Cutaneous leukocytoclastic vasculitis: the role of lymphocytes and related immune markers

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Abstract

Introduction: Apart from neutrophils, other immune cells may play a significant pathogenetic role in cutaneous leukocytoclastic vasculitis (CLV).

Aim: To investigate lymphocytes and related immunological factors in patients with CLV requiring systemic glucocorticoid treatment.

Material and methods: Fourteen patients with severe idiopathic CLV were treated with systemic prednisolone in a tapered dose regimen. Ten healthy individuals served as controls. At baseline and post-treatment, we studied inducer/helper and suppressor/cytotoxic T lymphocytes, B lymphocytes, natural killer cells, CD4+CD25++CD127– cells, CD4+CD25+CD39+ cells and FOXP3, transforming growth factor β1 (TGF-β1) and interleukin-10 (IL-10) mRNA levels in the blood using flow cytometry and real time polymerase chain reaction (RT-PCR), respectively. On immunohistochemistry, we studied CD4, CD8, granzyme B, TGF-β1, and IL-10.

Results: Flow cytometry did not show significant differences. The RT-PCR revealed that TGF-β1 mRNA expression was significantly higher after therapy when compared to baseline and controls. On immunohistochemistry, baseline CLV lesions showed significantly more CD4+ lymphocytes than post-treated CLV and controls. CD8+ expression was significantly higher after therapy when compared to baseline and controls. Baseline granzyme B was significantly increased when compared to treated CLV and controls. The IL-10 expression of treated CLV was significantly increased when compared to baseline CLV and; baseline CLV IL-10 expression was significantly increased as compared to controls.

Conclusions: Circulating T regulatory cells do not play a significant role in the pathogenesis of CLV. T helper cells and granzyme B seem to be involved in the inflammatory cutaneous process of CLV. A resolution of CLV observed after glucocorticosteroid treatment may be mediated via up-regulation of TGF-β1 and IL-10 in different compartments.

Key words: cutaneous vasculitis, lymphocytes, cytokines.

Introduction

Cutaneous leukocytoclastic vasculitis (CLV), also known as hypersensitivity vasculitis and hypersensitivity angiitis, is a histopathologic term commonly used to denote a small-vessel vasculitis. The CLV is usually not associated with systemic vasculitis or glomerulonephritis etc. [1]. The main clinical feature of CLV is palpable purpura, primarily affecting dependent areas such as legs, ankles, feet and buttocks. Based on current data, CLV is associated with the following conditions: idiopathic (45–55%), infection (15–20%), inflammatory disease (15–20%), drug intake (10–15%), and malignancies (5%) [2]. Acute stage CLV is characterized by a predominate infiltrate of neutrophils in and around the vessel wall and the lumen, but without evidence of new capillary formation and fibrous proliferation in and around the vessel. Another feature is that the internal elastic lamina remains intact. In the subacute stage, CLV is characterized by a mixed infiltrate of neutrophils, lymphocytes and histiocytes with the formation of a fibrinoid deposit (i.e., fibrinoid necrosis) in the intima of the affected arteries. The subacute stage is then followed by a reparative (i.e., granulation tissue) and healed stage (i.e., scarring) [1]. Usually, vascular immunoglobulin (IgM, IgG) and complement (C3) deposits are found by direct immunofluorescence assessments implicating immune complex deposition in the pathogenesis of CLV. However, the exact pathomechanism of CLV is obscure [2].

So far, there have been no systematic studies investigating T cell subsets and other immune cells in CLV. In particular, T regulatory cells (Tregs) are currently of great interest in the pathogenesis of many human diseases, particularly such as autoimmune disorders. Tregs are now recognized as the mediators of peripheral tolerance and potent suppressors of excessive immune responses. Several Treg subtypes with distinct phenotypes, cytokine production...
profiles, and modes of action have been described [3, 4].
In the CD4+ regulatory T cell compartment, CD4+CD25+ T cells and interleukin-10-producing type 1 T regulatory
cells have been described. Other markers for Tregs include
CD4+CD25++CD127– and CD4+CD25+CD39 [3, 4].

