGCTGTACCTGCA	TTGATGGCCTTCGATTCTGGA
CXCL11	GCTATAGCCTTGGCTGTGATATTGTG	CTGCCACTTTCACTGCTTTTACC
FLG	AAGGTTCACATTTATTGCCAAA	GGATTTGCCGAAATTCCTTT
LOR	CCAGGGTACCACGGAGGCGAAGGA	TGAGGCACTGGGGTTGGGAGGTAG

TABLE E3. Correlations between mRNA expression induced in keratinocytes by supernatants from psoriatic cocultures and respective cytokine concentrations

Parameter	Cytokines from Sup-CLA$^+$/Epi/SE (n = 10-11)	*r*	*P* value
DEFB4	IL-17A	**0.71**	**.02**
	IFN-γ	0.40	.23
S100A7	IL-17A	**0.83**	**.002**
	IFN-γ	0.34	.31
LCN2	IL-17A	**0.70**	**.02**
	IFN-γ	0.47	.26
IL36G	IL-17A	**0.70**	**.02**
	IFN-γ	0.18	.29
IL8	IL-17A	**0.75**	**.009**
	IFN-γ	0.46	.18
CXCL9	IL-17A	0.52	.09
	IFN-γ	0.20	.55
CXCL10	IL-17A	0.49	.12
	IFN-γ	0.31	.36
CXCL11	IL-17A	0.45	.16
	IFN-γ	0.27	.41
FLG	IL-17A	**−0.64**	**.04**
	IFN-γ	−0.51	.14
LOR	IL-17A	−0.48	.16
	IFN-γ	−0.3	.39

Pearson *r* coefficient analysis for log-transformed values for cytokines (in picograms per milliliter) and mRNA expression (fold increase). Values in boldface indicate statistical significance. *Sup-CLA+/Epi/SE*, Supernatants from CLA$^+$/Epi cocultures.

IL-9 is produced by *S. pyogenes*-activated psoriatic CLA+ T cells in the presence of epidermal cells and supports IL-17A secretion

Ester Ruiz-Romeu[1], Marta Ferran[2], Pablo García[2], Marc Sagristà[3], Anca Chiriac[4], Antonio Celada[5], Ramon M Pujol[2], Luis F Santamaria-Babí[1]*

[1] Translational Immunology, Department of Cellular Biology, Physiology and Immunology, Faculty of Biology, Universitat de Barcelona, Spain

[2] Department of Dermatology, Hospital del Mar, IMIM, Universitat Autònoma de Barcelona, Spain

[3] Hospital Sant Jaume de Calella, Barcelona, Spain

[4] Department of DermatoPhysiology, Apollonia University, Iasi, Nicolina Medical Center, Dermatology Department, Iasi, Romania

[5] Macrophage Biology, Department of Cellular Biology, Physiology and Immunology, Faculty of Biology, Universitat de Barcelona, Spain

*Corresponding author: luis.santamaria@ub.edu

ABSTRACT

Background: The putative role of IL-9 in skin diseases has recently been described, since many Th9 cells constitute a subset of cutaneous lymphocyte-associated antigen (CLA)-positive memory T cells in humans. However, IL-9 responses against clinically relevant pathogenic triggers in psoriasis, such as *Streptococcus pyogenes*, which causes throat infections that precede the onset or exacerbations of guttate and plaque psoriasis, respectively, have not been characterized.

Objective: This study aims to investigate the production of IL-9 in a psoriatic *ex vivo* approach in response to a disease-associated innate stimulus, the *S. pyogenes*, and to evaluate its function over other coexisting effector immune responses.

Methods: The *ex vivo* assays are based on a coculture of peripheral memory T cells with autologous epidermal cells from samples from either psoriatic (n=31) or healthy control subjects (n=10). After activation with an extract of *S. pyogenes* (SE), IL-9 production was evaluated in the coculture-derived supernatants, together with IL-17A and IFN-γ, which are known to be key mediators in psoriasis development. In order to establish cellular contribution to the model and the potential paracrine activity of IL-9 on other cytokines production, blocking assays were performed.

Results: *S. pyogenes* triggered the simultaneous production of IL-9, together with IL-17A and IFN-γ, that depended on CLA^+ T cell and epidermal cell interaction, and through HLA class I and II presentation. Neutralization of IL-9 in cultures of CLA^+ T cells and epidermal cells activated by SE, reduced IL-17A production but had no effect on IFN-γ production. Cocultures from guttate or plaque psoriasis patients indistinctly produced similar amounts of IL-9 levels.

Conclusion: Our results support a potential role of IL-9 in promoting psoriasis inflammation through memory CLA^+ T cells, when activated by a clinically relevant innate trigger in psoriasis, and especially by up-regulating IL-17A production.

Key messages:

- T-cell production of IL-9, through the interaction with autologous epidermal cells and *S. pyogenes* activation, is preferentially exerted by psoriatic circulating CLA^+ T cells.

- IL-9 might be functionally significant since it supports IL-17A production, suggesting a potential contribution to psoriasis immunopathology.

Capsule Summary:

IL-9 is preferentially produced by psoriatic skin-homing CLA^+ T cells upon activation with *S. pyogenes*, through the interaction with autologous lesional epidermal cells in a MHC-dependent manner, and simultaneously enhances IL-17A production, thus suggesting a pathogenic role in psoriasis.

Key Words: IL-9, psoriasis, CLA, *Streptococcus pyogenes*, IL-17A

Abbreviations:

CA: *Candida albicans*

CLA: Cutaneous lymphocyte-associated antigen

CLA^+/Epi or CLA^-/Epi: Cocultures of either CLA^+ or CLA^- T cells and autologous epidermal cells.

SE: *Streptococcus pyogenes* extract

SEB: Superantigen staphylococcal enterotoxin B

INTRODUCTION

Th9 cells participate in different inflammatory processes such as allergy, infection, tumor immunity, and autoimmune diseases (1-3). IL-9R is expressed on immune cells and on non-hematopoietic cells, as lung and gut epithelia, and keratinocytes (4-6). Thus, on the one hand, studies of experimental autoimmune encephalomyelitis models revealed that IL-9 may contribute to autoimmune disease development, especially by facilitating Th17 cells expansion, which express high levels of IL-9R (7,8). On the other hand, recent studies in a murine model of ulcerative colitis suggest that IL-9 promotes gut inflammation through epithelial barrier impairment (9).

In the last years, mounting evidence of clinical significance of IL-9 and Th9 cells in the human has been suggested, especially regarding chronic autoimmune and inflammatory systemic diseases. For instance, in ulcerative colitis, IL-9 and its receptor are predominantly involved in its pathogenesis (10). Also, IL-9 has been found to be increased in sera and synovial fluids of rheumatoid arthritis patients (11), and *IL9R* polymorphism has been linked to this disease (12). In psoriatic arthritis, circulating numbers of Th9 cells correlates with disease activity, and interestingly they decrease after anti–TNF-α and ustekinumab treatments, suggesting a clinical involvement (13).

Recently, an association between Th9 and skin in human has been described, since healthy blood-derived Th9 cells are primarily an effector skin-homing CLA^+ T cell population that selectively respond to *Candida albicans* (CA) presented by autologous monocytes (3). Interestingly, circulating CLA^+ T cells constitute cellular biomarkers for T-cell–mediated skin diseases since their phenotype and number correlate with the cutaneous clinical conditions (14). In addition, IL-9 has been suggested to have a putative involvement in psoriasis. The *IL9* gene is present in the psoriatic susceptibility region (5q31.1) (15, 16), but its functional role in psoriasis patients is poorly characterized. IL-9 was found in supernatants from $CD4^+$ T cells from psoriasis patients after polyclonal activation, and higher numbers of $IL\text{-}9R^+$ and $IL\text{-}9^+$ cells were found in psoriatic skin lesions than in healthy samples (3,17). We have recently established an *ex vivo* model of psoriasis that uses circulating memory CLA^+ T cells together with autologous lesional epidermal cells. Streptococcal throat infections can trigger or exacerbate guttate and plaque psoriasis, respectively, and several studies support the benefit of tonsillectomy (18). Then, activation of these cocultures with an extract of *S. pyogenes* (SE) resulted in the production of psoriatic inflammatory Th17/Th1 responses, both in guttate and

plaque psoriasis samples (19,20). Thus, the components of these cultures make this model an optimal scenario for the characterization of IL-9 production in an *ex vivo* disease environment that allows for expansion of current understanding of the IL-9 role in psoriasis (3,17), as well as of its relationship with IL-17A. Here, we report that IL-9 was mostly produced by CLA^+ T cells, depended on epidermal cells interaction and HLA class I and class II presentation in *S. pyogenes*-activated psoriatic cocultures. Such response followed a similar pattern to that of IL-17A, the production of which was in turn enhanced by IL-9. This is the first study showing how an innate stimulus that trigger psoriasis in the clinic, generates an IL-9 response selectively through CLA^+ T cells and epidermal cells interaction, and contributing to IL-17A production, but not IFN-γ.

MATERIALS AND METHODS

Patients

This study was performed with human samples and in accordance with the Declaration of Helsinki. A total of 31 psoriasis patients and 10 healthy individuals were recruited from three different hospitals (see supplementary Table S1). All participants contributed voluntarily and provided written informed consent. Psoriatic sample collection included patients with guttate (n=19) or plaque (n=12) lesions, without any age or gender restriction. Patients that received any systemic treatment for the last 6 weeks were excluded in order not to obtain underestimated cellular activation. Patients and healthy subjects underwent two skin biopsies, which were punched in lesions in psoriatic cases, and a blood extraction. All experimental procedures were run with fresh isolated samples in an external non-hospital laboratory.

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 separations as described previously (19). Skin biopsies were incubated overnight in Dispase (Corning, Bedford, Mass) at 4°C, then epidermal sheet was peeled off from the dermis. The epidermis was cut in smaller pieces that were incubated in trypsin solution (Biological Industries, Kibbutz Beit Haemek, Israel) for 30 minutes at 37°C. Equal volume of RPMI media (SIGMA-Aldrich, St Louis, Mo) containing 10% of FBS (Gibco, Grand Island, NY) was added to inhibit

trypsin action. Epidermal tissue was then mechanically disaggregated by gently up and down pipetting. Epidermal cell suspension was transferred to fresh media [RPMI, 10% FBS, 1% penicillin-streptomycin (SIGMA-Aldrich)], and the remaining tissue scaffold was discarded.

Cultures and pathogen activation

Ex vivo cocultures consisted of the culture of $5x10^4$ CLA^+ or CLA^- T cells with $3x10^4$ autologous epidermal cells (CLA^+/Epi or CLA^-/Epi, respectively), in 96-well flat-bottom plates (Nunc, Rosklide, Denmark), in the culture media described above. Cocultures were left untreated or activated for 5 days with pathogens preparations: an extract of *Streptococcus 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 staphylococcal enterotoxin B (SEB) (SIGMA-Aldrich) at 100 ng/mL. In cultures containing T cells alone, the above-mentioned amounts of each cell type were used, and activation with SE was equally performed. For the time course, supernatants from SE-activated cocultures 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 cocultures from psoriatic samples prior to activation, at a final concentration of 10 µg/mL.

Cytokine quantification

Supernatants were kept frozen prior to cytokine quantification. Multiplex fluorescent bead-based immunoassay with Diaclone DIAplex kit (Gen-Probe, Besançon, France) was used for IL-17A and IFN-γ quantification. IL-9 concentration was measured by ELISA with pre-coated plates (Biolegend).

Statistical Analysis

Data are genetally represented as individual dots and the median. For multiple comparison, one-way analysis of variance (ANOVA) Kruskall-Wallis with Dunn's post-test was used. Differences between two groups were analyzed by Mann-Whitney test, and for paired comparisons Wilcoxon match pairs test was used. Pearson's correlation coefficient was used to assess cytokine similarity. Differences were considered

significant at a P value of less than 0.05, and represented by symbols as follows: (*) or (#): P<0.05; (**) or (##): P<0.01; and (***) or (###): P<0.001.

RESULTS

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

Supernatants generated from 31 cocultures derived from psoriasis samples and 10 healthy controls were collected at day 5 for IL-9 quantification, along with IL-17A and IFN-γ (Fig 1). IL-9 was preferentially induced in psoriatic-derived cocultures containing CLA$^+$ T cells (101.3 ± 40.3/275.7 pg/mL) over non-skin homing CLA$^-$ T cells (11.88 ± 0/42.51 pg/mL) or CLA$^+$/Epi cocultures from healthy controls samples (27.03 ± 0/83.7 pg/mL). Similar response profile was observed for IL-17A production (Fig 1, B), and IFN-γ (Fig 1, C), this latter also showing a higher production by activated CLA$^-$/Epi cocultures than that from healthy controls (P<0.05; Fig 1, C).

Other pathogens are likely to activate this coculture system, as it is CA and SEB (19). However, when CLA$^+$/Epi or CLA$^-$/Epi cocultures were directly challenged with CA, IL-9 production was not observed in healthy donors, and it was specific of psoriatic-derived CLA$^+$/Epi cocultures (Fig 1, D), as it was observed for SE. These selective responses from CLA$^+$/Epi cocultures to SE and CA in psoriatic samples were not due to poor response from those coming from healthy controls, as SEB activation exerted the same IL-9 induction capacity in both type of donors (Fig 1, E).

As CLA$^+$ T cells cultured with autologous epidermal cells produced the higher amounts of either IL-9, IL-17A and IFN-γ, we sought to analyze the correlation between them to check proportionality in the inflammatory response against SE stimulation. No significant correlation was found between IL-9 and IL-17A production, and dot plot distribution showed differing profiles in some supernatants, being IL-9 or IL-17A–predominant (Fig 1, F). Conversely, IL-9 and IFN-γ amounts significantly correlated, somehow indicating a similar proportion in both cytokines production in each coculture (Fig 1, G). Then, as expected, IL-17A and IFN-γ production did not correlate neither (Fig 1, H), and differing responses were also observed, as it was observed for IL-9 vs. IL-17A.

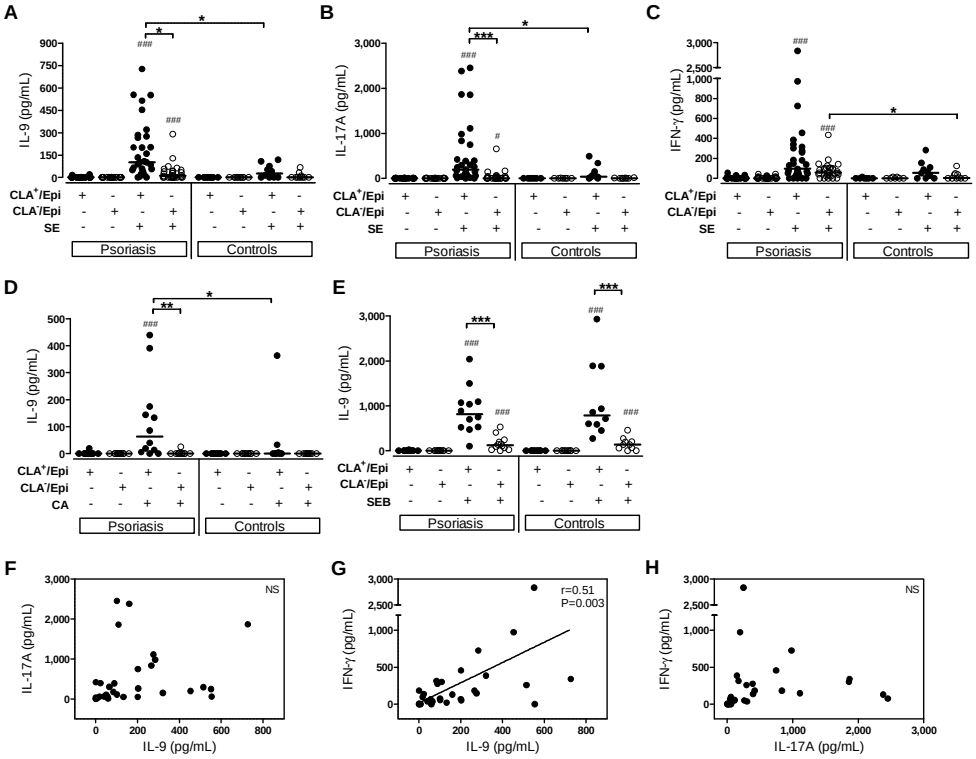


Figure 1. Psoriatic circulating memory CLA⁺ T cells produce IL-9, together with IL-17A and IFN-γ, upon *S. pyogenes* activation. Peripheral circulating psoriatic (n=31) or helathy (n=10) CD45RA⁻ CLA⁺ or CLA⁻ T cells were cocultured with autologous epidermal cells, and IL-9 (A), IL-17A (B) and IFN-γ (C) were measured in supernatants after 5 days of stimulation with SE. IL-9 levels were measured in 5 days supernatants from psoriatic (n=12) or healthy controls (n=10) cocultures activated by CA (D) or SEB (E). Cell types and pathogens presence (+) or absence (-) for each coculture condition are defined below the x-axis. Data are represented by dot plots, each one representing the values of one patient-derived coculture supernatant, and the median. P values symbols (#) indicate differences with basal CLA⁺/Epi or CLA⁻/Epi untreated conditions. Linear correlations between CLA⁺/Epi/SE-produced cytokines; IL-9 vs IL-17A (F); IL-9 vs IFN-γ (G); and IFN-γ vs IL-17A (H). NS: non-significant.

SE-induced IL-9 production by circulating CLA⁺ T cells requires of autologous epidermal cells and depends on HLA-mediated presentation

In order to understand the contribution of epidermal cells in IL-9 production in the coculture, IL-9 content, along with that of IL-17A and IFN-γ, was evaluated in cultures with either T cells alone or with lesional epidermal cells (Fig 2, A–C). SE activity was minimal in T cells cultures, while the presence of autologous lesional epidermal cells were actually responsible for the production of IL-9, IL-17A and IFN-γ by T cells, highly

enhancing IL-9 and IL-17A production by CLA^+ T cells over CLA^- T cells ($P<0.05$, Fig 2, A and B). Such effector responses were dependent on HLA class I and II molecules, which led to about 50% and 90–100% of inhibition of cytokine production when they were respectively neutralized in the coculture (Fig 2, D–F, and see Fig S1). Thus, potential interactions with SE-derived antigen/s, either through HLA class I or II molecules, may be involved in IL-9 production (Fig 2, D), as well as in that of IL-17A and IFN-γ (Fig 2, E and F, respectively), which was consistent with previously reported results (19).

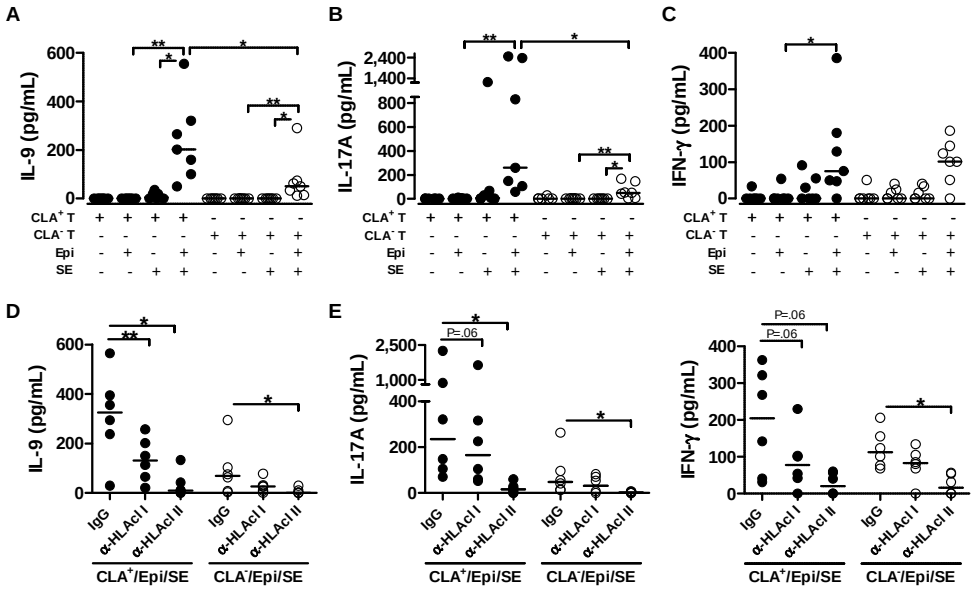


Figure 2. *S. pyogenes*-associated IL-9, IL-17A and IFN-γ production by psoriatic cocultures is enhanced by the presence of epidermal cells and depends on HLA class I and II molecules. CLA^+ or CLA^-T cells alone or cocultured with autologous epidermal cells and were activated with SE or left untreated. IL-9 (A), IL-17A (B) and IFN-γ (C) were measured in each cell culture supernatants after 5 days (n=7). Cell types and SE presence (+) or absence (-) for each culture condition are defined below the x-axis. HLA class I and class II molecules were neutralized at day 0 in the SE-activated cocultures, or cocultures were treated with control IgG isotype. IL-9 (D), IL-17A (E) and IFN-γ (F) measured at day 5 are shown (n=6), and pairwise compared respect to isotype values. Data are represented by dot plots, each one representing the values of one patient-derived coculture supernatant, and the median.

Kinetics of the SE-dependent CLA^+ production of IL-9 and contribution to IL-17A production in psoriasis

The kinetics of IL-9 production upon SE-stimulation of psoriatic-derived CLA^+/Epi cocultures were evaluated 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 (Fig 3, A). Despite

IL-9 displayed no peak preceding IL-17A and IFN-γ production, blocking assays showed that IL-17A production depended by 50% on IL-9, while no such clear effect was observed in IFN-γ production (Fig 3, B, and Fig S2).

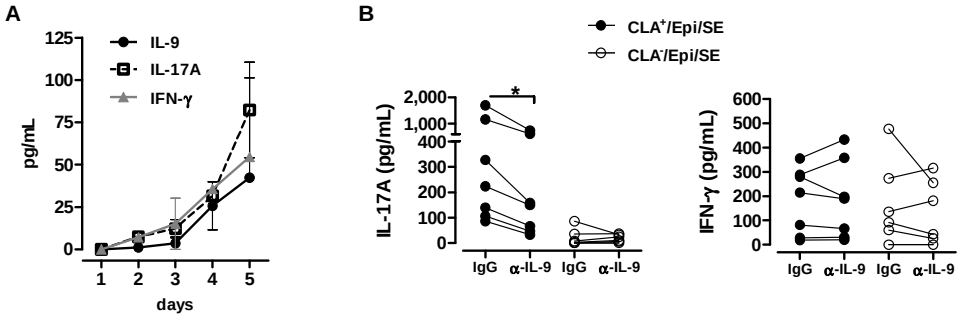


Figure 3. IL-9 production induced by *S. pyogenes* follows similar IL-17A and IFN-γ production kinetics and enhance IL-17A production. (A) IL-9, IL-17A and IFN-γ were measured in supernatants from psoriatic CLA^+/Epi cocultures that were activated for 1 (n=2-3), 2 (n=1-2), 3 (n=2-3), 4 (n=1-2) and 5 (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. Then, IL-17A and IFN-γ levels were measured in 5 days supernatants from psoriatic CLA^+/Epi and CLA^-/Epi cocultures (n=7) and paired results were compared respect to isotype values. Each dot represents the values of one patient-derived coculture supernatant.

IL-9 production by SE in CLA^+/Epi cocultures is similarly induced in both guttate and plaque psoriasis-derived samples

In this study, two types of psoriasis patients were included, guttate (n=19) and plaque psoriasis (n=12), and both responded to *S. pyogenes* activation in the *ex vivo* model. Cytokine profile was evaluated in these two different forms of psoriasis (Fig 4). In guttate psoriasis-derived cocultures activated by SE, significant paired difference was found for IL-17A (293.6 ± 48.5/979.4 pg/mL) compared with IL-9 (101.3 ± 22.6/275.7 pg/mL) or IFN-γ (75.5 ± 35.2/300.9 pg/mL) values within the same supernatants, confirming a clear predominant Th17 profile. Conversely, no clear preferential response towards IL-9, IL-17A or IFN-γ production was found in plaque psoriasis, as respective measured amounts were similar among them (110 ± 42.6/406.3; 139 ± 56.35/352.1; and 111.4 ± 24.9/304.2 pg/mL, respectively).

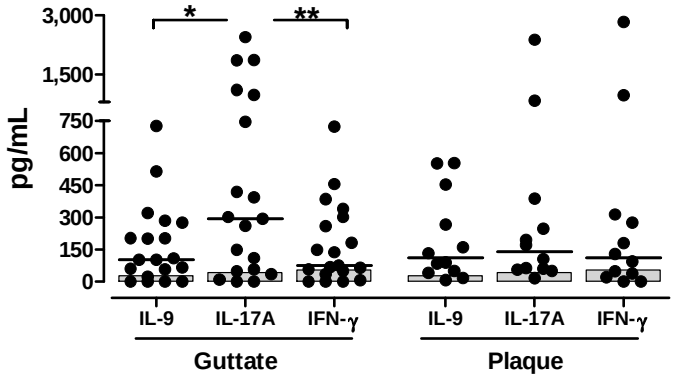


Figure 4. *S. pyogenes*-induced IL-9 by CLA⁺ T cells is produced by guttate and plaque psoriasis-derived cocultures. Paired IL-9, IL-17A and IFN-γ production in SE-activated CLA⁺/Epi cocultures were compared within samples obtained from guttate psoriasis patients (n=19) or plaque psoriasis patients (n=12). Grey chart bars indicate median cytokine levels produced by healthy controls cocultures (n=10). Data are represented by dot plots, each one representing the values of one patient-derived coculture supernatant, and the median.

DISCUSSION

The role of IL-9 in psoriasis in humans is poorly characterized. IL-9–producing T cells, namely Th9, and IL-9R⁺ cells have been found to be increased in psoriasis lesions vs. healthy skin (3,17), thus suggesting a potential involvement in this disease. Additionally, polyclonal activation of CD4⁺ T cells isolated from psoriatic patients' blood produced higher levels of IL-9 than controls (17), however it is not known whether clinically associated triggers of psoriasis can induce its production.

Our results extend current understanding of the role of IL-9 in psoriasis by studying its role in an antigen-specific *ex vivo* model of psoriasis, where circulating CLA⁺ T cells and lesional epidermal cells are activated with *S. pyogenes*, a pathogen clinically validated as a trigger in this disease (19,20). In this model, IL-9 was preferentially produced by memory CLA⁺ T cells, together with IL-17A and IFN-γ upon activation with an extract of *S. pyogenes* in the presence of autologous lesional epidermal cells. Such *S. pyogenes*-induced IL-9 production by CLA⁺ T cells was in a specific manner, since activation with SEB equally induced IL-9 production by psoriasis and healthy controls. The activation capacity of CA was also tested. In contrast to recent studies performed with CLA⁺ T cells

activated by autologous pulsed monocytes from healthy donors (3), we found that IL-9 production was mainly associated with the cocultures containing CLA^+ T cells and epidermal cells from psoriasis patients, but not from healthy controls. This difference may be due to the use of autologous monocytes instead of autologous epidermal cells, and the use of direct pathogen activation.

Usually, those cocultures which are strong responders to SE produce high amounts of either IL-9, IL-17A and IFN-γ. However, lack of correlation between IL-9 and IL-17A was found, as well as between IL-17A and IFN-γ, while IL-9 correlated with IFN-γ. The exact reason of the existence of differing or matching proportionality between such production of cytokines, whether it is depending on T-cell phenotypes isolated from peripheral circulation and/or the specificity for SE, has yet to be elucidated.

Optimal production of IL-9, as well as IL-17A and IFN-γ, depended on HLA class I and HLA class II presentation, and on the presence of epidermal cells as a possible source of antigen presenting cells. The IL-9, IL-17A and IFN-γ production was completely reduced by the addition of a blocking antibodies against HLA class II molecules and supports the role of $CD4^+$ T cells as source of IL-9, as it has been proposed (3). Interestingly, about 50% of IL-9, IL-17A and IFN-γ depended on HLA class I presentation, which indicates a noteworthy role by $CD8^+$ T cells, if it is taken into account the fact that this subset constitutes a minor fraction of the lymphocyte population in the coculture, even in activated conditions (data not shown). These results are in the line of our previous results (19), and deserve further studies.

Previous reports about re-activation of *in vitro* polarized Th9 cells showed a peak of IL-9 production at day 3 (21), and more recently, a transient increase of $IL\text{-}9^+$ cells with a peak at day 2 was observed in polyclonal-activated CLA^+ T cells from healthy individuals (3). This phenomenon preceded the up-regulation of other effector cells, including $IL\text{-}17^+$ and $IFN\text{-}\gamma^+$ cells (3). For these reasons, we analyzed cytokine production in the supernatants of SE-activated CLA^+/Epi cocultures at different days, but the same accumulating tendency over time was found for IL-9, IL-17A and IFN-γ production, thereby supporting that CLA^+ T cells producing IL-9 were responding to SE in the same fashion as those producing IL-17A and IFN-γ did in the *ex vivo* cocultures.

IL-9 has been shown to be associated with increased IL-17 production by different ways. In the K5.hTGF-β1 transgenic mouse, IL-9 injection increased mRNA expression of IL-

17A, but not that of IFN-γ (17). Using purified human $CD4^+$ T cells from psoriasis patients, IL-9 enhanced IL-17A production, and in healthy CLA^+ T cells, IL-9 was required for a maximal activation-induced increase of $IL\text{-}17^+$ $CD4^+$ T cells (3,17). However, it is not known whether IL-9 plays a role in the innate-induced production of IL-17A by antigen-specific responding T cells in psoriasis. Interestingly, when IL-9 was neutralized, a 50% reduction in SE-induced IL-17A production was found, whereas IFN-γ amounts were not altered.

Frequently, psoriasis is described to be predominated by Th17 and Th1 immune responses (23), although IFN-γ it is not clinically validated in psoriasis since neutralizing monoclonal antibody against IFN-γ in the clinic does not improve lesions (24). Considering our results and other published data, we suggest that Th9 cells could additionally contribute to psoriatic inflammation. Furthermore, in two of the existing forms of disease, guttate and plaque psoriasis, SE-activation of circulating memory CLA^+ T cells cultured with autologous lesional epidermal cells resulted in a similar response regarding to IL-9 production. When samples were not separately analyzed according to type of disease, IL-17A levels were higher than those of IL-9 and IFN-γ. However, independent characterization led us to understand that the IL-17A predominance was due to the guttate psoriasis response, probably due to its acute profile, thus confirming our previous reported data defining a predominant Th17 response in guttate psoriasis (20). In plaque psoriasis, though, no predominant response was found, as levels of IL-17A were slightly diminished, but without significance, compared to guttate levels, and presented a mixed Th9/Th17/Th1 response to SE. Still, IL-9 and IFN-γ levels were alike within and among each of these two psoriasis subtypes.

Limitations of the current study include the indeterminacy of the exact cellular and molecular mechanisms underlying SE-activation of the cocultures, specially concerning to which specific epitopes could be recognized by T cells. Also, whether a real uniqueness of IL-9 T cell source exists in the cocultures has not been characterized. Last, the real significance of IL-9 in the clinics has to be further studied, and though we report that either guttate and plaque psoriasis patients are carrying skin-tropic memory CLA^+ T cells with IL-9 effector properties, the impact on real lesion development is not clarified.

In summary, an IL-9 effector response is preferentially exerted in psoriatic circulating CLA^+ T cells cultured with autologous epidermal lesional cells through *S. pyogenes* activation, along with IL-17A and IFN-γ, and following the same production pattern as well. Furthermore, IL-9 might be functionally significant by simultaneously promoting twice the IL-17A levels, suggesting a potential contribution to psoriasis immunopathology.

ACKNOWLEDGMENTS: We thank D. Cabrerizo for his technical support.

FUNDING: The study was funded by FIS/ISCIII 2013 (Ministerio de Economía y Competitividad e Instituto de Salud Carlos III; PI09/2222, PI13/01845 and PI13/01716) and PhD fellowship to E. R. R is granted by the Ministerio de Educación, Cultura y Deporte of the Spanish Government (FPU13/02308).

CONFLICTS OF INTERESTS

None declared.

REFERENCES

1. Noelle RJ, Nowak EC. Cellular sources and immune functions of interleukin-9. *Nat Rev Immunol* 2010; **10**:683–7.

2. Wilhelm C, Turner JE, Van Snick J, *et al*. Stockinger B. The many lives of IL-9: a question of survival? *Nat Immuno*. 2012; **13**:637–41.

3. Shlapbach C, Gehad A, Yang C, *et al*. Human Th9 cells are skin-tropic and have autocrine and paracrine proinflammatory capacity. *Sci Transl Med* 2014; **6**:219ra8.

4. Little FF, Cruikshank WW, Center DM, IL-9 stimulates release of chemotactic factors from human bronchial epithelial cells. *Am J Respir Cell Mol Biol* 2001; **25**:347–52.

5. Goswami R, Kaplan MH, A brief history of IL-9. *J Immunol* 2011; **186**:3283–8.

6. Hong CH, Chang KL, Wang HJ, *et al*. IL-9 induces IL-8 production via STIM1 activation and ERK phosphorylation in epidermal keratinocytes: A plausible mechanism of IL-9R in atopic dermatitis. *J Dermatol Sci* 2015; **78**:206–14.

7. Elyaman W, Bradshaw EM, Uyttenhove C, *et al*. IL-9 induces differentiation of Th17 cells and enhances function of FOXP3+ natural regulatory T cells. *Proc Natl Acad Sci USA* 2009; **106**:12885–90.

8. Nowak EC, Weaver CT, Turner H, *et al*. IL-9 as a mediator of Th17-driven inflammatory disease. *J Exp Med* 2009; **206**:1653–60.

9. Gerlach K, Hwang Y, Nikolaev A, *et al*. Th9 cells that express the transcription factor PU.1 drive T cell–mediated colitis via IL-9 receptor signaling in intestinal epithelial cells. *Nat Immunol* 2014; **15**:676–86.

10. Nalleweg N, Chiriac MT, Podstawa E, *et al*. IL-9 and its receptor are predominantly involved in the pathogenesis of UC. *Gut* 2015; **64**:743–55.

11. Ciccia F, Guggino G, Rizzo A, *et al*. Potential involvement of IL-9 and Th9 cells in the pathogenesis of rheumatoid arthritis. *Rheumatology (Oxford)* 2015; **54**:2264–72.

12. Burkhardt J, Petit-Teixeira E, Teixeira VH, *et al*. Association of the Xchromosomal genes TIMP1 and IL9R with rheumatoid arthritis. *J Rheumatol* 2009; **36**:2149–57.

13. Ciccia F., Guggino G, Ferrante A, *et al*. IL-9 over-expression and Th9 polarization characterize the inflamed gut, the synovial tissue and the peripheral blood of patients with psoriatic arthritis. *Arthritis Rheumatol* 2016; **68**:1922–31.

14. Ferran F, Romeu ER, Rincón C, *et al*. Circulating CLA+ T lymphocytes as peripheral cell biomarkers in T-cell-mediated skin diseases. *Exp Dermatol* 2013; **22**:439–42.

15. Modi WS, Pollock DD, Mock BA, *et al*. Regional localization of the human glutaminase (GLS) and interleukin-9 (IL9) genes by in situ hybridization. *Cytogenet Cell Genet* 1991; **57**:114–116.

16. Friberg C, Björck K, Nilsson S, *et al*. Analysis of chromosome 5q31–32 and psoriasis: confirmation of a susceptibility locus but no association with SNPs within SLC22A4 and SLC22A5. *J Invest Dermatol* 2006; **126**:998–1002.

17. Singh TP, Schön MP, Wallbrecht K, *et al*. Involvement of IL-9 in Th17-Associated Inflammation and Angiogenesis of Psoriasis. *PLOS One* 2013; **8**:e51752.

18. Thorleifsdottir RH, Eysteinsdottir JH, Olafsson JH, *et al*. Throat infections are associated with exacerbation in a substantial proportion of patients with chronic plaque psoriasis. *Acta Derm Venereol* 2016; **96**:788–91.

19. Ferran M, Galván AB, Rincón C, *et al*. Streptococcus Induces Circulating CLA+ memory T-cell-dependent epidermal cell activation in psoriasis. *J Invest Dermatol* 2013; **133**:999–1007.

20. Ruiz-Romeu E, Ferran M, Sagristà M, *et al*. Streptococcus pyogenes-induced cutaneous lymphocyte antigen-positive T cell-dependent epidermal cell activation triggers Th17 responses in patients with guttate psoriasis. *J Allergy Clin Immunol* 2016; **138**:491–9.

21. Tan C, Aziz KMK, Lovaas JD, *et al*. Antigen-specific Th9 cells exhibit uniqueness in their kinetics of cytokine production and short retention at the inflammatory site. *J Immunol* 2010; **185**:6795–801.

22. Talamonti M, Botti E, Galluzzo M, *et al*. Pharmacogenetics of psoriasis: HLA-Cw6 but not LCE3B/3C deletion nor TNFAIP3 polymorphism predisposes to clinical response to interleukin 12/23 blocker ustekinumab. *Br J Dermatol* 2013; **169**:458–63.

23. Harden JL, Johnson-Huang LM, Chamian MF, *et al*. Humanized anti–IFN-γ (HuZAF) in the treatment of psoriasis. *J Allergy Clin Immunol* 2015; **135**:553–6.

SUPPLEMENTARY MATERIAL

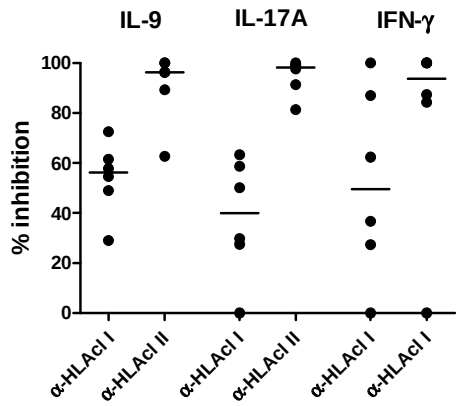


Figure S1. IL-9, IL-17A and IFN-γ production by SE is HLA class I and II dependent. Percentage of inhibition in cytokine production after 5 days of SE-activated CLA⁺/Epi cocultures with HLA class I or II blockade respective to isotype control (n=6). Data are represented by dot plots, each one representing the values of one patient-derived coculture, and the median.

