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

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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.

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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

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

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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

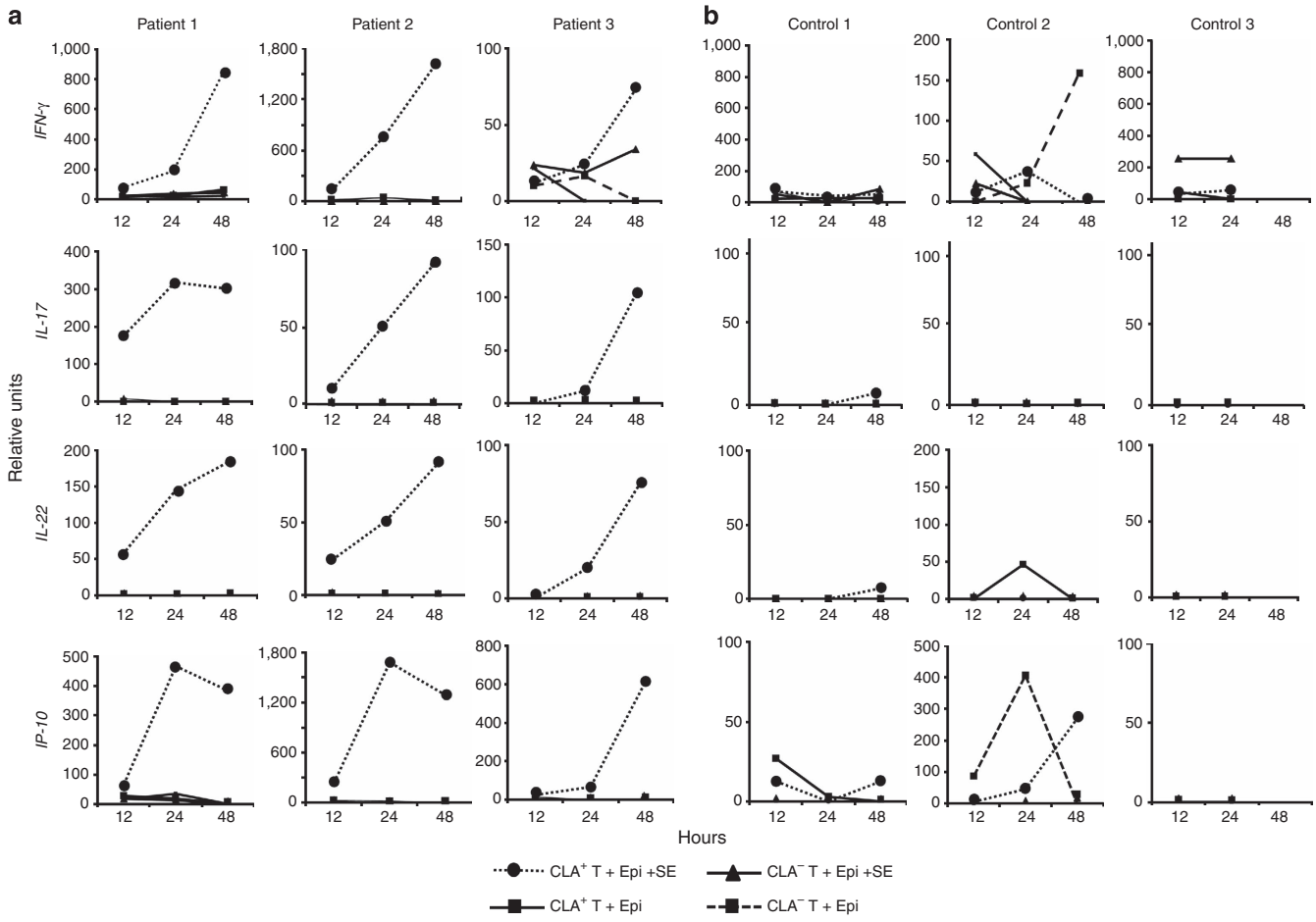
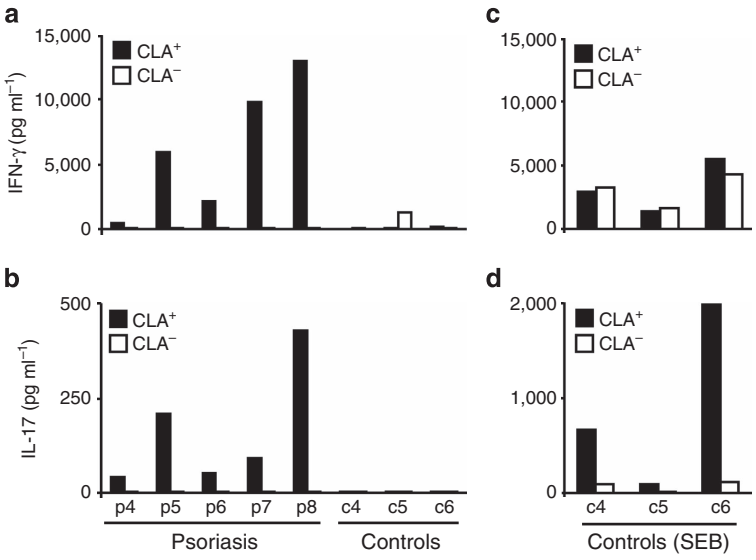