Aim
Hence, we aimed to focus on the assessment and comparison of lymphocytes – in particular Tregs – and related
immune markers in patients with CLV and controls who underwent treatment with systemic prednisolone.

Material and methods
Subjects
Fourteen patients (10 males, 4 females; mean ± SD
age: 42.6 ± 13.7 years) with severe (ulceration, skin ne-
crosis etc.) and/or recurrent flares of CLV were enrolled
in the study after giving informed consent. The diagno-
sis of CLV was verified by patient’s history, clinical symp-
toms, histological findings, and laboratory studies [1, 2].
Systemic manifestations of vasculitis were excluded by
means of laboratory tests (e.g. urine creatinine, ANA, ANCA, cryoglobulins) and abdomen ultra-
sonography. In all patients, direct immunofluorescence
studies were performed. Patients were treated with an
initial dose of intravenous prednisolone (1.5 mg/kg body
weight) which was slowly tapered. Treatment outcome
was estimated using a simple score: 0 = none improve-
ment/worsening; 1 = slight improvement; 2 = moderate
improvement; 3 = good to excellent improvement. Ad-
ditionally, we included 10 healthy sex/age-matched con-
trols (age: 45.8 ± 15.3 years). The study was approved by
the ethics review board of the Ruhr-University Bochum
and conducted according to the Declaration of Helsinki principles.

Flow cytometry
In order to identify and determine the absolute counts
of lymphocytes in the peripheral blood, flow cytometric
methods were applied using antibodies for T lymphocytes
(CD3+), B lymphocytes (CD19+), helper/inducer T lympho-
cytes (CD3+CD4+), suppressor/cytotoxic T lymphocytes
(CD3+CD8+), natural killer (NK) cells (CD3–CD16+CD56+),
and Tregs (CD4+CD25++CD127– and CD4+CD25+CD39). The
FACSCanto II™ cytometer (BD Biosciences, San Jose, CA,
USA), equipped with 633 nm and 488 nm red and blue
lasers, together with computer hardware and FACSDiva-
software™ were used to analyze the aforementioned lympho-
cyte populations and subsets.

Real-time polymerase chain reaction (RT-PCR)
Quantitative analysis of real-time RT-PCR was per-
formed in duplicate as previously suggested [5]; total
cellular RNA was isolated from blood samples of CLV
patients and controls. The quantities of FOXP3, trans-
growing factor β1 (TGF-β1) and interleukin-10 (IL-10) mRNA levels were determined using the median
log transform of the gene expression. Relative mRNA ex-
pression levels were calculated using the comparative
ΔΔCT method as previously suggested by Livak and
Schmittgen [5]. Quantities of all targets in the test sam-
ple were normalized to the corresponding RPL38 RNA
transcript in the blood samples.

Routine histology, direct immunofluorescence and
immunohistochemistry
Four millimetre punch biopsies were performed in
lesional skin of patients (at baseline and after 7 days)
and healthy skin of controls. Biopsy sites were the lower
legs. In order to confirm clinical diagnosis, routine his-
ology (haematoxylin-eosin staining) and direct immuno-
fluorescence were performed in all CLV patients. For im-
munohistochemistry, paraffin-embedded sections were
mounted on silanized slides and stored for 1 h in a humid
chamber at 60°C. Sections were deparaffinized in xylene
and washed with 100%, 96%, 70% and 50% ethanol for
5 min each and rinsed with demineralized water. Immu-
nohistochemical staining was eventually performed for
CD4 and CD8, granzyme B, transforming growth factor β1
(TGF-β1, Abcam, Cambridge, UK), and interleukin 10 (IL-10,
Antibodies-online, Atlanta, USA). Immunohistochemistry
specimens were analysed using a Zeiss (Jena, Germany)
light microscope at 400× magnification in 5 fields of view.
Quantitative results were expressed as the relative propor-
tion of positive dermal infiltrating cells (0 = 100%). The
CD4/CD8 ratio was calculated from the mean percentage
of positive cells for each case. Mean values of immunore-
activity scores were used for statistical analysis.