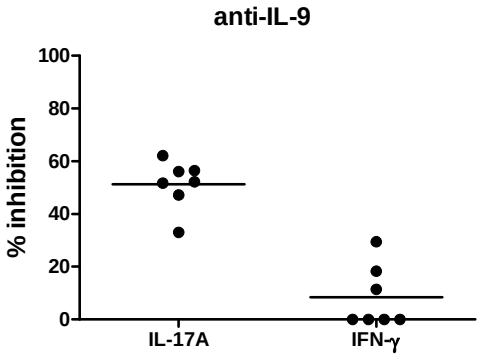


Figure S2. IL-9 selectively enhances IL-17A by 50%, when both are produced by CLA⁺/Epi coculture activated by SE. Percentage of inhibition produced by IL-9 blockade in IL-17A and IFN-γ production after 5 days of SE-activated CLA⁺/Epi cocultures respective to isotype control (n=7). Data are represented by dot plots, each one representing the values of one patient-derived coculture, and the median.

Participants	Age	Diagnostic
26/02/2013	38	Guttate
09/10/2013	23	Guttate
10/10/2013	20	Guttate
17/02/2014	34	Guttate
08/04/2014	34	Guttate
09/04/2014	30	Guttate
07/05/2014	23	Guttate
03/07/2014	26	Guttate
25/09/2014	32	Guttate
14/01/2015	30	Guttate
15/01/2015	34	Guttate
19/01/2015	25	Guttate
26/02/2015	--	Guttate
27/05/2015	19	Guttate
25/06/2015	21	Guttate
09/11/2015	38	Guttate
04/02/2016	30	Guttate
11/05/2016	20	Guttate
23/05/2016	33	Guttate
19/12/2013	21	Plaque
12/02/2014	28	Plaque
02/12/2014	43	Plaque
10/12/2014	59	Plaque
15/10/2015	32	Plaque
27/10/2015	42	Plaque
03/11/2015	65	Plaque
02/03/2016	43	Plaque
26/04/2016	58	Plaque
03/05/2016	39	Plaque
10/05/2016	59	Plaque
19/05/2016	45	Plaque
20/10/2011	--	Control
19/12/2012	--	Control
16/10/2013	47	Control
22/10/2013	--	Control
14/01/2014	51	Control
20/01/2014	--	Control
07/11/2014	50	Control
21/11/2014	20	Control
16/06/2015	47	Control
14/07/2015	--	Control

Table S1. List of all participants in the study.

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

Ester Ruiz-Romeu,[1] Marta Ferran,[2] Marc Sagristà,[3] Pablo García,[2] JM Casanova,[4] JM Fernandez,[4] Anca Chiriac,[5] Péter Hóllo,[6] Antonio Celada,[7] Ramon M Pujol[2] and Luis F Santamaria-Babí[1]*

[1] Translational Immunology, Department of Cellular Biology, Physiology and Immunology, Faculty of Biology, Universitat de Barcelona, Spain
[2] Department of Dermatology, Hospital del Mar, IMIM, Universitat Autònoma de Barcelona, Spain
[3] Hospital Sant Jaume de Calella, Barcelona, Spain
[4] Hospital del Arnau de Vilanova, Lleida, Spain
[5] Department of DermatoPhysiology, Apollonia University, Iasi, Nicolina Medical Center, Dermatology Department, Iasi. Romania
[6] Department of Dermatovenerology and Oncodermatology, Semmelweis Egyetem, Budapest, Hungary
[7] Macrophage Biology, Department of Cellular Biology, Physiology and Immunology, Faculty of Biology, Universitat de Barcelona, Spain

*Corresponding author: luis.santamaria@ub.edu

ABSTRACT

Background: On the one hand, the association between throat infections with *Streptococcus pyogenes* and guttate psoriasis flares is well established, a process in which raised titers of anti-streptolysin O (ASO) in serum are usually found. On the other hand, circulating skin-homing CLA^+ T cells constitute peripheral cellular biomarkers for human T-cell–mediated skin diseases, thus providing a tool for translational evaluation of effector responses upon contact with cognate pathogens that match the pathogenesis. In this regard, *S. pyogenes* elicits cytokine production by CLA^+ T cells in the presence of epidermal cells. However, further evaluation of the association of cytokine secretion with ASO levels, as well as with symptoms severity in those patients, would support the importance of the direct implication of strep-responsiveness of CLA^+ T cells with clinical severity of the disease.

Objective: To evaluate the patterns in ASO levels, psoriasis area severity index (PASI) score and cytokine production, through ex vivo cultures, in guttate psoriasis patients with different durations of disease at the moment of sampling.

Methods: The study included 16 patients with guttate psoriasis, 11 with plaque psoriasis, and 10 healthy subjects. Psoriasis patients were examined for ASO titers and PASI score. Through an *ex vivo* approach, stimulation with an extract of *Streptococcus pyogenes* (SE) of cocultures containing memory T cells from peripheral blood, either CLA^+ or CLA^-, and autologous epidermal cells isolated from skin punches, was evaluated at cytokine levels in the supernatants.

Results: We observed a peak at cytokine level in SE-stimulated CLA^+/Epi cocultures from guttate psoriasis patients with skin rashes of 1 to 2 months of duration, and that were preceded by a pharyngitis episode, which matched the highest ASO blood levels and an increased PASI score.

Conclusion: The common peak between raised ASO titers, PASI score, and the capacity of circulating CLA^+ T cells to be re-stimulated with *S. pyogenes*, suggests the existence of a period of time during which the more strep-sensitized those cells, the more severity of the disease is reflected.

Key messages:

- High effector capacity of circulating CLA^+ T cells during strep-derived guttate psoriasis may imply more severe disease, or vice versa, especially during 1-2 months after disease onset.
- Such effector capacity varies in accordance with the streptococcal antigen exposure, and thus prospective valuable studies in the guttate course of psoriasis should be focused on peaks of streptococcal-sensitization periods.

Capsule Summary:

CLA^+ T cell-dependent high cytokine responses to *ex vivo* activation with *S. pyogenes* in guttate psoriasis patients with a 1–2 months period of disease duration, parallels with peaks in ASO and PASI levels, which supports defining CLA^+ T cell as a functional link between streptococcal infection and lesion severity.

Key Words: guttate psoriasis, CLA, *Streptococcus pyogenes*, ASO, PASI

Abbreviations:

ASO: anti-streptolysin O

CLA: Cutaneous lymphocyte-associated antigen

CLA^+/Epi or CLA^-/Epi: Cocultures of either CLA^+ or CLA^- T cells and autologous epidermal cells

PASI: Psoriasis area severity index

SE: *Streptococcus pyogenes* extract

INTRODUCTION

Psoriasis is an inflammatory immune-mediated skin disease which usually occurs in individuals with genetic susceptibility in conjunction with environmental triggers, as it is common for multifactorial diseases. Affected skin is classically characterized by a focal formation of sharply demarcated, red, and slightly raised lesions with constantly shedding silvery-white scales. Several clinical phenotypes of psoriasis are recognized that share such histopathological changes in the skin but with highly variable morphology, distribution, severity and course. In this regard, guttate-type of psoriasis is generally an acute form presented as small drop-shaped lesions and, generally, self-limiting (1). Interestingly, almost 40% of guttate psoriasis patients will later develop chronic plaque psoriasis, which is the commonest form of the disease, since it accounts for 90% of the cases, and guttate flares are seen in already existing plaque-affected patients (2), thus contributing to its natural history.

The onset of guttate psoriasis is often preceded by tonsillar infection with *Streptococcus pyogenes*, a group A β-hemolytic streptococci, which constitutes a well-characterized environmental trigger. Interestingly, streptococcal superantigens can induce the surface expression of the cutaneous lymphocyte-associated antigen (CLA) on T cells (3). In addition, identical skin-homing CLA^+ T-cell clonotypes, but not CLA^-, have been found in streptococcal angina and in skin lesions in patients with streptococcal-driven psoriasis, thereby supporting a pathogenic link between those cells generated in the infected tonsils and subsequent skin lesion immune response, which implies their migration from tonsils to cutaneous sites (4). Such CLA^+ T cells infiltrating psoriatic lesions would contain potentially cross-reactive clones that could persist for a long time, potentially by recognizing self-epitopes via molecular mimicry (5,6). The fact that these effector memory CLA^+ T cells have been suggested to recirculate between skin and blood during cutaneous inflammation due to the observed effects upon skin T-cell trafficking blockade with anti–LFA-1 treatments (7–10), and that phenotypical changes in circulating CLA^+ T cells are related to severity and clinical evolution in T-cell–mediated skin diseases (11,12), indicates that their study may provide valuable translational information. For instance, we recently developed an *ex vivo* model of psoriasis in which isolated peripheral memory CLA^+ T cells and autologous lesional epidermal cells are cocultured in presence of an extract of *S. pyogenes* (SE). Such experiments have revealed a high production of psoriasis-associated cytokines, including the main

signature products of Tc17/Th17 and Tc1/Th1 cells, IL-17A and IFN-γ, respectively (13,14), as well as IL-9 (unpublished data), in both guttate and plaque psoriasis samples.

Effector memory CLA^+ T cells may represent the functional link between pharyngitis episodes and subsequent lesion development. However, at present it is not known to what extent the streptococcal-specific T-cell immune–mediated response in guttate psoriasis relates to the clinical status. Generally, ASO blood levels are raised in those patients, however variable values can be found, either because of intrinsic constitutional factors that affect the antibody response and/or the duration of disease at the moment of sampling. Here, we aim to evaluate a possible association of raising ASO titers, as a systemic evidence of streptococcal infection, and PASI score, with the effector responses exerted by isolated CLA^+ T cells against *S. pyogenes*, by the same time of sampling.

MATERIALS AND METHODS

Patients

The study included 16 guttate patients: 13 of which had reported a prior pharyngitis episode and within a duration of disease that ranged from 15 days to 4 months, and 3 patients with a chronic course with lesions that remained in guttate form for more than a year. 11 chronic plaque psoriasis patients with no history of streptococcal infections and 10 healthy controls were also included. All participants provided written consent. Psoriatic and healthy subjects underwent 2 skin punches, that were lesional in psoriatic cases, and a blood extraction. None of the psoriasis patients had received any treatment for at least 6 weeks prior to samples obtainment and PASI measurement. The study was conducted in accordance with the Declaration of Helsinki.

Cells isolation

Circulating $CD45RA^-$ CLA^+ T cells and CLA^- T cells were isolated from peripheral blood mononuclear cells using immunomagnetic separations as described by Ferran and colleagues (14). Skin punches were incubated overnight in Dispase solution (Corning, Bedford, Mass) at 4ºC, and epidermal sheets were peeled off and cut into smaller pieces before trypsin (Biological Industries, Kibbutz, Beit Haemek, Israel) treatment for 20 minutes at 37ºC. Mechanically dissociation by pipetting against the tissue was performed until cloudy epidermal cell suspensions were obtained. Trypsin activity was

then stopped by adding RPMI (SIGMA-Aldrich, St Louis, Mo) containing 10% of FBS (Gibco BRL-Life Technologies, Grand Island, NY). Remaining undigested tissue fragments were discarded by sedimentation under gravity, and cell suspensions were transferred to new tubes, then centrifuged and resuspended in new media (RPMI, 10% FBS) containing 1% of penicillin-streptomycin (SIGMA-Aldrich).

T cells and epidermal cells cocultures

Ex vivo cocultures were performed by seeding $5x10^4$ CLA^+ or CLA^- T cells and $3x10^4$ autologous epidermal cells in a 96-well flat-bottom microwell plate (Nunc, Roskilde, Denmark) in culture medium (RPMI, 10% FBS, 1% penicillin-streptomycin). An extracted of *Streptococcus pyogenes* (SE), that consisted of a sonicated pooled preparation from *S. pyogenes* isolated from throat swabs of infected tonsils of psoriasis patients, was added to the cocultures at a final concentration of 1 µg/ml.

Cytokine quantification

Supernatants were collected after 5 days of culture. Multiplex fluorescent bead-based immunoassay with the Diaclone DIAplex kit (Gen-Probe, Besançon, France). IL-9 was measured by ELISA with pre-coated plates (Biolegend, San Diego, CA).

Data analysis

Statistical analysis was conducted using Graphpad Prism v5 (Graphpad Software Inc., La Jolla, CA). Demographic and clinical characteristics were compared with unpaired t-test. Cytokine production in small groups, according with duration of guttate psoriasis, were compared with Two-way ANOVA with Bonferroni post-test. Mann Whitney U test was used for cytokine production in larger groups of samples (plaque psoriasis vs. healthy controls). Correlation analysis was performed with Pearson coefficient analysis (r). The significance threshold was set at $P<0.05$.

RESULTS

Patients samples characteristics

On one hand, a total of 16 patients with guttate psoriasis were selected for the study, which included 13 patients with a guttate outbreak associated with throat infections, and 3 patients of long disease duration without a complete involution of guttate lesions. On the other hand, 11 psoriasis patients with chronic plaques that had no association with throat infection were chosen as an opposite psoriasis symptomatology. As shown in Table 1, a little study of both populations revealed that guttate patients had significant higher levels of anti-streptolysin O (ASO), and 14 of the 16 of them had raised positive titers, compared to the 2 of the 11 plaque psoriasis, as expected by their abovementioned respective existence or not of a prior pharyngitis episode. Also, in accordance to the commonly described characteristic of each type of disease, those guttate patients were consistently younger than plaque psoriasis patients (mean age of 24.7 and 43.3, respectively, $P<0.0001$), and had lower mean PASI scores than those with plaque psoriasis (6.8 vs 15.5, respectively, $P=0.0002$).

Table 1. Symptoms, clinical parameters, severity and age of the studied psoriasis population.

	Guttate Psoriasis (n=16)	Plaque Psoriasis (n=11)	P-value*
Associated pharyngitis (%)	13 (81.3)	0 (0)	-
ASO titre (IU/mL) mean (±SEM)	507.9 (±80.82)	154 (±45.1)	0.002
n ASO ≥ 200 (%)	14 (87.5)	2 (18.2)	-
PASI mean (±SEM)	6.8 (±0.9)	15.5 (±2)	0.0002
Age mean (±SEM)	24.7 (±1.3)	43.3 (±13.6)	<0.0001

*: T-test mean comparison. SEM: standarad error of the mean.

ASO and PASI variability and duration of disease

The minimal stated interval between the upper respiratory infection and the onset of guttate flares in some of our recruited patients has been of one week, which also has been reported in other studies (15). Also, first raised peaks in ASO levels are generally observed in 3 weeks post-infection. Therefore, in order to find potentially positive ASO

titers, only guttate patients with lesion flares later than 15 days were chosen, from which they were positive for ASO levels. Then, variabilities in ASO titers were explored along different duration of disease ranges, with a top length of 4 months, in which there was still guttate flares cases with evidence of a prior pharyngitis (Table 2). All guttate patients comprised within this interval had positive ASO titers (≥200 IU/ml), but those with a length of disease of 1 month (n=5) to 2 months (n=3) presented the highest levels. Interestingly, measuring PASI in guttate patients, although it is generally a tool used to evaluate plaque psoriasis severity, also revealed the highest scores within the same duration of disease-group of patients. Negative ASO titers were found in 2 of 3 long-lasting guttate patients (>1 year) with no clear history of streptococcal infection and, despite of an uncomplete resolution of their lesions, those patients presented a milder severity in terms of PASI score.

Most of plaque psoriasis patients of the study had chronic diseases for years, except for 3 patients who had developed plaque lesions between 5 months and less than a year. Although no prior tonsils infections were reported, 2 of these 3 patients had positive ASO titers but it did not imply higher PASI scores than the rest of ASO-negative chronic plaque psoriasis patients (n=8) (Table 2).

Table 2. Temporal distribution and clinical implications in the studied psoriasis patients.

	Guttate Psoriasis				Plaque Psoriasis			
Duration of disease	n	ASO titre mean (n ≥ 200)	Associated pharyngitis	PASI mean	n	ASO titre mean (n ≥ 200)	Associated pharyngitis	PASI mean
15 d < 1 mo	3	397.3 (3)	Yes	5.6	-	-	-	-
1 mo	5	680.2 (5)	Yes	7.7	-	-	-	-
1 mo - 2 mo	3	777.3 (3)	Yes	10.8	-	-	-	-
2 mo - 4 mo	2	339.5 (2)	Yes	4.1	-	-	-	-
5 mo < 1 y	-	-	-	-	3	345 (2)	No	12.1
> 1 y	3	174.3 (1)	No/Unclear	4	8	84.3 (0)	No	16.6

d: day; mo: month; y: year

Clinical parameters correlate with cytokine production in *S. pyogenes*-activated guttate CLA$^+$/Epi cocultures

Next, we sought to examine the effector capacity of skin-homing CLA$^+$ T cells in the presence of autologous lesional epidermal cells in comparison with non-skin tropic CLA$^-$ T cells, after an *ex vivo* activation with SE, within the same ranges of duration of disease. IL-17A, IFN-γ and IL-9 were evaluated after 5 days of activation. Again, the highest values were found in those cocultures derived from guttate samples of 1 to 2 months of disease length, especially significant for IL-17A (1 month: P<0.01; 1-2 months: P<0.001), and IL-9 values (1 month: P<0.05; 1-2 months: P<0.001) (Table 3.1). Interestingly, plaque psoriasis-derived cocultures still responded upon SE stimulation compared to healthy controls (Table 3.2).

Finally, based on the observation of coinciding peaks in the CLA-dependent effector responses, ASO titers and PASI score, linear correlations were performed in guttate subjects for a better evaluation of individual proportionality. Thus, ASO and cytokine levels finely correlated (Fig 1, A), showing significance in case of IL-17A (r=0.55; P=.02), and IL-9 (r=0.68; P=.003) production, or near significance for that of IFN-γ (r=0.45; P=.07). Similarly, PASI score correlated almost significantly with IL-17A levels (r=0.48; P=.06), and significantly with IFN-γ (r=0.55; P=.02) and IL-9 (r=0.61; P=.01) (Fig 1, B).

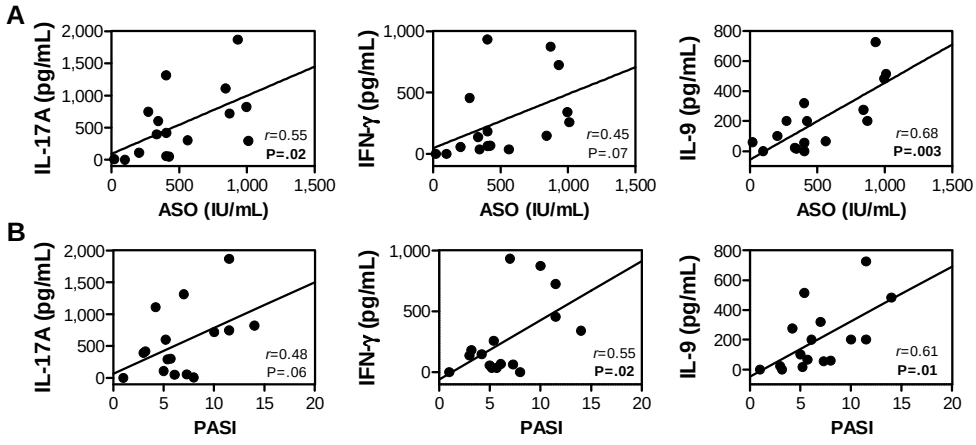


Figure 1. Clinical parameters correlate with cytokines production by *S. pyogenes*-activated guttate CLA$^+$/Epi cocultures. Cytokine values produced by SE-activated CLA$^+$/Epi co-cultures from guttate patients were linearly correlated with their respective ASO (A) and PASI levels (B). r: Pearson coefficient.

Table 3.1. IL-17A, IFN-γ and IL-9 responses: guttate psoriasis and time of disease.

GP (n=16)	IL-17A mean (±SEM)			IFN-γ mean (±SEM)			IL-9 mean (±SEM)		
Duration of disease	CLA^+/Epi/SE	CLA^-/Epi/SE	P-value§	CLA^+/Epi/SE	CLA^-/Epi/SE	P-value§	CLA^+/Epi/SE	CLA^-/Epi/SE	P-value§
15 d < 1 mo (n=3)	153.4 (±76)	2.6 (±2.6)	ns	53.2 (±9.5)	26.7 (±14.6)	ns	122.3 (±40.3)	28.5 (±14.3)	ns
1 mo (n=5)	584.2 (±184.7)	10.1 (±6.7)	P < 0.01	359.8 (±144.1)	142.2 (±83.1)	ns	250 (±75.2)	19.5 (±12.1)	P<0.05
1 mo - 2 mo (n=3)	1,334 (±302.4)	226.3 (216.2)	P < 0.001	665.4 (±173.2)	188.2 (±62.7)	P < 0.01	510.2 (±118)	76.8 (±27.6)	P < 0.001
2 mo - 4 mo (n=2)	497.5 (±104.8)	14.1 (±0.4)	ns	86.48 (±50.2)	9.1 (±9.1)	ns	19.6 (±2.9)	0 (±0)	ns
> 1 y (n=3)	142.4 (±137.9)	0 (±0)	ns	60.6 (±60.6)	25.4 (±16.6)	ns	19.8 (±19.8)	0 (±0)	ns
Time factor§			*P<0.05*			*ns*			*P < 0.01*

Table 3.2. IL-17A, IFN-γ and IL-9 responses: plaque psoriasis and healthy controls.

	IL-17A mean (±SEM)			IFN-γ mean (±SEM)			IL-9 mean (±SEM)		
	CLA^+/Epi/SE	CLA^-/Epi/SE	P-value*	CLA^+/Epi/SE	CLA^-/Epi/SE	P-value*	CLA^+/Epi/SE	CLA^-/Epi/SE	P-value*
Plaque psoriasis (n=11)	413.5 (±193.3)	28.6 (±13.8)	0.0004	446 (±252.8)	80.6 (±12.6)	ns	217.9 (±62.3)	47.1 (±25.3)	0.009
Controls (10)	117.8 (±53)	3.9 (±1.7)	0.01	76.34 (±27.9)	25 (±12.6)	ns	41 (±14.7)	11.3 (±7.2)	ns
P-value*	0.04	ns		ns	0.01		0.009	0.04	

§ : Two-way ANOVA with Bonferroni post-test. Variables: cell cultures and duration of disease (Time factor)

*: Mann Whitney U test (CLA^+ vs CLA^- or Plaque psoriasis vs Controls).

CLA^+/Epi or CLA^-/Epi: cocultures of either CLA^+ or CLA^- T cells and autologous epidermal cells; SEM: standard error of the mean.

DISCUSSION

Guttate psoriasis eruptions are usually self-limiting and resolve in 4 to 8 months (1,15). Interestingly, about 33–40% of patients with guttate psoriasis develop plaque-type disease in the future, and chronic plaque psoriasis can suffer guttate flares (2,15). Guttate-type eruptions are often preceded by a streptococcal throat infection, and therefore ASO titers are usually raised in patients with guttate psoriasis. Despite a rapid involuting course tends to be associated with young patients and elevated ASO titers (15), it has been previously suggested that streptococcal infections prior to the onset of guttate psoriasis would be a common preceding manifestation of future chronic plaque psoriasis (16). Overall, better understanding of the relations between streptococcal infection-associated clinical diagnostic tools, and disease severity indexes, with effector streptococcal-dependent responses exerted by disease-involved T cells, may lead to unravel relevant translational information about the course in psoriatic disease.

Varying degrees in raised ASO titers may be justified by the time period after throat infections, but also may reflect other individual factors such as an increased exposure to *S. pyogenes* due to persistent throat colonization or repeated infections. Then streptococcal antigens are released into the bloodstream, and thus prompting an enhanced peripheral immune response against this pathogen. When guttate psoriasis patients were recruited after 1 to 2 months after lesions onset, a peak in ASO titers was found, and such raised peak-associated interval was similar to that found in PASI scores. The functional association between *S. pyogenes* and psoriasis skin lesions has been determined to be effector memory skin-tropic CLA^+ T cells, which might be generated in the tonsils and migrate to the skin, as identical CLA^+ T cell clones have been found in both tonsils and cutaneous lesional sites in psoriasis patients (4). We previously described that CLA^+ T cells, cocultured with autologous lesional epidermal cells, preferentially responded to the SE, either in guttate and plaque samples (13,14). Such responses include key T cell-derived cytokines such as IL-17A and IFN-γ, and more recently found, also IL-9 (unpublished data). We suggest then that the effector responses against *S. pyogenes* antigens exerted by streptococcal-sensitized CLA^+ T cells that are found in circulation may be associated with an increased exposure to the pathogen, and consequently with the disease severity in guttate eruptions preceded by throat infections. This was further supported by the fact that cocultures from samples taken after 1–2 months of guttate-type disease duration, showed a peak response to

an *ex vivo* stimulation with SE, and by significant correlations found between cytokines levels with ASO titers and PASI scores.

Conversely, CLA^+ T cells and epidermal cells cocultures from most of plaque patients included in the study, even though they had no prior throat infections and mostly presented negative ASO titers (<200 IU/ml), showed significant responses against SE compared to healthy controls. This phenomenon could be explained by an asymptomatic carrier status in those patients, in which *S. pyogenes* could possibly colonize the oropharyngeal mucosa forming intracellular reservoirs (17,18), which would suggest that other diagnostic tools rather than ASO, could reveal a possible streptococcal burden in those patients. However, the long interval since disease onset and/or the possible uncontrolled exposure to antibiotics during this period, makes it difficult to establish a temporal link between a possible ancient infection and chronic disease symptoms.

In summary, these results support the functional relationship between the response capacity of circulating effector memory CLA^+ T cells with a potential higher exposition to streptococcal antigens and a higher severity in guttate psoriasis flares. Therefore, studying cellular responses in guttate psoriasis might have into consideration the streptococcal implication in its disease course to obtain more relevant results.

REFERENCES

1. Griffiths CEM, Barker JNWN. Pathogenesis and clinical features of psoriasis. *Lancet* 2007; **370**:263–71.

2. Langley R, Krueger G, Griffiths C. Psoriasis: epidemiology, clinical features, and quality of life. *Ann Rheum Dis* 2005;**64**(Suppl 2):ii18–ii23.

3. Leung DY, Gately M, Trumble A, *et al*. Bacterial Superantigens Induce T Cell Expression of the Skin-Selective Homing Receptor, the Cutaneous Lymphocyte-Associated Antigen, Via Stimulation of Interleukin 12 Production. *J Exp Med* 1995; **181**:747–53.

4. Diluvio L, Vollmer S, Besgen P, *et al*. Identical TCR β-Chain Rearrangements in Streptococcal Angina and Skin Lesions of Patients with Psoriasis Vulgaris. *J Immunol* 2006; **176**:7104–11.

5. Valdimarsson H, Thorleifsdottir RH, Sigurdardottir SL, *et al*. Psoriasis – as an autoimmune disease caused by molecular mimicry. *Trends Immunol* 2009; **30**:494–501.

6. Gudjonsson JE, Johnston A, Sigmundsdottir H, *et al*. Immunopathogenic mechanisms in psoriasis. *Clin Exp Immunol* 2004; **135**:1–8.

7. Ferran M, Romeu ER, Rincón C, *et al*. Circulating CLA+ T lymphocytes as peripheral cell biomarkers in T-cell-mediated skin diseases. *Exp Dermatol* 2013; **22**:439–42.

8. Johnson-Huang LM, Pensabene CA, Shah KR, *et al*. Post-Therapeutic Relapse of Psoriasis after CD11a Blockade Is Associated with T Cells and Inflammatory Myeloid DCs. *PLOS One* 2012; **7**:e30308.

9. Harper EG, Simpson EL, Takiguchi RH, *et al*. Efalizumab Therapy for Atopic Dermatitis Causes Marked Increases in Circulating Effector Memory CD4+ T Cells That Express Cutaneous Lymphocyte Antigen. *J Invest Dermatol* 2008; **128**:1173–81.

10. Vugmeyster Y, Kikuchi T, Lowes MA, *et al*. Efalizumab (anti-CD11a)-induced increase in peripheral blood leukocytes in psoriasis patients is preferentially mediated by altered trafficking of memory CD8+ T cells into lesional skin. *Clin Immunol* 2004; **113**:38–46.

11. Santamaría-Babí LF. Skin-Homing T Cells in Cutaneous Allergic Inflammation. *Chem Immunol Allergy* 2006; **91**:87–97.

12. Santamaria-babí LF. CLA $^{+}$ T cells in cutaneous diseases. *Eur J Dermatol* 2004; **14**:13–8.

13. Ruiz-Romeu E, Ferran M, Sagristà M, *et al*. Streptococcus pyogenes–induced cutaneous lymphocyte antigen–positive T cell–dependent epidermal cell activation triggers TH17 responses in patients with guttate psoriasis. *J Allergy Clin Immunol* 2016; **138**:491–499.

14. Ferran M, Galván AB, Rincón C, *et al*. Streptococcus Induces Circulating CLA+ Memory T-Cell-Dependent Epidermal Cell Activation in Psoriasis. *J Invest Dermatol* 2013; **133**:999–1007.

15. Ko HC, Jwa SW, Song M, *et al*. Clinical course of guttate psoriasis: Long-term follow-up study. *J Dermato* 2010; **37**:894–9.

16. Williams RC, McKenzie AW, Roger JH, *et al*. HL-A antigens in patients with guttate psoriasis. *Br J Dermatol* 1976; **95**:163–7.

17. Thorleifsdottir RH, Eysteinsdóttir JH, Olafsson JH, *et al*. Throat Infections are Associated with Exacerbation in a Substantial Proportion of Patients with Chronic Plaque Psoriasis. *Acta Derm Venereol* 2016; **96**:788–91.

18. Sigurdardottir SL, Thorleifsdottir RH, Valdimarsson H, *et al*. The association of sore throat and psoriasis might be explained by histologically distinctive tonsils and increased expression of skin-homing molecules by tonsil T cells. *Clin Exp Immunol* 2013; **174**:139–51.

ACKNOWLEDGEMENTS

We thank D. Cabrerizo for his technical support.

FUNDING SOURCES

The study was funded by FIS/ISCIII 2013 (Ministerio de Economía y Competitividad e Instituto de Salud Carlos III; PI09/2222, PI13/01845 and PI13/01716) and PhD fellowship to E. R. R is granted by the Ministerio de Educación, Cultura y Deporte of the Spanish Government (FPU13/02308).

CONFLICTS OF INTERESTS

None declared.

ORIGINAL ARTICLE

MCPIP1 RNase Is Aberrantly Distributed in Psoriatic Epidermis and Rapidly Induced by IL-17A

Ester Ruiz-Romeu[1], Marta Ferran[2], Ana Giménez-Arnau[2], Beata Bugara[3], Barbara Lipert[3], Jolanta Jura[3], Edwin F. Florencia[4], Errol P. Prens[4], Antonio Celada[5], Ramon M. Pujol[2] and Luis F. Santamaria-Babí[1]

ZC3H12A, which encodes the RNase monocyte chemotactic protein-induced protein 1 (MCPIP1), is up-regulated in psoriatic skin and reduced to normal levels after clinical treatments with anti–IL-17A/IL-17R neutralizing antibodies. In IL-17A–stimulated keratinocytes, MCPIP1 is rapidly increased at the transcript and protein levels. Also, IL-17A was found to be the main inducer of *ZC3H12A* expression in keratinocytes treated with supernatants derived from a *Streptococcus pyogenes*-activated psoriatic ex vivo model based on the co-culture of psoriatic cutaneous lymphocyte-associated antigen (CLA^+) T cells and lesional epidermal cells. Moreover, MCPIP1 was aberrantly distributed in the suprabasal layers of psoriatic epidermis. In psoriatic samples, IL-17A–stimulated epidermal cell suspensions showed an increased MCPIP1 expression, especially in the mid-differentiated cellular compartment. The knockdown of *ZC3H12A* showed that this RNase participates in the regulation of the mRNAs present in suprabasal differentiated keratinocytes. Furthermore, JAK/STAT3 inhibition prevented the IL-17A–dependent induction of MCPIP1. In the mouse model of imiquimod-induced psoriasis, *Zc3h12a* expression was abrogated in *Il17ra*$^{-/-}$ mice. These results support the notion that IL-17A–mediated induction of MCPIP1 is involved in the regulation of local altered gene expression in suprabasal epidermal layers in psoriasis.

Journal of Investigative Dermatology (2016) **136,** 1599–1607; doi:10.1016/j.jid.2016.04.030

INTRODUCTION

The relevance of the T helper 17 (Th17) response in psoriasis is clinically evidenced by the therapeutic efficacy rates achieved by selective biologics targeting IL-17A and IL-17R in psoriatic patients (Langley et al., 2014; Leonardi et al., 2012; Papp, Leonardi, et al., 2012). Moreover, keratinocytes are the dominant skin cell population expressing IL-17 receptor and thus a potential proinflammatory target of this cytokine (Chiricozzi et al., 2011). Therefore, unraveling the role of the genes induced by this cytokine in psoriatic lesions is highly relevant and may help us understand its pathogenesis. Gene expression arrays have allowed the determination of IL-17–induced genes in keratinocytes (Chiricozzi et al., 2011; Nograles et al., 2008). In addition, many genes have been identified whose mRNA expression is increased in psoriatic lesions versus healthy or nonlesional skin and decreased after clinical suppression of the IL-17 pathway (Krueger et al., 2012; Russell et al., 2014). Those studies included sets of previously known psoriasis-related genes along with other poorly characterized markers such as *ZC3H12A*, which is the only RNase within the 10 most up-regulated transcripts in the IL-17A–induced signature in keratinocytes. Remarkably, *ZC3H12A* has been described as a differentially expressed gene that may participate in distinguishing nonlesional from normal and lesional skin samples (Xie et al., 2014). However, its characterization has not been addressed in this disease in depth.

ZC3H12A encodes for the monocyte chemotactic protein-1 (MCP-1)–induced protein (MCPIP1), which is an RNase that regulates mRNA stability and diminishes the levels of several inflammatory cytokines such as IL-1β, IL-6, and IL-12p40 and also its own transcript (Matsushita et al., 2009; Mizgalska et al., 2009). Furthermore, its RNase activity has been linked to microRNA degradation by counteracting the Dicer enzyme and directly cleaving the terminal loops of precursor microRNAs (Suzuki et al., 2011). Initially, it was described to be up-regulated by MCP-1, lipopolysaccharides, and IL-1β (Kasza et al., 2010; Liang et al., 2008; Mizgalska et al., 2009; Zhou et al., 2006). More recently, it was reported to be also induced by IL-17 through NF-κB activation and mRNA stabilization, and it might represent a mechanism by which this proinflammatory cytokine regulates other genes (Somma et al., 2015; Sønder et al., 2012; Sparna et al., 2010). Recent studies point to feedback inhibitory activity of IL-17 signaling through

[1]*Translational Immunology, Department of Cellular Biology, Physiology and Immunology, Faculty of Biology, Universitat de Barcelona, Barcelona, Spain;* [2]*Department of Dermatology, Hospital del Mar, Universitat Autònoma de Barcelona, Barcelona, Spain;* [3]*Department of General Biochemistry, Faculty of Biochemistry, Biophysics and Biotechnology, Jagiellonian University, Krakow, Poland;* [4]*Department of Dermatology, Erasmus MC, University Medical Center, Rotterdam, The Netherlands; and* [5]*Macrophage Biology Group, Department of Cellular Biology, Physiology and Immunology, Faculty of Biology, Universitat de Barcelona, Barcelona, Spain*

Correspondence: Luis F. Santamaria-Babí, Translational Immunology Group, PCB/Universitat de Barcelona, Baldiri i Reixac, 10 Barcelona, Barcelona 08028, Spain. E-mail: luis.santamaria@ub.edu

Abbreviations: CLA, cutaneous lymphocyte-associated antigen; KRT, keratin; MCPIP1, monocyte chemotactic protein-induced protein 1; TNF, tumor necrosis factor

Received 11 August 2015; revised 18 March 2016; accepted 4 April 2016; accepted manuscript published online 12 May 2016; corrected proof published online 21 June 2016

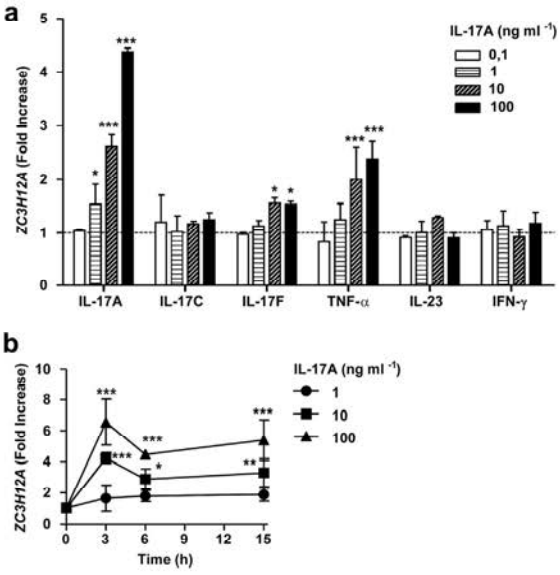


Figure 1. IL-17A induces the up-regulation of *ZC3H12A* expression in keratinocytes. (**a**) Keratinocytes were cultured and stimulated at confluence with a range of doses of IL-17A, IL-17C, IL-17F, TNF-α, IL-23, or IFN-γ. *ZC3H12A* mRNA expression was evaluated at 15 hours of treatment (n = 3). (**b**) mRNA from IL-17A–stimulated (1, 10, and 100 ng/ml) keratinocytes was extracted at various early and late time points for *ZC3H12A* expression analysis (n = 3). Data are presented as mean ± standard deviation. Significant differences were analyzed with two-way analysis of variance *t* test versus untreated $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$. h, hours; TNF, tumor necrosis factor.

MCPIP1 in non-hematopoietic cells, in which this RNase is also vital for maintaining immune homoeostasis in vivo (Garg et al., 2015). Furthermore, additional functions of MCPIP1 in other biological processes, such as angiogenesis, cell migration, and differentiation, have been reported (Chao et al., 2015; Lipert et al., 2014; Niu et al., 2008; Vrotsos et al., 2009; Wang et al., 2011).

