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

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

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

INTRODUCTION

The onset of guttate psoriasis is preceded by tonsil infection by Streptococcus pyogenes in 56–97% of patients (Prinz, 2001), and raised anti-streptolysin O (ASO) titers are commonly detected. Furthermore, the streptococcal influence is not limited to this acute subtype of psoriasis. Indeed, there is evidence that throat infection by this microbe in patients with plaque psoriasis exacerbates the symptoms of the disease (Gudjonsson et al., 2003). Also, such infections are approximately 10 times more frequent in individuals with chronic plaque psoriasis than in a control population.

1Translational Immunology, Department of Cellular Biology, Physiology and Immunology, Faculty of Biology, Universitat de Barcelona, Spain;
2Department of Dermatology, Hospital del Mar, IMIM, Universitat Autònoma de Barcelona, Spain; 3Hospital Sant Jaume de Calella, Barcelona, Spain; 4Hospital del Arnau de Vilanova, Lleida, Spain; 5Department of Dermatovenerology and Oncodermatology, SemmelweisEgyetem, Budapest, Hungary; and 6Department of Dermatovenerology and Oncodermatology, SemmelweisEgyetem, Budapest, Hungary; and 7Macrophage Biology, Department of Cellular Biology, Physiology and Immunology, Faculty of Biology, Universitat de Barcelona, Spain.

Correspondence: Luis F. Santamaria-Babi, Parc Cientific de Barcelona, Translational Immunology, Baldiri i Reixa, 10, 08028 Barcelona, Spain. E-mail: luis.santamaria@ub.edu

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

Received 22 December 2016; revised 7 August 2017; accepted 16 August 2017; accepted manuscript published online 17 October 2017 (Gudjonsson et al., 2003). The co-culture of circulating skin-homing cutaneous lymphocyte-associated antigen (CLA)$^+$ T cells with autologous epidermal cells and activation by an extract of S. pyogenes (SE) provides an ex vivo model through which to study psoriasis. Such co-cultures have shown a high production of psoriasis-associated cytokines, including the main signature products of T cytotoxic (Tc) 17 and T helper type (Th) 17 and Tc1/Th1 cells, namely IL-17A and IFN-γ, respectively (Ferran et al., 2013b; Ruiz-Romeu et al., 2016). Therefore, the components of this ex vivo approach provide an optimal scenario in which to address other less known inflammatory mediators in psoriasis.

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

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

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

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

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

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

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

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

![Figure 1. Circulating psoriatic memory CLA⁺ T cells produce IL-9, together with IL-17A and IFN-γ, upon activation with Streptococcus pyogenes.](https://www.jidonline.org)
IFN-γ (Figure 2e and f, respectively). This notion is consistent with previously reported results (Ferran et al., 2013b). Activation of CD4-depleted psoriatic co-cultures with SE or CA did not lead to the detectable production of IL-9, IL-17A, or IFN-γ (see Supplementary Figure S4a–c online). However, IL-17F, which is usually produced in higher amounts than IL-17A and is also known to be generated by the co-cultures (Xue et al., 2016), was detected in two of four experiments with SE (see Supplementary Figure S4d).

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

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

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

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

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

Guttate flares with positive ASO titers (≥200 IU/ml) and preceded by throat infections (n = 13) showed variable ASO levels and PASI scores when they were distributed on the basis of duration of disease, from 15 days to 4 months, in which still there was evidence of a prior pharyngitis (Figure 5a). The highest ASO blood levels and PASI scores were found among guttate flares between 1 and 2 months after disease onset. Lower ASO titer results (mean < 200 IU/ml) were found in two of the three patients with long-duration (>1 year) guttate psoriasis with no clear history of streptococcal infection. Despite incomplete resolution of their lesions, these patients presented milder severity in
terms of PASI score. In contrast, patients with plaque psoriasis, who had long-duration chronic disease, presented high PASI scores but had negative ASO titer results, thus confirming the reported lack of prior throat infections in this group (n = 10) (Figure 5a). Such clinical temporal variability observed in guttate psoriasis samples was also reproduced in terms of cytokine responses, in the CLA⁺/Epi condition for IL-9, IL-17A, and IFN-γ (Figure 5b), and the highest values were found again in CLA⁺/Epi co-cultures derived from guttate samples from patients with 1–2 months of disease duration. Consequently, ASO and cytokine levels finely correlated (Figure 5c, upper panels), showing significance in the case of IL-9 (r = 0.68, P = .003) and IL-17A (r = 0.55, P = .02) production and near significance for that of IFN-γ (r = 0.45, P = .07). Similarly, the PASI score significantly correlated with IL-9 (r = 0.61, P = .01) and IFN-γ (r = 0.55, P = .02) production and near significance was found with IL-17A levels (r = 0.48, P = .06) (Figure 5c, lower panels).

