The inflammation in cutaneous lichen planus is dominated by IFN-γ and IL-21—A basis for therapeutic JAK1 inhibition

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Abstract
Cutaneous lichen planus (CLP) and psoriasis (PSO) are both common chronic inflammatory skin diseases for which development of new treatments requires the identification of key targets. While PSO is a typical Th17/IL-17-disorder, there is some evidence that Th1/IFN-γ dominate the inflammatory process in CLP. Nonetheless, the immunopathogenesis of CLP is not fully explained and key immunological factors still have to be recognized. In this study, we compared the immune signature of CLP lesions with the well-characterized inflammation present in PSO skin. First, we analysed the histological and immunohistological characteristics of CLP and PSO. Second, we assessed the cytokine expression (IL1A, IL1B, IL4, IL6, IL8, IL10, IL12A, IL12B and IL36G) of lesional skin of CLP with PSO by qPCR. Histology revealed a similar epidermal thickness in CLP and PSO. Immunohistochemically, both diseases presented with an inflammatory infiltrate mainly composed by CD3+CD4+ T cells rather than CD3+CD8+. Importantly, mRNA analysis showed a distinct cytokine signature: while levels of IL12B, IL1A, IL6 and IL23 were similar between the two groups, the characteristic PSO-associated cytokines IL8, IL17A, IL22, IL19 and IL36G were expressed at very low levels in CLP. In contrast, CLP lesional skin was dominated by the expression of IFNG, IL21, IL4, IL12A and TNF. Immunohistochemistry confirmed the dominance of IL-21, IFN-γ and also pSTAT1 in the dermal infiltrate of CLP, while IL-17A was more present in PSO. Collectively, this study improves our understanding of the immunological factors dominating CLP. The dominating cytokines and signalling proteins identified suggest that anti-cytokine therapeutics like JAK inhibitors may be beneficial in CLP.

Keywords
IL-17, immune signature, JAK inhibitors, lichen planus, psoriasis
Lichen planus (LP) and psoriasis (PSO) are both chronic inflammatory skin diseases with pronounced discomfort that impair the quality of life of patients.1,2 LP may affect the skin and the nails (cutaneous LP, CLP), the oral and/or genital mucosa (oral LP, OLP) or the scalp with a risk for scar formation (lichen planopilaris, LPP).3 LP—predominantly CLP—and PSO share some features. Both skin diseases can be provoked by external factors like physical factors (Köbner phenomenon) or certain drug trigger. However, clinical differences between the two suggest the presence of a different immunological pathogenesis. PSO never affects the mucosal sites. Scalp inflammation during PSO presents with erythema and scaling but without persistent hair loss or scarring, whereas LPP has a scarring tendency.3,4 This suggests that the inflammation in each skin disease is composed by different cytokines.