To the best of our knowledge, this study is the first to show MCPIP1 expression in primary human keratinocytes and its presence in differentiated epidermal cells in situ and ex vivo. In addition, we show that *ZC3H12A* is induced in keratinocytes in an IL-17A–dependent manner under a psoriasis-like proinflammatory milieu (Ferran et al., 2013; Ruiz-Romeu et al., 2016), as well as in suprabasal lesional psoriatic epidermal cells activated by recombinant IL-17A. Finally, no induction of *Zc3h12a* expression was found in *Il17ra*$^{-/-}$ mice with a psoriasis phenotype developed by imiquimod. Overall, our findings point to a critical role of the IL-17A/IL-17RA/MCPIP1 axis in the regulation of local altered gene expression in suprabasal epidermal layers in psoriasis.

RESULTS

IL-17A induces *ZC3H12A*/MCPIP1 expression in keratinocytes

Primary human keratinocytes were incubated with several proinflammatory cytokines that are increased in psoriatic skin. Among some IL-17 isoforms, only IL-17A highly induced *ZC3H12A* expression in a dose-dependent fashion ($P < 0.05$) (Figure 1a), whereas a much lower increase was detected in response to IL-17F. *ZC3H12A* was also triggered by tumor necrosis factor (TNF)-α but at a lower intensity ($P < 0.001$), and no effect was observed upon stimulation with IL-23 or IFN-γ (Figure 1a). With respect to MCPIP1 RNase activity, the IL-17A–dependent induction of *ZC3H12A* was further characterized by the evaluation of its mRNA expression kinetics. The highest expression of *ZC3H12A* was rapidly enhanced within 3 hours of exposure to higher IL-17A doses, and this elevated expression was maintained for at least 15 hours, whereas lower doses of IL-17A resulted in a moderate induction of *ZC3H12A* after 6 and 15 hours (Figure 1b). HaCaT keratinocytes stimulated with IL-17A for 6, 15, and 24 hours showed an increase in MCPIP1 protein levels, thus suggesting that the translation of *ZC3H12A* mRNA to its protein product had already occurred at 6 hours or earlier posttreatment (see Supplementary Figure S1 online).

The IL-17A contained in the supernatants of co-cultures of psoriatic CLA^{+} T cells and epidermal cells activated by streptococcal extract induces *ZC3H12A* in keratinocytes

We previously established a psoriatic ex vivo model in which the activation of co-cultures of psoriatic CLA^{+} memory T cells with autologous lesional epidermal cells (EPI) through a *Streptococcus pyogenes* extract (SE) (CLA^{+}/EPI/SE) resulted in the production of key psoriatic proinflammatory cytokines (Ferran et al., 2013). These cytokines include, among others, IL-17A and TNF-α, which induced the highest *ZC3H12A* expression, and IFN-γ, which was used as a negative control. Keratinocytes stimulated with these supernatants showed an induction of *ZC3H12A* expression (Figure 2a). Conversely, the supernatants from co-culture conditions with non-skin homing CLA^{-} T cells or healthy control cells did not cause this induction. By using neutralizing antibodies, we detected that the preferential component of the supernatants inducing *ZC3H12A* was IL-17A, whereas no such reduction was obtained when antibodies against TNF-α or IFN- γ were used (Figure 2b and see Supplementary Figure S2 online). The amount of IL-17A, but not of TNF-α or IFN-γ, in the CLA^{+}/EPI/SE co-culture–derived supernatants correlated significantly with its corresponding induction of *ZC3H12A* expression (Figure 2c). This *ZC3H12A* induction, which was driven mainly by the IL-17A produced by streptococcal activation, correlated with anti-streptolysin O titer, a clinical parameter indicating streptococcal infection (Figure 2d).

MCPIP1 is aberrantly expressed in the suprabasal epidermis and is rapidly up-regulated by IL-17A in psoriasis

Next, we sought to determine the in situ expression of MCPIP1 in the skin. The specific MCPIP1 immunohistochemical staining showed that the expression was limited to a narrow band in the upper granular layer in control healthy skin, similar to what was seen in atopic dermatitis lesions. However, in psoriatic samples, MCPIP1 staining showed predominant expression within the epidermis with a broad distribution throughout suprabasal keratinocytes, especially beneath the apical side of the cells (Figure 3). To further

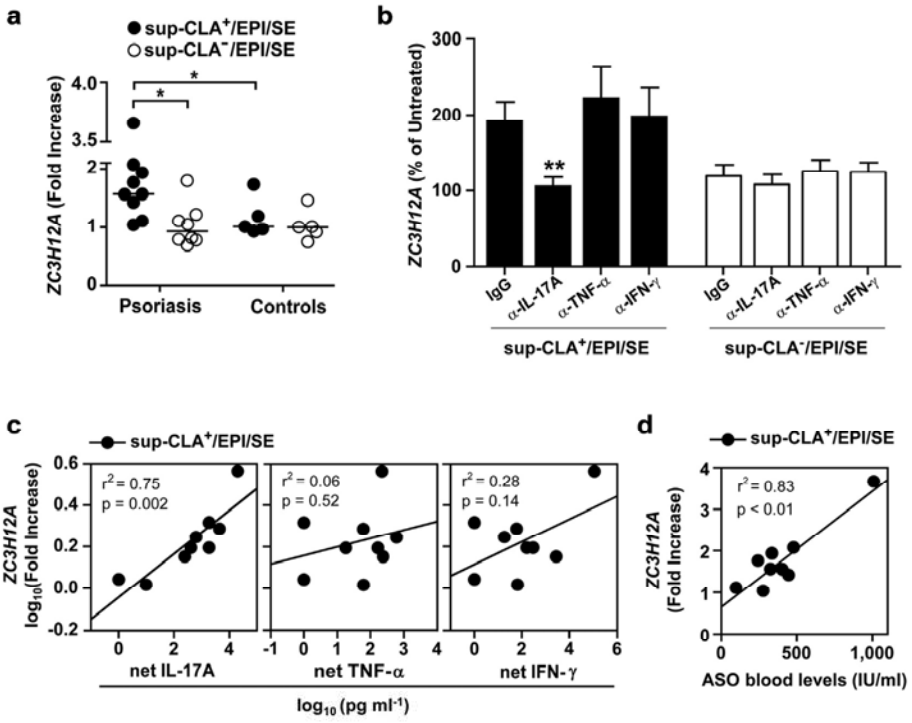


Figure 2. *ZC3H12A* induction by supernatants enriched with psoriasis-associated inflammatory mediators depends mainly on the effect of IL-17A. (**a**) *ZC3H12A* mRNA expression was analyzed in keratinocytes stimulated with supernatants derived from SE-activated co-cultures with CLA^+ or CLA^- T cells and autologous epidermal lesional cells (nine psoriatic patients and five control subjects; both are represented by dots). Horizontal bar shows median induction. Differences were analyzed with the Mann-Whitney *U* test (*$P < 0.05$). (**b**) The supernatants from five psoriasis patients with neutralized IL-17A, TNF-α, and IFN-γ were used for subsequent activation of keratinocytes and *ZC3H12A* mRNA analysis. Bar chart represents the percentage of *ZC3H12A* expression in relation to that found in untreated keratinocytes expression (mean ± standard error of the mean). A Student *t* test was used to check differences versus isotype conditions (**$P < 0.01$). (**c**) Correlation between IL-17A, TNF-α, or IFN-γ contents of supernatants derived from SE-activated CLA^+/EPI co-cultures and respective *ZC3H12A* mRNA up-regulation in keratinocytes. (**d**) Correlation between *ZC3H12A* mRNA up-regulation in keratinocytes induced by supernatants from SE-activated CLA^+/EPI co-cultures with anti-streptolysin O blood levels from respective patients. RNA extraction was done at 15 hours after stimulation. ASO, anti-streptolysin O; CLA, cutaneous lymphocyte-associated antigen; EPI, epidermal cells; IU, international units; SE, *Streptococcus pyogenes* extract; sup, supernatant; TNF, tumor necrosis factor.

characterize MCPIP1 distribution, we developed an intracellular flow cytometry staining in freshly isolated epidermal cells from skin biopsy samples (Figure 4a). Specific quantification of MCPIP1 expression by various epidermal cell subsets was achieved by a triple staining with two additional markers: CD29, a β1 integrin expressed by basal-proliferative epidermal keratinocytes (Staquet et al., 1989), and keratin 10 (KRT10), a differentiation keratin expressed by suprabasal keratinocytes (Leigh et al., 1993) (Figure 4b). Compared with control skin, psoriatic samples showed an overrepresentation of the proliferative compartment ($CD29^+$) (Figure 4b and see Supplementary Figure S3a online), which is a distinctive feature of this disease. This proliferative compartment contained basal $KRT10^-CD29^+$ keratinocytes and a transiently amplifying $KRT10^+CD29^+$ subset, the latter previously identified by flow cytometry (Bata-Csorgo et al., 1993). These observations were also consistent with previous studies showing patches of suprabasal CD29 expression in psoriatic epidermis (Horrocks et al., 1991). The $MCPIP1^+$ population showed a distinct distribution in control and psoriasis samples, being present mostly in $KRT10^+CD29^-$ cells in the former (95.67 ± 2.8%), whereas in the latter this population was distributed in both $KRT10^+CD29^-$ and $KRT10^+CD29^+$ subsets (64.6 ± 10.57% and 30.93 ± 4.7%, respectively) (Figure 4c and see Supplementary Figure S2b). In addition, the mean fluorescence intensity of MCPIP1 in $KRT10^+CD29^-$ cells in controls was higher than in psoriatic samples, but the opposite was found for the $KRT10^+CD29^+$ population (see Supplementary Figure S3c and d). These data support the distinct MCPIP1 distribution observed in skin immunohistochemistry.

Next, we used the epidermal cell suspensions to assess the capacity of recombinant human IL-17A to induce MCPIP1 expression ex vivo. After 4 hours of incubation, we found no evident modifications in controls ($n = 3$), whereas in psoriasis samples a dose-dependent increase in $MCPIP1^+$ cells was observed, especially in the suprabasal $KRT10^+CD29^+$ subset, being significant at 50 ng/ml of IL-17A treatment ($n = 5$; $P = 0.007$) (Figure 4d and e). Similarly, an increase in the mean fluorescence intensity was also significant ($P = 0.02$) for $KRT10^+CD29^+$ cells, and an

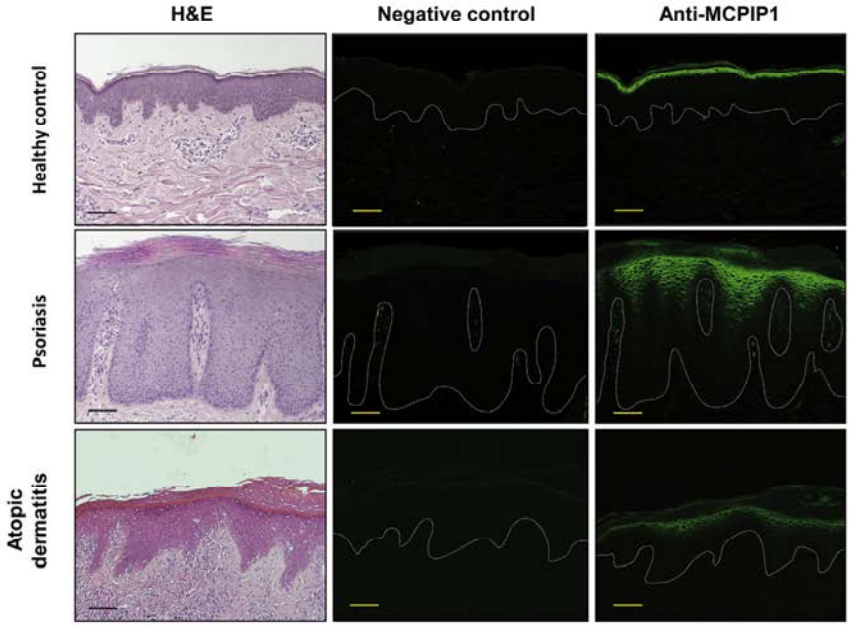


Figure 3. Psoriatic skin shows aberrant MCPIP1 expression. MCPIP1 localization by immunohistochemistry visualized by fluorescence microscopy. Skin samples from healthy controls and from psoriatic and atopic dermatitis patients were fixed with 4% formaldehyde for paraffin embedding. Tissue sections were stained with hematoxylin and eosin (left column) or were deparaffinized for immunostaining without primary antibody (center column) and with specific anti-MCPIP1 primary antibody (right column). Scale bars = 100 μm. H&E, hematoxylin and eosin; MCPIP1, monocyte chemotactic protein-induced protein 1.

increasing trend was observed in KRT10^{-}CD29^{+} cells (see Supplementary Figure S3e).

Silencing *ZC3H12A* alters the expression of IL-17–regulated genes in the suprabasal epidermal layer

To determine the functional activity of the MCPIP1 RNase in the regulation of other genes in psoriatic lesions, we used HaCaT keratinocytes transfected with a plasmid expressing small hairpin RNA sequence toward *ZC3H12A*. Knockdown was confirmed at both the mRNA and protein level (see Supplementary Figure S4a and b online). Psoriasis-associated genes were checked, such as transcript levels of antimicrobial protein members *S100A8* and *S100A9* and the early differentiation markers involucrin (*IVL*) and keratin-1 (*KRT1*). *ZC3H12A* knockdown altered basal gene expression, increasing *S100A8* and *S100A9* expression, as well as their IL-17–mediated induction (see Supplementary Figure S4c and d), in line with other antimicrobial proteins reported to be down-regulated by MCPIP1, such as *Lcn2* (Garg et al., 2015). Remarkably, *IVL*, an early precursor of the cornified envelope that is up-regulated in psoriasis, was tonically down-regulated in *ZC3H12A*-silenced cells, and its IL-17–dependent induction was also reduced (see Supplementary Figure S3e). In contrast, *KRT1*, which is usually reduced in psoriasis, was increased in silenced conditions (see Supplementary Figure S4f).

MCPIP1 induction in keratinocytes is dependent on signal transducer and activator of transcription (STAT)-3 phosphorylation

IL-17 regulates *ZC3H12A* expression through activation of NF-κB (Somma et al., 2015; Sønder et al., 2012). However, given that epidermal keratinocytes of psoriatic lesions show phosphorylated STAT3 (Sano et al., 2005), a transcription factor through which other IL-17–mediated genes are induced in keratinocytes (Shi et al., 2011; Simanski et al., 2013), we addressed the potential role of STAT3 in regulating MCPIP1 expression. Keratinocytes treated with IL-17A showed strong STAT3 phosphorylation, which was even higher than that of the NF-κB p65 subunit (see Supplementary Figure S5 online). Inhibition of STAT3 phosphorylation by AZD1480 decreased the induction of MCPIP1 by IL-17A (Figure 5a). Accordingly, keratinocytes treated with IL-6, which also activates STAT3, showed an increased expression of MCPIP1 (Figure 5b). However, induction of *ZC3H12A* transcript levels in keratinocytes by IL-6 was less evident, because only a slight increase was observed at shorter times of treatment (Figure 5c).

Zc3h12a expression is induced in an imiquimod-induced psoriasis model in mice, and it is IL-17R dependent

To further support the notion of the relation between altered *ZC3H12A* expression in psoriasis and its induction by IL-17A, we carried out experiments using a mouse model of innate imiquimod-induced psoriasis (van der Fits et al., 2009). Inflamed skin from Balb/c mice showed the up-regulation of *Zc3h12a* expression, which was reduced after application of potent topical corticosteroids (Figure 6a). In addition, the strong induction of *Zc3h12a* in wild-type mice (C57BL/6 strain) treated with imiquimod was not observed in *Il17ra*$^{-/-}$ mice (Figure 6b), a condition that has been reported to also block this imiquimod-induced disease (van der Fits et al., 2009).

DISCUSSION

IL-17–induced genes are of relevance in lesional epidermal cells in psoriasis because clinical blockade treatments of both anti–IL-17A/IL-17RA in this disease led to rapid

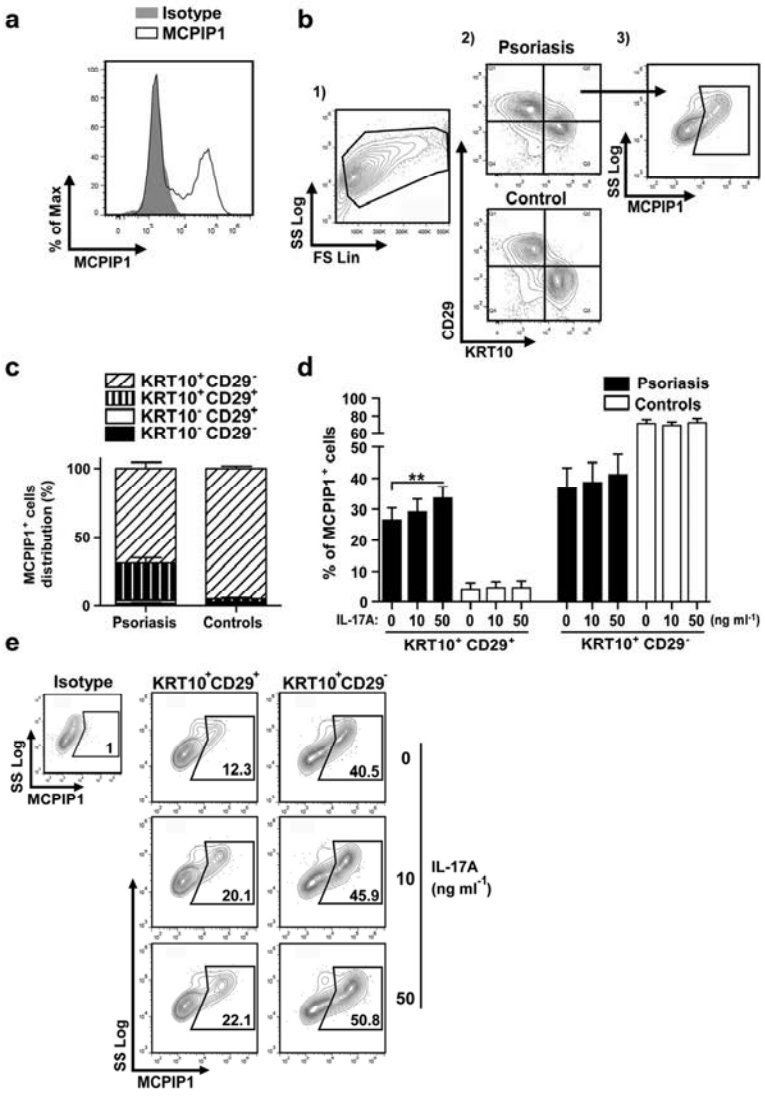


Figure 4. Abnormal MCPIP1 expression in psoriatic skin is distributed in highly and mid-differentiating suprabasal epidermal population subsets and is inducible by IL-17A. (**a**) Intracellular MCPIP1 detection by flow cytometry in epidermal cell suspension from a representative healthy skin sample. (**b**) Gating strategy for the evaluation of MCPIP1 expression in basal (CD29) and suprabasal differentiated (KRT10) cells (1 = epidermal cells, 2 = KRT10/CD29 quadrant, 3 = MCPIP1 gating within each quadrant). (**c**) Distribution of MCPIP1$^+$ cells in epidermal cell subsets in psoriatic (n = 4) and control (n = 3) samples. (**d**) Epidermal cell suspensions were cultured for 4 hours with or without IL-17A (0, 10, or 50 ng/ml), and MCPIP1 expression was analyzed by flow cytometry. Paired *t* test comparison analysis was used between stimuli conditions (**$P < 0.01$). (**e**) MCPIP1 induction in KRT10$^+$ subsets in one representative psoriatic sample after 4 hours of IL-17A activation. Data are presented as mean ± standard error of the mean. FSLin, forward scatter linear; KRT10, keratin 10; MCPIP1, monocyte chemotactic protein-induced protein 1; SS Log, side scatter logarithmic.

transcriptional changes initially in keratinocyte-expressed genes. This included the normalization of the *ZC3H12A* RNase expression as early as 2 weeks after treatment and preceding the clinical efficacy achieved through a progressive decrease in the Psoriasis Area and Severity Index score (Krueger et al., 2012; Russell et al., 2014). However, the association of *ZC3H12A* with psoriasis is limited to the transcript level. Recently, the participation of MCPIP1 in the negative regulation of IL-17 signal transduction has been reported (Garg et al., 2015). As in most altered immune situations, psoriasis is a question of balance between activators and inhibitors. However, in chronic inflammatory conditions, the persistent presence of activating stimuli overwhelms these inhibitory feedback signals. Because MCPIP1 is also involved in regulating a number of biological processes in various cell types (Jura et al., 2012), constant MCPIP1 activity may also participate in other aspects of the psoriasis pathogenesis.

We observed that keratinocytes underwent a potent induction of *ZC3H12A* by IL-17A and, to a lesser extent, by TNF-α, a cytokine also related to the induction of this gene (Chiricozzi et al., 2011). Supernatants from a previously developed ex vivo psoriatic model, composed of a co-culture of memory CLA$^+$ T cells with autologous epidermal lesional cells and stimulated by SE (Ferran et al., 2013; Ruiz-Romeu et al., 2016), induced *ZC3H12A* expression in keratinocytes in an IL-17A–dependent manner. Consequently, this model shows that, in an inflammatory psoriasis-associated environment, IL-17A is the key mediator inducing *ZC3H12A* in keratinocytes. Of note, *ZC3H12A* mRNA expression was also positively correlated with anti-streptolysin O blood levels, thereby strengthening the notion that exposure to

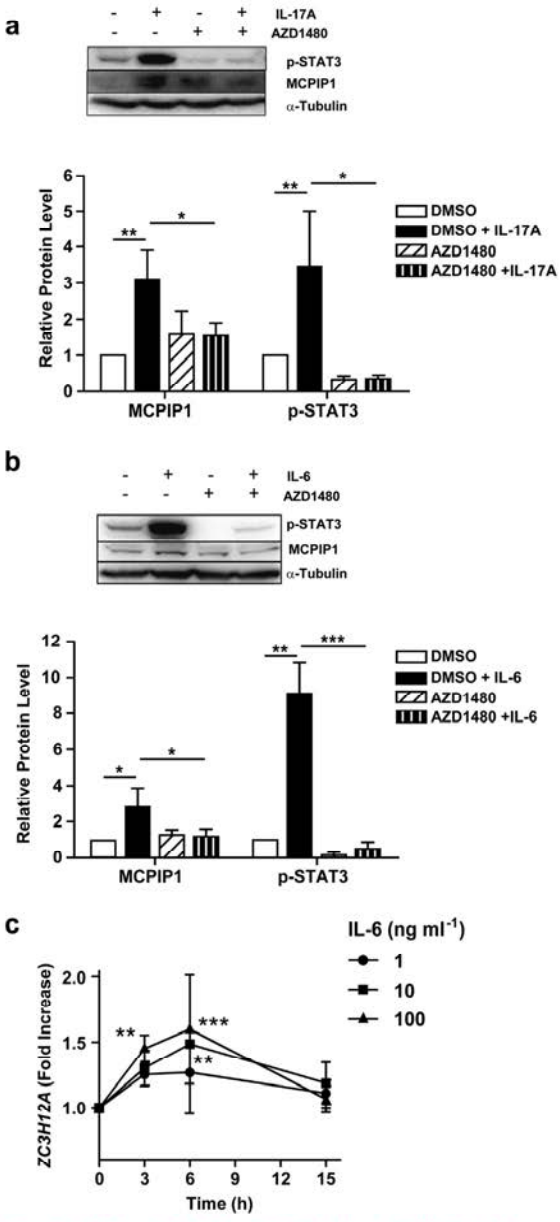


Figure 5. Inhibition of STAT3 by AZD1480 reduces MCPIP1 induction. Primary keratinocytes were incubated with 1 μmol/L of pharmacological inhibitor AZD1480 or DMSO (0.02%) before stimulation with (**a**) IL-17A or (**b**) IL-6 for 15 hours, and phosphorylated STAT3 and MCPIP1 protein were detected and quantified by densitometry. Statistical analysis was done on the basis of results from three independent experiments. A Student *t* test was used for data analysis. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. (**c**) *ZC3H12A* expression from IL-6–stimulated (1, 10, and 100 ng/ml) keratinocytes (n = 3) was analyzed at various early and late time points. Significant differences were analyzed with two-way analysis of variance *t* test versus untreated. $^{**}P < 0.01$, $^{***}P < 0.001$. Data are presented as mean ± standard deviation. h, hours; MCPIP1, monocyte chemotactic protein-induced protein 1; p-STAT, phosphorylated signal transducer and activator of transcription; STAT, signal transducer and activator of transcription.

streptococcal infections can lead to a cutaneous inflammatory reaction that gives rise to the altered *ZC3H12A* expression in psoriatic lesions.

MCPIP1 cytoplasmic staining was found in the granular layer keratinocytes of healthy epidermis, whereas in hyperproliferated psoriatic epidermis this was located in the suprabasal to upper spinous additional layers. An intermediate profile was observed in atopic dermatitis. This expression pattern in psoriasis is similar to that reported for other gene products involved in terminal differentiation and IL-17A–induced products (Kulke et al., 1998; Tsuchida et al., 2004) and is consistent with the psoriatic epidermal distribution of IL-17RA (Chiricozzi et al., 2014; Peric et al., 2008). Using epidermal cell suspensions, we observed that the suprabasal rapid-cycling cells co-expressing KRT10 and CD29 (Bata-Csorgo et al., 1993) showed an increase in MCPIP1 expression after IL-17A stimulation. Conversely, control epidermal cells expressing MCPIP1 de novo were generally imperceptible. These results suggest that MCPIP1 is rapidly induced by IL-17A in psoriatic differentiating keratinocytes and that its expression in healthy epidermis is basically constitutive.

MCPIP1 has been reported to inhibit the expression of *Lcn2* and other proinflammatory products (Garg et al., 2015). This inhibitory behavior was also observed in the increased expression of the antimicrobial proteins *S100A8* and *S100A9*, in *ZC3H12A* knockdown keratinocytes. In addition, these cells showed an altered expression of genes associated with early differentiation and cornification, namely *IVL* and *KRT1*, which were decreased and increased, respectively, thereby revealing a potential regulatory function of MCPIP1 in the keratinocyte differentiation program. These data support the role of MCPIP1 in regulating tonic expression regulation (Garg et al., 2015). In addition, IL-17–dependent modulation of these transcripts was also altered by the knockdown of *ZC3H12A*. Thus, the impact of MCPIP1 activity under chronic exposure to a proinflammatory milieu could be of relevance for the study of the altered gene expression in suprabasal cells in psoriatic epidermis, as this RNase might act mainly on transcripts undergoing active translation (Mino et al., 2015).

In contrast to previous reports (Somma et al., 2015; Sønder et al., 2012), our data suggest that the IL-17A–dependent induction of MCPIP1 in keratinocytes is highly dependent on the STAT3 pathway. Interestingly, *Stat3* overexpression in transgenic mouse epidermis leads to the development of psoriatic lesions (Sano et al., 2005) and has been proposed to regulate the lack of subcorneal cell compaction (Honma et al., 2006). IL-6 stimulation, which also activates STAT3 signaling, also induced MCPIP1 expression. However, the true impact of IL-6 on the induction of MCPIP1 in situ requires further study, because the expression of the IL-6 receptor in psoriatic lesions and the efficacy of targeting IL-6 signaling are still unclear (Ghoreschi and Gadina, 2014; Ohta et al., 1991; Papp, Menter, et al., 2012).

Finally, we observed that the up-regulation of *Zc3h12a* mRNA in the inflamed skin of imiquimod-induced psoriasis in mice was decreased after topical application of a potent corticosteroid. This treatment decreases IL-23/IL-17A axis,

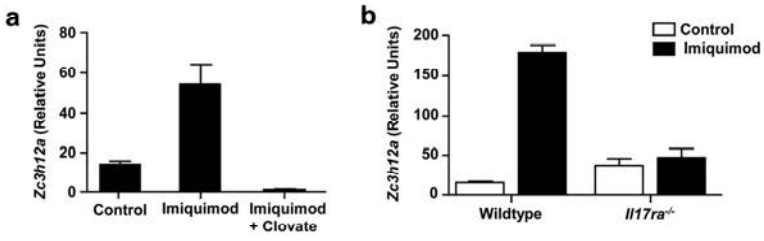


Figure 6. Induction of *Zc3h12a* in imiquimod-induced psoriasiform skin in mice is abrogated by topical corticosteroid and is dependent on IL-17R signaling. (**a**) Balb/c mice were left untreated (n = 3) or treated with Aldara (imiquimod) cream on dorsal skin for 6 days (n = 4). A group was additionally treated with corticosteroid-containing Clovate cream (n = 4). (**b**) Wild-type and *Il17ra*$^{-/-}$ C57BL/6 mice (minimum of two animals per group) were treated with Aldara cream for 5 days. Relative units of mRNA expression of *Zc3h12a* in skin samples were assessed by quantitative real-time PCR. Data are presented as mean ± standard deviation.

which is crucial for the development of this model (Sun et al., 2013; van der Fits et al., 2009). In fact, imiquimod-treated *Il17ra*$^{-/-}$ mice showed no increase in *Zc3h12a* expression, in line with normalized levels achieved with IL-17/IL-17RA neutralizing therapies in human subjects (Krueger et al., 2012; Russell et al., 2014).

Our data support the strong IL-17A–dependent enhancement of MCPIP1 expression. MCPIP1 activity, in turn, may regulate pathogenic mechanisms in suprabasal epidermis, thereby contributing to an altered balance of the IL-17A effects in psoriasis.

MATERIALS AND METHODS

Keratinocyte in vitro culture and activation

Primary human keratinocytes (1×10^5) were cultured in a 24-well plate in CnT-07 medium (CELLnTEC, Bern, Switzerland) for 48 hours. Cells were then washed with phosphate buffered saline and stimulated with recombinant human IL-17A, IL-17F, IL-6, and IL-23 (Myltenyi Biotech, Berisch Gladbach, Germany) and IFN-γ and IL-17C (R&D Systems, Minneapolis, MN) at 0.1, 1, 10, and 100 ng/ml for 15 hours. Additional time points were considered. For the blocking cytokine assay, 10 μg/ml of neutralizing antibodies against human IL-17A (eBiosciences, San Diego, CA), TNF-α and IFN-γ (Biolegend, San Diego, CA), or isotype (Biolegend) were incubated with medium containing diluted supernatant (see ex vivo psoriatic model in the Supplementary Materials online) at room temperature for 30 minutes before stimulation.

Patients

Skin biopsy samples or/and blood extractions were obtained from eleven psoriatic and seven healthy subjects. All participants contributed voluntarily and gave written informed consent. The samples were collected after a minimum period of 6 weeks without any systemic treatment. The study was approved by the Medical Ethics Committee of Hospital del Mar (Barcelona, Spain) and was conducted following the principles set out in the Declaration of Helsinki.

Histology

Skin samples from mouse back and human skin biopsies were fixed in 4% formaldehyde and embedded in paraffin. Tissue sections were stained with hematoxylin and eosin or were deparaffinized for immunostaining. Primary antibody against ZC3H12A/MCPIP1 protein (1:100; GeneTex, Irvine, CA) was detected with an anti-rabbit secondary antibody labeled with AF488 (Abcam, Cambridge, UK). Slides were visualized with a Nikon Eclipse E600 microscope (Nikon, Melville, NY). Slides and staining were performed by the Plataforma de Recerca Aplicada en Animals de Laboratori (PRAAL) at the Parc Científic of Barcelona (PCB) (Barcelona, Spain).

Ex vivo epidermal cell suspension stimulation by IL-17A

Epidermal cell suspensions were obtained from punch skin biopsy samples from same individuals after an overnight incubation at 4 °C in dispase solution (Discovery Labwere Inc, Bedford, MA) to peel off the epidermal sheets. These sheets were then cut and incubated in trypsin for 20 minutes at 37 °C and were mechanically disaggregated. For IL-17A stimulation, 7×10^4 cells were cultured in a round-bottom, 96-well plate and stimulated at 10 or 50 ng/ml in culture medium (RPMI, 10%; SIGMA-Aldrich, St. Louis, MO), 1% penicillin-streptomycin (SIGMA-Aldrich) and fetal bovine serum (Gibco BRL-Life Technologies, Grand Island, NY).

Flow cytometry

Epidermal cell suspensions were incubated with FcR Binding Inhibitor (eBioscience, San Diego, CA) and stained with anti-CD29-AF700 (Biolegend, San Diego, CA), then fixed in 1% formalin solution and permeabilized with 0.5% saponin (SIGMA-Aldrich). For intracellular staining, anti-MCPIP1 (1:1,000, GeneTex), anti-rabbit IgG–PE (eBioscience), and anti-KRT10-APC (Abcore, Ramona, CA) were used.

Imiquimod-induced psoriasis skin inflammation in mice

Balb/c mice were treated with 62.5 mg of Aldara cream (Meda Pharmaceuticals, Leicester, UK) containing 5% imiquimod (water-in-water), which was topically applied to shaved dorsal skin, as described previously (van der Fits et al., 2009) on six consecutive mornings. Control mice were also shaved and left untreated. Otherwise, a group of mice were additionally treated with 0.5 mg/g of Clovate cream (UCB Pharma, Madrid, Spain) in the evenings. Mice were killed on day 7, and samples from dorsal skin were taken for RNA extraction and histology procedures. *Il17ra*$^{-/-}$ C57BL/6 mice were kindly provided by Dr. Erik Lubberts, and a 5-day application of Aldara cream procedure and subsequent RNA isolation and complementary DNA synthesis were performed by Dr. E.P. Prens's group (Erasmus MC, Rotterdam, The Netherlands). This study was approved by the Dutch Animal Welfare Committee.

RNA isolation and quantitative real-time PCR

RNA was isolated using the Reliaprep RNA Cell Miniprep System (Promega, Madison, WI) and PureLink RNA Mini Kit (Ambion, Life Technologies, Carlsbad, CA), following the manufacturer's instructions. For mouse skin RNA, a first step with TRIzol reagent (SIGMA-Aldrich) was used. Complementary DNA synthesis and quantitative real-time PCR were performed as described by Ferran et al. (2013). The housekeeping genes *ACTB*, *RPS26*, and *Rpl14*

were used for human primary keratinocytes, HaCaT keratinocytes, and mouse samples, respectively. Sequences of primers are shown in Supplementary Tables S1 and S2 online. Fold increase of mRNA levels was obtained using the expression of keratinocytes under stimulation with supernatants of CLA^+ or CLA^- T cells with epidermal cells without SE for each patient as individual baseline.

Statistical analysis

Analysis of variance and *t* test were used to compare mean values. A Mann-Whitney *U* test was used for median comparison of *ZC3H12A* expression in keratinocytes treated with ex vivo co-culture–derived supernatants from psoriatic or healthy control samples. For linear regression analysis, log-transformation of cytokine values was applied to make their distribution approximately normal, and Pearson's coefficient correlation was used. For this purpose, non-detectable cytokine concentrations were assigned to 1 pg/ml for inclusion in the analysis.

CONFLICT OF INTEREST

The authors state no conflict of interest.

ACKNOWLEDGMENTS

This work was supported by European Regional Development Fund grants from the Instituto de Salud Carlos III (PI09/2222 and PI13/01845 [to LFSB]; PI09/07811 [to AGA], and PI13/01716 [to RP]). Projects and grants were coordinated by LFSB. ERR was the recipient of a PhD fellowship from the Ministerio de Educación, Cultura y Deporte. We acknowledge the support of the COST BM0903 Network/SKINBAD (Skin Barrier in Atopic Dermatitis) to LFSB and JJ. Special thanks go to Maria Marcuello for technical support.

SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at www.jidonline.org, and at http://dx.doi.org/10.1016/j.jid.2016.04.030.

REFERENCES

Bata-Csorgo Z, Hammerberg C, Voorhees JJ, Cooper KD. Flow cytometric identification of proliferative subpopulations within normal human epidermis and the localization of the primary hyperproliferative population in psoriasis. J Exp Med 1993;178:1271–81.

Chao J, Dai X, Peña T, Doyle DA, Guenther TM, Carlson MA. MCPIP1 regulates fibroblast migration in 3-D collagen matrices downstream of MAP kinases and NF-κB. J Invest Dermatol 2015;135:2944–54.

Chiricozzi A, Guttman-Yassky E, Suárez-Fariñas M, Nograles KE, Tian S, Cardinale I, et al. Integrative responses to IL-17 and TNF-α in human keratinocytes account for key inflammatory pathogenic circuits in psoriasis. J Invest Dermatol 2011;131:677–87.

Chiricozzi A, Nograles KE, Johnson-Huang LM, Fuentes-Duculan J, Cardinale I, Bonifacio KM, et al. IL-17 induces an expanded range of downstream genes in reconstituted human epidermis model. PLoS One 2014;9(2):e90284.

Ferran M, Galván AB, Rincón C, Romeu ER, Sacrista M, Barboza E, et al. Streptococcus induces circulating CLA+ memory T-cell-dependent epidermal cell activation in psoriasis. J Invest Dermatol 2013;133:999–1007.

Garg AV, Amatya N, Chen K, Cruz JA, Grover P, Whibley N, et al. MCPIP1 Endoribonuclease activity negatively regulates interleukin-17-mediated signaling and inflammation. Immunity 2015;43:475–87.

Ghoreschi K, Gadina M. Jakpot! New small molecules in autoimmune and inflammatory diseases. Exp Dermatol 2014;23:7–11.

Honma M, Benitah SA, Watt FM. Role of LIM kinases in normal and psoriatic human epidermis. Mol Biol Cell 2006;17:1888–96.

Horrocks C, Duncan JI, Oliver M, Thomson AW. Adhesion molecule expression in psoriatic skin lesions and the influence of cyclosporin A. Clin Exp Immunol 1991;84:157–62.

Jura J, Skalniak L, Koj A. Monocyte chemotactic protein-1-induced protein-1 (MCPIP1) is a novel multifunctional modulator of inflammatory reactions. Biochim Biophys Acta 2012;1823:1905–13.

Kasza A, Wyrzykowska P, Horwacik I, Tymoszuk P, Mizgalska D, Palmer K, et al. Transcription factors Elk-1 and SRF are engaged in IL1-dependent regulation of ZC3H12A expression. BMC Mol Biol 2010;11:14.