Statistical analysis
Data analysis was performed using the statistical
package MedCalc Software (Mariakerke, Belgium). Dis-
tribution of data was assessed by means of the D’Agostino-
Pearson test. Based on distribution data were expressed
as medians (range) or means ± SD. The results were
analyzed using the Mann-Whitney test, Wilcoxon test,
and paired and un-paired t-test. Correlation analysis was
performed using the Pearson and Spearman procedure.
This was an explorative pilot study without sample size
calculation and adjustment for multiple testing. Differ-
ences were considered significant when p < 0.05.

Results
Clinical data
In all patients, the diagnosis of CLV was confirmed
by histology and direct immunofluorescence. In-patient
treatment was performed for median 7 days (5–13 days).
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After treatment clinical improvement was estimated by one observer (M.K.) as follows: 1 (8.3%) patient had slight improvement, 2 (16.7%) patients had moderate and 9 (75%) showed good to excellent improvement. Baseline C-reactive protein levels (28.4 mg/l (5.6–69.4 mg/l)) were significantly \( (p = 0.039) \) increased when compared to post-treatment levels of 5.8 (1–29.4). Baseline (9150/µl (6670–12570/µl)) and post-treatment (10850/µl (8190–14250/µl)) blood leukocyte levels did not significantly \( (p = 0.084) \) differ, although there was a trend for higher post-treatment leukocytes (likely because of prednisolone intake).

**Flow cytometry**

As shown in Table 1 in detail, flow cytometry for CD4+, CD8+, CD4+CD25++CD127–, or CD4+CD25+CD39+ T lymphocytes, B lymphocytes and natural killer cells did not show significant differences between the groups assessed.

**Real time polymerase chain reaction**

RT-PCR revealed that TGF-β1 mRNA expression was significantly higher after therapy (13.3 ±8.1) when compared to baseline (6 ±4.3, \( p = 0.0065 \)) and controls (5.6 ±3.9, \( p = 0.026 \)). mRNA expression of FOXP3 and IL-10 did not significantly differ between the groups (Table 1). Baseline TGF-β1 and IL-10 mRNA levels of CLV patients significantly \( (p = 0.0051) \) correlated as indicated by a high coefficient of correlation of 0.83 (Figure 1).

**Immunohistochemistry**

On immunohistology (Table 2 and Figure 2), baseline CLV lesions (42% (6.3–70%)) showed significantly more CD4+ lymphocytes than post-treated CLV (20.8% (8.2–37.5%, \( p = 0.0068 \)) and controls (18.4% (6.5–26%), \( p = 0.024 \) and controls (16.5% (8.2–31%, \( p = 0.0055 \)). After treatment, the CD4/CD8 (0.68% (0.22–0.94) ratio was significantly decreased when compared to baseline CLV (2.6 (0.43–5.7), \( p = 0.001 \) and controls (1.31 (0.9–1.55), \( p = 0.0061 \). Baseline granzyme B expression (21.9% (2–40%)) was significantly increased when compared to treated CLV (0% (0–8%), \( p = 0.0005 \) and controls (0% (0–4%), \( p = 0.0004 \). IL-10 expression of treated CLV (84.4% (70–91)) was significantly increased when compared to baseline CLV (75.2% (54.6–84%), \( p = 0.0005 \) and controls (14.2% (8–24%), \( p = 0.0004 \); baseline CLV IL-10 expression was also significantly increased as compared to controls (\( p = 0.0004 \).

**Discussion**

Overexpression of FOXP3 has been shown to lead to a hypoactive immune state suggesting that it is a central regulator of T cell activity. FOXP3 is a more specific marker of Tregs than most cell surface markers such as