Histologically, both skin diseases show a marked infiltration of immune cells, predominantly in the dermis. However, the quality and the distribution of immune cells is different in CLP and PSO. In PSO, the leukocytic infiltration is mainly found at perivascular sites, while in LP the leukocytes typically show a band-like infiltration in the upper dermis.5 Immunophenotyping in PSO resulted in the identification of Th17 cells and associated cytokines like IL-17A and IL-23 as key players. As a consequence, several Th17-targeting therapeutics have been developed and approved.5,7 These therapeutics include antibodies directly neutralizing IL-17A or IL-23 and small molecules indirectly affecting the Th17 response by interfering with the JAK/STAT pathway.8-10 In contrast, the immunological facts on the pathogenesis of LP are limited and this is emphasized by the lack of approved targeted treatments.11 The identification of druggable cytokines or cytokine signalling pathways critical in the pathogenesis of LP will help to initiate clinical studies and to develop effective treatments for patients. Earlier reports described a Th1/IFN-γ-dominated immune response in LP.12,13 Additionally, more recent data suggested the involvement of the Th17 pathway and related cytokines in CLP, OLP and LPP.12,14-18 Yet, the exact pathogenic role of Th17 cells in LP still needs to be clarified. First case studies indicate that targeting antibodies neutralizing IL-17A or IL-23 may be beneficial for patients with OLP.19-21 If CLP would also benefit from IL-17A or IL-23 neutralization is uncertain. In this study, we aimed to better understand the cytokine signature in CLP. Specifically, we were interested in similarities and differences of cytokine expression in lesional skin of CLP patients compared with the well-determined inflammation in psoriatic plaques. We analysed biopsies from affected skin of patients with CLP, PSO and healthy skin. In detail, we studied the T-cell infiltrate, the expression of cytokines by quantitative PCR and immunohistochemistry (IHC), and the activation of signal transducer and activator of transcription (STAT) proteins. Our results show new Insights regarding the cytokine signature of CLP. As previously reported, we can confirm that IFN-γ is predominant in CLP. Yet, our analysis unveil that other cytokines such as IL-12, IL-6 and IL-21 are overexpressed in CLP lesional skin. Importantly, STAT1 showed higher activation in the dermal cellular infiltrate of CLP compared to PSO. Our analysis unveil that cytokines like IFN-γ and IL-21 and their signalling factor STAT1 are active in CLP lesional skin and possibly critically involved in its pathogenesis.

Between August 2017 and May 2018, tissue samples of seven patients with plaque-type PSO and seven patients suffering from CLP were collected. Diagnosis of LP and PSO was based on the clinical phenotype and histopathological finding. Patients’ demographics and history were also collected. Informed consent was obtained from each individual prior to the collection of skin samples and data. Ethical approval was obtained by the ethics committee of the Eberhard Karls University (309/2017BO2). After giving their written informed consent intralesional skin samples of patients with plaque-type PSO or CLP were obtained by 4 mm punch biopsies. Clinically healthy spare skin of individuals undergoing surgery was used as control tissue.

Total RNA was purified from punch biopsies with PeqGOLD total RNA Kit (VWR, Darmstadt, Germany). For reverse transcription into cDNA Maxima First Strand cDNA Synthesis Kit for RT-qPCR (Thermo Fisher, Schwerte, Germany) was used according to manufacturer’s instructions. RT-qPCR was performed using Roche LightCycler480 system (Roche, Mannheim, Germany). TaqMan probes and primers for IL1A, IL1B, IL4, IL6, IL8, IL10, IL17A, IL19, IL21, IL22, IL23A, IL13, IFNG, TNF, IL12A, IL12B, IL36G, ACTB and POLR2A were synthesized by TIB MolBiol, Berlin, Germany. Human primers and probes used for real-time PCR are summarized in Table S4.

PCR reactions were performed in duplicates, POLR2A and ACTB were used as housekeeping genes and aqua as blank sample. The relative expression of the indicated genes was calculated relative to the expression of POLR2A and ACTB. Expression of genes in skin from healthy donors was used as control condition and set as 1.0. Gene expression was determined by the ΔΔCt method.

### 2.3 | Histology and immunohistochemistry

Evaluation of epidermal thickness and immunohistochemistry was performed by a board-certified dermatopathologist (FCG). Skin biopsies were fixed in parafomaldehyde, embedded in paraffin, and further processed for H&E and immunohistochemical analysis of the inflammatory infiltrate as recently described. 4 µm sections were processed by the automated IHC stainer BOND-MAX (Leica) and Autostainer Plus automated immunostaining device (Dako). Standard protocols were used for CD3 (A. Menarini diagnostics), CD4 (Cell Marque) and CD8 (Dako) staining. Visualization was carried out using the Bond Polymer Refine Detection Kit (Leica; CD3, CD4, CD8) and Liquid
Permanent Red substrate-chromogen (Dako). Images of stained tissue were taken using Nikon Eclipse 80i microscope and NIS-Elements software (Nikon). Immunohistochemical stainings for IL-17, IL-21, IFN-γ, MX1, pSTAT1 and pSTAT3 were performed on an automated immunostainer (Ventana Medical Systems, Inc) according to the company’s protocols for open procedures with slight modifications. The slides were stained with the following antibodies: IL-17/IL-17A (R&D systems), IL-21, IFN-γ (clone INFNG/466), MX1, pSTAT1 (clone EPR3146), all Abcam) and pSTAT3 (Y705) (Cell signalling). Positive and negative controls were stained. Glass slides were converted to high-resolution digital data with a digital light scanner (Hamamatsu photonics, Hamamatsu, Japan). Images of stained tissue were taken with NPD.view2 software (Hamamatsu photonics). To quantify levels of positive infiltrating cells, six defined dermal fields (field of vision) per slide were investigated for histological analysis. Positive and negative cells were counted using Image J software at 100× magnification.