Krueger JG, Fretzin S, Suárez-Fariñas M, Haslett PA, Phipps KM, Cameron GS, et al. IL-17A is essential for cell activation and inflammatory gene circuits in subjects with psoriasis. J Allergy Clin Immunol 2012;130:145–54.

Kulke R, Bornscheuer E, Schlüter C, Bartels J, Rowert J, Sticherling M, et al. The CXC receptor 2 is overexpressed in psoriatic epidermis. J Invest Dermatol 1998;110:90–4.

Langley RG, Elewski BE, Lebwohl M, Reich K, Griffiths CE, Papp K, et al. Secukinumab in plaque psoriasis—results of two phase 3 trials. N Engl J Med 2014;371:326–38.

Leigh IM, Purkis PE, Whitehead P, Lane EB. Monospecific monoclonal antibodies to keratin 1 carboxy terminal (synthetic peptide) and to keratin 10 as markers of epidermal differentiation. Br J Dermatol 1993;129:110–9.

Leonardi C, Matheson R, Zachariae C, Cameron G, Li L, Edson-Heredia E, et al. Anti–interleukin-17 monoclonal antibody ixekizumab in chronic plaque psoriasis. N Eng J Med 2012;366:1190–9.

Liang J, Wang J, Azfer A, Song W, Tromp G, Kolattukudy PE, et al. A novel CCCH-zinc finger protein family regulates proinflammatory activation of macrophages. J Biol Chem 2008;283:6337–46.

Lipert B, Wegrzyn P, Sell H, Eckel J, Winiarski M, Budzynski A, et al. Monocyte chemoattractant protein-induced protein 1 impairs adipogenesis in 3T3-L1 cells. Biochim Biophys Acta 2014;1843:780–8.

Matsushita K, Takeuchi O, Standley DM, Kumagai Y, Kawagoe T, Miyake T, et al. Zc3h12a is an RNase essential for controlling immune responses by regulating mRNA decay. Nature 2009;458:1185–90.

Mino T, Murakawa Y, Fukao A, Vandenbon A, Wessels HH, Ori D, et al. Regnase-1 and roquin regulate a common element in inflammatory mRNAs by spatiotemporally distinct mechanisms. Cell 2015;161:1058–73.

Mizgalska D, Węgrzyn P, Murzyn K, Kasza A, Koj A, Jura J, et al. Interleukin-1-inducible MCPIP protein has structural and functional properties of RNase and participates in degradation of IL-1β mRNA. FEBS J 2009;276:7386–99.

Niu J, Azfer A, Zhelyabovska O, Fatma S, Kolattukudy PE. Monocyte chemotactic protein (MCP)-1 promotes angiogenesis via a novel transcription factor, MCP-1-induced protein (MCPIP). J Biol Chem 2008;283:14542–51.

Nograles KE, Zaba LC, Guttman-Yassky E, Fuentes-Duculan J, Suárez-Fariñas M, Cardinale I, et al. Th17 cytokines interleukin (IL)-17 and IL-22 modulate distinct inflammatory and keratinocyte-response pathways. Br J Dermatol 2008;159:1092–102.

Ohta Y, Katayama I, Funato T, Yokozeki H, Nishiyama S, Hirano T, et al. In situ expression of messenger RNA of interleukin-1 and interleukin-6 in psoriasis: interleukin-6 involved in formation of psoriatic lesions. Arch Dermatol Res 1991;283:351–6.

Papp KA, Leonardi C, Menter A, Ortonne JP, Krueger JG, Kricorian G, et al. Brodalumab, an anti–interleukin-17–receptor antibody for psoriasis. N Engl J Med 2012a;366:1181–9.

Papp KA, Menter A, Strober B, Langley RG, Buonanno M, Wolk R, et al. Efficacy and safety of tofacitinib, an oral Janus kinase inhibitor, in the treatment of psoriasis: a phase 2b randomized placebo-controlled dose-ranging study. Br J Dermatol 2012b;167:668–77.

Peric M, Koglin S, Kim SM, Morizane S, Besch R, Prinz JC, et al. IL-17A enhances vitamin D3-induced expression of cathelicidin antimicrobial peptide in human keratinocytes. J Immunol 2008;181:8504–12.

Ruiz-Romeu E, Ferran M, Sagristà M, Gómez J, Giménez-arnau A, Herszenyi K, et al. *Streptococcus pyogenes*-induced CLA+ T cell-dependent epidermal cell activation triggers Th17 responses in guttate psoriasis [e-pub ahead of print]. J Allergy Clin Immunol 2016; http://dx.doi.org/10.1016/j.jaci.2016.02.008 (accessed 4 April 2016).

Russell CB, Rand H, Bigler J, Kerkof K, Timour M, Bautista E, et al. Gene expression profiles normalized in psoriatic skin by treatment with brodalumab, a human anti-IL-17 receptor monoclonal antibody. J Immunol 2014;192:3828–36.

Sano S, Chan KS, Carbajal S, Clifford J, Peavey M, Kiguchi K, et al. Stat3 links activated keratinocytes and immunocytes required for development of psoriasis in a novel transgenic mouse model. Nat Med 2005;11:43–9.

Shi X, Jin L, Dang E, Chang T, Feng Z, Liu Y, et al. IL-17A upregulates keratin 17 expression in keratinocytes through STAT1- and STAT3-dependent mechanisms. J Invest Dermatol 2011;131:2401–8.

Simanski M, Rademacher F, Schröder L, Schumacher HM, Gläser R, Harder J. IL-17A and IFN-γ synergistically induce RNase 7 expression via STAT3 in primary keratinocytes. PLoS One 2013;8:e59531.

Somma D, Mastrovito P, Grieco M, Lavorgna A, Pignalosa A, Formisano L, et al. CIKS/DDX3X interaction controls the stability of the Zc3h12a mRNA induced by IL-17. J Immunol 2015;194:3286–94.

Sønder SU, Paun A, Ha HL, Johnson PF, Siebenlist U. CIKS/Act1-mediated signaling by IL-17 cytokines in context: Implications for how a CIKS gene variant may predispose to psoriasis. J Immunol 2012;188:5906–14.

Sparna T, Rétey J, Schmich K, Albrecht U, Naumann K, Gretz N, et al. Genome-wide comparison between IL-17 and combined TNF-alpha/IL-17 induced genes in primary murine hepatocytes. BMC Genomics 2010;11:226.

Staquet MJ, Dezutter-Dambuyant C, Zambruno G, Schmitt D. Human epidermal basal keratinocytes express CDw29 antigens. Br J Dermatol 1989;121:577–85.

Sun J, Zhao Y, Hu J. Curcumin inhibits imiquimod-induced psoriasis-like inflammation by inhibiting IL-1beta and IL-6 production in mice. PLoS One 2013;8:e67078.

Suzuki HI, Arase M, Matsuyama H, Choi YL, Ueno T, Mano H, et al. MCPIP1 ribonuclease antagonizes dicer and terminates microRNA biogenesis through precursor microRNA degradation. Mol Cell 2011;44:424–36.

Tsuchida S, Bonkobara M, McMillan JR, Akiyama M, Yudate T, Aragane Y, et al. Characterization of Kdap, a protein secreted by keratinocytes. J Invest Dermatol 2004;122:1225–34.

van der Fits L, Mourits S, Voerman JS, Kant M, Boon L, Laman JD, et al. Imiquimod-induced psoriasis-like skin inflammation in mice is mediated via the IL-23/IL-17 axis. J Immunol 2009;182:5836–45.

Vrotsos EG, Kolattukudy PE, Sugaya K. MCP-1 involvement in glial differentiation of neuroprogenitor cells through APP signaling. Brain Res Bull 2009;79:97–103.

Wang K, Niu J, Kim H, Kolattukudy PE. Osteoclast precursor differentiation by MCPIP via oxidative stress, endoplasmic reticulum stress, and autophagy. J Mol Cell Biol 2011;3:360–8.

Xie S, Chen Z, Wang Q, Song X, Zhang L. Comparisons of gene expression in normal, lesional, and non-lesional psoriatic skin using DNA microarray techniques. Int J Dermatol 2014;53:1213–20.

Zhou L, Azfer A, Niu J, Graham S, Choudhury M, Adamski FM, et al. Monocyte chemoattractant protein-1 induces a novel transcription factor that causes cardiac myocyte apoptosis and ventricular dysfunction. Circ Res 2006;98:1177–85.

Supplementary material

***Ex vivo* coculture psoriatic model**

Ex vivo cocultures with T cells and epidermal cells were established, as described by Ferran *et al.* (2013). Briefly, memory CLA^+ and CLA^- T cells were isolated from blood samples and epidermal cells were obtained from skin biopsies from the same individuals. $3x10^4$ epidermal cells and $5x10^4$ CLA^+ or CLA^- T cells were seeded together in flat bottom 96-well plate, and stimulated with a sonicated preparation of *Streptococcus pyogenes* (5 µg/ml). Five days after stimulation supernatants were collected and cytokines were measured by multiplex fluorescent bead-based immunoassay.

HaCaT stimulation and Western blot

$2x10^5$ HaCaT cells were seeded in a 12 well plate for 48 h and the stimulated for 6, 15 and 24 h with aan optimum concentration of IL-17A (33 ng/ml). HaCaT cells were washed with PBS and harvested in RIPA buffer supplemented with Protease Inhibitor Cocktail (SIGMA-Aldrich). Protein concentration in cell lysates was measured with the bicinchoninic acid assay. Cell lysates containing 40 µg of total protein were separated in 10% SDS-PAGE and electrotransfered to a PVDF membrane (Merck Millipore). Membranes were blocked for 1h in 3% non-fat milk dissolved in Tris-buffered saline containing 0.1% Tween-20 (BioShop). Membranes were then incubated with primary antibody overnight at 4 °C. After incubation with secondary antibody (1h, RT), chemiluminescence was detected using an enhanced chemiluminescence reagent Luminata Crescendo (Merck Millipore) and ChemiDoc XRS chemiluminescence detector (Bio-Rad). The following antibodies were used: rabbit anti-MCPIP1 (1:2,000, GeneTex), mouse anti-tubulin (1:1,000; Calbiochem), peroxidase-conjugated

anti-rabbit (1:30,000; SIGMA-Aldrich) and peroxidase- conjugated anti-mouse (1:20,000, BD Pharmingen).

Analysis of signaling pathways activation after IL-17-stimulation.

Primary keratinocytes ($2x10^5$per well) were seeded on a 12-well culture plate. After 48h, cells were stimulated with IL-17 in a final concentration of 40 ng/ 600µl, for 15h. Cells were then washed with PBS, harvested and lysed in RIPA buffer supplemented with Protease Inhibitor Cocktail (SIGMA-Aldrich). For phosphorylated protein detection phosphatase inhibitor cocktail (PhosSTOP, Roche) was added to RIPA buffer. Protein concentration in cell lysates was measured with the bicinchoninic acid assay. 40 µg of total protein was separated on SDS/PAGE 10% polyacrylamide gel, electrotransfered to a PVDF membrane (Millipore) and blocked in 5% non-fat milk dissolved in Tris-buffered saline containing 0.05% Tween (BioShop). Membranes were then incubated with primary antibody overnight at 4°C. After incubation with secondary antibody (1 h, RT), chemiluminescence was detected using Luminata Crescendo (Millipore) substrate and a chemiluminescence detector ChemiDoc (Bio-Rad). The following antibodies were used: rabbit anti-MCPIP1 (1:2,000, GeneTex), rabbit anti-phospho-STAT3 (1:500, Cell Signaling), rabbit anti-phospho-p65 (1:1,000, Cell Signaling), rabbit polyclonal anti-phospho-p38 (1:1,000, Cell Signaling), rabbit anti-phospho-ERK (1:1,000, Cell Signaling), mouse anti-tubulin (1:1,000, Sigma), peroxidase-conjugated anti-rabbit (1:30,000; SIGMA-Algrich) and peroxidase-conjugated anti-mouse (1:20,000, SIGMA-Aldrich). The densitometry analysis was performed with IMAGEJ 1.40G software. All measures values were normalized to alpha-tubulin expression level.

JAK2/STAT activation

Primary keratinocytes ($2x10^5$) were seeded in a 12-well plate for 48 h and then incubated for 1 h with 1 μM AZD1480 (SelleckChem, Houston, TX, USA) or dimethyl sulfoxide (DMSO, 0.02%). Cells were then stimulated with IL-6 (40 ng/ml; PromoKine, Heidelberg, Germany) or with IL-17A (67 ng/ml; Myltenyi Biotech) for 15 h, washed with PBS, harvested and lysed in RIPA buffer supplemented with Protease Inhibitor Cocktail (SIGMA-Aldrich). 40 μg of total protein was separated on SDS/PAGE 10% polyacrylamide gel, electrotransfered to PVDF membrane (Millipore, Billerica, MA, USA) and blocked in 5 % non-fat milk dissolved in Tris-buffered saline containing 0.05% Tween (BioShop, Canada). Membranes were then incubated with primary antibody overnight at 4 °C. After incubation with secondary antibody (1 h, RT), chemiluminescence was detected using Luminata Crescendo (Millipore) substrate and chemiluminescence detector ChemiDoc (Bio-Rad, Hercules, CA, United States). The antibodies were as follow: rabbit anti-MCPIP1 (1:2,000, GeneTex), rabbit anti-phospho-STAT3 (1:500, Cell Signaling, Beverly, MA, USA), mouse anti-tubulin (1:1,000, SIGMA-Aldrich), peroxidase-conjugated anti-rabbit (1:30,000; SIGMA-Aldrich); and peroxidase-conjugated anti-mouse (1:20,000, SIGMA-Aldrich). The densitometry analysis was performed with IMAGEJ 1.40G software. All measures values were normalized to alpha-tubulin expression level.

Silencing of *ZC3H12A* expression in HaCaT cells

To obtain stable MCPIP1-silenced HaCaT cell line we used a lentiviral system. Lentiviruses were generated using a second generation packaging system. Packaging plasmid psPAX2 (Addgene plasmid # 12260) and an envelope plasmid pMD2.G (Addgene plasmid # 12259) were gifts from Didier Trono. For silencing, a plasmid expressing shRNA sequence towards *ZC3H12A* was used (SIGMA-Aldrich). As a control of silencing we used plasmid carrying unspecific scrambled

sequence (SIGMA-Aldrich). Packaging was performed in HEK293 cells by co-transfection of $5x10^6$ cells with 15 µg of psPAX2, 6 µg of pMD2.G and 20 µg of adequate expression plasmid. Transfection was facilitated by addition of PEI in a 3:1 (w/w) ratio of PEI:DNA. Infection medium was harvested 2 days later, quickly frozen in liquid nitrogen and stored at -80 °C until use in later experiments. A relative titer of generated lentiviruses was measured by real-time PCR using primers targeting coding sequence of puromycin N-acetyl-transferase (FW: CCGACTACCCCGCCACG, RV: GACCCACACCTTGCCGATG). HaCaT cells transduction was carried out in suspension. 10^5 cells were seeded on an 8-cm^2 culture dish in growth medium mixed 1:1 with infection medium and 6 µg/ml polybrene. After 24h, medium was changed for normal growth medium. Three days after transduction, puromycin was added to the medium (1µg/ml) in order to select positively-transduced cells. After 7 days of selection the knockdown was verified by Western blotting.

Stimulation assay with HaCaT cells transduced with shRNA

$1x10^5$ were seeded at 1 ml/well in a 12 well plate and stimulated after 24h with 33 ng/ml of IL-17A. After 15 h of incubation protein or RNA extraction was performed. Western blot procedure was followed as mentioned above.

Supplementary Figure S1

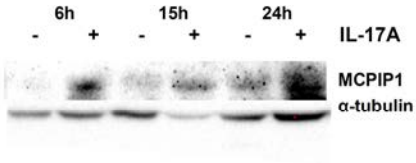


Figure S1. Protein levels of MCPIP1 are induced by IL-17A in HaCaT cells at early and late time points. Protein extracts from HaCaT cells were analyzed by Western blot at 6, 15 and 24 h after IL-17A stimulation (33 ng/ml).

Supplementari Figure S2

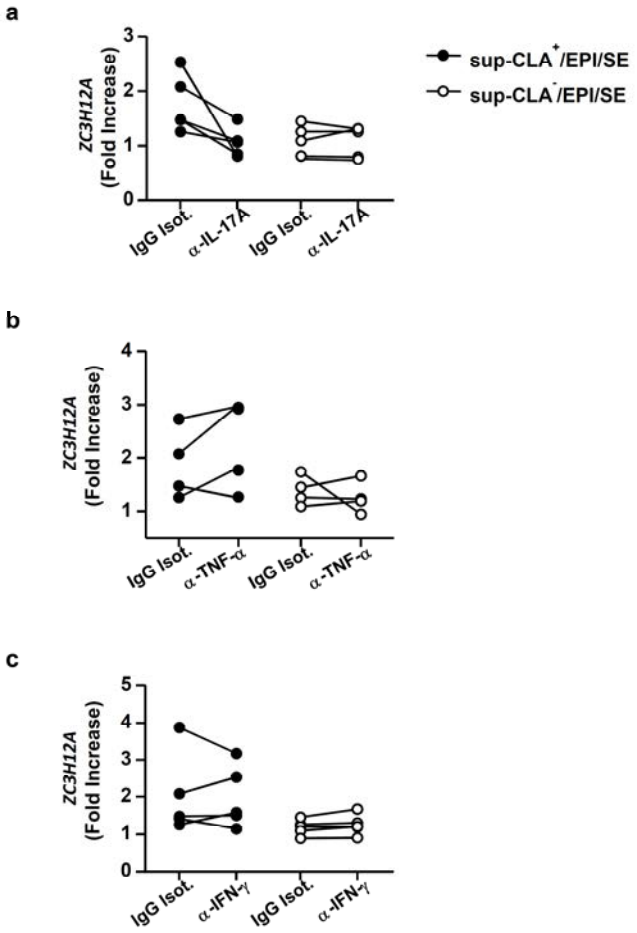


Figure S2. *ZC3H12A* mRNA expression in keratinocytes stimulated by pre-treated supernatants with neutralizing antibodies against IL-17A, TNF-α or IFN-γ. Supernatants from ex vivo SE-activated CLA^+/EPI or CLA^-/EPI cocultures from psoriatic patients were pre-incubated with IgG isotype control, anti-IL-17A (**a**), anti-TNF-α (**b**), or anti-IFN-γ (**c**) for 30 min before being used to stimulate *in vitro* cultured keratinocytes (n=4-5). Each dot represents a supernatant from one *ex vivo* coculture from a psoriatic patient.

Supplementary Figure S3

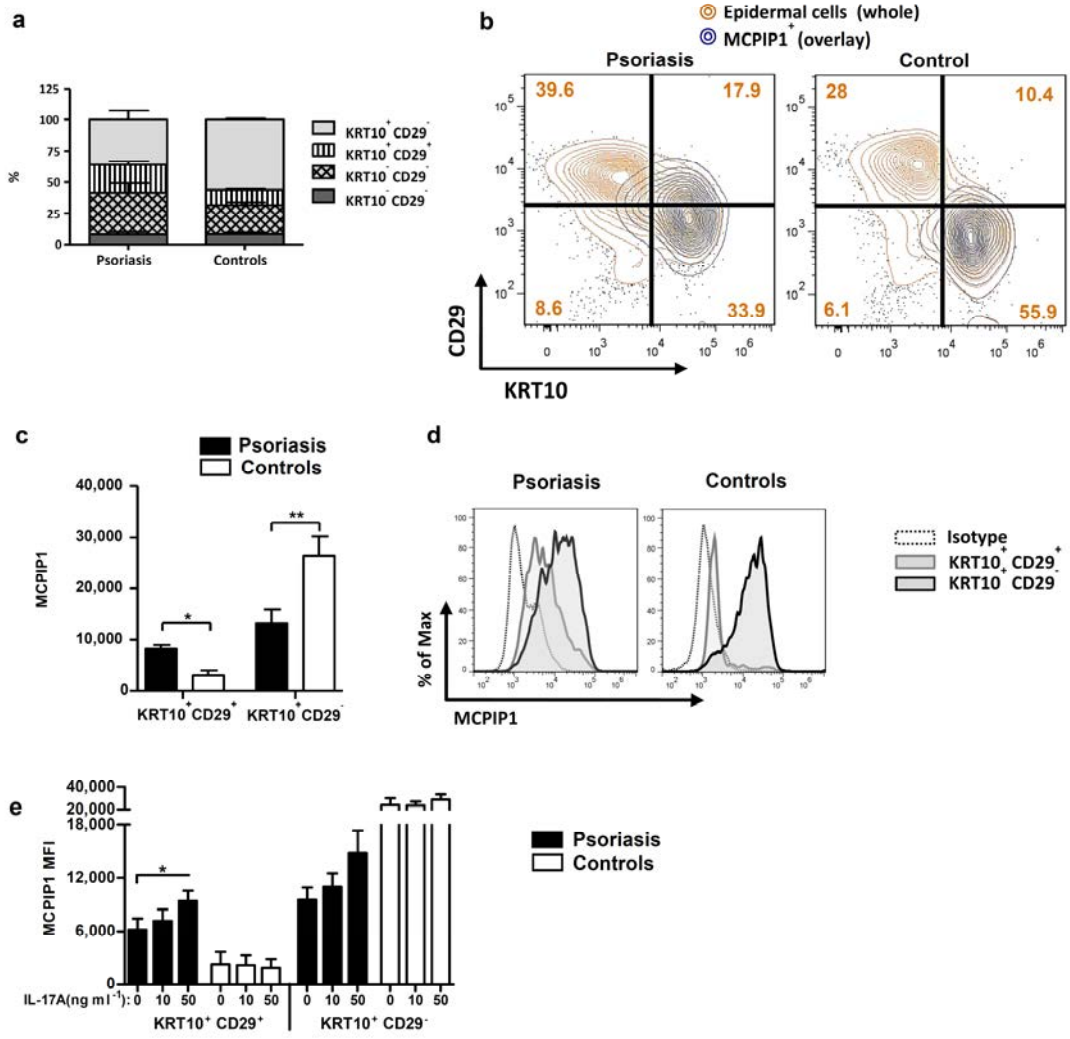


Figure S3. Flow cytometry analysis of KRT10, CD29 and MCPIP1 expression in epidermal cell suspensions. (**a**) Cumulative graph shows distribution of distinct epidermal cell subsets on the basis of KRT10 and CD29 expression from psoriatic (n=4) or control (n=3) cell suspensions. (**b**) Overlay of MCPIP1^{+} cells (blue contour) over whole epidermal cells (orange contour) acquired on a KRT10/CD29 quadrant graph (orange bold numbers refers to all epidermal population subsets within each quadrant). One representative psoriatic and healthy sample is shown. (**c**) MFIs for MCPIP1 expression in transient mid-differentiating cells (KRT10^{+}CD29^{+}) and in highly differentiated cells (KRT10^{+}CD29^{-}), represented by histograms in a representative psoriatic and control sample (**d**). A Student t-test was used to check differences versus isotype conditions (*p<0.05 and **p<0.01). (**e**) MCPIP1 MFI in epidermal cell suspensions from 5 psoriatic and 3 controls samples stimulated with IL-17A (10 and 50 ng/ml) for 4 h. Paired t-test comparison analysis was used between stimuli conditions. Data are presented as mean ± SEM.

Supplementary Figure S4

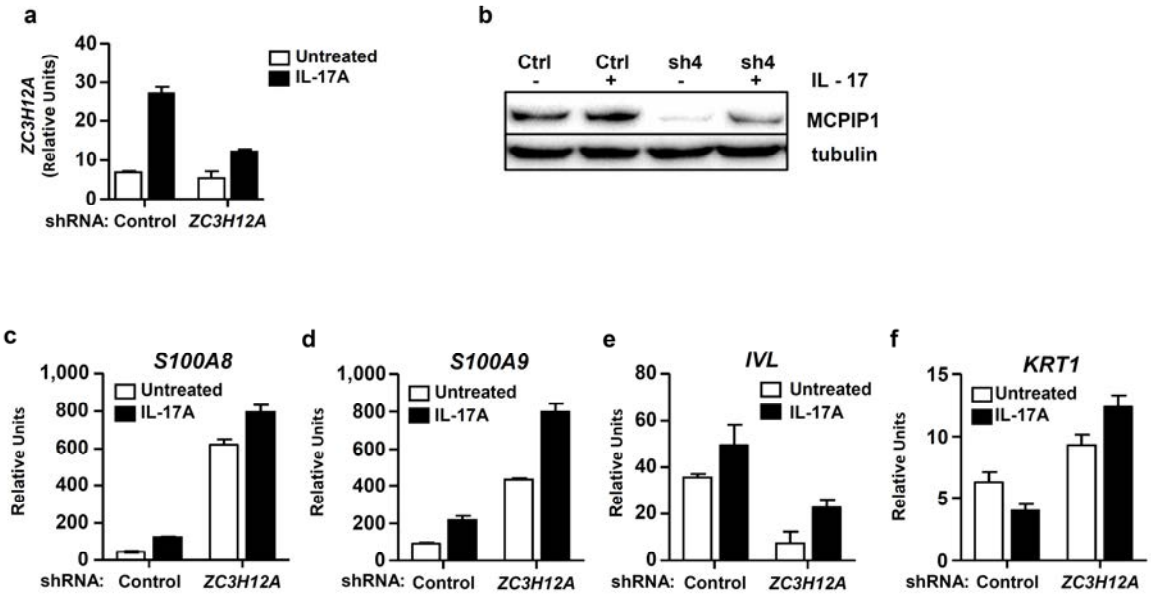


Figure S4. MCPIP1 might modulate IL-17A-target suprabasal epidermis genes in psoriasis. MCPIP1-silencing in HaCaT keratinocytes by lentiviral plasmid transfection was verified at mRNA (qRT-PCR) (**a**) and protein (Western blot) (**b**) levels after selection of positively-transduced cells which were stimulated or not with IL-17A for 15 h. Extracted RNA from the same IL-17A-stimulated or untreated silenced HaCaT cells was used to quantify mRNA levels of *S100A8* (**c**), *S100A9* (**d**), *IVL* (**e**) and *KRT1* (**f**), all epidermal genes that show altered expression in psoriasis. Data presented as mean ± SD of qRT-PCR triplicates of one silencing experiment.

Supplementary Figure S5

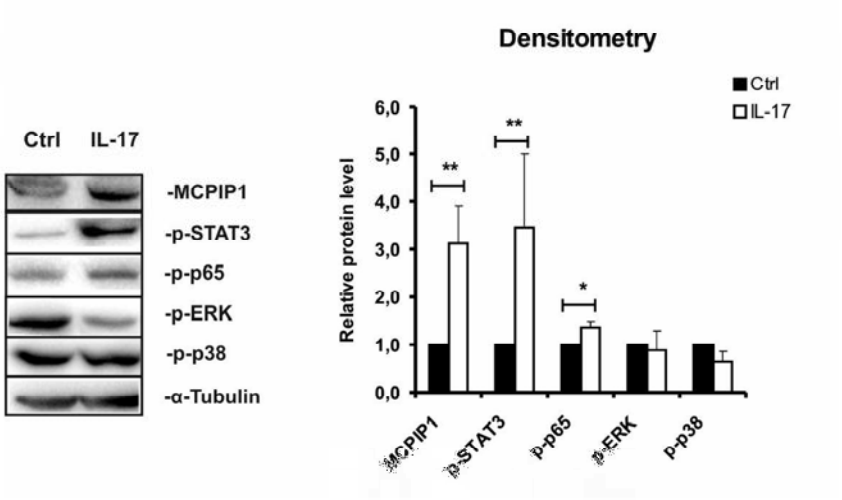


Figure S5. Analysis of signaling pathways activation after IL-17 stimulation. Primary keratinocytes were stimulated with IL-17A for15 h. MCPIP1 and phosphorylated-STAT3, -p65, -ERK, and -p-38 were detected from total protein. Measures were normalized to alpha-tubulin expression level. Statistical analysis was done on the basis of results from three independent experiments and a two-tailed Student's t test was used for data analysis.

Table S1. Human primers sequences.

	5' →3' Forward	5' →3' Reverse
ACTB	AGCCTCGCCTTTGCCGATCC	ACCATCACGCCCTGGTGCCT
RPS26	GCAGCAGTCAGGGACATTTCTG	TTCACATACAGCTTGGGAAGCA
ZC3H12A	GGCAGTGAACTGGTTTCTGGA	GATCCCGTCAGACTCGTAGG
S100A8	GAATTTCCATGCCGTCTACAGG	GCCACGCCCATCTTTATCACCAG
S100A9	GCTCCTCGGCTTTGACAGAGTGCAAG	GCATTTGTGTCCAGGTCCTCCATGATGTGT
KRT1	ATTTCTGAGCTGAATCGTGTGATC	CTTGGCATCCTTGAGGGCATT
IVL	GATGTCCCAGCAACACACAC	TGCTCACATTCTTGCTCAGG

Table S2. Mouse primers sequences.

	5' →3' Forward	5' →3' Reverse
L14	TCCCAGGCTGTTAACGCGGT	GCGCTGGCTGAATGCTCTG
Zc3h12a	ACGAAGCCTGTCCAAGAATCC	TAGGGGCCTCTTTAGCCACA

SUMMARY OF RESULTS AND GENERAL DISCUSSION

SUMMARY OF RESULTS

The main purpose of this thesis has been to take advantage of an *ex vivo* model to study psoriasis immunopathology and deepen in the contribution of key cellular and inflammatory mediators to this pathogenesis. Essentially, this model is sustained by the use of circulating skin-homing CLA^+ T cells, which due to their recirculation capacity between the skin and peripheral blood, and their effector memory phenotype, allows obtaining relevant translational information of the disease when these cells respond to an *ex vivo* re-stimulation after their isolation from blood of psoriasis patients. Additionally, in order to reproduce the *in situ* interaction between those T cells and epidermal cells, the *ex vivo* approach contains autologous epidermal cells isolated from psoriatic lesions as a possible source for antigen presentation. Then, the effector function from T cells was evaluated under the activation exerted by *Streptococcus pyogenes*, a relevant innate trigger in psoriasis which has been clinically associated with the onset or exacerbation of guttate and plaque psoriasis, respectively. Thus, this model allows the use of cells from either guttate and plaque psoriasis samples.

In answering the first objective, several studies were performed in reference to the effector responses exerted by the cocultures. First, guttate psoriasis-derived samples were recruited in order to assess differences depending on the underlying environmental and genetic conditions from donors. Guttate-type onset is characterized by acute development of small rounded psoriatic lesions. This psoriasis form is mostly confined to HLA-Cw6 allele carriers, and streptococcal pharyngitis often precedes the onset of this particular psoriasis phenotype (Prinz, 2001). Therefore, we found that CLA^+/Epi cocultures from the cohort of guttate psoriasis patients whose flares were associated with a prior throat infection, and who were also HLA-Cw6–positive, displayed the highest responses to *S. pyogenes*, and presented a predominant Th17 (IL-17A and IL-17F) response. Such predominance was also supported by the fact that keratinocytes treated with enriched supernatants had increased expression levels of IL-17– targeted genes.

Following to these first results, in which we describe a particular response from guttate psoriasis patients, several psoriasis patients were used, either with guttate or chronic plaque-type psoriasis, to evaluate the IL-9 role in our cocultures. Many Th9 cells are tropic for the skin (CLA^+), and an aberrant activation of those cells may contribute to

inflammatory diseases of the skin (Schlapbach et al., 2014). In addition, IL-9, is known to play a role as a mediator of Th17-driven inflammatory diseases (Nowak et al., 2009). *Candida albicans* was shown to selectively induce IL-9 production by CLA^+ T cells (Schlapbach et al., 2014). Using the *ex vivo* model of psoriasis with *S. pyogenes* as a trigger, IL-9 production was also preferentially produced by CLA^+/Epi cocultures from psoriasis patients, thus extending the list of pathogens capable of such IL-9–produced CLA-selectivity in psoriasis. Furthermore, when IL-17A and IFN-γ production was simultaneously evaluated in the cocultures in the presence of neutralizing anti–IL-9 antibody, only IL-17A amounts were supported by 50% by IL-9.

Characterization of cytokine responses in the *ex vivo* model has then established three key T-cell–derived cytokine against *S. pyogenes*: IL-17A, IFN-γ and IL-9. Once again focusing in guttate psoriasis, a third study was performed to evaluate further translational value of circulating CLA^+ T cells regarding disease severity and ASO levels, as a reflection of streptococcal infection. Common peaks were observed when subjects with raised ASO titers (>15 days of disease onset) were compared in parallel with PASI score and *ex vivo* cytokine responses in CLA^+/Epi cocultures.

With regard to the second objective, the use of enriched supernatants, generated by the *ex vivo* coculture system, on *in vitro* cultured keratinocytes was assessed in order to identify abnormally regulated genes potentially involved in the disease. An IL-17A–induced transcript was identified, the transcript *ZC3H12A*, which encodes for the ribonuclease monocyte chemotactic protein-1–induced protein (MCPIP1). MCPIP1 expression was localized *in situ* in the epidermis from both healthy and psoriasis skin, however its distribution in psoriasis samples was found to be abnormal, and its expression was increased with the exogenous addition of recombinant IL-17A. Genes involved in the epidermal differentiation, along with other differentiated expressed genes in psoriasis, were altered by the silencing of *ZC3H12A*. In addition, we describe an unknown up-stream mechanism in the induction of MCPIP1, which had been commonly associated with nuclear-factor kappa B (NF-κB), that also involves phosphorylation of signal transducer and activator (STAT)-3 signaling in keratinocytes.

Overall, it is demonstrated that this *ex vivo* model of psoriasis is a useful tool that provides relevant translational information and allows the characterization of novel mechanisms involved in the disease.

GENERAL DISCUSSION

Psoriasis is an inflammatory immune-mediated skin disease that usually occurs in individuals with genetic susceptibility in conjunction with environmental triggers. Circulating effector memory skin-tropic CLA^+ T cells have been shown to participate in the development of several immune cell-mediated cutaneous diseases, due to its selective recruitment into the tissue and the subsequent rapid effector response upon cognate antigen recognition. Several studies point to the capacity of recirculation of CLA^+ T cells between skin and blood, which suggests that in skin diseases implying chronicity, such as psoriasis, the study of circulating CLA^+ T cells could constitute a tool for translational evaluation of current pathology status (Ferran et al., 2013a). In this regard, we proposed an *ex vivo* model that uses isolated memory CLA^+ T cells from patient's blood, which then are cultured together with autologous epidermal cells, in order to match more accurately a proper intercellular interaction in the cutaneous context of skin lesions. Once established the cellular basis, this experimental approach is completed by the addition of a clinical relevant trigger. *Streptococcus pyogenes* may contribute to the onset and exacerbation of guttate and plaque-type psoriasis, respectively, and many reports refer to a close functional relation between streptococcal throat infections and subsequent CLA^+ T cell generation and sensitization as an initial factor of lesions development (Prinz, 2001). Actually, both guttate and chronic plaque psoriasis-derived cocultures responded to an extract of *S. pyogenes* (SE), leading to the production of several psoriasis-associated Th1/Th17/Th22-signature cytokines (Ferran et al., 2013b). The SE-specificity in the inflammatory coculture responses produced by the CLA^+ subset in psoriasis was further supported by the fact that the use of a non-specific stimulus, such as SEB, was able to induce an immune reaction either in CLA^+ and CLA^- T-cell subsets, and from psoriasis or healthy samples.

In an attempt to go further, this *ex vivo* approach has been used for the characterization of diverse features of the disease, including the observation of differential effector profiles based on the characteristics of patients, and the study of less characterized inflammatory mediators and molecules potentially involved in psoriasis.

Guttate psoriasis is characterized by an acute onset of small rounded psoriatic lesions and is mostly confined to those carrying the HLA-Cw6 allele (Asumalahti et al., 2003), which consists on a high-risk allele revealed by numerous genetic linkage studies

(Griffiths and Barker, 2007). Furthermore, among HLA-Cw6–positive patients with psoriasis, streptococcal pharyngitis, mainly by *Streptococcus pyogenes*, may contribute to the onset or exacerbation of the inflammatory process of lesions (Mallbris et al., 2009). Given that streptococcal pharyngitis often precedes guttate lesions outbreaks, it has been proposed that patients who suffer this particular phenotype of disease may display a genetically determined sensitivity to streptococcal infection (Weisenseel et al., 2002). Also, such microbial-triggered events in guttate psoriasis provide a useful tool to study a clear starting point of psoriasis disease, and to examine early events in its development (Leung et al., 1995b). In this context, and given that more than one-third of guttate patients develop chronic plaque disease in the future (Ko et al., 2010; Langley et al., 2005), the study of guttate-derived samples in our *ex vivo* model might help elucidating initial immune responses in psoriasis development. Here, the evaluation of the cytokines produced in SE-activated CLA^+/Epi cocultures first revealed that those subjects that were carriers of the HLA-Cw6 allele, and had a previous pharyngitis episode, displayed higher Th17 responses, in terms of IL-17A and IL-17F production, than other inflammatory-associated T-cell signatures, as it is the Th1-derived IFN-γ. These results are in line with high serum IL-17 levels found in patients with psoriasis with early spreading guttate morphology (Choe et al., 2012), and accords with a reported psoriasis bimodal immunopathology theory, proposing that psoriasis is first initiated by IL-1/Th17 dominated response (Christophers et al., 2014). Therefore, these results support that circulating CLA^+ T cells from patients with guttate psoriasis preceded by streptococcal infection, should preferentially respond to SE, and producing high levels of IL-17A and IL-17F.

The enriched cytokine content within the collected supernatants, which results from an adaptive (CLA^+ T cell) and innate (epidermal cells) interaction, and in the presence of SE, represents an inflammatory milieu that could itself induce a switch in gene expression in normal keratinocytes, resembling how non-lesional keratinocytes are affected by the surrounding inflammatory focus in an active lesion. With this aim, it was found that IL-17–targeted transcripts, *DEFB4*, *S100A7*, *LCN2*, *IL8*, and *IL36G*, which are up-regulated in psoriasis, were strongly induced by the supernatants in normal keratinocytes. Interestingly, filaggrin *(FLG)* and loricrin *(LOR)* transcript levels were reduced by the same supernatants, thereby suggesting that the inflammatory mediators induced by SE-activation of CLA^+/Epi cocultures from the responding-cohort of guttate patients would also participate in the skin barrier impairment, which

also contributes to the development of skin lesions. In fact, low and altered expression of *FLG* and *LOR* are found in psoriatic lesions, which could be modulated by the underlying inflammatory milieu (Kim et al., 2011; Roberson and Bowcock, 2010), and remarkably, *FLG* expression can be decreased by IL-17 in keratinocytes (Gutowska-Owsiak et al., 2012). Although psoriasis as a chronic phase is usually described to be predominated by Th17 and Th1 immune responses (Lowes et al., 2008), our results point to a clear predominance by the Th17 profile in predisposed guttate-type subjects, and, by extrapolation, in the initial stages of disease. Such Th17/Th1 cytokine unbalanced production seems to be lost in chronic plaque patients, in which IL-17A production is slightly decreased to reach similar levels to that of IFN-γ in the cocultures.