Figure 1. Streptococcal extract (SE) induces upregulation of mRNA for *ifn-γ*, *il-17*, *il-22*, and *ip-10* in psoriatic cutaneous lymphocyte–associated antigen (CLA)$^+$ T cells cultured with lesional autologous epidermal cells. Culture of freshly isolated circulating CLA$^+$/CLA$^-$ CD45RO$^+$CD3$^+$ 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.



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(pg ml^{-1})	Patient 10		Patient 11		Patient 12	
	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

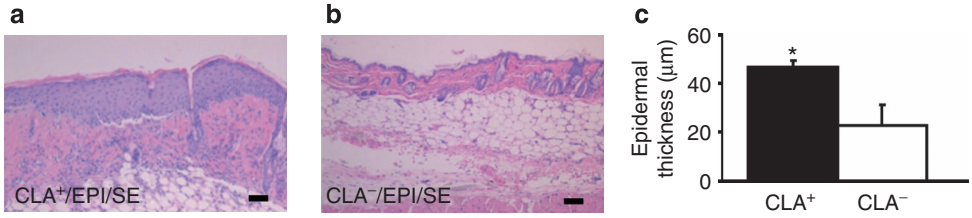

Figure 3. Supernatants of streptococcal extract (SE)–activated cutaneous lymphocyte–associated antigen (CLA)$^+$ T cells and epidermal cells generate epidermal hyperplasia *in vivo*. Intradermal injection of supernatants from CLA$^+$ T cells cultured with lesional epidermal cells activated with SE induces *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.

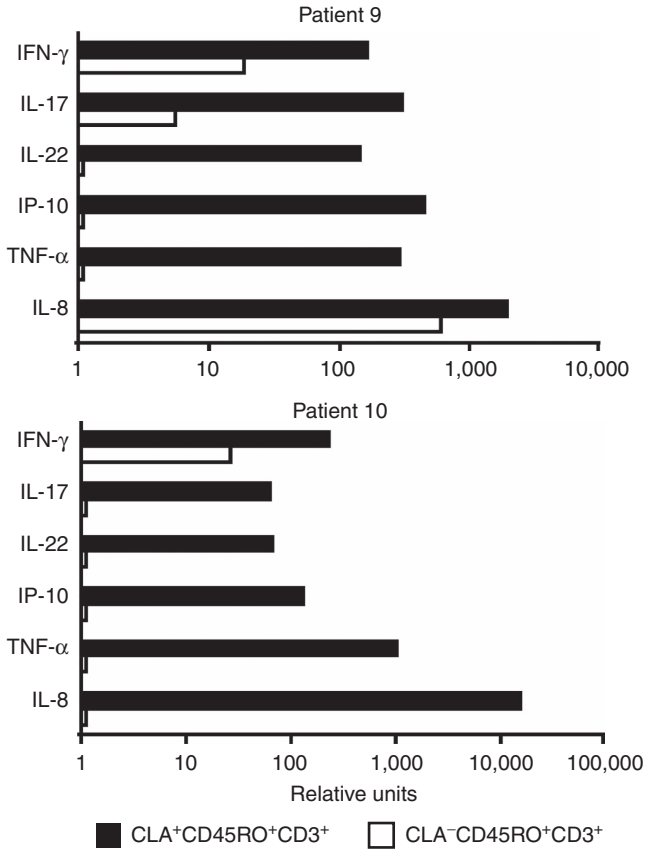

Figure 4. Streptococcal extract–induced upregulation of mRNA for ifn-γ, il-17, il-22, ip-10, tnf-α, and il-8 in cultures of cutaneous lymphocyte–associated antigen (CLA)$^+$/CLA$^-$ T cells of nonlesional epidermal cells of psoriasis patients. Upregulated gene transcription in the culture 24 hours after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