| Parameter | Cutaneous leukocytoclastic vasculitis | Cutaneous leukocytoclastic vasculitis | Controls |
|-----------|--------------------------------------|--------------------------------------|----------|
| Flow cytometry: | | | |
| CD4+/µl (n.s.) | 799 (123–2980) | 1066 (218–2112) | 644 (433–1329) |
| CD8+/µl (n.s.) | 274 (48–887) | 331 (75–836) | 296 (147–631) |
| CD4/CD8 ratio (n.s.) | 3 (0.56–11.5) | 2.9 (0.9–15.6) | 2.5 (0.9–4.4) |
| CD4+CD25++CD127– (%) (n.s.) | 6.4 (3.5–17) | 7.6 (1.3–11.9) | 5.7 (2.4–8.9) |
| CD4+CD25+CD39+ (%) (n.s.) | 4 (0.6–13.1) | 3.4 (1.7–10.8) | 2 (0.7–6.2) |
| B lymphocytes/µl (n.s.) | 190 (35–876) | 473 (67–670) | 186 (55–577) |
| Natural killer cells/µl (n.s.) | 162 (59–691) | 158 (44–827) | 183 (108–337) |
| Real-time RT-PCR§: | | | |
| FOXP3 (n.s.) | 0.067 ±0.051 | 0.045 ±0.091 | 0.099 ±0.068 |
| Transforming growth factor-β1* | 6 ±4.3 | 13.3 ±8.1 | 5.6 ±3.9 |
| A vs. B, \( p = 0.0065 \) | | | |
| B vs. C, \( p = 0.026 \) | | | |
| Interleukin 10 (n.s.) | 0.017 ±0.005 | 0.011 ±0.006 | 0.02 ±0.02 |

n.s. = differences not significant; pair-wise comparisons for dependent and independent data (Wilcoxon, Mann-Whitney, paired and unpaired t-tests) did not reveal statistically significant differences, except for *. Quantities of analyzed genes were determined using the median log-transformation of the gene expression. Relative mRNA expression levels were calculated using the comparative \( Δ–ΔCt \) method. Gene expression was normalized to the homogenously expressed housekeeping gene RPL38.
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CD4 and CD25. By contrast, CD4+CD25++CD127– T cells are the best known membrane markers for Tregs which can be used for natural occurring Treg isolation in human blood. Moreover, CD4+CD25+CD39+ T cells represent activated/memory Tregs [3, 4]. Tregs have relatively rarely been studied in vasculitic disorders. Klapa et al. [6] as well Morgan et al. [7] have shown that patients with granulomatosis with polyangiitis – an ANCA-associated systemic small vessel vasculitis – demonstrate a decrease in the number and function of Tregs in the blood and this is most pronounced in those with most active disease [7]. Olivito et al. [8] reported that the frequencies of CD4+CD25+FOXP3+ Tregs in the blood were significantly lower during the acute phase of Kawasaki’s disease than healthy donors and a similar tendency was revealed for FOXP3 mRNA transcripts. FOXP3 expression increased significantly, at both protein and mRNA levels, after intravenous immunoglobulin therapy treatment [8].

Yang et al. [9] studied patients with Henoch-Schönlein purpura (HSP) which is a small vessel vasculitis closely related to CLV. They found that compared with healthy controls, the proportions of T helper 2 (Th2) cells in patients with HSP were significantly higher, and the ratio of Th1/Th2 was remarkably decreased. The proportions of Tregs in patients with HSP were significantly lower than those of controls. Moreover, Yang et al. [9] observed that the mRNA expression of FOXP3 was significantly decreased in patients with HSP when compared to controls. Sengar et al. [10] have shown that during the active phase, HSP patients developed significant lymphocytosis, whereby the absolute numbers of CD4+ cells were significantly increased but absolute numbers of CD8+ cells were not altered.

Unlike in systemic vasculitides, such as granulomatosis with polyangiitis, CLV does not seem to be associated with abnormalities of circulation Tregs, In the present study, neither flow cytometry nor RT-PCR revealed significant differences in circulating Tregs among the groups compared. Moreover, the number of cytotoxic cells (CD8+), B lymphocytes and natural killer cells did not change significantly.