Aside of the well-known incidence of IL-17 and IFN-γ cytokines in psoriasis, the exploration of other T-cell–derived cytokines could help understanding the disease, especially those that could promote IL-17 production, such as IL-9. Up to this point, the *ex vivo* model has proven to reveal psoriasis-associated events by the use of both, guttate and plaque-type psoriasis samples, thus the IL-9 presence in the supernatants as a response to SE would support its relevant role in the disease, which has already been suggested by a few publications. First, $CD4^+$ T cells isolated from blood of psoriasis patients produce higher levels of IL-9 than healthy controls (Singh et al., 2013), and second, skin-homing CLA^+ T cells were described to be the main IL-9 producers in human (Schlapbach et al., 2014). Both concepts are contemplated in the *ex vivo* approach, psoriasis and CLA^+ T cells, but additionally integrating a clinically associated triggering pathogen. In the same way as IL-17A, SE-dependent production of IL-9 by the system was high selective for the psoriatic CLA^+/Epi cocultures but did not show a prevalence within guttate or plaque psoriasis. IL-17A, IFN-γ and IL-9 were simultaneously produced by the CLA^+/Epi coculture, though only IL-17A levels were decreased by a 50% upon IL-9 neutralization. These results reveal a putative role of IL-9 function in psoriasis development, at least by enhancing IL-17A production.

CLA^+ T cells represents the functional link between streptococcal throat infection and psoriasis lesions. If these cells recirculate, the immune response exerted by streptococcal-sensitized CLA^+ T cells to a strep re-stimulation could reflect more accurately some of the clinical features in patients that had reported a pharyngitis episode, and shortly after developed psoriasis, a sequence of facts that can be more easily traced in guttate-type psoriasis. In such patients, elevated ASO titers are usually found, especially after a 15-days period since lesion onset, at least in participants

included in this thesis. Following this period, varying degrees of raised ASO titers among guttate psoriasis patients may be justified by individual factors, such as an increased exposure to *S. pyogenes* due to persistent throat colonization or repeated infections, as well as by the time period after throat infections. In this regard, the highest ASO values were found in patients with 1 to 2 months of disease duration. Interestingly, this peak was also observed in disease severity, in terms of PASI score, as well as in IL-17A, IFN-γ and IL-9 levels produced by the *ex vivo* SE-activation of CLA^+/Epi cocultures from those patients. This suggests that the *ex vivo* behavior against re-stimulation with *S. pyogenes* of circulating CLA^+ T cells, within a particular period of time, may reflect the clinical status of those patients, that is streptococcal antigens exposure (ASO) and severity of guttate eruptions (PASI).

Conversely, in an opposite situation, cocultures from plaque psoriasis patients without any recent history of throat infection, as it is also stated by their negative ASO titers, still responded more to SE than healthy controls. However, the possibility of the existence of a streptococcal burden in those patients cannot be discarded (Sigurdardottir et al., 2013; Thorleifsdottir et al., 2016), and it might be unraveled by other diagnostic tools rather than ASO titers, an issue that needs further investigation. Also, the long interval since disease onset, the possible uncontrolled exposure to antibiotics during this period, and the lack of an acute activity of disease stage, make it difficult to establish a link between possible ancient infections, disease symptoms, and circulating CLA^+ T cells activity against SE in long-lasting plaque-type disease.

The complexity of streptococcal components contained in the *S. pyogenes* sonicated extract, as well as the frequency of cellular components configuring the coculture, might determine the variability in the *ex vivo* immune response. Regarding to the cellular interaction, one may question the relevance of the epidermal component. In a previous publication, it was already discussed that cytokine response and epidermal activation in the SE-activated CLA^+/Epi cocultures would not be attributed to those immune cells within the epidermal cell suspension, since CLA^-/Epi cocultures displayed much less or none activity (Ferran et al., 2013b). However, here it is demonstrated that optimal production of IL-17A, IFN-γ and IL-9 depended on the presence of autologous epidermal cells, thereby suggesting that intercellular interactions may occur. One possible molecular interaction between T cells and epidermal cells would be the presentation through HLA molecules of SE-derived peptides, or other self-epitopes exposed due to the inflammatory milieu (Besgen et al., 2010; Shi et al., 2011). $CD4^+$ T cells accounts for nearly 80% of total T-lymphocyte population in the cocultures (see

page 175, Figure A1 in Appendix II), and thus it could explain the drastic 90–100% reduction in IL-17A, IFN-γ and IL-9 production when HLA class II was blocked. Remarkably, the blocking of HLA class I molecules, which interact with $CD8^+$ T cells, resulted in a 50% reduction of cytokine production, indicating a noteworthy role of $CD8^+$ T cells, despite their minor presence in the cocultures. Although antigen-driven T cells might have a major role in psoriasis lesions, especially in chronic plaques (Valdimarsson et al., 2009), the use of circulating memory T cells in the *ex vivo* system may imply a higher frequency of T cells activated by SAgs, rather than by conventional processed antigen (Yarwood et al., 2000), especially in guttate psoriasis, in which expansion of SAg reactive T-cell populations has been demonstrated (Leung et al., 1995b). In fact, the SAg interaction between the HLA-DR, either from professional APCs (LCs, DCs or macrophages), and/or from non-professional APCs (keratinocytes, melanocytes), and the TCR, cannot be discarded since it could be blocked by those antibodies to HLA-DR used in the coculture. In any case, the strongest decrease in activity found upon blockage of HLA-DR, suggests an early mechanism dependent on HLA class II-associated activation and inflammatory effects. Consequently, self-antigens exposure, autoreactive CLA^+ $CD8^+$ T-cell expansion, and/or diminished self-recognition threshold by those T cells, may contribute to a secondary HLA class I-dependent wave of events.

Finally, the use of enriched supernatants from psoriatic-derived CLA^+/Epi cocultures to create a psoriasis-like inflammatory milieu for activation of normal keratinocytes, provided a tool to investigate other altered genes that have not been characterized in psoriasis. Since the expression of IL-17A–signatures genes in the skin of psoriatic patients are the first to be normalized after effective biological therapies that target IL-17A or IL-17RA (Krueger et al., 2012; Russell et al., 2014), it was clear that a gene or molecule potentially involved in the disease should be regulated by IL-17A. *ZC3H12A* was present between the normalized transcripts in those studies, and, interestingly, it constitutes the only ribonuclease that has been included in the top 10 up-regulated transcripts by IL-17A in keratinocytes (Chiricozzi et al., 2011). In fact, the supernatants from psoriatic cocultures induced *ZC3H12A* expression in normal keratinocytes in an IL-17A–dependent manner. Consequently, this indicated that, in an inflammatory psoriasis-associated environment induced by SE, IL-17A would be the key mediator of the *ZC3H12A*-induced expression in keratinocytes. Such IL-17–dependence was further supported by the lack of *Zc3h12a* induction in inflamed skin of *Il17ra*$^{-/-}$ mice in a

psoriasis-like inflammation model with IMQ. Although MCPIP1 function in the epidermis has to be determined, the silencing of *ZC3H12A* in HaCaT keratinocytes points to a potential modulation of other IL-17A–associated transcripts, as well as epidermal differentiation-associated genes.

In summary, the study of the circulating memory CLA^+ T-cell subset as a potential carrier of disease-associated information, and therefore as a peripheral cellular biomarker, in an *ex vivo* model, has allowed the characterization of different aspects of psoriasis disease. Importantly, all these studies were carried out based on an antigen-specific innate stimulus, the *S. pyogenes*, which is a clinically validated trigger in psoriasis. Such activation induces, among others, high levels of IL-17 response by CLA^+ T cells, which is predominant in case of guttate psoriasis, and correlates with IL-17–regulated transcripts levels in keratinocytes as well, resembling a psoriatic-associated gene expression pattern. Interestingly, IL-17A has been shown to be the key disease-associated cytokine responsible of psoriasis severity in patients. Therefore, the finding of a CLA^+ T-cell–selective production of IL-9 upon activation with *S. pyogenes*, which in turn is necessary for optimal production of IL-17A from CLA^+ T cells, suggests a significant Th17-supportive role from other less-characterized skin-homing T-cell lineages in psoriasis. Additionally, we have found a synchronicity in guttate psoriasis-derived CLA^+T-cell *ex vivo* responses to *S. pyogenes*, the rate of streptococcal exposure (ASO) and disease severity (PASI), thereby supporting that clinical outcomes can be reflected by peripheral CLA^+ T cells following encounter of an antigen that initially triggered the disease. However, the assessment of the relation of *S. pyogenes*-exerted effector function in plaque psoriasis-derived samples with their clinical status, has still to be elucidated. Finally, the early determination in keratinocytes of the IL-17A–targeted gene *ZC3H12A*, which encodes for the ribonuclease MCPIP1, through the strep-induced production of IL-17A by the CLA^+/Epi coculture, led to the succeeding characterization of MCPIP1 aberrant expression in psoriatic lesions and its rapid induction in epidermal cells, in which its ribonuclease activity could be modulating other altered-expressed genes.

CONCLUSIONS

Premise 1): Psoriatic-associated events are reproduced in the *ex vivo* model in a CLA- and SE-dependent manner and provides a tool to expand knowledge and understanding of immune responses in psoriasis. **Hence,**

1.1) Clinical, genetic and environmental factors can be contemplated as a source of responses variability.

1.2) Guttate psoriasis samples from HLA-Cw6–positive patients with early onset associated to streptococcal pharyngitis present a predominant Th17 response against SE. No predominance in cytokine response is presented by plaque psoriasis samples.

1.3) High cytokine responses to SE in cocultures derived from guttate psoriasis associates with raised ASO and PASI levels, especially in a temporary window of 1–2 months of disease length.

1.4) High CLA^+ T-cell–selective IL-9 production induced by SE, and its supporting role for optimal SE-induced IL-17A levels, suggests a potential contribution of IL-9 in psoriasis.

Premise 2): Enriched supernatants from CLA^+/Epi cocultures activated with SE may represent an inflammatory milieu close to that produced in active psoriatic lesions. In addition, the modulation of the expression of IL-17–signature genes in the skin constitute the key early event in psoriasis epidermal changes. **Hence,**

2.1) We can identify and characterize IL-17A–modulated genes that previously have not been related to psoriasis on the basis of the effect of SE-induced mediators by CLA^+/Epi on cutaneous resident cells, such as keratinocytes.

2.2) The ribonuclease *ZC3H12A*/MCPIP1 is highly up-regulated by IL-17A in keratinocytes and is aberrantly distributed in epidermal cells in psoriasis.

2.3) MCPIP1 could be modulating other IL-17A–associated transcripts, as well as other genes involved in the differentiation of the epidermis.

BIBLIOGRAPHY

Abernathy-Carver, K.J., Sampson, H.A., Picker, L.J., and Leung, D.Y. (1995). Milk-induced eczema is associated with the expansion of T cells expressing cutaneous lymphocyte antigen. J. Clin. Invest. *95*, 913–918.

Abrams, J.R., Kelley, S.L., Hayes, E., Kikuchi, T., Brown, M.J., Kang, S., Lebwohl, M.G., et al. (2000). Blockade of T lymphocyte costimulation with cytotoxic T lymphocyte-associated antigen 4-immunoglobulin (Ctla4ig) reverses the cellular pathology of psoriatic plaques, including the activation of keratinocytes, dendritic cells and endothelial cells. J. Exp. Med. *192*, 681–694.

Agace, W.W. (2006). Tissue-tropic effector T cells: generation and targeting opportunities. Nat. Rev. Immunol. *6*, 682–692.

Aggarwal, S., Ghilardi, N., Xie, M.H., Sauvage, F.J. de, and Gurney, A.L. (2003). Interleukin-23 promotes a distinct CD4 T Cell activation state characterized by the production of interleukin-17. J. Biol. Chem. *278*, 1910–1914.

Akdis, M., Akdis, C.A., Weigl, L., Disch, R., and Blaser, K. (1997). Skin-homing, CLA+ memory T cells are activated in atopic dermatitis and regulate IgE by an IL-13-dominated cytokine pattern: IgG4 counter-regulation by CLA- memory T cells. J. Immunol. *159*, 4611–4619.

Antoniu, S.A. (2012). Discontinued drugs 2011: pulmonary, allergy, gastrointestinal and arthritis. Expert Opin. Investig. Drugs *21*, 1607–1618.

Antúnez, C., Torres, M.J., López, S., Rodriguez-Pena, R., Blanca, M., Mayorga, C., and Santamaría-Babi, L.F. (2009). Calcitonin gene-related peptide modulates interleukin-13 in circulating cutaneous lymphocyte-associated antigen-positive T cells in patients with atopic dermatitis. Br. J. Dermatol. *161*, 547–553.

Arakawa, A., Siewert, K., Stöhr, J., Besgen, P., Kim, S.M., Rühl, G., Nicjel, J., et al. (2015). Melanocyte antigen triggers autoimmmunity in human psoriasis. J. Exp. Med. *212*, 2203–2212.

Asumalahti, K., Ameen, M., Suomela, S., Hagforsen, E., Michaëlsson, G., Evans, J., Munro, M., et al. (2003). Genetic analysis of PSORS1 distinguishes guttate psoriasis and palmoplantar pustulosis. J. Invest. Dermatol. *120*, 627–632.

Babi, L.F.S., Moser, R., Soler, M.T.P., Picker, L.J., Blaser, K., and Hauser, C. (1995). Migration of skin-homing T Cells across cytokine-activated human endothelial cell layers involves interaction of the cutaneous lymphocyte-associated antigen (CLA), the very late antigen-4 (VLA-4), and the lymphocyte function-associated antigen-1 (LFA-1). J. Immunol. *154*, 1543–1550.

Baker, B., Laman, J., Powles, A., van der Fits, L., Voerman, J., Melief, M.J., and Fry, L. (2006). Peptidoglycan and peptidoglycan-specific Th1 cells in psoriatic skin lesions. J. Pathol. *209*, 174–181.

Baker, B.S., Brown, D.W., Fischetti, V.A., Ovigne, J.M., Porter, W., Powles, A., and Fry, L. (2001). Skin T cell proliferative response to M protein and other cell wall and membrane proteins of group A streptococci in chronic plaque psoriasis. Clin. Exp. Immunol. *124*, 516–521.

Bartlett, H.S., and Million, R.P. (2015). Targeting the IL-17-T(H)17 pathway. Nat. Rev. Drug Discov. *14*, 11–12.

Benson, J.M., Sachs, C.W., Treacy, G., Zhou, H., Pendley, C.E., Brodmerkel, C.M., Shankar, G., and Mascelli, M.A. (2011). Therapeutic targeting of the IL-12/23 pathways: generation and characterization of ustekinumab. Nat. Biotechnol. *29*, 615–624.

Berg, E.L., Yoshino, T., Rott, L.S., Robinson, M.K., Warnock, R.A., Kishimoto, T.K., Picker, L.J., and Butcher, E.C. (1991). The cutaneous lymphocyte antigen is a skin lymphocyte homing receptor for the vascular lectin endothelial cell-leukocyte adhesion molecule 1. J. Exp. Med. *174*, 1461–1466.

Bergboer, J.G.M., Zeeuwen, P.L.J.M., and Schalkwijk, J. (2012). Genetics of psoriasis: evidence for epistatic interaction between skin barrier abnormalities and immune deviation. J. Invest. Dermatol. *132*, 2320–2331.

Besgen, P., Trommler, P., Vollmer, S., and Prinz, J.C. (2010). Ezrin, maspin, peroxiredoxin 2, and heat shock protein 27: potential targets of a streptococcal-induced autoimmune response in psoriasis. J. Immunol. *184*, 5392–5402.

Bissonnette, R., Papp, K., Maari, C., Yao, Y., Robbie, G., White, W.I., Le, C., et al. (2010). A randomized, double-blind, placebo-controlled, phase I study of MEDI-545, an anti–interferon-alfa monoclonal antibody, in subjects with chronic psoriasis. J. Am. Acad. Dermatol. *62*, 427–436.

Borowitz, M.J., Weidner, A., Olsen, E.A., and Picker, L.J. (1993). Abnormalities of circulating T-cell subpopulations in patients with cutaneous T-cell lymphoma: cutaneous lymphocyte-associated antigen expression on T cells correlates with extent of disease. Leukemia *7*, 859–863.

Bos, J.D, Hulsebosch, H.J., Krieg, S.R., Bakker, P.M., and Cormane, R.H. (1983). Immunocompetent cells in psoriasis. In situ immunophenotyping by monoclonal antibodies. Arch. Dermatol. Res. *275*, 181–189.

Bowcock, A.M., and Krueger, J.G. (2005). Getting under the skin: the immunogenetics of psoriasis. Nat. Rev. Immunol. *5*, 699–711.

Boyman, O., Hefti, H.P., Conrad, C., Nickoloff, B.J., Suter, M., and Nestle, F.O. (2004). Spontaneous development of psoriasis in a new animal model shows an essential role for resident T cells and tumor necrosis factor-α. J. Exp. Med. *199*, 731–736.

Burkhardt, J., Petit-Teixeira, E., Teixeira, V.H., Kirsten, H., Garnier, S., Ruehle, S., Oeser, C., et al. (2009). Association of the X-chromosomal genes TIMP1 and IL9R with rheumatoid arthritis. J. Rheumatol. *36*, 2149–2157.

Capon, F., Munro, M., Trembath, R., and Barker, J. (2002). Searching for the Major Histocompatibility Complex Psoriasis Susceptibility Gene. J. Invest. Dermatol. *118*, 745–751.

Cargill, M., Schrodi, S.J., Chang, M., Garcia, V.E., Brandon, R., Callis, K.P., Matsunami, N., et al. (2007). A large-scale genetic association study confirms IL12B and leads to the identification of IL23R as psoriasis-risk genes. Am. J. Hum. Genet. *80*, 273–390.

Carretero, G., Ferrandiz, C., Dauden, E. Vanaclocha Sebastián, F. Gómez-García, F.J., Herrera-Ceballos, E., De la Cueva-Dobao, P., et al. (2015). Risk of adverse events in psoriasis patients receiving classic systemic drugs and biologics in a 5-year observational study of clinical practice: 2008-2013 results of the Biobadaderm registry. J. Eur. Acad. Dermatol. Venereol. *29*, 156–163.

Chang, H.C., Sehra, S., Goswami, R., Yao, W., Yu, Q., Stritesky, G.L., Jabeen, R., et al. (2010). The transcription factor PU.1 is required for the development of IL-9-producing T cells and allergic inflammation. Nat. Immunol. *11*, 527–534.

Cheung, K.L., Jarrett, R., Subramaniam, S., Salimi, M., Gutowska-Owsiak, D., Chen, Y.L., Hardman, C. (2016). Psoriatic T cells recognize neolipid antigens generated by mast cell phospholipase delivered by exosomes and presented by CD1a. J. Exp. Med. *213*, 2399–2412.

Chiricozzi, A., Guttman-Yassky, E., Suárez-Fariñas, M., Nograles, K.E., Tian, S., Cardinale, I., Chimenti, S., et al. (2011). Integrative responses to IL-17 and TNF-α in human keratinocytes account for key inflammatory pathogenic circuits in psoriasis. J. Invest. Dermatol. *131*, 677–687.

Choe, Y.B., Hwang, Y.J., Hahn, H.J., Jung, J.W., Jung, H.J., Lee, Y.W., Ahn, K.J., et al. (2012). A comparison of serum inflammatory cytokines according to phenotype in patients with psoriasis. Br. J. Dermatol. *167*, 762–767.

Christophers, E., Metzler, G., and Röcken, M. (2014). Bimodal immune activation in psoriasis. Br. J. Dermatol. *170*, 59–65.

Ciccia, F., Guggino, G., Rizzo, A., Manzo, A., Vitolo, B., Manna, M.P.L., Giardina, G., et al. (2015). Potential involvement of IL-9 and Th9 cells in the pathogenesis of rheumatoid arthritis. Rheumatology *54*, 2264–2272.

Ciccia, F., Guggino, G., Ferrante, A., Cipriani, P., Giacomelli, R., and Triolo, G. (2016). Interleukin-9 and T helper type 9 cells in rheumatic diseases. Clin. Exp. Immunol. *185*, 125–132.

Czarnowicki, T., Santamaria-Babí, L.F., and Guttman-Yassky, E. (2016). Circulating CLA^+ T-cells in atopic dermatitis and their possible role as peripheral biomarkers. Allergy doi:10.1111/all.13080.

Davison, S., Allen, M., Harmer, A., Vaughan, R., and Barker, J.N.W.N. (1999). Increased T-cell receptor Vβ2 chain expression in skin homing lymphocytes in psoriasis. Br. J. Dermatol. *140*, 845–848.

Davison, S.C., Allen, M.H., Mallon, E., and Barker, J.N.W.N. (2001a). Contrasting patterns of streptococcal superantigen-induced T-cell proliferation in guttate vs. chronic plaque psoriasis. Br. J. Dermatol. *145*, 245–251.

Davison, S.C., Ballsdon, A., Allen, M.H., and Barker, J.N.W.N. (2001b). Early migration of cutaneous lymphocyte-associated antigen (CLA) positive T cells into evolving psoriatic plaques. Exp. Dermatol. *10*, 280–285.

Diluvio, L., Vollmer, S., Besgen, P., Ellwart, J.W., Chimenti, S., and Prinz, J.C. (2006). Identical TCR β-Chain rearrangements in streptococcal angina and skin lesions of patients with psoriasis vulgaris. J. Immunol. *176*, 7104–7111.

Di Meglio, P., Perera, G.K., and Nestle, F.O. (2011). The multitasking organ: recent insights into skin immune function. Immunity *35*, 857–869.

Duffin, K.C., and Krueger, G.G. (2009). Genetic variations in cytokines and cytokine receptors associated with psoriasis found by genome-wide association. J. Invest. Dermatol. *129*, 827–833.

Elder, J.T., Bruce, A.T., Gudjonsson, J.E., Johnston, A., Stuart, P.E., Tejasvi, T., Voorhees, et al. (2010). Molecular dissection of psoriasis: integrating genetics and biology. J. Invest. Dermatol. *130*, 1213–1226.

El-Rachkidy, R.G., Hales, J.M., Freestone, P.P.E., Young, H.S., Griffiths, C.E.M., and Camp, R.D.R. (2007). Increased blood levels of IgG reactive with secreted Streptococcus pyogenes proteins in chronic plaque psoriasis. J. Invest. Dermatol. *127*, 1337–1342.

Elyaman, W., Bradshaw, E.M., Uyttenhove, C., Dardalhon, V., Awasthi, A., Imitola, J., Bettelli, E., et al. (2009). IL-9 induces differentiation of TH17 cells and enhances function of FoxP3+ natural regulatory T cells. Proc. Natl. Acad. Sci. U. S. A. *106*, 12885–12890.

Engelhardt, B., Jagasia, M., Savani, B., Bratcher, N., Greer, J., Jiang, A., Kassim, A., et al. (2011). Regulatory T cell expression of CLA or α4β7 and skin or gut acute GVHD outcomes. Bone Marrow Transplant. *46*, 436–442.

Engelhardt, B.G., Sengsayadeth, S.M., Jagasia, M., Savani, B.N., Kassim, A.A., Lu, P., Shyr, Y., et al. (2012). Tissue-specific regulatory T cells: biomarker for acute graft-vs-host disease and survival. Exp. Hematol. *40*, 974–982.e1.

Fahlén, A., Engstrand, L., Baker, B.S., Powles, A., and Fry, L. (2012). Comparison of bacterial microbiota in skin biopsies from normal and psoriatic skin. Arch. Dermatol. Res. *304*, 15–22.

Fanti, P.A., Dika, E., Vaccari, S., Miscial, C., and Varotti, C. (2006). Generalized psoriasis induced by topical treatment of actinic keratosis with imiquimod. Int. J. Dermatol. *45*, 1464–1465.

Ferran, M., Giménez-Arnau, A.M., Bellosillo, B., Pujol, R.M., and Santamaria-Babi, L.F. (2008). Circulating CLA $^{+}$ T cell subsets inversely correlate with disease severity and extension in acute psoriasis but not in chronic plaque psoriasis. Eur. J. Dermatol. *18*, 647–650.

Ferran, M., Romeu, E.R., Rincón, C., Sagristà, M., Giménez Arnau, A.M., Celada, A., Pujol, R.M., et al. (2013a). Circulating CLA+ T lymphocytes as peripheral cell biomarkers in T-cell-mediated skin diseases. Exp. Dermatol. *22*, 439–442.

Ferran, M., Galván, A.B., Rincón, C., Romeu, E.R., Sacrista, M., Barboza, E., Giménez-Arnau, A., et al. (2013b). Streptococcus induces circulating CLA+ memory T-cell-dependent epidermal cell activation inpsoriasis. J. Invest. Dermatol. *133*, 999–1007.

Flutter, B., and Nestle, F.O. (2013). TLRs to cytokines: Mechanistic insights from the imiquimod mouse model of psoriasis. Eur. J. Immunol. *43*, 3138–3146.

Friberg, C., Björck, K., Nilsson, S., Inerot, A., Wahlström, J., and Samuelsson, L. (2006). Analysis of chromosome 5q31–32 and psoriasis: Confirmation of a susceptibility locus but no association with SNPs within SLC22A4 and SLC22A5. J. Invest. Dermatol. *126*, 998–1002.

Fuhlbrigge, R.C., Kieffer, J.D., Armerding, D., and Kupper, T.S. (1997). Cutaneous lymphocyte antigen is a specialized form of PSGL-1 expressed on skin-homing T cells. Nature *389*, 978–981.

Fuhlbrigge, R.C., King, S.L., Sackstein, R., and Kupper, T.S. (2006). CD43 is a ligand for E-selectin on CLA+ human T cells. Blood *107*, 1421–1426.

Fuschiotti, P., Larregina, A.T., Ho, J., Feghali-Bostwick, C., and Medsger, T.A. (2013). Interleukin-13–producing CD8+ T cells mediate dermal fibrosis in patients with systemic sclerosis. Arthritis Rheum. *65*, 236–246.

Gaffen, S.L., Jain, R., Garg, A.V., and Cua, D.J. (2014). The IL-23-IL-17 immune axis: from mechanisms to therapeutic testing. Nat. Rev. Immunol. *14*, 585–600.

Gerlach, K., Hwang, Y., Nikolaev, A., Atreya, R., Dornhoff, H., Steiner, S., Lehr, et al. (2014). TH9 cells that express the transcription factor PU.1 drive T cell-mediated colitis via IL-9 receptor signaling in intestinal epithelial cells. Nat. Immunol. *15*, 676–686.

Ghannam, S., Dejou, C., Pedretti, N., Giot, J.P., Dorgham, K., Boukhaddaoui, H., Deleuze, V., et al. (2011). CCL20 and β-defensin-2 induce arrest of human Th17 cells on inflamed endothelium in vitro under flow conditions. J. Immunol. *186*, 1411–1420.

Gibson, S.J., Lindh, J.M., Riter, T.R., Gleason, R.M., Rogers, L.M., Fuller, A.E., Oesterich, J.L., et al. (2002). Plasmacytoid dendritic cells produce cytokines and mature in response to the TLR7 agonists, imiquimod and resiquimod. Cell. Immunol. *218*, 74–86.

Griffiths, C.E.M., and Barker, J.N.W.N. (2007). Pathogenesis and clinical features of psoriasis. Lancet *370*, 263–271.

Gottlieb, S.L., Gilleaudeau, P., Johnson, R., Estes, L., Woodworth, T.G., Gottlieb, A.B., and Krueger, J.G. (1995). Response of psoriasis to a lymphocyte-selective toxin (DAB389IL-2) suggests a primary immune, but not keratinocyte, pathogenic basis. Nat. Med. *1*, 442–447.

Gottlieb, A.B., Krueger, J.G., Wittkowski, K., Dedrick, R., Walicke, P.A., and Garovoy, M. (2002). Psoriasis as a model for T-cell-mediated disease: immunobiologic and clinical effects of treatment with multiple doses of efalizumab, an anti-CD11a antibody. Arch. Dermatol. *138*, 591–600.

Gudjonsson, J.E., Thorarinsson, A.M., Sigurgeirsson, B., Kristinsson, K.G., and Valdimarsson, H. (2003). Streptococcal throat infections and exacerbation of chronic plaque psoriasis: a prospective study. Br. J. Dermatol. *149*, 530–534.

Gudjonsson, J.E., Johnston, A., Sigmundsdottir, H., and Valdimarsson, H. (2004). Immunopathogenic mechanisms in psoriasis. Clin. Exp. Immunol. *135*, 1–8.

Gudjonsson, J.E., Karason, A., Runarsdottir, E.H., Antonsdottir, A.A., Hauksson, V.B., Jónsson, H.H., Gulcher, J., et al. (2006). Distinct clinical differences between HLA-Cw*0602 positive and negative psoriasis patients – An analysis of 1019 HLA-C- and HLA-B-typed patients. J. Invest. Dermatol. *126*, 740–745.

Gudmundsdottir, A.S., Sigmundsdottir, H., Sigurgeirsson, B., Good, M.F., Valdimarsson, H. and Jonsdottir, I. (1999). Is an epitope on keratin 17 a major target for autoreactive T lymphocytes in psoriasis? Clin. Exp. Immunol. *117*, 580–586.

Gutowska-Owsiak, D., Schaupp, A.L., Salimi, M., Selvakumar, T.A., McPherson, T., Taylor, S., et al. (2012). IL-17 downregulates filaggrin and affects keratinocyte expression of genes associated with cellular adhesion. Exp. Dermatol. *21*, 104–110.

Guttman-Yassky, E., and Krueger, J.G. (2007). Psoriasis: evolution of pathogenic concepts and new therapies through phases of translational research. Br. J. Dermatol. *157*, 1103–1115.

Harden, J.L., Johnson-Huang, L.M., Chamian, M.F., Lee, E., Pearce, T., Leonardi, C.L., Haider, A., et al. (2015). Humanized anti–IFN-γ (HuZAF) in the treatment of psoriasis. J. Allergy Clin. Immunol. *135*, 553–556.e3.

Harper, E.G., Simpson, E.L., Takiguchi, R.H., Boyd, M.D., Kurtz, S.E., Bakke, A.C., and Blauvelt, A. (2008). Efalizumab therapy for atopic dermatitis causes marked increases in circulating effector memory CD4+ T cells that express cutaneous lymphocyte antigen. J. Invest. Dermatol. *128*, 1173–1181.

Heald, P.W., Yan, S.-L., Edelson, R.L., Tigelaar, R., and Picker, L.J. (1993). Skin-selective lymphocyte homing mechanisms in the pathogenesis of leukemic cutaneous T-cell lymphoma. J. Invest. Dermatol. *101*, 222–226.

Homey, B., Alenius, H., Müller, A., Soto, H., Bowman, E.P., Yuan, W., McEvoy, L., et al. (2002). CCL27-CCR10 interactions regulate T cell-mediated skin inflammation. Nat. Med. *8*, 157–165.

Hong, C.H., Chang, K.L., Wang, H.J., Yu, H.S., and Lee, C.H. (2015). IL-9 induces IL-8 production via STIM1 activation and ERK phosphorylation in epidermal keratinocytes: A plausible mechanism of IL-9R in atopic dermatitis. J. Dermatol. Sci. *78*, 206–214.

Hudak, S., Hagen, M., Liu, Y., Catron, D., Oldham, E., McEvoy, L.M., and Bowman, E.P. (2002). Immune surveillance and effector functions of CCR10+ skin homing T cells. J. Immunol. *169*, 1189–1196.

Johnson-Huang, L.M., Pensabene, C.A., Shah, K.R., Pierson, K.C., Kikuchi, T., Lentini, T., Gilleaudeau, P., et al. (2012). Post-therapeutic relapse of psoriasis after CD11a blockade is associated with T cells and inflammatory myeloid DCs. PLoS One *7*, e30308.

Johnston, A., Gudjonsson, J.E., Sigmundsdottir, H., Love, T.J., and Valdimarsson, H. (2004). Peripheral blood T cell responses to keratin peptides that share sequences with streptococcal M proteins are largely restricted to skin-homing CD8+ T cells. Clin. Exp. Immunol. *138*, 83–93.

Kauffman, C.L., Aria, N., Toichi, E., McCormick, T.S., Cooper, K.D., Gottlieb, A.B., Everitt, D.E., Frederick, B., Zhu, Y., Graham, M.A., et al. (2004). A phase I study evaluating the safety, pharmacokinetics, and clinical response of a human IL-12 p40 antibody in subjects with plaque psoriasis. J. Invest. Dermatol. *123*, 1037–1044.

Kim, B.E., Howell, M.D., Guttman, E., Gilleaudeau, P.M., Cardinale, I.R., Boguniewicz, M., Krueger, J.G., et al. (2011). TNF-α downregulates Filaggrin and Loricrin through c-Jun N-terminal kinase: Role for TNF-α antagonists to improve skin Barrier. J. Invest. Dermatol. *131*, 1272–1279.

Ko, H.C., Jwa, S.W., Song, M., Kim, M.B., and Kwon, K.S. (2010). Clinical course of guttate psoriasis: Long-term follow-up study. J. Dermatol. *37*, 894–899.

Koelle, D.M., Liu, Z., McClurkan, C.M., Topp, M.S., Riddell, S.R., Pamer, E.G., Johnson, A.S., et al. (2002). Expression of cutaneous lymphocyte-associated antigen by CD8+ T cells specific for a skin-tropic virus. J. Clin. Invest. *110*, 537–548.

Kopp, T., Riedl, E., Bangert, C., Bowman, E.P., Greisenegger, E., Horowitz, A., Kittler, H., et al. (2015). Clinical improvement in psoriasis with specific targeting of interleukin-23. Nature *521*, 222–226.

Krueger, J.G. (2005). Psoriasis pathophysiology: current concepts of pathogenesis. Ann. Rheum. Dis. *64*, ii30-ii36.

Krueger, J.G. (2012). Hiding under the skin: A welcome surprise in psoriasis. Nat. Med. *18*, 1750–1751.

Krueger, J.G., Fretzin, S., Suárez-Fariñas, M., Haslett, P.A., Phipps, K.M., Cameron, G.S., McColm, J., et al. (2012). IL-17A is essential for cell activation and inflammatory gene circuits in subjects with psoriasis. J. Allergy Clin. Immunol. *130*, 145–154.

Kupper, T.S., and Fuhlbrigge, R.C. (2004). Immune surveillance in the skin: mechanisms and clinical consequences. Nat. Rev. Immunol. *4*, 211–222.

Laggner, U., Meglio, P.D., Perera, G.K., Hundhausen, C., Lacy, K.E., Ali, N., Smith, C.H., et al. (2011). Identification of a novel proinflammatory human skin-homing Vγ9Vδ2 T cell subset with a potential role in psoriasis. J. Immunol. *187*, 2783–2793.

Lande, R., Botti, E., Jandus, C., Dojcinovic, D., Fanelli, G., Conrad, C., Chamilos, G., et al. (2014). The antimcrobial peptide LL37 is a T-cell autoantigen in psoriasis. Nat. Commun. *5*, 5621.

Langley, R., Krueger, G., and Griffiths, C. (2005). Psoriasis: epidemiology, clinical features, and quality of life. Ann. Rheum. Dis. *64*, ii18-ii23.

Lee, B. (2016). Practical psoriasis. [ONLINE]. Available at: http://slideshare.net/BenjaminLee22/practical-psoriasis.

Lee, E., Trepicchio, W.L., Oestreicher, J.L., Pittman, D., Wang, F., Chamian, F., Dhodapkar, M., et al. (2004). Increased expression of interleukin 23 p19 and p40 in lesional skin of patients with psoriasis vulgaris. J. Exp. Med. *199*, 125–130.

Leung, D.Y., Gately, M., Trumble, A., Ferguson-Darnell, B., Schlievert, P.M., and Picker, L.J. (1995a). Bacterial superantigens induce T cell expression of the skin-selective homing receptor, the cutaneous lymphocyte-associated antigen, via stimulation of interleukin 12 production. J. Exp. Med. *181*, 747–753.

Leung, D.Y., Travers, J.V., Giorno, R., Norris, D.A., Skinner, R., Aelion, J., Kazemi, L.V., et al. (1995b). Evidence for a streptococcal superantigen-driven process in acute guttate psoriasis. J. Clin. Invest. *96*, 2106–2112.

Little, F.F., Cruikshank, W.W., and Center, D.M. (2001). IL-9 stimulates release of chemotactic factors from human bronchial epithelial cells. Am. J. Respir. Cell Mol. Biol. *25*, 347–352.

Liu, Y., Krueger, J.G., and Bowcock, A.M. (2006). Psoriasis: genetic associations and immune system changes. Genes Immun. *8*, 1–12.

Lowes, M.A., Bowcock, A.M., and Krueger, J.G. (2007). Pathogenesis and therapy of psoriasis. Nature *445*, 866–873.

Lowes, M.A., Chamian, F., Abello, M.V., Fuentes-Duculan, J., Lin, S.L., Nussbaum, R., Novitskaya, I., Carbonaro, H., et al. (2005). Increase in TNF-alpha and inducible nitric oxide syntase-expressing denritic cells in psoriasis and reduction with efalizumab (anti-CD11a). Proc. Natl. Acad. Sci. U.S.A. *102*, 19057–19062.

Lowes, M.A., Kikuchi, T., Fuentes-Duculan, J., Cardinale, I., Zaba, L.C., Haider, A.S., Bowman, E.P., et al. (2008). Psoriasis vulgaris lesions contain discrete populations of Th1 and Th17 T cells. J. Invest. Dermatol. *128*, 1207–1211.