2.4 | Statistical analysis

All data were analysed and plotted using GraphPad Prism 7.03 and 8.4.3 software. Statistical analyses were performed by using the Mann–Whitney test, P values of the PCR data were adjusted with the Holm-Sidak method for analysing a stack of P values with an alpha of 0.05. Values of P < 0.05 (*) were considered significant.

3 | RESULTS

3.1 | Patients’ demographics

In this study, we included seven patients with CLP and seven patients with PSO (Figure 1, Table S1–S3). At initial diagnosis, patients with CLP had an average age of 49 years (range 26 to 73 years), the mean age of PSO patients at initial diagnosis was 39 years (range 11–60 years). Female to male ratio in the CLP group was 5 to 2 and in the PSO group 3 to 4. Besides cutaneous characteristics (7/7), some patients with CLP had mucosal lesions (2/7) and nail involvement (3/7). Previous positive history of hepatitis B was found in 1 of 7 patients with CLP. In the PSO group, all patients suffered from plaque-type PSO, some patients also had psoriatic arthritis (2/7) and nail involvement (3/7). Biopsies were taken from active non-treated lesions (for a summary of clinical information please see Tables S1 and S2).

3.2 | Epidermal thickness and T-cell infiltration in cutaneous lichen planus and psoriasis

Histological examination revealed similar average epidermal thickness in CLP samples (0.4929 mm ± 0.1298) as compared to PSO samples (0.4714 mm ± 0.04206) (Table S3). As expected, all CLP samples showed a dense infiltration with a typical lichenoid pattern, while the infiltrating lymphocytes in PSO samples were less abundant compared to CLP and showed a perivascular formation. By immunohistochemistry, we further characterized the T-cell infiltration. Both diseases showed a predominance of CD3+ and CD4+ T cells, while CD8+ T cells were less frequent in CLP as well as in PSO (Figure 2).

3.3 | Innate cytokine expression in CLP compared to PSO

To better understand the immune signature of CLP compared to PSO, we performed qPCR analysis for innate and adaptive cytokines expressed in lesional skin. The expression of each cytokine was set

FIGURE 1 Clinical comparison of lichen planus to psoriasis vulgaris. Cutaneous lichen planus (upper row) presents with flat topped, red to brown polygonal purple papules with fine white scales (Wickham’s striae). Psoriasis plaques (lower row) are characterized by well demarcated red plaques with prominent white scaling.
into relation to healthy skin samples of control individuals. First, we studied the expression of typical innate cytokines. Here, we found negligible levels of IL1A in CLP and PSO samples. In contrast, PSO samples showed a stronger expression of IL1B and of IL36G compared with CLP skin lesions, which showed no or very low expression (Figure 3A). Although IL12A showed similar expression levels in CLP as in healthy skin, its expression was suppressed in PSO when compared to CLP. As expected, IL12B was strongly expressed...
in PSO, but also in CLP skin samples (Figure 3A). IL6 was found in both skin diseases, while TNF was stronger expressed in CLP than in PSO samples, although at low level (Figure 3A).