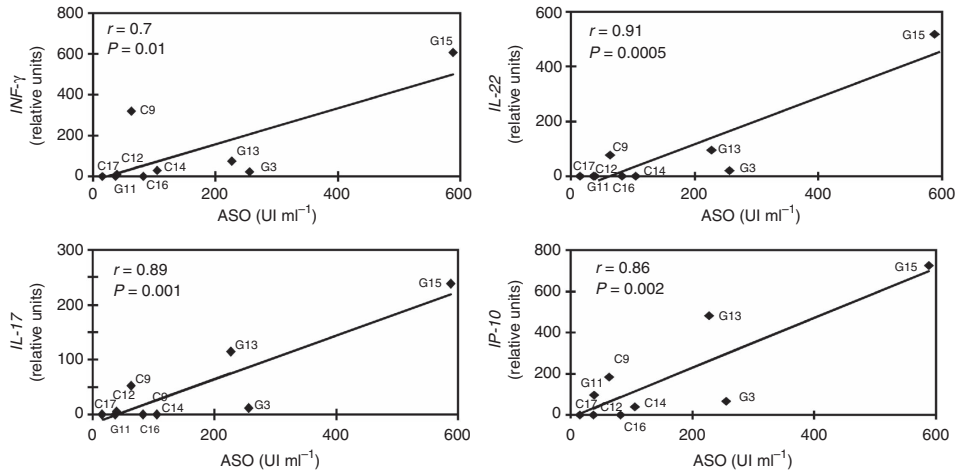

Figure 5. Streptococcal extract–induced upregulation in gene transcription for ifn-γ, il-17, il-22, and ip-10 after 24 hours of activation of cutaneous lymphocyte–associated antigen (CLA)$^+$ memory T cells and epidermal cells correlates with antistreptolysin O (ASO) serum levels in psoriatic patients.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

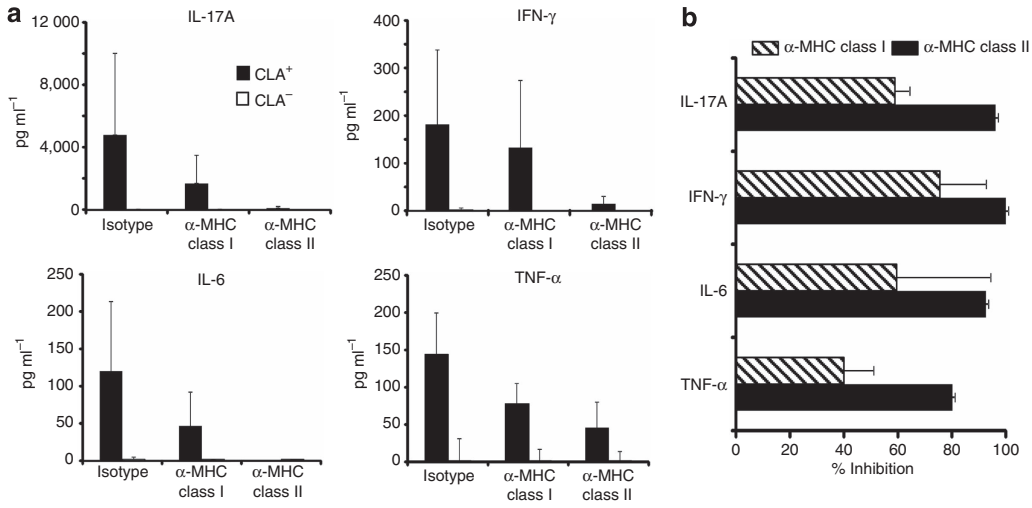

Figure 6. Streptococcal extract (SE)–induced upregulation of IL-17A, IFN-γ, IL-6, and TNF-α in cultures of cutaneous lymphocyte–associated antigen (CLA)$^+$ T cells and epidermal cells depends on HLA class I and HLA class II. Three experiments conducted with patients p18, p19, and p23 are presented. (**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$^-$CD45R0$^+$ 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, CD45R0$^+$ 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 μg ml^{-1} final), which was kindly provided by Drs Johann Gudjonsson and Helki Valdimarsson, *C. albicans* at 40 μg 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 μg 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.

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SUPPLEMENTARY MATERIAL

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

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