Mahil, S.K., Capon, F., and Barker, J.N. (2016). Update on psoriasis immunopathogenesis and targeted immunotherapy. Semin. Immunopathol. *38*, 11–27.

Mallbris, L., Larsson, P., Bergqvist, S., Vingård, E., Granath, F., and Ståhle, M. (2005). Psoriasis phenotype at disease onset: Clinical characterization of 400 adult cases. J. Invest. Dermatol. *124*, 499–504.

Mallbris, L., Wolk, K., Sánchez, F., and Ståhle, M. (2009). HLA-Cw*0602 associates with a twofold higher prevalence of positive streptococcal throat swab at the onset of psoriasis: a case control study. BMC Dermatol. *9*, 5.

Marelli-Berg, F.M., Cannella, L., Dazzi, F., and Mirenda, V. (2007). The highway code of T cell trafficking. J. Pathol. *214*, 179–189.

Mayeux, R. (2004). Biomarkers: potential uses and limitations.

Menssen, A., Trommler, P., Vollmer, S., Schendel, D., Albert, E., Gürtler, L., Riethmüller, G., et al. (1995). Evidence for an antigen-specific cellular immune response in skin lesions of patients with psoriasis vulgaris. J. Immunol. *155*, 4078–4083.

Modi, W.S., Pollock, D.D., Mock, B.A., Banner, C., Renauld, J.C., and Van Snick, J. (1991). Regional localization of the human glutaminase (GLS) and interleukin-9 (IL9) genes by in situ hybridization. Cytogenet. Cell. Genet. *57*, 114–116.

Nalleweg, N., Chiriac, M.T., Podstawa, E., Lehmann, C., Rau, T.T., Atreya, R., Krauss, E., et al. (2015). IL-9 and its receptor are predominantly involved in the pathogenesis of UC. Gut *64*, 743–755.

Naylor, S. (2003). Biomarkers: current perspectives and future prospects. Expert Rev. Mol. Diagn. *3*, 525–529.

Nestle, F.O., Conrad, C., Tun-Kyi, A., Homey, B., Gombert, M., Boyman, O., Burg, G., et al. (2005). Plasmacytoid predendritic cells initiate psoriasis through interferon-α production. J. Exp. Med. *202*, 135–143.

Nestle, F.O., Kaplan, D.H., and Barker, J. (2009). Psoriasis. N. Engl. J. Med. *361*, 496–509.

Noda, S., Krueger, J.G., and Guttman-Yassky, E. (2015) The translational revolution and use of biologics in patients with inflammatory skin diseases. J. Allergy Clin. Immunol. *135*, 324–336.

Noelle, R.J., and Nowak, E.C. (2010). Cellular sources and immune functions of interleukin-9. Nat. Rev. Immunol. *10*, 683–687.

Nowak, E.C., Weaver, C.T., Turner, H., Begum-Haque, S., Becher, B., Schreiner, B., Coyle, A.J., et al. (2009). IL-9 as a mediator of Th17-driven inflammatory disease. J. Exp. Med. *206*, 1653–1660.

Ogg, G.S., Dunbar, P.R., Romero, P., Chen, J.L., and Cerundolo, V. (1998). High frequency of skin-homing melanocyte-specific cytotoxic T lymphocytes in autoimmune vitiligo. J. Exp. Med. *188*, 1203–1208.

Oppenheim, J.J., Biragyn, A., Kwak, L.W., and Yang, D. (2003). Roles of antimicrobial peptides such as defensins in innate and adaptive immunity. Ann. Rheum. Dis. *62*, ii17-ii21.

Oppmann, B., Lesley, R., Blom, B., Timans, J.C., Xu, Y., Hunte, B., Vega, F., et al. (2000). Novel p19 protein engages IL-12p40 to form a cytokine, IL-23, with biological activities similar as well as distinct from IL-12. Immunity *13*, 715–725.

Österlund, A., Popa, R., Nikkilä, T., Scheynius, A., and Engstrand, L. (1997). Intracellular reservoir of Streptococcus pyogenes in vivo: A possible explanation for recurrent pharyngotonsillitis. The Laryngoscope *107*, 640–647.

Owen, C.M., Chalmers, R.J.G., O'Sullivan, T., and Griffiths, C.E.M. (2001). A systematic review of antistreptococcal interventions for guttate and chronic plaque psoriasis. Br. J. Dermatol. *145*, 886–890.

Pasparakis, M., Haase, I., and Nestle, F.O. (2014). Mechanisms regulating skin immunity and inflammation. Nat. Rev. Immunol. *14*, 289–301.

Picker, L.J., Treer, J.R., Ferguson-Darnell, B., Collins, P.A., Bergstresser, P.R., and Terstappen, L.W. (1993). Control of lymphocyte recirculation in man. II. Differential regulation of the cutaneous lymphocyte-associated antigen, a tissue-selective homing receptor for skin-homing T cells. J. Immunol. *150*, 1122–1136.

Pinto-Almeida, T., and Torres, T. (2014). Biologic therapy for psoriasis - still searching for the best target. An. Bras. Dermatol. *89*, 365–367.

Pont-Giralt, M., Giménez-Arnau, A.M., Pujol, R.M., and Santamaria-Babi, L.F. (2006). Circulating CLA+ T cells from acute and chronic psoriasis patients manifest a different activation state and correlation with disease severity and extension. J. Invest. Dermatol. *126*, 227–228.

Prinz, J.C. (2001). Psoriasis vulgaris – a sterile antibacterial skin reaction mediated by cross-reactive T cells? An immunological view of the pathophysiology of psoriasis. Clin. Exp. Dermatol. *26*, 326–332.

Prinz, J.C., Vollmer, S., Boehncke, W.H., Menssen, A., Laisney, I., and Trommler, P. (1999). Selection of conserved TCR VDJ rearrangements in chronic psoriatic plaques indicates a common antigen in psoriasis vulgaris. Eur. J. Immunol. *29*, 3360–3368.

Proft, T., and Fraser, J.D. (2016). Streptococcal superantigens: Biological properties and potential role in disease. In: Ferretti, J.J., Stevens, D.L., and Fischetti, V.A., eds. Streptococcus Pyogenes: Basic biology to clinical manifestations. Oklahoma City (OK): University of Oklahoma Health Sciences Center. [ONLINE]. Available at: https://www.ncbi.nlm.nih.gov/books/NBK333435/.

Rachakonda, T.D., Dhillon, J.S., Florek, A.G., and Armstrong, A.W. (2015). Effect of tonsillectomy on psoriasis: A systematic review. J. Am. Acad. Dermatol. *72*, 261–275.

Roberson, E.D.O., and Bowcock, A.M. (2010). Psoriasis genetics: breaking the barrier. Trends Genet. *26*, 415–423.

Rottman, J.B., Smith, T.L., Ganley, K.G., Kikuchi, T., and Krueger, J.G. (2001). Potential role of the chemokine receptors CXCR3, CCR4, and the integrin alphaEbeta7 in the pathogenesis of psoriasis vulgaris. Lab. Invest. *81*, 335–347.

Russell, C.B., Rand, H., Bigler, J., Kerkof, K., Timour, M., Bautista, E., Krueger, J.G., et al. (2014). Gene expression profiles normalized in psoriatic skin by treatment with brodalumab, a human anti-IL-17 receptor monoclonal antibody. J. Immunol. *192*, 3828–3836.

Sabat, R., Philipp, S., Höflich, C., Kreutzer, S., Wallace, E., Asadullah, K., Volk, H.D., et al. (2007). Immunopathogenesis of psoriasis. Exp. Dermatol. *16*, 779–798.

Santamaria-Babí, L.F. (2004). CLA $^{+}$ T cells in cutaneous diseases. Eur. J. Dermatol. *14*, 13–18.

Santamaria-Babí, L.F., Picker, L.J., Perez-Soler, M.T., Drzimalla, K., Flohr, P., Blaser, K., and Hauser, C. (1995). Circulating allergen-reactive T cells from patients with atopic dermatitis and allergic contact dermatitis express the skin-selective homing receptor, the cutaneous lymphocyte-associated antigen. J. Exp. Med. *181*, 1935–1940.

Schlapbach, C., Gehad, A., Yang, C., Watanabe, R., Guenova, E., Teague, J.E., Campbell, L., et al. (2014). Human TH9 cells are skin-tropic and have autocrine and paracrine proinflammatory capacity. Sci. Transl. Med. *6*, 219ra8-219ra8.

Schön, M.P., and Boehncke, W.H. (2005). Psoriasis. N. Engl. J. Med. *352*, 1899–1912.

Shen, Z., Wang, G., Fan, J.Y., Li, W., and Liu, Y.F. (2005). HLA DR B1*04, *07-restricted epitopes on Keratin 17 for autoreactive T cells in psoriasis. J. Dermatol. Sci. *38*, 25–39.

Shi, X., Jin, L., Dang, E., Chang, T., Liu, Y., and Wang, G. (2011). IL-17^{a} upregulates keratin 17 expression in keratinocytes through STAT1- and STAT3-dependent mechanisms. J. Invest. Dermatol. *131*, 2401–2408.

Sigurdardottir, S.L., Thorleifsdottir, R.H., Valdimarsson, H., and Johnston, A. (2013). The association of sore throat and psoriasis might be explained by histologically distinctive tonsils and increased expression of skin-homing molecules by tonsil T cells. Clin. Exp. Immunol. *174*, 139–151.

Singh, T.P., Schön, M.P., Wallbrecht, K., Gruber-Wackernagel, A., Wang, X.J., and Wolf, P. (2013). Involvement of IL-9 in Th17-associated inflammation and angiogenesis of psoriasis. PLoS One *8*, e51752.

Smith, C.H., and Barker, J.N.W.N. (2006). Psoriasis and its management. BMJ *333*, 380–384.

Spits, H., and Di Santo, J.P. (2011). The expanding family of innate lymphoid cells: regulators and effectors of immunity and tissue remodeling. Nat. Immunol. *12*, 21–27.

Staudt, V., Bothur, E., Klein, M., Lingnau, K., Reuter, S., Grebe, N., Gerlitzki, B., et al. (2010). Interferon-regulatory factor 4 is essential for the developmental program of T helper 9 cells. Immunity *33*, 192–202.

Suzuki, H., Wang, B., Shivji, G.M., Toto, P., Amerio, P., Sauder, D.N., Tomai, M.A., et al. (2000). Imiquimod, a topical immune response modifier, induces migration of langerhans cells1. J. Invest. Dermatol. *114*, 135–141.

Talamonti, M., Botti, E., Galluzzo, M., Teoli, M., Spallone, G., Bavetta, M., Chimenti, S., et al. (2013). Pharmacogenetics of psoriasis: HLA-Cw6 but not LCE3B/3C deletion nor TNFAIP3 polymorphism predisposes to clinical response to interleukin 12/23 blocker ustekinumab. Br. J. Dermatol. *169*, 458–463.

Teng, M.W.L., Bowman, E.P., McElwee, J.J., Smyth, M.J., Casanova, J.L., Cooper, A.M., and Cua, D.J. (2015). IL-12 and IL-23 cytokines: from discovery to targeted therapies for immune-mediated inflammatory diseases. Nat. Med. *21*, 719–729.

Thorleifsdottir, R.H., Sigurdardottir, S.L., Sigurgeirsson, B., Olafsson, J.H., Sigurdsson, M.I., Petersen, H., Arnadottir, S., et al. (2012). Improvement of psoriasis after tonsillectomy is associated with a decrease in the frequency of circulating T cells that recognize streptococcal determinants and homologous skin determinants. J. Immunol. *188*, 5160–5165.

Thorleifsdottir, R.H., Eysteinsdóttir, J.H., Olafsson, J.H., Sigurdsson, M.I., Johnston, A., Valdimarsson, H., and Sigurgeirsson, B. (2016). Throat infections are associated with exacerbation in a substantial proportion of patients with chronic plaque psoriasis. Acta Derm. Venereol. *96*, 788–791.

Tonel, G., Conrad, C., Laggner, U., Di Meglio, P., Grys, K., McClanahan, T.K., Blumenschein, W.M., et al. (2010). Cutting edge: A critical functional role for IL-23 in psoriasis. J. Immunol. *185*, 5688–5691.

Trembath, R.C., Clough, R.L., Rosbotham, J.L., Jones, A.B., Camp, R.D.R., Frodsham, A., Browne, J., et al. (1997). Identification of a major susceptibility locus on chromosome 6p and evidence for further disease loci revealed by a two stage genome-wide search in psoriasis. Hum. Mol. Genet. *6*, 813–820.

Valdimarsson, H., Sigmundsdóttir, H., and Jónsdóttir, I. (1997). Is psoriasis induced by streptococcal superantigens and maintained by M-protein-specific T cells that cross-react with keratin? Clin. Exp. Immunol. *107 Suppl 1*, 21–24.

Valdimarsson, H., Thorleifsdottir, R.H., Sigurdardottir, S.L., Gudjonsson, J.E., and Johnston, A. (2009). Psoriasis – as an autoimmune disease caused by molecular mimicry. Trends Immunol. *30*, 494–501.

van der Fits, L. van der, Mourits, S., Voerman, J.S.A., Kant, M., Boon, L., Laman, J.D., Cornelissen, F., et al. (2009). Imiquimod-induced psoriasis-like skin inflammation in mice is mediated via the IL-23/IL-17 axis. J. Immunol. *182*, 5836–5845.

Vissers, W.H.P.M, Arndtz, C.H.M., Muys, L., Van Erp, P.E.J., De Jong, E.M.G., and Van De Kerkhof, P.C.M. (2004). Memory effector (CD45RO+) and cytotoxic (CD8+) T cells appear early in the margin zone of spreading psoriatic lesions in contrast to cells expressing natural killer receptors, which appear late. Br. J. Dermatol. *150*, 852–859.

Vollmer, S., Menssen, A., and Prinz, J.C. (2001). Dominant lesional T cell receptor rearrangements persist in relapsing psoriasis but are absent from nonlesional skin: evidence for a stable antigen-specific pathogenic T cell response in psoriasis vulgaris. J. Invest. Dermatol. *117*, 1296–1301.

Vugmeyster, Y., Kikuchi, T., Lowes, M.A., Chamian, F., Kagen, M., Gilleaudeau, P., Lee, E., et al. (2004). Efalizumab (anti-CD11a)-induced increase in peripheral blood leukocytes in psoriasis patients is preferentially mediated by altered trafficking of memory CD8+ T cells into lesional skin. Clin. Immunol. *113*, 38–46.

Wagner, E.F., Schonthaler, H.B., Guinea-Viniegra, J., and Tschachler, E. (2010). Psoriasis: what we have learned from mouse models. Nat. Rev. Rheumatol. *6*, 704–714.

Walter, A., Schäfer, M., Cecconi, V., Matter, C., Urosevic-Maiwald, M., Belloni, B., Schönewolf, N., et al. (2013). Aldara activates TLR7-independent immune defence. Nat. Commun. *4*, 1560.

Weinberg, A., Jin, G., Sieg, S., and McCormick, T.S. (2012). The yin and yang of human beta-defensins in health and disease. Front. Immunol. *3*, 294.

Weisenseel, P., and Prinz, J.C. (2005). Incidental detection of S. pyogenes-DNA in psoriatic skin by PCR. Arch. Dermatol. Res. *296*, 573–576.

Weisenseel, P., Laumbacher, B., Besgen, P., Ludolph-Hauser, D., Herzinger, T., Roecken, M., Wank, R., and Prinz, J.C. (2002). Streptococcal infection distinguishes different types of psoriasis. J. Med. Genet. *39*, 767–768.

Weishaupt, C., Munoz, K.N., Buzney, E., Kupper, T.S., and Fuhlbrigge, R.C. (2007). T-cell distribution and adhesion receptor expression in metastatic melanoma. Clin. Cancer Res. *13*, 2549–2556.

Weninger, W., Ulfman, L.H., Cheng, G., Souchkova, N., Quackenbush, E.J., Lowe, J.B., and von Andrian, U.H. (2000). Specialized contributions by α(1,3)-fucosyltransferase-IV and fucT-VII during leukocyte rolling in rermal microvessels. Immunity *12*, 665–676.

Wilhelm, C., Turner, J.E., Van Snick, J., and Stockinger, B. (2012). The many lives of IL-9: a question of survival? Nat. Immunol. *13*, 637–641.

Yarwood, J.M., Leung, D.Y., Schlievert, P.M. (2000). Evidence for the involvement of bacterial superantigens in psoriasis, atopic dermatitis, and Kawasaki syndrome. FEMS Microbiol. Lett. *192*, 1–7.

Yawalkar, N., Karlen, S., Hunger, R., Brand, C.U., and Braathen, L.R. (1998). Expression of interleukin-12 is increased in psoriatic skin. J. Invest. Dermatol. *111*, 1053–1057.

Zaba, L.C., Cardinale, I., Gilleaudeau, P., Sullivan-Whalen, M., Suárez-Fariñas, M., Fuentes-Duculan, J., Novitskaya, I., et al. (2007). Amelioration of epidermal hyperplasia by TNF inhibition is associated with reduced Th17 responses. J. Exp. Med. *204*, 3189–3194.

DIRECTOR'S REPORT

In connection with the present doctoral thesis, entitled "**Characterization of a New *Ex Vivo* Model for the Study of Psoriasis Immunopathology**", by the candidate Ester Ruiz Romeu, the director, Dr. Luis Francisco Santamaria, reports the following items that here are presented:

In "*Streptococcus pyogenes*-induced cutaneous lymphocyte antigen-positive T cell-dependent epidermal cell activation triggers TH17 responses in patients with guttate psoriasis", the candidate carried out most of the experiments, actively participated with the majority of manuscript's writing, and performed the graphs and tables.

Published in: Journal of Allergy and Clinical Immunology, 2016. (#1 in Allergy Category)

Impact factor: 12,485

In "MCPIP1 RNase is aberrantly distributed in psoriatic epidermis and rapidly induced by IL-17A", the candidate carried out most of the experiments. Some experiments regarding to the molecular biology content were assisted by Dr. Jolanta Jura, in Poland. The manuscript was written by the candidate.

Published in: Journal of Investigative Dermatology, 2016. (#1 in Dermatology Category).

Impact factor: 6,915

"IL-9 is produced by *S. pyogenes*-activated psoriatic CLA^+ T cells in the presence of epidermal cells and supports IL-17A secretion", and "Peripheral CLA^+ T cells effector response to *S. pyogenes* in guttate psoriasis parallels anti-streptolysin O levels and PASI score with a common peak after disease onset", were predominantly carried out and written by the candidate. The submission of these manuscripts is pending.

Dr. Luis Francisco Santamaria Babí

APPENDIX I: Supporting publications

DOI: 10.1111/exd.12154
www.wileyonlinelibrary.com/journal/EXD

Viewpoint

Circulating CLA+ T lymphocytes as peripheral cell biomarkers in T-cell-mediated skin diseases

Marta Ferran[1], Ester R. Romeu[2], Catalina Rincón[2], Marc Sagristà[1], Ana M. Giménez Arnau[1], Antonio Celada[2], Ramon M. Pujol[1], Péter Holló[3], Hajnalka Jókai[3] and Luis F. Santamaria-Babí[1,2]

[1]Department of Dermatology, Hospital del Mar, Research Group in Inflammatory Dermatologic Diseases, IMIM, Universitat Autònoma de Barcelona, Barcelona, Spain; [2]Department of Physiology and Immunology, Universitat de Barcelona, Barcelona, Spain; [3]Department of Dermatovenerology and Dermatooncology, Semmelweis University, Budapest, Hungary
Correspondence: Luis F. Santamaria-Babi, Departament of Fisiologia I Immunologia, Universitat de Barcelona, Facultat de Biologia, UB Avda Diagonal 643, Barcelona 08028, Spain, Tel.: 0034-677375160, e-mail: Luis.santamaria@ub.edu

Abstract: T lymphocytes expressing the CLA antigen constitute a subset of effector memory lymphocytes that are functionally involved in T-cell-mediated cutaneous diseases. Skin-seeking lymphocytes recirculate between inflamed skin and blood during cutaneous inflammation. Many studies in different T-cell-mediated inflammatory cutaneous diseases have clearly related their pathologic mechanisms to CLA+ T cells. Based on common features of these cells in different cutaneous disorders mediated by T cells, we propose that circulating CLA+T cells could constitute very useful peripheral cellular biomarkers for T-cell-mediated skin diseases.

Key words: associated antigen – biomarker – cutaneous lymphocyte – skin-homing – T lymphocyte

Accepted for publication 16 April 2013

Introduction

Most of the cytokine producing T cells during cutaneous inflammation belong to the memory phenotype (1). Memory T lymphocytes present in the circulation are not distributed randomly to peripheral tissues (2). Specific mechanisms guide those cells to cutaneous locations under homoeostatic conditions in inflammation, infections and cancer. The cutaneous lymphocyte-associated antigen (CLA) is a cell-surface carbohydrate induced by the fucosyltransferase VII-dependent post-translational modification of platelet (P)-selectin glycoprotein ligand 1 (PSGL-1) and interacts with endothelial (E)-selectin. CLA distinguishes human effector memory CD45R0+ T cells (3,4) with cutaneous tropism involved in pathologic mechanisms of T-cell-mediated skin diseases (5). CLA+ T cells are present in approximately 15% to 20% of CD3+ cells (6), and most infiltrating T cells in cutaneous lesions are CLA+, whereas T cells infiltrating non-cutaneous tissues are CLA− (3). Healthy skin also harbours CLA+ T cells (7).

The CLA antigen is part of a complex multistep molecular interaction between circulating lymphocytes and cutaneous vascular endothelium that takes place during lymphocyte migration to the skin. Proinflammatory mediators expressed under inflammatory conditions, like IL-1 or TNF-α, upregulate the expression of adhesion molecules and chemokines on the surface of endothelial cells (5).

Some studies have shown numerical and phenotypical changes in circulating CLA+ T cells that are related to cutaneous disease severity and clinical evolution in T-cell-mediated skin diseases (5,8). However, in such cases, CLA+ T-cell recirculation between affected skin and blood during cutaneous inflammation has not been fully demonstrated in humans. As mentioned, beside other adhesion molecules and chemokines, CLA+ T cells require the LFA-1/ICAM-1 interaction during their transendothelial migration *in vitro* (9,10). Information regarding the mode of action of anti-LFA-1 treatments has been obtained from clinical trials in psoriasis and atopic dermatitis (11–13), providing translational evidence in favour of effector memory CLA+ T-cell recirculating capacity between skin and blood during cutaneous inflammation. If continuous recirculation occurs between inflamed skin and blood during cutaneous inflammation in psoriasis and atopic dermatitis, blockade of extravasation of T cells from blood into skin with anti-LFA-1 treatment should affect normal circulating levels of CLA+ T cells. In fact, in both diseases, a rise in circulating CLA+ T cells is observed in parallel to the clinical improvement of inflammation due to treatment (12,13).

Such clinical phenomena might be explained by two mechanisms. First, the LFA–1/ICAM-1 interaction is involved in the transendothelial migration of CLA+ T cells; second, infiltrating CLA+ T cells recirculate from the lesion to the blood through the thoracic duct and accumulate in the blood (Fig. 1a). Skin-activated CLA+ T cells that recirculate to blood could not extravasate due to the blockade of their adhesion mechanisms and they would accumulate, inducing CLA+ T-cell lymphocytosis (Fig. 1b). Such lymphocytosis present during treatment with anti-LFA-1 can have clinical relevance: a skin relapse may develop after stopping the treatment (11). A similar phenomenon has been observed for a different memory T-cell subset when blocking the alpha4 integrin in multiple sclerosis, leading to lymphocytosis (14).

T-cell skin-homing mechanisms have been extensively studied in the mouse and recently reviewed (15). However, the study of those cells in patients with chronic cutaneous inflammatory diseases is a more complex situation. Understanding immunological mechanisms responsible for skin flares in patients with long-standing atopic dermatitis, or psoriasis, is different from those induced *de novo* by delayed-type hypersensitivity in a sensitized

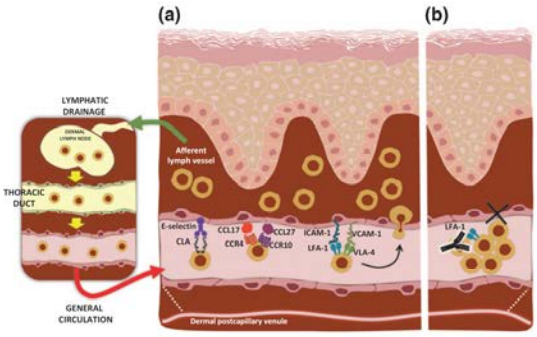


Figure 1. Circulating CLA+ T cells as cellular biomarkers. CLA+ T cells recirculate between blood and skin (a) through specific adhesion and chemotactic mechanisms. Anti-LFA-1 blocking antibodies inhibit the migration of CLA+ T cells from blood into skin and cause accumulation of lesion-derived CLA+ T cells in blood (b). Studying peripheral CLA+ T cells allows obtaining information about immune-inflammatory processes taking place in the skin.

mouse to haptens or antigens. The chronicity and maintenance of those T-cell-mediated diseases can reside on antigen-specific memory T cells present in blood and involved in the skin flares during cutaneous effector phases. For example, in patients with atopic dermatitis or contact dermatitis, circulating effector memory CLA+ T cells, but not CLA−, respond to house dust mites or nickel, suggesting a compartmentalization of the immune response present in periphery (16).

Analysis of the peripheral blood CLA+ T-cell populations provides opportunity to better understand human T-cell-mediated skin diseases. Circulating CLA+ T cells have recirculating capacity, show specificity towards cutaneous antigens/allergens/autoantigens, and their phenotype is influenced by the cutaneous environment to which they have been exposed. Our point of view is that there are evidences from different cutaneous diseases that support the relevance of circulating CLA+ T cells as cellular peripheral biomarkers and as a source of translational knowledge in T-cell-mediated skin diseases.

Psoriasis

CLA+ T cells are considered key cellular elements in the mechanism involved in psoriasis lesion formation (17–20). They are present at the initial stage of plaque psoriasis before epidermal hyperplasia takes place (21,22). The percentage of CLA+ T cells in peripheral blood correlates inversely with Psoriasis Area and Severity Index (PASI) and body surface area (BSA) in acute but not in chronic psoriasis (23), suggesting a sequestration of those cells from blood into inflamed skin. In addition, the number of circulating CD4 + CLA + and CD3 + CLA + cells expressing the activation marker HLA-DR is increased in peripheral blood of psoriatic patients with active disease compared to patients with chronic lesions (24). Recently, a novel subset of human skin-homing CLA + CCR6 + Vγ9Vδ2 T cells has been identified (25). As in previous studies, the number of circulating CLA + Vγ9Vδ2 T cells correlated inversely with PASI score and increased with successful treatment.

In psoriasis, CLA+ T cells not only migrate to skin but participate in the initiation of lesion. It has been suggested that CLA+ T cells are present in non-lesional psoriatic skin before epidermal hyperplasia takes place (21). Some infections have been described as relevant triggers of psoriasis, especially Streptococcus pyogenes (18,26) or cytomegalovirus (27). Streptococcal tonsillar infection can induce the onset of the disease or trigger relapses (26). CLA+ T cells are generated, upon activation by *Streptococcus pyogenes* superantigens, in tonsils and migrate to psoriatic cutaneous lesions. In fact, an identical and highly restricted TCR usage has been found in CLA+ T cells present in tonsils and psoriatic lesion express of the same patient, suggesting migration of those cells from tonsil to skin lesion (28). Circulating CLA + CD8 + T cells react with keratin peptides that share sequence with streptococcal M proteins (29) and their percentage in peripheral blood correlates with disease severity (30). Interestingly, in psoriatic patients with a previous history of exacerbation after sore throat, a close correlation has been recently demonstrated between the degree of clinical improvement after tonsillectomy and the reduction in the frequency of circulating CLA + CD8 + lymphocytes specific to peptides shared by streptococcal M proteins and skin keratins (31). The relevance of streptococcal throat infection has been known for more than 50 years (32); recently, it has been demonstrated that psoriatic circulating effector memory CLA+ T, but not CLAnegative, upon activation with streptococcal extract secrete Th1/Th17 cytokines and induce keratinocyte activation an hyperplasia (33).

All these studies underline the relevance of studying circulating CLA+ T cells in psoriasis because they are related to pathologic mechanisms in this chronic cutaneous inflammation. This is not surprising because psoriatic lesions produce a great variety of chemokines that can attract circulating CLA+ T cells to cutaneous psoriatic lesions such as CCL20, CCL27, CXCL8, CXCL9, CXCL10, CXCL11 (34) and CXCL16 (35). Several studies have demonstrated that the amount of circulating CLA+ T cells is altered during effective antipsoriatic treatments (25,31,36). It has been shown in a murine psoriasis model that therapeutic doses of PUVA resulted in a sequestration of CLA + CD25 + CD4 + T cells in lymph nodes, suggesting the migration from peripheral nodes to skin (and vice-versa), where they would induce PUVA therapeutic effect (37). In humans, changes of CLA expression in the periphery have been found to correlate with long-term clinical responsiveness to synchronous balneophototherapy using narrow band UVB light (38). Similarly, regarding TNF-alpha inhibitor biological therapy, a significant difference has been recently found between peripheral CLA expression tendencies of responder and relapsing patients in the induction treatment period (39). These results highlight a potential predictive importance of the CLA molecule in different psoriasis therapies.

Other cutaneous inflammatory diseases

Several studies have reported that, in different T-cell-mediated cutaneous diseases, the clinical status can induce changes in phenotype, cell numbers or related mechanisms of CLA+ T cells. In atopic dermatitis, circulating CLA+ T cells recognize allergens or bacterial molecules involved in the pathogenesis of eczema development. Their role in atopic dermatitis and other allergic diseases has been recently reviewed (8,40). Circulating CLA+ T cells are attracted into cutaneous lesions by different chemokines, whose specific receptors are present on the CLA+ T-cell surface. Serum levels of CCL17, CCL22 and CCL27 have been correlated with the clinical severity of atopic dermatitis. Circulating CLA+ T cells

from patients with active atopic dermatitis express T-cell activation markers and spontaneously produce IL-4 (16) and IL-13 (41), as expected for a lymphocyte population that recirculates between inflamed skin and blood.

Neuropeptides are thought to influence the mechanism of cutaneous flares in atopic dermatitis, and stress is a well-known trigger of disease flares. A recent study has demonstrated how calcitonin gene-related peptide, but not other neuropeptides, selectively activates circulating CLA+ T cells from atopic dermatitis patients, inducing IL-13 production in a manner independent of T-cell receptors (42). Considering that CLA+ T cells preferentially produce IL-31, an important cytokine involved in pruritus generation (43), circulating skin-homing T cells can also be of value as cellular biomarkers of molecular processes involved in T-cell-mediated pruritus.

In non-immediate cutaneous reactions to drugs such as beta-lactams and anticonvulsants, CLA+ T cells are the subset of memory T cells that respond to those antigens (44). Under re-exposure, those cells express activation markers in parallel to the cutaneous symptoms (45). The CLA antigen is also expressed on the surface of regulatory T cells (T-regs) implicated in the prevention of acute graft-versus-host disease (aGVHD) after allogeneic stem cell transplantation (ASCT). Increased amount of CLA+ T-regs at engraftment is associated with the prevention of skin aGVHD (46). A threshold value for CLA+ T-regs could be of use to predict important hematopoietic cell transplant (HCT) outcomes and may help to direct the rational use of tissue-specific preventive therapies to decrease clinical aGVHD and improve HCT survival (47). In scleroderma, a new venue of research has been initiated by studying circulating CD8 + CLA+ T cells in early stages of the disease. Those cells produce IL-13, accumulate in the lesions and produce cytotoxic granules that may be involved in the early vascular damage present in scleroderma (48).

Skin tumors

CLA+ T lymphocytes homing to cutaneous lesions play a central role in anti-tumoral immune responses (49). Although the heterogeneous subset of tumor-infiltrating lymphocytes (TILs) in malignant melanoma involves cells with both tumor-stimulating and tumor-inhibiting properties (50), a common feature of these cells is CLA expression (51) accompanied by special combinations of chemokine receptors (52,53). In metastatic lesions, endothelial cell adhesion molecule expression (e.g. E-selectin, the pair of the CLA antigen) markedly declines, which explains decreased skin- and tumor-infiltrating capacity of circulating CLA+ reactive lymphocytes as well as unimpeded tumor progression (54). Similarly, a downregulation of endothelial adhesion molecules and the consequent inhibition of CLA+ lymphocyte extravasation have been reported in epithelial skin cancers (squamous cell carcinoma and basal cell carcinoma) (55,56).

In primary cutaneous T-cell lymphomas, co-expression of CLA and skin-specific chemokine receptors on T lymphocytes is a requisite for T-cell skin infiltration (49). The distinct clinical behaviour of mycosis fungoides (MF) and Sézary syndrome (SS) might be explained by a different origin of the tumor cells. In MF, the main skin addressins, CLA and chemokine receptor CCR4 guide malignant effector memory T lymphocytes to the skin. These cells typically lack lymph node homing receptors (CCR7, L-selectin) explaining why tumor cells are primarily found in the skin without circulating in the periphery and infiltrating lymph nodes (57). However, in parallel with progression, tumor cells downregulate skin-specific homing molecules, accumulate in blood and express great amounts of lymph node–homing markers (58). A variable degree of circulating tumor cell CLA expression has been demonstrated in SS with general co-expression of lymph node addressins, referring to the central memory phenotype of malignant lymphocytes. A subset of these cells showing prominent expression of CLA and CCR4 migrates to cutaneous areas (57). A strong correlation has been observed between percentage of CLA+ circulating lymphoma cells and extent of skin symptoms (59,60). These major differences in tumor cell–homing abilities may explain why MF is confined to skin and has an overall better prognosis than SS, with involvement of skin, peripheral circulation, lymph nodes and a general poor clinical outcome (57).

All in all, tumor progression is facilitated by decreased expression of endothelial adhesion molecules in malignant tumors. Enhancing migratory capacity of CLA+ reactive immune cells could be beneficial in the therapy. Skin infiltration of CLA+ tumor cells is a key feature of primary cutaneous T-cell lymphomas. Targeted inhibition of tissue-specific tumor cell homing might be of notable therapeutic importance in the future.

In summary, results obtained from different T-cell-mediated skin diseases highlight the relevance of circulating CLA+ T cells. These cells may be a source of information as peripheral cell biomarkers, both in a predictive or retrospective way, as their number and phenotype parallel local cutaneous events and correlate with clinical activity and treatments. In addition, the study of different subsets of CLA+ T cells can bring added value in the biomarker area such as those recently activated lymphocytes (24) or expressing certain chemokine receptors on their surface. Future clinical trials are required to validate the relevance of circulating CLA+ T cells as clinical biomarkers for T-cell-mediated cutaneous diseases.

Acknowledgements

This work was supported by a grant from the *Instituto de Salud Carlos III* FEDER (PS09/0222 and PS09/01811). We acknowledge the support of the COST BM0903 Network/SKINBAD (Skin Barrier in Atopic Diseases).

Author contributions

MF, ER, CR, MS, PH, HJ performed the research; LS, PH, HJ designed research studies; AG, RP contributed essential reagents and tools; LS, PH, HJ analysed the data; LS and PH wrote the paper.

Conflict of interests

The authors have declared no conflicting interests.

References

1 Robert C, Kupper T S. N Engl J Med 1999: **341**: 1817–1828.
2 Sigmundsdottir H, Butcher E C. Nat Immunol 2008: **9**: 981–987.
3 Picker L J, Michie S A, Rott L S *et al.* Am J Pathol 1990: **136**: 1053–1068.
4 Sallusto F, Lenig D, Forster R *et al.* Nature 1999: **401**: 708–712.
5 Santamaria-Babi L F. Eur J Dermatol 2004: **14**: 13–18.
6 Picker L J, Martin R J, Trumble A *et al.* Eur J Immunol 1994: **24**: 1269–1277.
7 Clark R A, Chong B, Mirchandani N *et al.* J Immunol 2006: **176**: 4431–4439.
8 Santamaria-Babi L F. Chem Immunol Allergy 2006: **91**: 87–97.
9 Santamaria Babi L F, Moser R, Perez Soler M T *et al.* 1995: J Immunol **154**: 1543–1550.