**DISCUSSION**

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

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

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**Figure 3.** IL-9 production induced by *Streptococcus pyogenes* follows similar kinetics to the production of IL-17A and IFN-γ and enhances IL-17A production. (a) IL-9, IL-17A, and IFN-γ were measured in supernatants from psoriatic CLA⁺/Epi co-cultures that were activated for 1 (n = 2–3), 2 (n = 1–2), 3 (n = 2–3), 4 (n = 1–2), and 5 (n = 2–3) days with SE. Each symbol represents the median ± interquartile range. (b) Neutralizing antibodies against IL-9 or IgG isotype control were added at day 0 of culture and were activated by SE. Next, IL-17A and IFN-γ levels were measured in 5-day supernatants from psoriatic CLA⁺/Epi and CLA⁻/Epi co-cultures (n = 7), and paired results were compared with respect to isotype values. (c) CLA⁺ and CLA⁻ T cells were cultured with or without IL-9 for 5 days. An increase or decrease in living or early apoptotic cells, respectively, is shown (n = 4). Each dot represents the values of a supernatant from a patient-derived co-culture. *P < 0.05. CLA, cutaneous lymphocyte-associated antigen; CLA⁺/Epi, co-cultures of CLA⁺ T cells and autologous epidermal cells; CLA⁻/Epi, co-cultures of CLA⁻ T cells and autologous epidermal cells; SE, *S. pyogenes* extract.
produced by CLA⁻ T cells was higher than that by CLA⁺ T cells. Also, IL-17A, which is highly associated with cutaneous defense, was produced in higher amounts by epidermal cells than by DCs when co-cultures were activated by SE and SEB, even though the former are a more heterogeneous population of cells. Thus, SE-induced IL-9 production by psoriatic CLA⁺/Epi co-cultures occurred in a specific manner, because activation with SEB equally induced IL-9 in both psoriasis and healthy control co-cultures (n = 10). Data are represented by dot plots, each representing the values of a supernatant from a patient-derived co-culture, and by the median. *P < 0.05, **P < 0.01. CLA, cutaneous lymphocyte-associated antigen; CLA⁺/Epi, co-cultures of CLA⁺ T cells and autologous epidermal cells; SE, S. pyogenes extract.

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

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

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

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

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

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

**MATERIALS AND METHODS**

**Patients**

This study was performed with human samples and in accordance with the Declaration of Helsinki. A total of 39 nontreated psoriasis patients and 10 healthy individuals were recruited from three hospitals (see Supplementary Table S1 online). All participants contributed...
voluntarily and provided written informed consent. Psoriatic samples were from patients with guttate (n = 19) and plaque (n = 12) lesions, without any age or sex restriction. Patients who had received systemic treatment in the 6 weeks before sample collected were excluded to avoid the underestimation of cell activation. Patients and healthy subjects underwent two skin biopsies, which were punched in lesions in psoriatic patients, and a blood extraction.

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

Generation of DCs from peripheral blood monocytes
CD14+ cells purified by immunomagnetic separation were cultured at a final density of 105/ml with 50 ng/ml of IL-4 and GM-CSF (Miltenyi Biotec, Bergisch Gladbach, Germany). Half the medium was replaced by new supplemented medium at day 4. At day 7, cells were washed and counted for proper culture conditions.

Cultures and pathogen activation
Ex vivo co-cultures involved the culture of total or CD4-depleted 5 × 104 CLA+ or CLA− T cells with 3 × 104 autologous epidermal cells (CLA+/Epi or CLA−/Epi, respectively) in 96-well flat-bottom plates (Nunc, Roskilde, Denmark) in the medium described. Also, co-cultures were performed with 5 × 104 autologously generated DCs, which were seeded with thawed 5 × 104 CLA+ or CLA− T cells. Co-cultures were left untreated or activated for 5 days with the following pathogen preparations: an extract of S. pyogenes obtained from sonicated S. pyogenes isolated from throat swabs from patients with psoriasis, used at 1 µg/ml; commercial CA preparation (Greer Labs, Lenoir, NC) at 72 µg/ml; and SEB (Sigma-Aldrich) at 100 ng/ml. In cultures containing only T cells, the mentioned amounts of each cell type were used, and activation with SE was performed in the same way. For the time course experiments, supernatants from SE-activated co-cultures were collected each day from replicated wells. For blocking assays, azide-free HLA-A/B/C (class I), HLA-DR (class II), or IL-9 neutralizing antibodies, or respective isotype IgG controls (Biolegend, San Diego, CA), were added at day 0 to co-cultures from psoriatic patient samples before activation at a final concentration of 10 µg/ml.

Apoptosis assay
Isolated 106 CLA+ and CLA− T cells from patients with psoriasis were cultured in the medium described supplemented or not with 20 ng/ml of IL-9 (Miltenyi Biotec) for 5 days. Cells were then washed with cold phosphate buffered saline and stained with Annexin-V-APC (BD Bioscience, San Jose, CA) and DAPI and analyzed by flow cytometry.

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

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

CONFLICT OF INTEREST
The authors state no conflict of interest.

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

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