3.4 | The psoriasis-associated factors IL8 and IL19 are not expressed in CLP

Next, we analysed the expression of two mediators that are typically produced during psoriatic inflammation, the neutrophil chemoattractant IL-8 and the IL-10-family cytokine IL-19. As expected, both mediators were highly expressed in psoriatic skin samples. Interestingly, only little amounts were present in the skin of CLP (Figure 3B).

3.5 | Higher expression of IFNG in CLP compared to PSO

The mRNA expression of the Th1-lineage defining cytokine IFN-γ was found in both PSO and CLP samples with significant higher levels in CLP (Figure 4A). As expected, psoriatic skin lesions showed no or only little expression of the Th2-associated cytokines IL4, IL13 and the T regulatory (Treg) cell-associated cytokine IL10. (Figure 4A).

3.6 | IL21 is predominant in CLP, while IL17A and IL22 are predominant in PSO

Finally, we focused on Th17-associated factors IL17A, IL21, IL22 and IL23A, given the importance of these cytokines in psoriasis and, based on some preliminary reports, in CLP. As expected, we found high expression levels for IL-17A, IL-22 and IL-23A in PSO skin samples. Although previous reports described high levels of Th17 cytokines to be present in OLP, levels of IL-17A, IL-22 and IL-23A mRNA in CLP samples were very low. Surprisingly, IL-21 mRNA showed strong expression in CLP with significant higher levels than in the skin of patients with PSO (Figure 4B). Our study shows that immunophenotyping of CLP reveals a type I-dominated inflammation accompanied by IL-21 expression.

3.7 | The cellular dermal infiltrate in CLP is characterized by IL-21, IFN-γ and STAT1 activation

To confirm the mRNA data by protein data, we performed IHC and focused on the dermal infiltrate in CLP and PSO. As depicted

![Graphs showing cytokine expression](image_url)
in Figure 5, significant higher numbers of IL-17+ dermal cells were found in PSO compared with CLP. We also found significant higher numbers of IL-21+ and IFN-γ+ dermal cells in CLP compared with PSO, demonstrating that the results of the cytokine mRNA expression are reflected by protein data. The type I interferon-dependent inducible protein MX1 showed no difference when studying the dermal infiltrating cells in CLP and PSO. Finally, we studied the activation of STAT1 and STAT3 by phospho-STAT staining. We found significant higher numbers of pSTAT1+ cells within the dermis of CLP samples compared to PSO samples, while the numbers of STAT3+ dermal cells were comparable in both entities. Taken together, the expression of STAT1-activating cytokines IFN-γ and IL-21 and the presence of high STAT1 activation in CLP indicate that inhibiting the signalling of these cytokines by an inhibitor that should at least target JAK1—which is used by the receptors for IFN-γ and IL-21, but also by IL-6 and type I interferon—could be a promising therapeutic approach.

4 | DISCUSSION

So far, only few studies tried to elucidate the cytokine signature in lesional skin of CLP.12,13,21 In contrast, a large body of evidence exists about the cytokine network in PSO.4,6,7 Both diseases, CLP and PSO are considered as inflammatory autoimmune diseases predominantly driven by T cells. In agreement with previous studies, our immunohistological examination confirms that CD3+ T cells, especially CD4+ T cells rather than CD8+ T cells dominate the cellular infiltration in lesional skin of both diseases, CLP as well as PSO (Table S3, Figure 2). Yet, in PSO one histological hallmark is the abundance of neutrophils, a cell type that is almost absent in CLP lesional skin, being principally infiltrated by T cells. These histomorphological characteristics are underlined by IL8 and IL36γ expression, which are highly present in PSO samples.22,23 But almost absent in CLP.24 (Figure 3B). IL-36γ is produced by psoriatic keratinocytes and induces the expression of various inflammatory factors, among them IL-8.24 IL-8 itself is produced by keratinocytes and neutrophils and further attracts the latter cell type to the skin, for example, via CXCL16.25-27 Another important factor for mediating neutrophil recruitment to the site of inflammation is IL-17A. As shown, IL-17A mRNA and protein are more present in PSO rather than in CLP skin (Figure 4B, Figure 5). Of note, there is evidence of a feedback loop between IL-17A and IL-36γ, in psoriasis.28