10 Mora J R, Von Andrian U H. Curr Top Microbiol Immunol 2006: **308**: 83–116.
11 Johnson-Huang L M, Pensabene C A, Shah K R *et al.* PLoS ONE 2012: **7**: e30308.
12 Harper E G, Simpson E L, Takiguchi R H *et al.* J Invest Dermatol 2008: **128**: 1173–1181.
13 Vugmeyster Y, Kikuchi T, Lowes M A *et al.* Clin Immunol 2004: **113**: 38–46.
14 Khademi M, Bornsen L, Rafatnia F *et al.* Eur J Neurol 2009: **16**: 528–536.
15 Egawa G, Kabashima K. J Invest Dermatol 2011: **131**: 2178–2185.
16 Santamaria Babi L F, Picker L J, Perez Soler M T *et al.* J Exp Med 1995: **181**: 1935–1940.
17 Guttman-Yassky E, Nograles K E, Krueger J G. J Allergy Clin Immunol 2011: **127**: 1420–1432.
18 Nestle F O, Kaplan D H, Barker J. N Engl J Med 2009: **361**: 496–509.
19 Bowcock A M, Krueger J G. Nat Rev Immunol 2005: **5**: 699–711.
20 Schon M P, Boehncke W H. N Engl J Med 2005: **352**: 1899–1912.
21 Davison S C, Ballsdon A, Allen M H *et al.* Exp Dermatol 2001: **10**: 280–285.
22 Vissers W H, Arndtz C H, Muys L *et al.* Br J Dermatol 2004: **150**: 852–859.
23 Ferran M, Gimenez-Arnau A M, Bellosillo B *et al.* Eur J Dermatol 2008: **18**: 647–650.
24 Pont-Giralt M, Gimenez-Arnau A M, Pujol R M *et al.* J Invest Dermatol 2006: **126**: 227–228.
25 Laggner U, Di M P, Perera G K *et al.* J Immunol 2011: **187**: 2783–2793.
26 Valdimarsson H, Thorleifsdottir R H, Sigurdardottir S L *et al.* Trends Immunol 2009: **30**: 494–501.
27 Weitz M, Kiessling C, Friedrich M *et al.* Exp Dermatol 2011: **20**: 561–567.
28 Diluvio L, Vollmer S, Besgen P *et al.* J Immunol 2006: **176**: 7104–7111.
29 Johnston A, Gudjonsson J E, Sigmundsdottir H *et al.* Clin Exp Immunol 2004: **138**: 83–93.
30 Sigmundsdottir H, Gudjonsson J E, Jonsdottir I *et al.* Clin Exp Immunol 2001: **126**: 365–369.
31 Thorleifsdottir R H, Sigurdardottir S L, Sigurgeirsson B *et al.* J Immunol 2012: **188**: 5160–5165.
32 Norrlind R. Acta Rheumatol Scand 1955: **1**: 135–144.
33 Ferran M, Galvan A B, Rincon C *et al.* J Invest Dermatol 2013: **133**: 999–1007.
34 Mabuchi T, Chang T W, Quinter S *et al.* J Dermatol Sci 2012: **65**: 4–11.
35 Gunther C, Carballido-Perrig N, Kaesler S *et al.* J Invest Dermatol 2012: **132**: 626–634.
36 Soyland E, Heier I, Rodriguez-Gallego C *et al.* Br J Dermatol 2011: **164**: 344–355.
37 Singh T P, Schon M P, Wallbrecht K *et al.* Exp Dermatol 2012: **21**: 228–230.
38 Hollo P, Marschalko M, Temesvari E *et al.* J Dermatol Sci 2005: **39**: 131–133.
39 Jokai H, Szakonyi J, Kontar O *et al.* Exp Dermatol 2013: **22**: 221–223.
40 Ferran M, Santamaria-Babi L F. World Allergy Organ J 2010: **3**: 44–47.
41 Akdis M, Akdis C A, Weigl L *et al.* J Immunol 1997: **159**: 4611–4619.
42 Antunez C, Torres M J, Lopez S *et al.* Br J Dermatol 2009: **161**: 547–553.
43 Bilsborough J, Leung D Y, Maurer M *et al.* J Allergy Clin Immunol 2006: **117**: 418–425.
44 Blanca M, Leyva L, Torres M J *et al.* Blood Cells Mol Dis 2003: **31**: 75–79.
45 Blanca M, Posadas S, Torres M J *et al.* Allergy 2000: **55**: 998–1004.
46 Engelhardt B G, Jagasia M, Savani B N *et al.* Bone Marrow Transplant 2011: **46**: 436–442.
47 Engelhardt B G, Sengsayadeth S M, Jagasia M *et al.* Exp Hematol 2012: **40**: 974–982.
48 Fuschiotti P, Larregina A T, Ho J *et al.* Arthritis Rheum 2012: **65**: 236–246.
49 Jokai H, Marschalko M, Csomor J *et al.* Pathol Oncol Res 2012: **18**: 749–759.
50 Oble D A, Loewe R, Yu P *et al.* Cancer Immun 2009: **9**: 3.
51 Adams D H, Yannelli J R, Newman W *et al.* Br J Cancer 1997: **75**: 1421–1431.
52 Harlin H, Meng Y, Peterson A C *et al.* Cancer Res 2009: **69**: 3077–3085.
53 Richmond A, Yang J, Su Y. Pigment Cell Melanoma Res 2009: **22**: 175–186.
54 Weishaupt C, Munoz K N, Buzney E *et al.* Clin Cancer Res 2007: **13**: 2549–2556.
55 Clark R A, Huang S J, Murphy G F *et al.* J Exp Med 2008: **205**: 2221–2234.
56 Verhaegh M, Beljaards R, Veraart J *et al.* Eur J Dermatol 1998: **8**: 252–255.
57 Campbell J J, Clark R A, Watanabe R *et al.* Blood 2010: **116**: 767–771.
58 Krieg C, Boyman O. Semin Cancer Biol 2009: **19**: 76–83.
59 Borowitz M J, Weidner A, Olsen E A *et al.* Leukemia 1993: **7**: 859–863.
60 Heald P W, Yan S L, Edelson R L *et al.* J Invest Dermatol 1993: **101**: 222–226.

ORIGINAL ARTICLE

Streptococcus Induces Circulating CLA$^+$ Memory T-Cell-Dependent Epidermal Cell Activation in Psoriasis

Marta Ferran[1], Ana B. Galván[1], Catalina Rincón[2], Ester R. Romeu[2], Marc Sacrista[1], Erika Barboza[2], Ana Giménez-Arnau[1], Antonio Celada[2], Ramon M. Pujol[1] and Luis F. Santamaria-Babí[1,2]

Streptococcal throat infection is associated with a specific variant of psoriasis and with HLA-Cw6 expression. In this study, activation of circulating psoriatic cutaneous lymphocyte–associated antigen (CLA)$^+$ memory T cells cultured together with epidermal cells occurred only when streptococcal throat extracts were added. This triggered the production of Th1, Th17, and Th22 cytokines, as well as epidermal cell mediators (CXCL8, CXCL9, CXCL10, and CXCL11). Streptococcal extracts (SEs) did not induce any activation with either CLA$^-$ cells or memory T cells cultured together with epidermal cells from healthy subjects. Intradermal injection of activated culture supernatants into mouse skin induced epidermal hyperplasia. SEs also induced activation when we used epidermal cells from nonlesional skin of psoriatic patients with CLA$^+$ memory T cells. Significant correlations were found between SE induced upregulation of mRNA expression for *ifn-γ*, *il-17*, *il-22*, *ip-10*, and serum level of antistreptolysin O in psoriatic patients. This study demonstrates the direct involvement of streptococcal infection in pathological mechanisms of psoriasis, such as IL-17 production and epidermal cell activation.

Journal of Investigative Dermatology (2013) **133,** 999–1007; doi:10.1038/jid.2012.418; published online 29 November 2012

INTRODUCTION

In recent years, the characterization of the molecular mechanisms of psoriasis has greatly improved and has revealed new therapeutic targets. However, many questions remain regarding how psoriatic lesions are triggered and initiated (Nestle *et al.*, 2009). A correlation between streptococcal infection and psoriasis has been identified using clinical and epidemiological data (Valdimarsson *et al.*, 2009; Leung *et al.*, 1995; Baker *et al.*, 2006; Thorleifsdottir *et al.*, 2012). In the blood of psoriatic patients, T cells that recognize determinants common to streptococcal M-proteins and keratins (Gudjonsson *et al.*, 2003) and other possible autoantigens have been identified (Besgen *et al.*, 2010). Circulating cutaneous lymphocyte–associated antigen (CLA)$^+$CD8$^+$ T cells respond with IFN-γ production to keratin peptides that share sequence with M-proteins (Johnston *et al.*, 2004) in HLA-Cw*0602(+) psoriasis patients, and skin-derived CD4$^+$ T cells produce IFN-γ in response to streptococcal antigen (Brown *et al.*, 2000). In addition, streptococcal DNA induces activation and proliferation of CLA$^+$ T cells in psoriasis (Cai *et al.*, 2009). Although the relevance of streptococcal throat infection is known for >50 years (Norrlind, 1955), no functional evidence has linked *Streptococcus* with the induction of Th17/Th22 cytokines and epidermal activation/hyperplasia, hallmarks of psoriatic lesion (Nestle *et al.*, 2009).

Circulating CLA$^+$ T cells, a subset of memory T cells with skin tropism, preferentially respond to antigens/allergens involved in cutaneous diseases mediated by T cells (Santamaria Babi *et al.*, 1995a). These CLA$^+$ T cells represent a subset of memory/effector T lymphocytes associated with the cutaneous immune system (Santamaria-Babi, 2004). CLA$^+$ T cells have been analyzed in psoriasis, suggesting an early migration of those cells into the skin before the plaque lesion is established (Davison *et al.*, 2001; Vissers *et al.*, 2004), as well as their participation in the acute stages of psoriasis, psoriatic score, and affected body surface area (Sigmundsdottir *et al.*, 2001; Pont-Giralt *et al.*, 2006; Ferran *et al.*, 2008). The recruitment of circulating CLA$^+$ T cells into the skin is considered one of the relevant features in the pathogenesis of psoriasis (Guttman-Yassky *et al.*, 2011; Laggner *et al.*, 2011). Chronic streptococcal stimulation in the tonsils provides a source of pathogenic CLA$^+$ T cells (Sigmundsdottir *et al.*, 2003) that could home into skin and participate together with innate immune mechanisms in the

[1]Department of Dermatology, Hospital del Mar, Research Group in Inflammatory Dermatologic Diseases, IMIM, Universitat Autònoma de Barcelona, Barcelona, Spain and [2]Institute for Research in Biomedicine (IRB Barcelona), and Departament of Fisiologia I Immunologia, Universitat de Barcelona, Barcelona, Spain

Correspondence: Luis F. Santamaria-Babí, Institute for Research in Biomedicine (IRB Barcelona), Baldiri Reixac, 10, Barcelona 08028, Spain. E-mail: luis.santamaria@ub.edu

Abbreviations: ASO, antistreptolysin O; CLA, cutaneous lymphocyte–associated antigen; SE, streptococcal extract; SEB, superantigen enterotoxin B

Received 4 November 2011; revised 7 August 2012; accepted 8 August 2012; published online 29 November 2012

initiation of psoriatic lesions (Nestle *et al.*, 2009; Guttman-Yassky *et al.*, 2011). The goal of this study has been to determine the direct involvement of *Streptococcus* in psoriasis through its interaction with circulating CLA$^+$ memory T cells and epidermal cells, two relevant cell populations involved in the pathogenesis of the disease.

RESULTS

Streptococcal extract (SE) induces upregulation of mRNA expression for ifn-γ, il-17, il-22, and ip-10 in circulating psoriatic CLA$^+$ T cells cultured with lesional autologous epidermal cells

Infiltrating CLA$^+$ memory T cells and epidermal cells are in close contact in psoriatic lesions (Robert and Kupper, 1999). We attempted to mimic this situation by *in vitro* culturing circulating CLA$^+$ memory T cells and autologous epidermal cells from psoriatic patients. Neither T cells nor epidermal cells were stimulated. However, after the addition of SE to the culture, a time-dependent upregulation in mRNA expression of *ifn-γ*, *il-17*, *il-22*, and *ip-10* was observed (Figure 1a). This activation seems to be specific and restricted to the subset of skin-homing memory T cells because it occurs with CLA$^+$ but not with CLA$^-$ T cells. Moreover, circulating CLA$^+$ or CLA$^-$ memory T cells and autologous epidermal cells from controls after SE activation showed a minimal increase in the transcription of these genes (Figure 1b).

SE induces IFN-γ and IL-17 production in CLA$^+$ T cells cultured with autologous lesional epidermal psoriatic cells

The increase in mRNA expression correlates with cytokine production (IFN-γ and IL-17), as measured in the supernatants after 48 hours of culture (Figure 2a and b). In four of the five patients tested, a marked increase of cytokines was observed because of the presence of SE. No such increase was produced when CLA$^-$ T cells were used or the experiment was conducted with skin from healthy donors. When we used a nonspecific stimulus, such as superantigen enterotoxin B (SEB), IL-17 and IFN-γ were produced in both CLA$^+$ and CLA$^-$ subsets from control subjects, indicating lack of response to SE, but not to SEB (Figure 2c and d). To further characterize mediators induced by the activation produced of

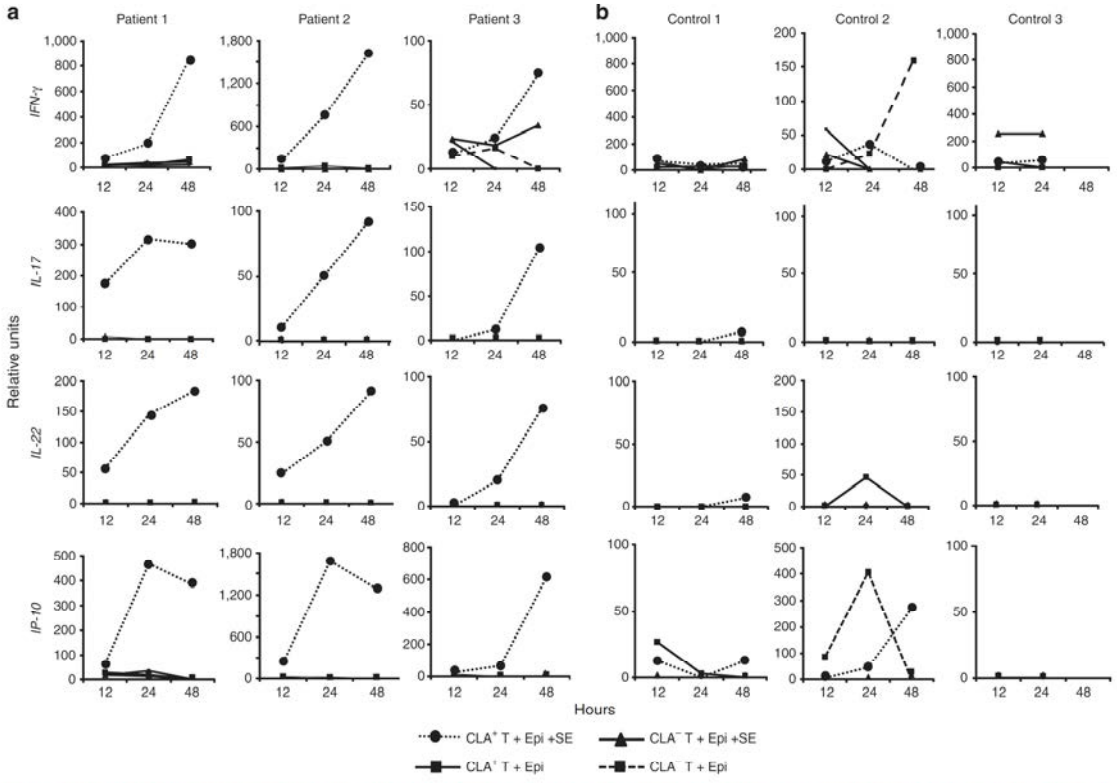
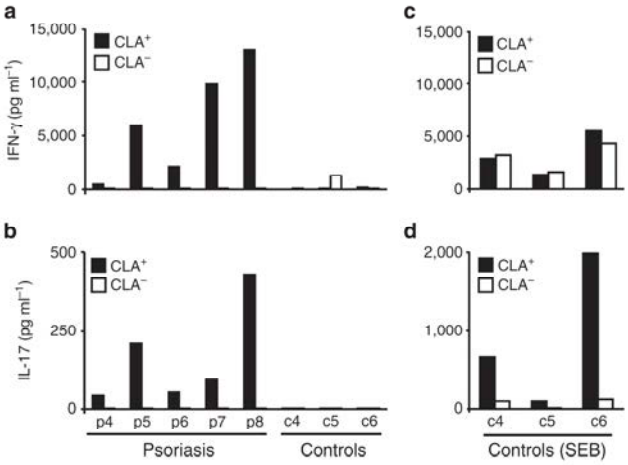


Figure 1. Streptococcal extract (SE) induces upregulation of mRNA for *ifn-γ*, *il-17*, *il-22*, and *ip-10* in psoriatic cutaneous lymphocyte–associated antigen (CLA)$^+$ T cells cultured with lesional autologous epidermal cells. Culture of freshly isolated circulating CLA$^+$/CLA$^-$ CD45RO$^+$CD3$^+$ cells with autologous epidermal cells obtained from lesional psoriatic skin was incubated with or without SE. A time-course analysis of *ifn-γ*, *il-17*, *il-22*, and *ip-10* mRNA expression was performed. RNA was taken at the indicated times after activation and determined by real-time PCR. (**a**) Data presented are from three psoriatic patients and (**b**) from three controls.



e

	Patient 10		Patient 11		Patient 12	
(pg ml^{-1})	CLA$^+$	CLA$^-$	CLA$^+$	CLA$^-$	CLA$^+$	CLA$^-$
IFN-γ	14,238	0	793	0	3,923	0
IL-17	15,404	0	300	0	1,884	0
IL-21	2,976	0	2,761	0	1,346	0
IL-22	0	0	0	0	0	0
IL-12p40	936	152	245	0	125	0
IL-12p70	116	0	11	0	29	0
IL-23	0	0	0	0	0	0
IL-6	611	0	30	0	71	0
TNF-α	57	0	0	0	0	0
CXCL11	95	67	156	71	94	0
CXCL10	1,688	0	281	0	214	0
CXCL9	136,440	567	19,486	100	16,544	0
CXCL8	257,111	7,828	88,689	4,085	95,311	0

Figure 2. Streptococcal extract (SE) induces IFN-γ and IL-17 production in cutaneous lymphocyte–associated antigen (CLA)$^+$ T cells cultured with autologous lesional epidermal psoriatic cells. Supernatants from cultured memory T cells (CLA$^+$/CLA$^-$) and autologous lesional epidermal psoriatic cells stimulated with SE or with *Staphylococcus aureus* enterotoxin B (SEB) for controls were analyzed for IFN-γ and IL-17 after 48 hours of culture by ELISA. (**a**–**d**) Data are presented from five psoriatic patients (p) and three controls (c) as net cytokine production.

SE in the culture of CLA$^+$ memory T cells and epidermal cells from psoriatic patients, we performed a quantitative antibody array for 13 cytokines (Figure 2e) using 5-day supernatants taken from three representative psoriatic patients who responded to SE. Cytokines produced by T cells and epidermal psoriatic cells, such as IFN-γ, IL-17 and IL-21, IL-12p40, IL-12p70, IL-6, CXCL11, CXCL10, CXCL9, and CXCL8, were significantly increased by SE in the cultures of CLA$^+$ T cells and epidermal cells in relation to CLA$^-$. As expected, the activation induced by SE produced a strong proliferation in the cultures of CLA$^+$ T cells with lesional epidermal cells (Supplementary Figure S2 online). Therefore, the SE induced an exacerbated immune response in our *in vitro* system. The SE response was restricted to the CLA$^+$CD45RO$^+$ T cells; however, *Candida Albicans* and SEB responses were not restricted to the skin-homing T-cell subset in pooled results from 5-day supernatants generated from four different patients (p24, p25, p26, and p27), as shown in Supplementary Figure S3 online. The dotted lines indicate the amount of cytokine production obtained by SE activation in the CLA$^-$ T-cell subset for each mediator.

Supernatants of SE-activated CLA$^+$ T cells and epidermal cells generate epidermal hyperplasia *in vivo*

Up to this point, we have found a significant increase in cytokines in the supernatants of the cultures. To assess the relevance of these *ex vivo* cultures on an *in vivo* model, we injected the supernatants intradermally in Balb/c mice. A significant epidermal hyperplasia, characteristic of psoriatic skin, was found when we injected supernatants of cultures of CLA$^+$ T cells incubated with lesional epidermal cells from psoriatic skin activated with SE (Figure 3a–c). However, the cultures containing CLA$^-$ of the same patient induced a minor epidermal thickness ($P<0.001$).

SE-induced upregulation of mRNA expression for ifn-γ, il-17, il-22, ip-10, tnf-α, and il-8 in cultures of CLA$^+$/CLA$^-$ T cells of nonlesional epidermal cells of psoriasis patients

CLA$^+$ T cells are present in the nonlesional marginal edge of psoriatic lesions before epidermal hyperplasia occurs (Davison *et al.*, 2001; Vissers *et al.*, 2004), and are considered to be relevant elements in the early events of plaque psoriasis formation (Guttman-Yassky *et al.*, 2011) together with other cell types, such as plasmocytoid dendritic cells (Albanesi *et al.*, 2009), thereby suggesting an initial involvement of skin-homing T cells in psoriatic lesion development. We assessed whether SE induces psoriatic gene expression in cultures of nonlesional epidermal cells and autologous circulating CLA$^+$ T cells of psoriatic patients. The expression of mRNA for *ifn-γ, il-17, il-22, tnf-α, and il-8 was* increased when nonlesional epidermal cells were cultured with CLA$^+$ T cells and SE (Figure 4), whereas those genes were not detected in cells from healthy donors (data not shown). Our results demonstrate that SE and CLA$^+$ T cells can induce upregulation of mRNA for some genes characteristic of the psoriatic lesion in epidermal cells obtained from non-lesional psoriatic skin.

Correlation between serum antistreptolysin O (ASO) antibody levels and SE-induced upregulation of mRNA of ifn-γ, il-17, il-22, and ip-10 in CLA$^+$ T cells cultured together with epidermal cells in psoriatic patients

As SE is able to induce CLA$^+$ T cell and epidermal cell activation in psoriasis, we explored whether this *in vitro* activity could be related to *in vivo* exposure to streptococcal infection in some patients. We compared anti-ASO antibody serum levels with the expression of *ifn-γ, il-17, il-22,* and *ip-10* obtained in the culture of circulating CLA$^+$ memory T cells and epidermal cells 24 hours after activation by SE. As shown

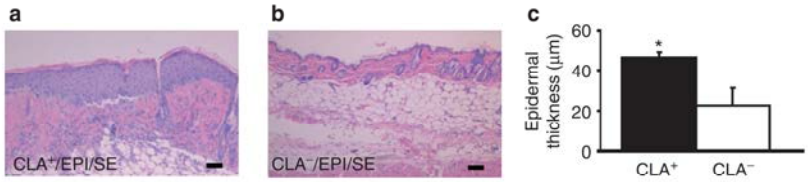


Figure 3. Supernatants of streptococcal extract (SE)–activated cutaneous lymphocyte–associated antigen (CLA)$^+$ T cells and epidermal cells generate epidermal hyperplasia *in vivo*. Intradermal injection of supernatants from CLA$^+$ T cells cultured with lesional epidermal cells activated with SE induces *in vivo* epidermal hyperplasia in Balb/c mice. Supernatant from day 6 of the culture was injected intradermally in Balb/c mice daily for 4 days, and epidermal hyperplasia was evaluated at day 4. (**a**–**c**) Results are shown with histological images of skin biopsies (hematoxylin and eosin) and a comparative graphic of epidermis thickness (in micrometers) after supernatant injections in four mice ($P<0.001$). Bar = 50 µm.

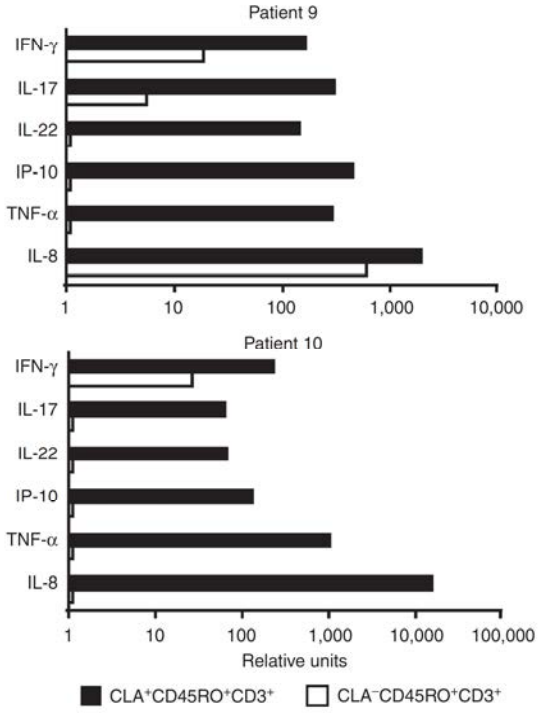


Figure 4. Streptococcal extract–induced upregulation of mRNA for ifn-γ, il-17, il-22, ip-10, tnf-α, and il-8 in cultures of cutaneous lymphocyte–associated antigen (CLA)$^+$/CLA$^-$ T cells of nonlesional epidermal cells of psoriasis patients. Upregulated gene transcription in the culture 24 hours after activation. Data are presented for two patients with nonlesional epidermal cells.

in Figure 5, significant correlation was found between anti-ASO levels and the expression of *ifn-γ* ($r=0.77$, $P=0.01$), *il-17* ($r=0.89$, $P=0.001$), *il-22* ($r=0.91$, $P=0.0005$), and *ip-10* ($r=0.86$, $P=0.002$). In addition, those ASO values also correlated with psoriasis area severity index scores ($r=0.68$, $P=0.04$), with a lower score in guttate patients (data not shown). The mRNA for those same genes was not amplified in cultures of CLA$^-$ memory T cells and epidermal cells activated by SE from the same patients.

Influence of major histocompatibility complex class I or class II on SE-activated cultures of memory T cells with epidermal cells
Our cultures are established using CD3$^+$CD45R0$^+$ cells. Therefore, we attempted to characterize the involvement of HLA class I or class II in the activation of cultures of CLA$^+$ memory T cells and epidermal cells using blocking antibodies (Figure 6 and Supplementary Figure S4C online). We simultaneously quantified IFN-γ, IL-17A, IL-6, and TNF-α in 5-day supernatants by flow cytometry. The SE-dependent activation of the culture of CLA$^+$ T cells and epidermal cells depends on the HLA class I and HLA class II molecules, because blocking antibodies reduces cytokine production; anti-HLA class II is significantly more active than anti-HLA class I: IL-17A ($P<0.0001$), IFN-γ ($P<0.03$), and TNF-α ($P<0.001$). As expected, the generation of new epidermal cells was also impaired. The generation of a new population of epidermal cells that appears only in the culture condition of CLA$^+$ memory T cell and epidermal cells activated by SE (Figure 4a), with reduced staining to CFSE compared with the whole population of epidermal cells after 4 days in culture (Figure 4b), and contains CD29$^+$HLA-DR$^+$ cells, was reduced by the effect of blocking antibodies (Figure 4c).

DISCUSSION

T cells are considered to represent a functional link between streptococcal tonsillitis and psoriatic inflammation (Diluvio *et al.*, 2006; Besgen *et al.*, 2010). Although clinical association between streptococcal infections and psoriasis are well established (Valdimarsson *et al.*, 2009), the inflammatory mechanisms involved are poorly characterized. A direct proof of the capacity of SE to induce hallmarks of psoriasis response, such as Th17 response, epidermal activation, and hyperplasia, *in vivo* selectively through circulating CLA$^+$ T cells in psoriasis would support this hypothesis (Valdimarsson *et al.*, 2009). To test this concept, we developed an *ex vivo* culture system where circulating memory CLA$^+$/CLA$^-$ T cells are cultured together with autologous lesional or nonlesional epidermal cells with/o SE in cells from patients with psoriasis or healthy controls. The SE we use is a mixture of four heat-killed and sonicated streptococcal throat isolates from patients

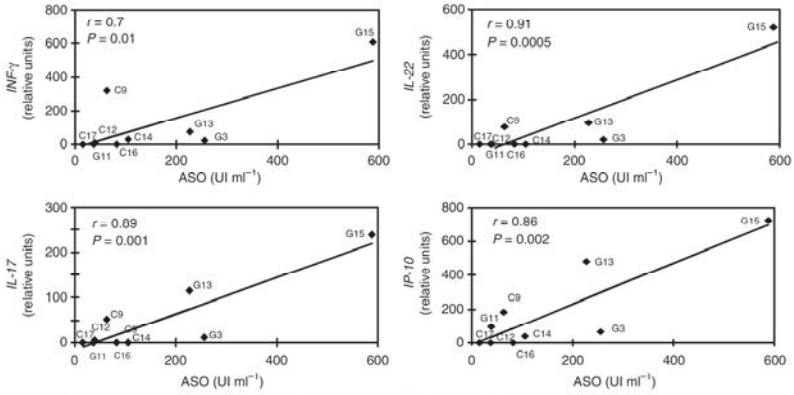


Figure 5. Streptococcal extract–induced upregulation in gene transcription for ifn-γ, il-17, il-22, and ip-10 after 24 hours of activation of cutaneous lymphocyte–associated antigen (CLA)$^+$ memory T cells and epidermal cells correlates with antistreptolysin O (ASO) serum levels in psoriatic patients. Patients are identified by number and psoriasis type (C, chronic; G, guttate).

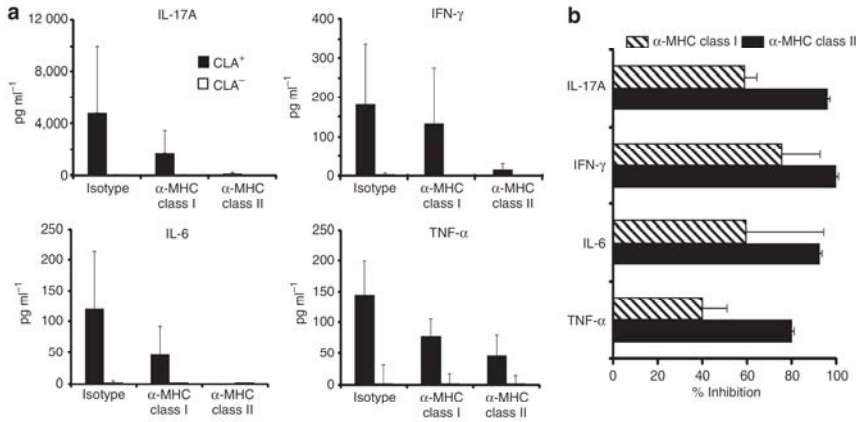


Figure 6. Streptococcal extract (SE)–induced upregulation of IL-17A, IFN-γ, IL-6, and TNF-α in cultures of cutaneous lymphocyte–associated antigen (CLA)$^+$ T cells and epidermal cells depends on HLA class I and HLA class II. Three experiments conducted with patients p18, p19, and p23 are presented. (**a**) Cytokines induced by SE on CLA$^+$ memory T cells and epidermal cells are inhibited with anti-HLA class I and anti class II blocking antibodies; data are presented as net values. (**b**) The mean percentage inhibition respect isotype is significantly higher for anti-HLA class II than anti-HLA class I; IL-17A ($P<0.0001$), IFN-γ ($P<0.03$), and TNF-α ($P<0.001$).

with psoriasis. Such preparation has been shown to induce CLA expression by T cells (Baker *et al.*, 1997).

The cytokines IFN-γ, IL-17, IL-21, and IL-22 are well-known inducers of epidermal activation and hyperplasia in psoriasis (Guttman-Yassky *et al.*, 2011). It is interesting to see that SE can trigger the expression of all those four mediators in psoriasis when circulating CLA$^+$ T cells and epidermal cells are cultured together. The difference found in the amount of IFN-γ, IL-17, and IL-21 compared to IL-22 in the antibody array deserves further exploration. Difference in the frequency of those T-cell subsets in the blood of psoriatic patients, and also the timing of supernatant collection in our model, might explain the lower levels of IL-22 found after 5 days. IL-21 is a cytokine recently associated with psoriasis (Caruso *et al.*, 2009) that is produced by circulating and infiltrating CLA$^+$ T cells in psoriasis and also induces epidermal hyperplasia. Cells producing IL-17, IL-21 of IL-22 are rarely found in circulating T lymphocytes (Eyerich *et al.*, 2009). However, the amount of IFN-γ, IL-17, and IL-21 obtained with our *ex vivo* culture, using only 5×10^4 circulating CLA$^+$ T cells and 3×10^4 epidermal cells activated by SE after 5 days of culture, are in the same range to the amount obtained using cloned 1×10^6 T cells polyclonally activated for 48 hours (Eyerich *et al.*, 2009). These results suggest that, in psoriasis, there is an enrichment of IFN-γ, IL-17, IL-21, and IL-22 producing T cells responding to SE within the subset of circulating CLA$^+$ T-cell

subset in psoriasis. This observation is in line with the general functional involvement of circulating CLA$^+$ T cells in other skin diseases (Santamaria-Babi, 2004). In contrast to the epidermal cells from controls, nonlesional epidermal cells could be activated with autologous CLA$^+$ T cells and SE inducing the expression of Th1 and Th17 cytokines, as well as epidermal cell mediators, such as *IP-10* (Gottlieb *et al.*, 1988) and *IL-8* (Sticherling *et al.*, 1991). These results are in accordance with the model in which SE activated specific circulating CLA$^+$ T cells that, upon migration to the skin, could participate in lesion induction (Valdimarsson *et al.*, 2009).

We did not observe IL-23 production in our cultures. This is not surprising because dermal dendritic cells are the main producers of IL-23 in psoriasis (Tonel *et al.*, 2010), and our cultures were devoid of dermal cells. It has been shown that keratinocytes can produce IL-12p40 and IL-12p70 (Aragane *et al.*, 1994). Interestingly, we observed that IL-12p40 and IL-12p70 are produced in the supernatants of activated epithelial cells, which concur with previous results in psoriatic lesions (Lee *et al.*, 2004). We also detected the production of the chemokines CXCL11, CXCL10, CXCL9, and CXCL8 (Figure 2e). These chemokines are expressed in psoriatic lesions (Nograles *et al.*, 2008) and are potent chemoattractants of immune cells found in the lesions.

Although the epidermal cell suspension from psoriasis lesions contained immune cells, we do not attribute our results to those cells. The same epidermal cell suspension is added to either circulating CLA$^+$ or CLA$^-$ memory T cells. If SEs were interacting with epidermal immune cells, the culture of CLA$^-$ memory T cells with SE and epidermal cells should also be activated. This is not the case. In addition, it is clear that the epidermal cell activation (CXCL11, CXCL10, CXCL9, and CXCL8) in our system depends on the presence of circulating CLA$^+$ memory T cells but not CLA$^-$.

Our results indicate that SEs trigger the activation of key psoriatic T-cell cytokines and epidermal chemokines through the interaction of CLA$^+$ T cells with either lesional or nonlesional skin of psoriatic patients. The correlation between *in vivo* and *in vitro* findings was confirmed by the development of epidermal hyperplasia after injection of supernatants of SE-activated CLA$^+$ T cells and epidermal cells from psoriatic patients in the mouse skin. Interestingly, in our culture, we identified an epidermal cell population that present decreased staining to CFSE, suggesting a proliferative state, and express HLA-DR/CD29 that only appears upon the activation of SE and in the condition with CLA$^+$ memory T cells and epidermal cells. This population decreases when blocking antibodies to major histocompatibility complex class I or class II are used. Although our culture system is not optimal for keratinocyte proliferation, our data suggest that it may be useful to study early activation of epidermal cells and T cells by SE. In addition, the production of IL-17A, IFN-γ, IL-6, and TNF-α in 5-day supernatants of cultures of CLA$^+$ T cells and epidermal cells activated with SE could also be inhibited by blocking antibodies against HLA class I and HLA class II, having stronger activity anti-HLA class II. One possible hypothesis for the activity of both blocking antibodies would be that SE antigens could be presented to CD4$^+$, and cytokines derived from CD4$^+$ T cells could induce autoantigens on epidermal cells (Shi *et al.*, 2011) that later can be recognized by CD8$^+$ T cells in HLA class I context. For this reason blockage of major histocompatibility complex class II could have stronger effect because it inhibits early mechanisms in our culture system.

In a high percentage of guttate psoriasis patients, streptococcal tonsillitis leads to relapses of psoriasis (Norrlind, 1955; Gudjonsson *et al.*, 2003; Mallbris *et al.*, 2005). For some of the patients in our cohort, serum levels of ASO were recorded at the moment of blood and biopsy extraction. We have found a significant correlation between ASO and SE-induced upregulation of mRNA expression at 24 hours *for ifn-γ*, *il-17*, *il-22*, and *ip-10* only in the culture of CLA$^+$ memory T cells and epidermal cells. Although this is a limited number of patients, the results suggest that SE induces higher activation of CLA$^+$ T cells and epidermal cells in the patients presenting higher ASO levels.

The HLA-Cw*0602 data of our patients suggest that streptococcal-induced activation of CLA$^+$ memory T cells and epidermal cells is not restricted to HLA-Cw*0602-positive patients. This concurs with early results, indicating that not all patients with streptococcal-related psoriasis carry HLA-CW*0602 (Fry *et al.*, 2006). Our current results do not establish any differences between guttate and chronic plaque psoriasis. However, patients 15 and 13 have guttate psoriasis with high ASO, onset associated with pharyngeal streptococcal infection, and higher upregulation of mRNA for ifn-γ, il-17, il-22, and ip-10 in CLA$^+$ T cells and epidermal cells (Figure 5). We would hypothesize that this type of patient may present the highest activation response to SE, in line with published data that indicate that HLA-Cw*0602-positive psoriasis patients have a more severe disease (Gudjonsson *et al.*, 2006).

Our separation methods obtain CLA$^+$/CLA$^-$ memory T cells that can be either α/β or γ/δ. Circulating CLA$^+$ Vγ9Vδ2 T cells can migrate from blood to skin, their number correlate with the clinical course of psoriasis and produce IL-17 (Laggner *et al.*, 2011). In addition, γδ-T cells are greatly increased in lesional psoriatic lesion and produce large amounts of IL-17 (Cai *et al.*, 2011). It could be hypothesized that our purified CLA$^+$ T-cell subsets contain some γδ-T cells that could react to some antigens present in the SE. In fact, streptococcal antigens can be recognized and activate γδ-T cells (Bender *et al.*, 1993; Bender and Kabelitz, 1992; Grinlinton *et al.*, 1993). We are currently exploring this interesting possibility.

To date, a direct effect of SE on circulating CLA$^+$ T cells that can explain how this induces new lesions in psoriatic patients has not been demonstrated. Our results from these culture experiments with psoriatic cells provide experimental evidence that *Streptococcus* is a relevant trigger of psoriasis lesions through its interaction with circulating skin-homing T cells and epidermal cells. The *ex vivo* culture model used in this study will contribute to clarifying the molecular mechanism involved in the proposed molecular mimicry of the autoimmune origin of psoriasis (Valdimarsson *et al.*, 2009;

Besgen *et al.*, 2010), and can be a valuable model to try new experimental therapies.

MATERIALS AND METHODS

Patients

The study included 27 psoriatic patients (15 with guttate psoriasis, 9 with chronic plaque psoriasis and 3 with acute plaque psoriasis) and 6 healthy controls. Detailed patients' characteristics can be found in Supplementary Table SI online. Patients and healthy controls participated voluntarily and gave written informed consent. Patients with erythrodermic pustular psoriasis or arthritis were excluded. We assessed psoriasis area severity index scores, disease extent (body surface area affected), and clinical characteristics. All the participants underwent a skin biopsy and a blood extraction. The samples were collected after a minimum period of 6 weeks without treatment of any kind. The study was approved by the Medical Ethics Committee of Hospital del mar, and written consent was obtained from all participants; this work was conducted according to the Declaration of Helsinki principles.