**Table 2. Immunohistochemistry of cutaneous leukocytoclastic vasculitis**

| Parameter                  | A Cutaneous leukocytoclastic vasculitis | B Cutaneous leukocytoclastic vasculitis | C Controls |
|----------------------------|----------------------------------------|----------------------------------------|------------|
| CD4*: A vs. B, p = 0.0068  | 42% (6.3–70%)                          | 20.8% (8.2–37.5%)                     | 18.4% (6.5–26%) |
| CD8*: B vs. A, p = 0.024  | 18.3% (5.8–34.7%)                      | 32.1% (12–48.9%)                      | 16.5% (8.2–31%) |
| CD4/CD8 ratio*: B vs. A, p = 0.001 | 2.6% (0.43–5.7%)              | 0.68% (0.22–0.94%)                    | 1.31% (0.9–1.55%) |
| Granzyme B*: A vs. B, p = 0.0005 | 21.9% (2–40%)                    | 0% (0–8%)                             | 0% (0–4%)  |
| Interleukin 10*: B vs. A, p = 0.0005 | 75.2% (54.6–84.2%)            | 84.4% (70–91%)                        | 14.2% (8–24%) |
| Transforming growth factor-β1: A vs. B, p = 0.0004 | 20.9% (2.3–80%)              | 12.9% (3.5–60.7%)                     | 25% (4–70%)  |

Pair-wise comparisons for dependent and independent data (Wilcoxon, Mann-Whitney, paired and unpaired t-tests) did not reveal statistically significant differences, except for *.
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**Figure 2.** Immunohistopathology image (magnification, 250×) showing increased CD4+ lymphocytes in cutaneous leukocytoclastic vasculitis (A) before treatment when compared to post-treatment skin lesions (B) and healthy controls (C). Granzyme B protein expression is significantly increased in cutaneous leukocytoclastic vasculitis (D) as compared to post-treatment skin lesions (E) and healthy controls (F).
not significantly differ in baseline blood of CLV patients and controls. The aforementioned data of the present study may support in part the fact that CLV is a condition in which systemic involvement is usually absent [11]. However, we observed that TGF-β1 mRNA expression was significantly increased after therapy when compared to baseline and controls. We explain the post-treatment increase in TGF-β1 expression in blood with systemic glucocorticosteroid therapy that can induce the expression of TGF-β1. Ossege et al. [12] reported that during and after treatment with systemic glucocorticosteroid therapy, the TGF-β1 mRNA blood levels significantly increased at day 3–5 and declined about 3 days after discontinuation of therapy. TGF-β1 is an immunosuppressant cytokine which blocks cytokine synthesis and hence T cell activation. It has been shown by Huss et al. [13] however that TGF-β signaling causes binding of Smad4 to the IL-10 promoter, providing molecular evidence for TGF-β signaling blocks cytokine synthesis and hence T cell activation. The latter is a pivotal mediator of apoptosis used by cytotoxic T lymphocytes and natural killer cells. The presence of granzyme B in CD4+ T cells was reported by several studies and was believed to be associated with their cytotoxic activity. However, granzyme B can also act upon the cells harboring them [14]. The relatively increased percentage of CD8+ cells after treatment is likely due to the decrease of CD4+ cells. Whether the marked reduction of T helper cells was caused by granzyme B-mediated apoptosis cannot be answered based on the present data. IL-10 was significantly higher expressed in untreated and treated CLV than in the skin of controls. Interestingly, IL-10 mRNA levels were increased even during therapy when compared to baseline. Glucocorticosteroids are known to induce immunotolerance via up-regulation of IL-10 [15]. However, limitations of the present study particularly include a small sample size and lack of functional studies. Moreover, we did not include a CLV control group not receiving systemic steroids. However, this pilot study was not designed to prove the efficacy of systemic prednisolone in CLV. Since CLV represents usually a self-limited, single-episode phenomenon, the treatment should rather consist of general measures such as leg elevation, warming, avoidance of standing, cold temperatures and tight fitting clothing, and therapy with antihistamines, aspirin, or non-steroidal anti-inflammatory drugs [16, 17]. Nevertheless, we studied more severe and/or recalcitrant cases requiring systemic treatment.

Conclusions

The data of the present study do not indicate a significant role of circulating Tregs in the pathogenesis of CLV. T helper cells and granzyme B seem to be involved in the inflammatory process of CLV. A resolution of CLV after glucocorticosteroid treatment may be mediated via up-regulation of TGF-β1 and IL-10 in different compartments.

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Thilo Gambichler and Magdalena A. Kulik equally contributed to this work.

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Conflict of interest

The authors declare no conflict of interest.

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