Classical innate cytokines like IL-1 or TNF are thought to play a role in the initial phase of skin inflammation like Th17-driven PSO. We found higher IL1B levels in PSO samples compared to CLP samples (Figure 3A). This observation fits with the Th17-promoting function of IL-1B in concert with other cytokines like IL-23, IL-6 or TGF-β.29,30 We found a tendency for higher TNF expression in CLP compared with PSO (Figure 3A). However, TNF blockade is well established for the treatment of PSO but is of no reproducible benefit in CLP. Anti-TNF-α blockade has even been reported to induce paradoxical manifestation of LP eruptions in skin and mucosa of single patients.31,32

The lichenoid infiltrate and the presence of apoptotic basal keratinocytes in CLP argue for a pathogenic role of an IFN-dominated type I immune responses.13 Compared to psoriatic skin, we found higher levels of IFN-γ mRNA and protein expression in CLP skin (Figures 4A and 5). In addition, we found more robust
IL12A and IL12B expression in CLP samples compared with PSO samples. Independent studies also reported that besides IFN-ɣ, IFN-associated chemokines like CXCL9 and signalling molecules like STAT1 and T-bet are present in LP.\textsuperscript{12,13,33,34} However, compared to PSO samples, which typically are free of Th2 cytokine expression, we also found some low expression of IL4, IL13 and IL10 in CLP (Figure 4A). We have no reasonable explanation for this, albeit others also reported from a sparse presence of Th2 factors like GATA3 in LP.\textsuperscript{12}

IL-10 expression is not restricted to Th2 responses. Tregs, Th1 and certain Th17 cells can also produce IL-10.\textsuperscript{35} The role of FOXP3\textsuperscript{+} Treg cells in LP is under debate. While some studies demonstrated an increase in FOXP3\textsuperscript{+} cells in OLP,\textsuperscript{37,36} others did not find significant differences when comparing LP to normal skin.\textsuperscript{37} In CLP, very low numbers of FOXP3\textsuperscript{+} cells have been found.\textsuperscript{13} Th1 cells can also produce high quantities of IL-10 in the presence of IL-12 and antigen presentation.\textsuperscript{38} IL-10 production by Th1 cells can be interpreted as a self-controlling mechanism to reduce a perpetuating inflammation. However, while clinical studies on cytokines like IL-10 or IL-4 exist with beneficial effects on PSO,\textsuperscript{3,39} there are no clinical data on IL-10 or IL-4 treatment for CLP.

To study the possible role of type I IFN, a known trigger of PSO and CLP, we evaluated the expression of MX1, which expression levels are controlled by type I and type III IFN.\textsuperscript{16} By IHC, we found comparable numbers of MX1-expressing cells in CLP and PSO (Figure 5). While MX1 expression has been reported to be expressed in active and resolved psoriatic skin,\textsuperscript{41,42} its expression in CLP seems to be restricted to lesional skin. In both diseases, plasmacytoid dendritic cells seem to be the main source of type I interferon production.\textsuperscript{33,43} In CLP, patients with hepatitis C have been shown to express higher levels of MX1 in their skin compared to hepatitis C negative patients.\textsuperscript{43} Although preliminary, these data suggest that type I IFN could play a pathogenic role in CLP pathogenesis. Yet, the exact function of type I interferon in CLP pathogenesis has to be further explored.