Purification of circulating peripheral blood CLA^+/CLA^- memory T cells

Circulating CLA^+ or $CLA^-CD45RO^+$ $CD3^+$ cells were purified from peripheral blood mononuclear cells obtained by Ficoll separation using 60 ml of blood. Three consecutive immunomagnetic separations were then carried out using antibodies and magnetic particle–conjugated antibodies from Miltenyi Biotec (Bergisch Gladbach, Germany) by a modified procedure originally described in Santamaria Babi *et al.*, 1995b. In the first separation, $CD14^+$ and $CD19^+$ were depleted, in the second separation, $CD16^+$ and $CD45RA^+$ lymphocytes were removed. Finally, in the third separation, $CD45RO^+$ memory T cells were divided into CLA^+ and CLA^- memory T-cell subpopulations by using the Anti-CLA MicroBead Kit (Miltenyi Biotec, Bergisch Gladbach, Germany). After the separation by two consecutive column separations to obtain high depletion and enrichment, the sample purity was routinely tested for the CLA-enriched T-cell preparations containing $\geqslant 95\%$ of CLA^+ cells and for the CLA-depleted preparations with $<10\%$ CLA^+ as assessed by FACS (Supplementary Figure S1 online).

Punch biopsies and epidermal cell suspension

Epidermal cells from cutaneous biopsies were isolated by incubating skin biopsy in dispase 37°C for 2 hours to peel off the epidermis from the dermis. The epidermal sheet was cut into small pieces and incubated with trypsin for 30 minutes to obtain epidermal cell suspensions.

Culture of epidermal cells together with $CLA^{+/-}$ memory T cells and stimulation with different antigens

The culture system was performed by seeding 50,000 circulating CLA^+/CLA^- memory T cells with 30,000 autologous epidermal cells in a 96-flat bottom microwell plate (Nunc, Roskilde, Denmark) with culture medium (RPMI 10% fetal calf serum) for 12, 24, 48 hours, 5, and 6 days. SE isolated from bacteria from the throat of psoriatic patients (1 $\mu g\,ml^{-1}$ final), which was kindly provided by Drs Johann Gudjonsson and Helki Valdimarsson, *C. albicans* at 40 $\mu g\,ml^{-1}$ (Greerlabs, Lenoir, NC), SEB at 100 $ng\,ml^{-1}$ (Sigma-Aldrich, St Louis, MO), or equivalent culture medium, was added in both conditions (CLA^+/CLA^-) to compare all the conditions. NaN_3 free blocking antibodies for HLA class I (clone W6/32) and class II (clone L243), as well as isotype control, were purchased from Biolegend (San Diego, CA) and used at 10 $\mu g\,ml^{-1}$.

Gene expression

The RNA of cell-cultured pellets was extracted using the Trizol (Invitrogen, Paisley, UK) and cDNA prepared using the High Capacity cDNA Reverse Transcription kit (Applied Biosystems, Carlsbad, CA), following the respective manufacturer's instructions. Subsequent analysis by real-time–PCR was performed with AB17900HT (Applied Biosystems) and the data were processed by SDS version 1.0 analysis program (Applied Biosystems). Primers and probes were purchased from Applied Biosystems. The reactions were run on an Applied Biosystems 7900HT system. Gene expression was calculated using the $\Delta - \Delta Ct$ method (using the mean cycle threshold value for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and the gene of interest for each sample). The equation $1.8\ e(Ct_{GAPDH} - Ct_{gene\ of\ interest}) \times 10^4$ was used to obtain the normalized values (Chan *et al.*, 2006). Increased values were calculated by subtracting normalized gene expression values in the culture for basal and SE-stimulated conditions.

Cytokine determination

Supernatants were taken after 48 hours of culture, and IFN-γ and IL-17 were measured by ELISA (Peprotech, London, UK). Data are presented from five psoriatic patients and three controls as net cytokine production. Customized arrays of antibodies (Tebu-bio, Le Perray en Yvelines, France) were used to simultaneously quantify the presence in cell culture of the following human mediators: IFN-γ, IL-17, IL-21, IL-22, IL-12p40, IL-12p70, IL-23, IL-6, TNF-α, CXCL11, CXCL10, CXCL9, and CXCL8. Supernatants were taken after 5 days of culture. Data are presented from three psoriatic patients as net cytokine production. Quantification of IFN-γ, IL-17A, IL-6, and TNF-α by flow cytometry was performed with a multiplex fluorescent bead-based immunoassay, Diaclone DIAplex kit (Gen-Probe, Besançon, France), to simultaneous quantify in the 4-day supernatants of all the culture conditions with a F500 Flow Cytometer (Beckman Coulter, Fullerton, CA), data are presented as net cytokine production.

Determination of ASO levels in serum

The same day that biopsies and blood were obtained, serum levels of ASO were analyzed by an immunoturbidimetric method.

Intradermal injection of supernatants into mouse skin

Fifty-µl supernatants (1/3 diluted) collected from cultures of four psoriatic patients on day 6 after SE activation, were intradermally injected into the back of anesthetized Balb/c mice (daily during 4 days). Epidermal hyperplasia was evaluated at day 4. Mouse skin was biopsied and sections from paraffin-embedded skin were prepared and stained with hematoxylin and eosin. Animal use was approved by the Animal Research Committee of the University of Barcelona (number 2523).

Data analysis

Data were analyzed with the Student's *t*-test. The analysis of variance test was used to assess the statistical significance in the regression analysis.

CONFLICT OF INTEREST

The authors state no conflict of interest.

ACKNOWLEDGMENTS

This work was supported by a grant from the Instituto de Salud Carlos III FEDER (PS09/0222 and PS09/01811) to LFSB and AGA. We acknowledge the support of the COST BM0903 Network/SKINBAD (Skin Barrier in Atopic Diseases) to LFSB. We thank Johann Gudjonsson (Department of Dermatology, University of Michigan Medical Center, Ann Arbor, Michigan, USA) and Helki Valdimarsson (Landspítali Háskólasjúkrahús, University Hospital, Hringbraut, 101 Reykjavík, Iceland), who kindly provided extract from streptococcal bacteria isolated from the throats of psoriatic patients.

SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at http://www.nature.com/jid

REFERENCES

Albanesi C, Scarponi C, Pallotta S *et al.* (2009) Chemerin expression marks early psoriatic skin lesions and correlates with plasmacytoid dendritic cell recruitment. *J Exp Med* 206:249–58

Aragane Y, Riemann H, Bhardwaj RS *et al.* (1994) IL-12 is expressed and released by human keratinocytes and epidermoid carcinoma cell lines. *J Immunol* 153:5366–72

Baker BS, Garioch JJ, Hardman C *et al.* (1997) Induction of cutaneous lymphocyte-associated antigen expression by group A streptococcal antigens in psoriasis. *Arch Dermatol Res* 289:671–6

Baker BS, Powles A, Fry L (2006) Peptidoglycan: a major aetiological factor for psoriasis? *Trends Immunol* 27:545–51

Bender A, Heckl-Ostreicher B, Grondal EJ *et al.* (1993) Clonal specificity of human gamma delta T cells: V gamma 9+ T-cell clones frequently recognize *Plasmodium falciparum* merozoites, *Mycobacterium tuberculosis*, and group-A streptococci. *Int Arch Allergy Immunol* 100:12–8

Bender A, Kabelitz D (1992) Preferential activation of peripheral blood V gamma 9 + gamma/delta T cells by group A, B and C but not group D or F streptococci. *Clin Exp Immunol* 89:301–4

Besgen P, Trommler P, Vollmer S *et al.* (2010) Ezrin, maspin, peroxiredoxin 2, and heat shock protein 27: potential targets of a streptococcal-induced autoimmune response in psoriasis. *J Immunol* 184:5392–402

Brown DW, Baker BS, Ovigne JM *et al.* (2000) Skin CD4+ T cells produce interferon-gamma in vitro in response to streptococcal antigens in chronic plaque psoriasis. *J Invest Dermatol* 114:576–80

Cai Y, Shen X, Ding C *et al.* (2011) Pivotal role of dermal IL-17-producing gammadelta T cells in skin inflammation. *Immunity* 35:596–610

Cai YH, Lu ZY, Shi RF *et al.* (2009) Enhanced proliferation and activation of peripheral blood mononuclear cells in patients with psoriasis vulgaris mediated by streptococcal antigen with bacterial DNA. *J Invest Dermatol* 129:2653–60

Caruso R, Botti E, Sarra M *et al.* (2009) Involvement of interleukin-21 in the epidermal hyperplasia of psoriasis. *Nat Med* 15:1013–5

Chan JR, Blumenschein W, Murphy E *et al.* (2006) IL-23 stimulates epidermal hyperplasia via TNF and IL-20R2-dependent mechanisms with implications for psoriasis pathogenesis. *J Exp Med* 203:2577–87

Davison SC, Ballsdon A, Allen MH *et al.* (2001) Early migration of cutaneous lymphocyte-associated antigen (CLA) positive T cells into evolving psoriatic plaques. *Exp Dermatol* 10:280–5

Diluvio L, Vollmer S, Besgen P *et al.* (2006) Identical TCR beta-chain rearrangements in streptococcal angina and skin lesions of patients with psoriasis vulgaris. *J Immunol* 176:7104–11

Eyerich S, Eyerich K, Pennino D *et al.* (2009) Th22 cells represent a distinct human T cell subset involved in epidermal immunity and remodeling. J. *Clin Invest* 119:3573–85

Ferran M, Gimenez-Arnau AM, Bellosillo B *et al.* (2008) Circulating CLA+ T cell subsets inversely correlate with disease severity and extension in acute psoriasis but not in chronic plaque psoriasis. *Eur J Dermatol* 18:647–50

Fry L, Powles AV, Corcoran S *et al.* (2006) HLA Cw*06 is not essential for streptococcal-induced psoriasis. *Br J Dermatol* 154:850–3

Gottlieb AB, Luster AD, Posnett DN *et al.* (1988) Detection of a gamma interferon-induced protein IP-10 in psoriatic plaques. *J Exp Med* 168: 941–8

Grinlinton FM, Skinner MA, Birchall NM *et al.* (1993) Gamma delta + T cells from patients with psoriatic and rheumatoid arthritis respond to streptococcal antigen. *J Rheumatol* 20:982–7

Gudjonsson JE, Karason A, Runarsdottir EH *et al.* (2006) Distinct clinical differences between HLA-Cw*0602 positive and negative psoriasis patients—an analysis of 1019 HLA-C- and HLA-B-typed patients. *J Invest Dermatol* 126:740–5

Gudjonsson JE, Thorarinsson AM, Sigurgeirsson B *et al.* (2003) Streptococcal throat infections and exacerbation of chronic plaque psoriasis: a prospective study. *Br J Dermatol* 149:530–4

Guttman-Yassky E, Nograles KE, Krueger JG (2011) Contrasting pathogenesis of atopic dermatitis and psoriasis-Part II: Immune cell subsets and therapeutic concepts. *J Allergy Clin Immunol* 127:1420–32

Johnston A, Gudjonsson JE, Sigmundsdottir H *et al.* (2004) Peripheral blood T cell responses to keratin peptides that share sequences with streptococcal M proteins are largely restricted to skin-homing CD8(+) T cells. *Clin Exp Immunol* 138:83–93

Laggner U, Di MP, Perera GK *et al.* (2011) Identification of a novel proinflammatory human skin-homing Vgamma9Vdelta2 T cell subset with a potential role in psoriasis. *J Immunol* 187:2783–93

Lee E, Trepicchio WL, Oestreicher JL *et al.* (2004) Increased expression of interleukin 23 p19 and p40 in lesional skin of patients with psoriasis vulgaris. *J Exp Med* 199:125–30

Leung DY, Travers JB, Giorno R *et al.* (1995) Evidence for a streptococcal superantigen-driven process in acute guttate psoriasis. *J Clin Invest* 96:2106–12

Mallbris L, Larsson P, Bergqvist S *et al.* (2005) Psoriasis phenotype at disease onset: clinical characterization of 400 adult cases. *J Invest Dermatol* 124:499–504

Nestle FO, Kaplan DH, Barker J (2009) Psoriasis. *N Engl J Med* 361: 496–9

Nograles KE, Zaba LC, Guttman-Yassky E *et al.* (2008) Th17 cytokines interleukin (IL)-17 and IL-22 modulate distinct inflammatory and keratinocyte-response pathways. *Br J Dermatol* 159:1092–102

Norrlind R (1955) The significance of infections in the origination of psoriasis. *Acta Rheumatol Scand* 1:135–44

Pont-Giralt M, Gimenez-Arnau AM, Pujol RM *et al.* (2006) Circulating CLA(+) T cells from acute and chronic psoriasis patients manifest a different activation state and correlation with disease severity and extension. *J Invest Dermatol* 126:227–8

Robert C, Kupper TS (1999) Inflammatory skin diseases, T cells, and immune surveillance. *N Engl J Med* 341:1817–28

Santamaria-Babi LF (2004) CLA(+) T cells in cutaneous diseases. *Eur J Dermatol* 14:13–8

Santamaria Babi LF, Picker LJ, Perez Soler MT *et al.* (1995a) Circulating allergen-reactive T cells from patients with atopic dermatitis and allergic contact dermatitis express the skin-selective homing receptor, the cutaneous lymphocyte-associated antigen. *J Exp Med* 181:1935–40

Santamaria Babi LF, Moser R, Perez Soler MT *et al.* (1995b) Migration of skin-homing T cells across cytokine-activated human endothelial cell layers involves interaction of the cutaneous lymphocyte-associated antigen (CLA), the very late antigen-4 (VLA-4), and the lymphocyte function-associated antigen-1 (LFA-1). *J Immunol* 154:1543–50

Shi X, Jin L, Dang E *et al.* (2011) IL-17A upregulates keratin 17 expression in keratinocytes through S. *J Invest Dermatol* 131:2401–8

Sigmundsdottir H, Gudjonsson JE, Jonsdottir I *et al.* (2001) The frequency of CLA+ CD8+ T cells in the blood of psoriasis patients correlates closely with the severity of their disease. *Clin Exp Immunol* 126:365–9

Sigmundsdottir H, Gudjonsson JE, Valdimarsson H (2003) Interleukin-12 alone can not enhance the expression of the cutaneous lymphocyte associated

antigen (CLA) by superantigen-stimulated T lymphocytes. *Clin Exp Immunol* 132:430–5

Sticherling M, Bornscheuer E, Schroder JM *et al.* (1991) Localization of neutrophil-activating peptide-1/interleukin-8-immunoreactivity in normal and psoriatic skin. *J Invest Dermatol* 96:26–30

Thorleifsdottir RH, Sigurdardottir SL, Sigurgeirsson B *et al.* (2012) Improvement of psoriasis after tonsillectomy is associated with a decrease in the frequency of circulating T cells that recognize streptococcal determinants and homologous skin determinants. *J Immunol* 188:5160–5

Tonel G, Conrad C, Laggner U *et al.* (2010) Cutting edge: A critical functional role for IL-23 in psoriasis. *J Immunol* 185:5688–91

Valdimarsson H, Thorleifsdottir RH, Sigurdardottir SL *et al.* (2009) Psoriasis as an autoimmune disease caused by molecular mimicry. *Trends Immunol* 30:494–501

Vissers WH, Arndtz CH, Muys L *et al.* (2004) Memory effector (CD45RO+) and cytotoxic (CD8+) T cells appear early in the margin zone of spreading psoriatic lesions in contrast to cells expressing natural killer receptors, which appear late. *Br J Dermatol* 150:852–9

SUPPLEMENTARY INFORMATION

Material and Methods

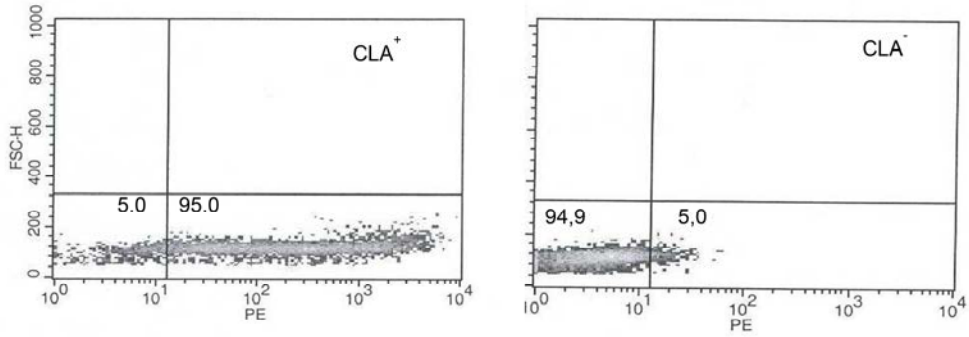
DNA extraction and HLA-C*0602 typing:

Purification of total DNA from cultured human cells was done using the DNeasy Blood & Tissue Kit (QIAGEN, Hilden, Germany) following a Spin-Column Protocol, using up to five million of either lymphocytes or fibroblasts from patients. After the procedure, eluted DNA was quantified by nanodrop spectophotometer and taken to concentrations of 100 ng/ul or 50 ng/ul. HLA C polymorphisms were examined using a PCR-SSO a technology. Briefly, the target DNA was amplified by PCR with biotinylated primers specifically designed for amplified exon 2 and exon 3 of HLA-C alleles. Then, the PCR amplicon was denatured and hybridized to complementary oligonucleotide probes immobilized on fluorescence-coated microsphere beads. At the same time, the biotinylated PCR product was labeled with phycoerythrin-conjugated streptavidin and immediately examined with Luminex 200 using the Quicktype software for analysis (LIFECODES. Gen-Probe Inc, San Diego, CA). The nomenclature of the HLA alleles followed the 2010 World Health Organization nomenclature Committee for factors regarding the HLA system.

Flow cytometry analysis

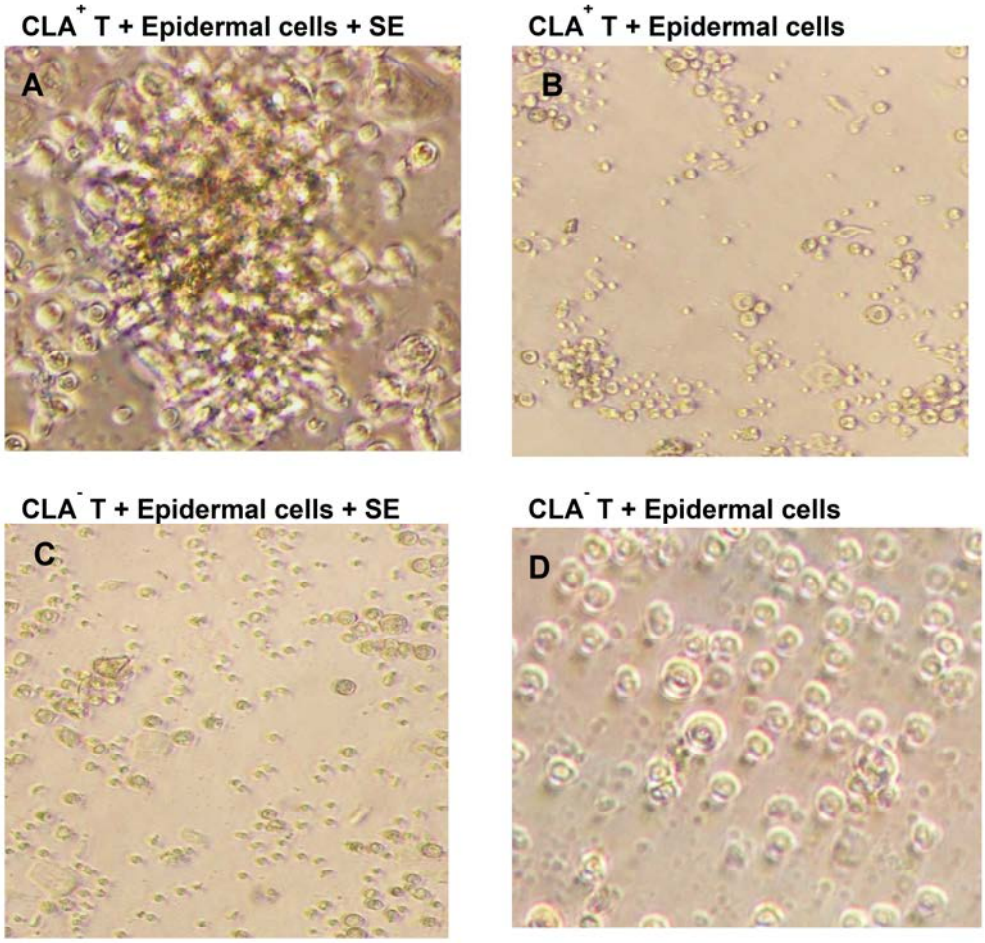
The following analysis were performed with a F500 Flow Cytometer (Beckman Coulter Inc, Fullerton, CA). *Epidermal cell proliferation with CFSE*. Epidermal cells were labeled at 37°C for 10 min using the CellTrace CFSE cell proliferationKIT (Life Technologies. Paisley, UK), washed and added to the different cell culture conditions. At day 4 of culture cells were analyzed for CFSE fluorescence. *Staining of $CD29^{+}HLA\text{-}DR^{+}$cultured epidermal cells*. Cells were stained with anti-human CD29-FITC (ebiosciences, San Diego, CA) and anti-human HLA-DR-PE (BD Biosciences, San Jose, CA).

Supplementary Figure 1:



Purity of immunomagnetic isolated CLA^+ and CLA^- T cell populations

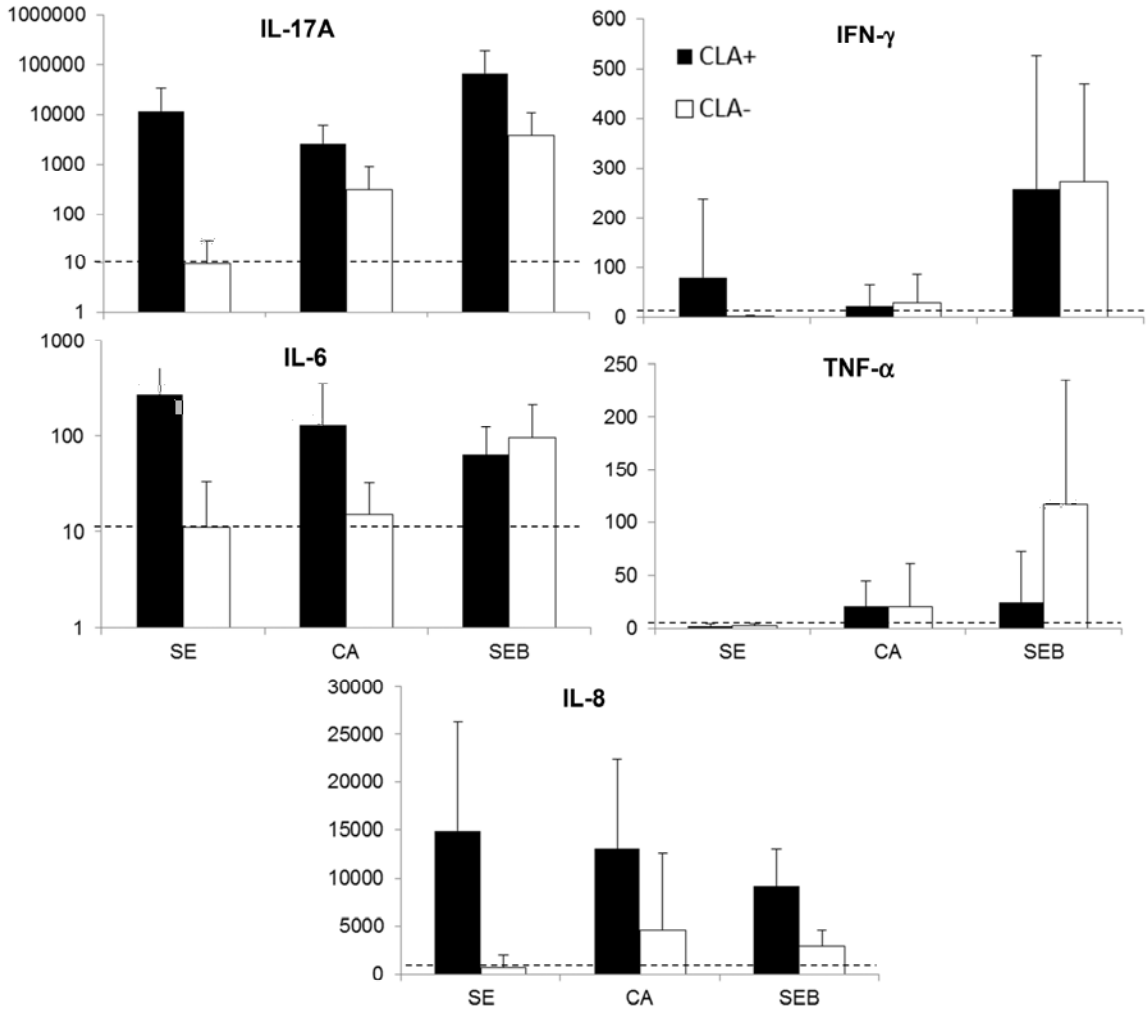
Supplementary Figure 2:



SE preferentially induces the activation of CLA^+ T cells cultured with autologous lesional epidermal psoriatic cells.

Culture of freshly isolated circulating CLA^+/CLA^- $CD45RO^+CD3^+$ cells with autologous epidermal cells obtained from lesional psoriatic skin was incubated w/o SE for 6 days: a) CLA^+T cells and epidermal cells with SE; b) CLA^+ T cells and epidermal cells; c) CLA^- T cells and epidermal cells with SE; and d) CLA^- T cells and epidermal cells.

Supplementary Figure 3:

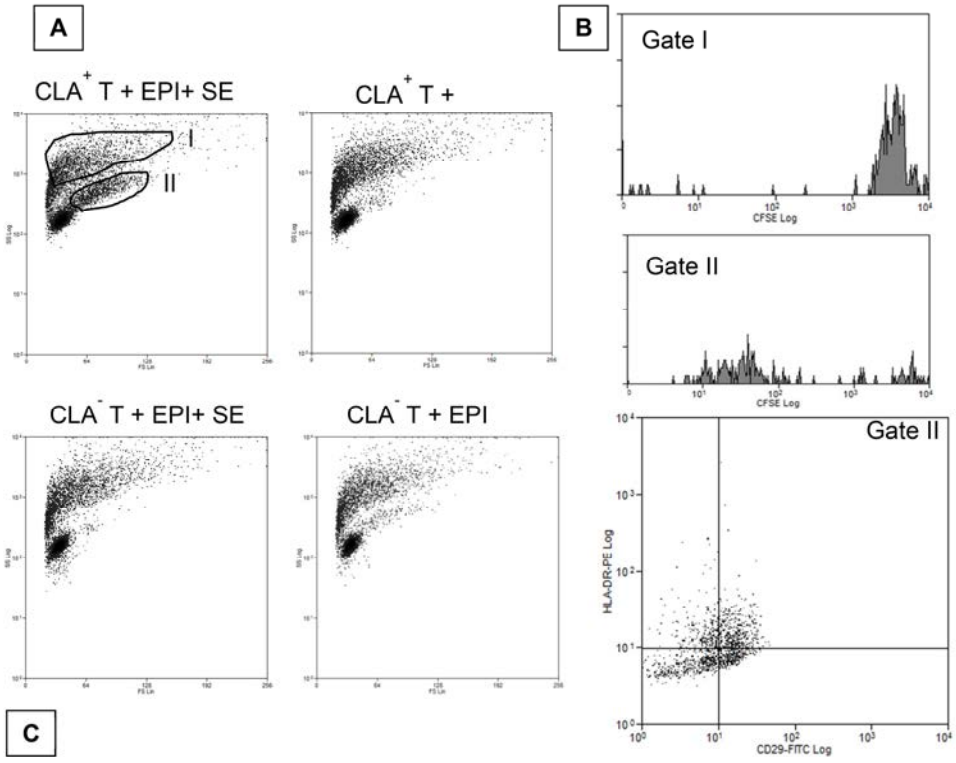


Antigen response to *C Albicans*, SEB and SE in memory T cell and epidermal cell culture

Results from 5 days supernatants generated from 4 different patients (p24, p256, p26 and p27). Quantification of cytokines (pg/ml) was performed by Diaplex and results are net values. The dotted lines indicate the amount of

cytokine production obtained in the CLA$^-$ T cell subset by SE activation for each cytokine

Supplementary Figure 4:



NETO VALUES FOR 4 DAYS CULTURE		CLA$^+$ + EPI + SE			CLA$^-$ + EPI + SE		
		Isotype	Anti-HLA-class I	Anti-HLA-Class II	Isotype	Anti-HLA-class I	Anti-HLA-Class II
p18	SE-induced epidermal cell (Gate II, % total)	4,1	2,6 *(36,6)*	1,4 *(65,8)*	0	0	0
	HLA-DR$^+$CD29$^+$ (Gate II)	17,1	5 *(70,7)*	0 *(100)*	0	0	0
p19	SE-induced epidermal cell (Gate II, % total)	9,7	5,2 *(46,3)*	0,9 *(90,7)*	0	0	0
	HLA-DR$^+$CD29$^+$ (Gate II)	26,4	20,9 *(79,1)*	0 *(100)*	0	O	0

SE together with CLA$^+$ memory T-cells induce proliferation of epidermal cells that express HLA-DR and CD29 and depends on HLA-Class I and class II interaction

A representative FACS result obtained with patient 19. In the culture condition with SE together with CLA$^+$ T cell an epidermal cells novel population (II)

appears after 4 days of culture (Fig 4A). In 4 independent experiments with patients (p22, p21, p20 and p18) the percentage of this subset compared to the CLA^- memory T cells + SE is 8,4 ± 1,8 vs 0,4 ± 0,4 ($p<0,01$), data not shown. Such population presents a decrease in CFSE staining compared to the main subset of epidermal cells (I) and contains cells expressing CD29 and HLA-DR (Fig 4B). The induction of population (II) could be inhibited by blocking antibodies against HLA-Class I or HLA-Class II (Fig 4C), patients 18 and 19.

SUPPLEMENTARY TABLE I. Patients of the study

Patient Number	Age (years)	Psoriasis Type	PASI	Duration of disease	*HLA-C*0602*	*Onset associated with pharyngeal strep infection*	*ASO (UI/ml)*
1	38	Guttate	8,5	>6 months	Negative	No	38
2	31	Chronic	9	years	Negative	No	181
3	42	Guttate	6	2 months	Positive	Yes	256
4	-----	Guttate	20	2 months	----	Yes	-----
5	-----	Guttate	30	-----	----	Yes	400
6	-----	Chronic	40	-----	-----	No	-----
7	-----	Chronic	50	-----	-----	No	-----
8	-----	Acute	30	-----	-----	No	-----
9	50	Chronic	12	7 years	Negative	No	63
10	28	Guttate	5	15 days	Positive	Yes	501
11	19	Guttate	10	3 months	Negative	No	39
12	24	Chronic	15	2 months	Negative	No	37
13	19	Guttate	6	1 month	Positive	Yes	227
14	33	Chronic	4	1 months	Negative	Yes	105
15	17	Guttate	4	4 moths	Positive	Yes	558
16	36	Chronic	14	1y	Positive	No	82
17	51	Chronic	11	>4months	Positive	No	15
18	23	Acute	7,6	2 months	Positive	Yes	443
19	46	Acute	25,2	3 months	Negative	No	58
20	38	Chronic	22	>2y	Positive	No	64
21	25	Guttate	14,8	1 months	Positive	No	278
22	41	Guttate	4	3 weeks	Positive	Yes	337
23	30	Guttate	8	25 days	Positive	Yes	243
24	33	Guttate	13,4	20 days	Positive	----	414
25	33	Guttate	6	7 days	Positive	----	-----
26	41	Guttate	4	5 weeks	Positive	----	480
27	65	Guttate	4,6	10 days	Positive	Yes	----

APPENDIX II: Supplementary figures

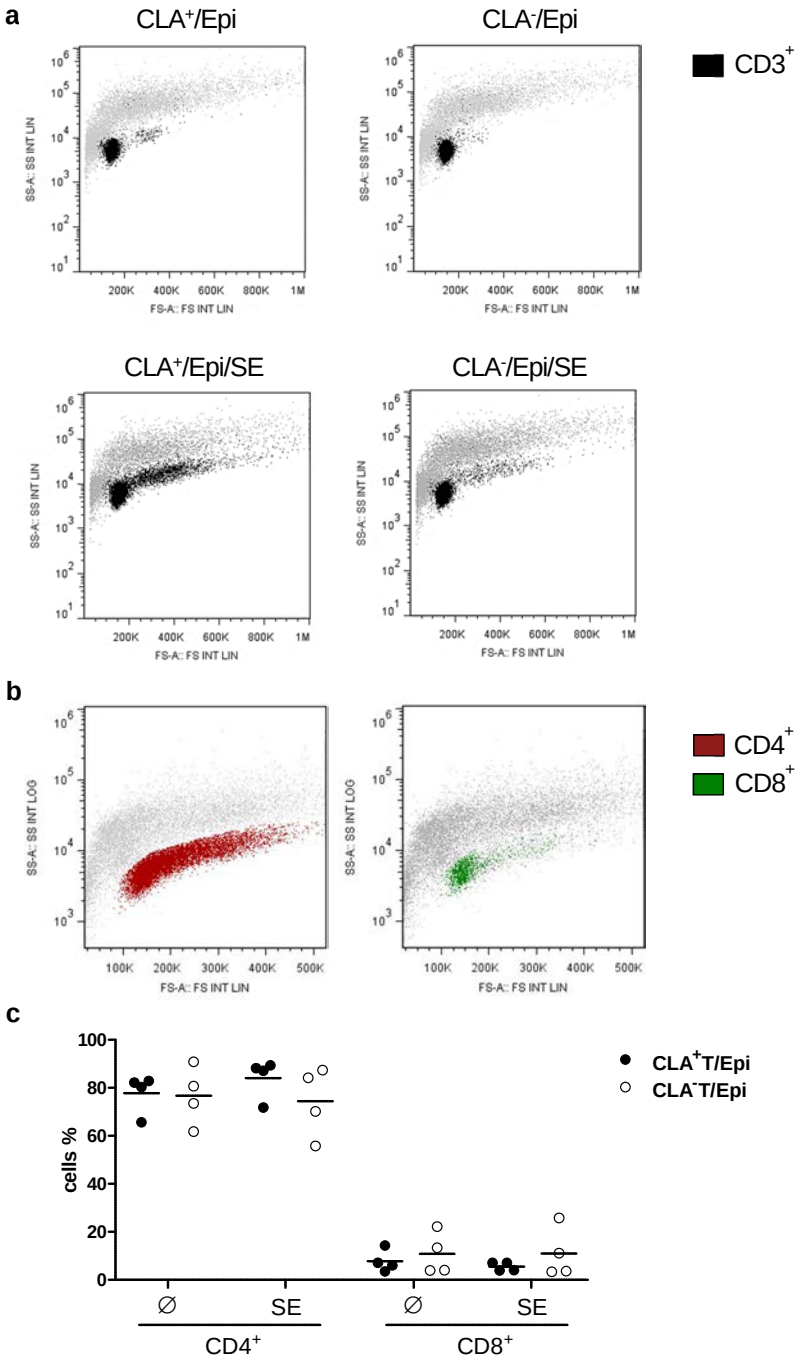


Figure A1. Coculture of T cells and epidermal cells through flow cytometry. Coculture cells were resuspended at day 5 and analyzed through flow cytometry. a) A small (SS-A low) $CD3^+$ lymphocyte population was detected, and increased in size when cocultures were stimulated with SE, especially in CLA^+/Epi/SE. b) A representative $CD4^+$ and $CD8^+$ cell staining in lymphocyte population in CLA^+/Epi/SE coculture at day 5. c) Percentage of $CD4^+$ and $CD8^+$ cells within the lymphocyte gate, at day 5 of culture (n=4).

THESIS DIRECTOR | **Dr. Luis Francisco Santamaria Babí**

SUMMARY | Most of the advances in understanding the immunopathology of psoriasis have occurred through translational science. Circulating skin-tropic CLA^+ T cells constitute peripheral cellular biomarkers for human T-cell–mediated skin diseases. The study of those CLA^+ T cells might reveal important translational information, for instance, through the evaluation of their effector responses upon contact with cognate pathogen that constitute a clinical relevant trigger, such as *Streptococcus pyogenes* in psoriasis. In fact, the guttate-type of psoriasis is often preceded by streptococcal throat infections. In this regard, and in the presence of autologous lesional epidermal cells, a *S. pyogenes* extract (SE) induced a Th17-predominant effector response by peripheral CLA^+ T cells, which in turn can induce an IL-17–signature genes modulation in normal keratinocytes. Other T-cell products, which also might represent relevant cytokines in psoriatic skin lesions, such as IFN-γ and IL-9, are also induced in this experimental approach. Interestingly, a common peak was observed between ASO titers, PASI score and *ex vivo* cytokine responses, between 1–2 months of disease duration in guttate psoriasis that reported previous pharyngitis. CLA^+ T cells and epidermal cells cocultures (CLA^+/Epi) from plaque psoriasis subjects displayed higher cytokine responses than healthy controls in response to SE as well, including IL-17A, IFN-γ and IL-9, this latter playing an essential role for optimal production of IL-17A, either in guttate or plaque psoriasis. Despite evidence that streptococcal infection lead to exacerbations in this psoriasis form, the assessment of the relation of SE-activation in plaque psoriasis with their clinical status has still to be elucidated. Finally, since IL-17A has been shown to be the key disease-associated cytokine responsible of psoriasis severity in patients, determination of IL-17A–targeted genes in keratinocytes, through SE-induced production of IL-17A by the CLA^+/Epi coculture, could unravel new genes potentially involved in psoriasis. In this way, the ribonuclease *ZC3H12A*/MCPIP1, was highly induced in keratinocytes in an IL-17A–dependent manner, and it was found to be aberrantly distributed in psoriatic epidermis, where its ribonuclease activity could be modulating other altered-expressed genes. Overall, psoriatic-associated events can be reproduced *ex vivo* in a CLA- and SE-dependent manner and provides a tool to expand knowledge and understanding of immune responses in psoriasis.

COVER | Hematoxylin & eosin staining of skin punch biopsy from plaque psoriasis.