The PSO key cytokines IL17A and IL22 were highly expressed in PSO (Figures 4B and 5), whereas in CLP only minimal expression was found. The exact role of IL-17 in LP is currently under investigation. Polymorphisms of the IL17A gene and increased serum levels of IL-17A have been associated with OLP.\textsuperscript{54,55} A recently published transcriptomic profiling detected low absolute levels of IL17A and IL22 in LP lesions, without any comparison to other inflammatory skin diseases.\textsuperscript{13} The IL-23 units IL12B and IL23A, which are important for the stimulation and pathogenicity of IL-17A and IL-22 by lymphocytes,\textsuperscript{30} were expressed in PSO and CLP samples (Figure 4B). Of note, recent case studies indicate that IL-23 neutralization may be effective in OLP.\textsuperscript{19,22} Yet, prospective and controlled studies are needed to determine, if IL-17A or IL-23 are feasible targets for the treatment of patients with CLP.

The most striking difference found in our study is the identification of IL-21 as a cytokine highly expressed in CLP but not in PSO (Figures 4B and 5). IL-21 has different effects on the immune system. IL-21 expression is regulated by IL-6, IL-12 and IL-21 itself and all three cytokines are found in the skin of patients with CLP.\textsuperscript{46} IL-21 stimulates the differentiation and function of CD4\textsuperscript{+} T cells, CD8\textsuperscript{+} T cells and NK cells, which are all present in CLP skin.\textsuperscript{3,12,47,48} For example, IL-21 promotes Th17 cell development. In concert with IL-6, IL-21 promotes the differentiation of T follicular helper cells (Tfh). Of note, Tfh cells have recently been shown to be increased in the peripheral blood of patients with OLP, although IL-21 serum concentrations were lower than in healthy controls.\textsuperscript{49,50} Tfh cells are also important for antibody-mediated B-cell responses. IL-21 exerts its intracellular effects in a STAT1/STAT3-dependent fashion and activated STAT1—a transcription factor also activated by IFN-ɣ—has been recently reported in LP skin.\textsuperscript{13} In addition, IL-21 can sustain IFN-ɣ-related gene expression and appears as a factor promoting the type I inflammation in CLP.\textsuperscript{51,52}

While IL-21 activates STAT3 and STAT1, IFN-ɣ activates primarily STAT1 and to a lesser extent STAT3. We found significant higher numbers of pSTAT1\textsuperscript{+} cells in the dermis of CLP samples compared to PSO samples, while the numbers of pSTAT3\textsuperscript{+} dermal cells were comparable (Figure 5). STAT1 can also be activated by type I IFN and IL-6.\textsuperscript{8} All these cytokines, IL-21, IFN-ɣ, IL-6 and type I IFN have in common that their receptors use JAK1. Of note, pJAK1 has been reported to be overexpressed in dermal skin of lichen planus.\textsuperscript{53} Therefore, JAK1 blockade could be beneficial for CLP. Recently, a JAK1/JAK3 inhibitor has been reported to improve LPP and OLP in single cases.\textsuperscript{54,55} In light of the results of Shao et al, revealing that JAK2/STAT1 pathway is pivotal in CLP, it would be interesting to compare the immunological effects of JAK1/JAK2 or JAK1/JAK3 inhibitors with selective JAK1 inhibitors in CLP. Lastly, it should be taken into consideration that in the setting of viral infections (hepatitis B or C or papillomavirus) associated with LP, systemic JAK inhibition that would suppress the antiviral IFN response could bear an oncogenic risk.\textsuperscript{56,57}

In summary, we found distinct cytokine based characteristics in CLP and PSO. The IL-17/IL-23 signature in PSO is different from the cytokine signature found in CLP. Our results demonstrate that the inflammation in CLP is dominated by IFN-ɣ, IL-6 and IL-21 and the pro-inflammatory milieu is dominated by STAT1 activation. A deeper understanding of the exact role of these cytokines in CLP pathogenesis is needed to establish anti-cytokine targeted therapies. Clinical studies using selective JAK inhibitors will show, if this approach will improve CLP in patients with severe disease.\textsuperscript{34}

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CONFLICT OF INTEREST

Authors declare there is no conflict of interests.
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