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SHORT COMMUNICATION

Altered regulation of the T-cell system in patients with CRPS

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Abstract
The aim of this study was to investigate T-cell subsets and immunomodulatory factors in patients with complex regional pain syndrome (CRPS). We found decreased numbers of pro-inflammatory Th17 cells in patients with CRPS as compared to healthy volunteers. The expression of Th17 related RORγT mRNA was also significantly decreased. Patients with CRPS showed an increased proportion of $CD39^+$ Tregs. CD39 is a known inhibitor of Th17 cell differentiation. Systemic cytokine levels were almost unchanged in patients with CRPS. These findings suggest that the decrease in Th17 cells in CRPS is regulated by an increase in $CD39^+$ Tregs and that this anti-inflammatory T-cell shift may be a mechanism to control inflammation in CRPS.
German clinical trial register Registration Trial DRKS00005954.

Keywords CRPS · Th17 cells · CD39 · Inflammation.

Introduction

Complex regional pain syndrome (CRPS) is a disorder distinguished by significant autonomic features [1]. It typically arises after trauma or surgery without obvious nerve injury (type I) or after trauma involving the nervous system (type II) [2]. Besides neuropathic pain characteristics, patients with CRPS also present a local inflammatory response as well as changes suggestive for an autonomic involvement [1]. These pathological changes are considered in the Budapest criteria, an important tool for the diagnosis of CRPS [3].

In the past, CRPS was understood as a sympathetically mediated peripheral pain condition [1]. Recent research indicates an involvement of immunological factors in the pathogenesis of pain and CRPS [1]. In fact, it has been shown that T cells and corresponding cytokines participate in the development and maintenance of pain behaviors in neuropathic rodent models [4, 5]. It is, therefore, not surprising that patients with CRPS show a disrupted Th1/Th2 balance [5]. Recently, the Th1/Th2 dichotomy has been expanded by pro-inflammatory Th17 and anti-inflammatory regulatory T cells (Treg) [6]. Tregs can be further divided into Tregs expressing the membrane bound endonucleotidase CD39 and in those which do not express CD39 [7]. It has been reported that $CD39^+$ Tregs have immune suppressive functions and can inhibit the development of Th17 cells [8]. Furthermore, recent research suggests also an important role for the approximately 18–22-nucleotide-long miRNAs in the regulation of T-cell differentiation as well as in pain development and maintenance [9].

CRPS represents a unique entity with neuropathic and inflammatory proportions. However, the role of the T-cell system (Th17, Tregs and CD39) in CRPS remains unknown. Previously, we found a modified Th17/Treg balance in patients suffering from neuropathic pain (NeP) or chronic low back pain (CLBP) and an involvement of miRNAs [6, 9, 10]; we, therefore, hypothesized that (1) the Th17/Treg balance is altered in patients suffering from CRPS, that (2) the amount of $CD39^+$ Tregs might be changed, and that (3) the miRNA expression is also modified.

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Methods

Ethics statement and participants

The study was approved by the Institutional Review Board of the Ludwig-Maximilians-Universität München (Ethical approval number: 331-10) and is in line with the principles of the Declaration of Helsinki (Trial registration: DRKS00005954).

12 patients with CRPS type I were included in the study (Table 1). CRPS was diagnosed according to the Budapest criteria [3]. 12 healthy volunteers (without pain), matched for gender and age served as controls. All participants gave written informed consent. None of the patients had been treated with corticosteroids or immunomodulatory drugs. Patients rated their average pain intensity using an 11-point numerical rating scale (NRS).

Cytokine assessment

Blood samples (peripheral) were taken between 9:00 and 9:30 a.m. For cytokine assessment, blood was centrifuged at 2000×*g* for 10 min to obtain cell-free serum. Samples were assessed for levels of T-cell-related cytokines using a human cytokine multiplex immunoassay (Myriad Rules-Based Medicine Inc., Austin, Texas, United States).

Flow cytometric staining and analysis

Peripheral blood mononuclear cells (PBMCs) were separated by density gradient preparation over Ficoll-Uropoline (Sigma Aldrich, Taufkirchen, Germany). Determination of Th17 cells and Treg numbers by flow cytometry was performed according to the manufacturer's protocol [6, 10]. $CD39^+$ Tregs were examined after staining with specific monoclonal antibodies (Biolegend, San Diego, California, United States) using flow cytometry. The gating strategy for Tregs and CD39 is displayed in Fig. 1a.

RNA isolation, cDNA synthesis and quantitative real-time PCR (qPCR)

RNA isolation from $CD4^+$ cells and cDNA synthesis was performed as described before [8]. qPCR was done in duplicates with a LightCycler 480 instrument (Roche Diagnostics, Mannheim, Germany) using LightCycler 480 Probes Master and RealTime ready single assays. Relative mRNA expression of Foxp3 and RORγT was calculated by Relative Quantification Software (Roche Diagnostics). SDHA and TBP were used for reference gene

Table 1 Characteristics of patients with CRPS

	Age (years)	Sex	BMI (kg/m^2)	NRS (rest)	NRS (motion)	Duration of CRPS (months)	Triggering cause	Edema	Allodynia	Increased skin temperature at the affected area
Patient 1	58	Male	25,3	6	8	5	Fracture of a metatarsal bone	+	+	+
Patient 2	43	Female	24,9	2	4	12	Fracture of the ankle joint	+	+	–
Patient 3	52	Male	31	6	9	8	Surgery of the hand	+	–	+
Patient 4	55	Female	18,7	4	6	7	Fracture of a metatarsal bone	+	–	–
Patient 5	35	Female	21	5	6	6	Fracture of the distal radius	+	+	+
Patient 6	24	Female	19,1	4	8	9	Bone bruise (hand)	–	–	+
Patient 7	20	Female	17,7	7	8	2	Surgery of the hand	+	–	+
Patient 8	21	Female	22,9	5	7	3	Soft tissue injury (hand)	+	+	+
Patient 9	49	Female	28,4	1	6	2	Fracture of the distal radius	+	–	–
Patient 10	70	Female	24,8	3	10	12	Fracture of the distal radius	–	+	+
Patient 11	19	Female	19,1	5	8	14	Bone bruise (food)	+	–	–
Patient 12	21	Female	21	3	8	8	Fracture of a carpal bone	+	+	+

BMI body mass index, *NRS* numeric rating scale, *CRPS* complex regional pain syndrome

normalization. Primer sequences and RT-Assays are listed in [6, 10].

Expression of miR-155, miR-206, and miR-210 in $CD4^+$ cells was quantified using TaqMan miRNA assays (Applied Biosystems) following the manufacturer's protocol. The cycling conditions were as follows: denaturing at 95 °C for 10 min, 45 cycles of 95 °C for 15 s and 60 °C for 60 s. U47 (small nuclear RNA) was used as endogenous control and for normalization of miRNA expression data.

Statistical analyses

Statistical analyses were performed with SigmaStat 12.0 (Systat Software, Chicago, USA). Testing for normal distribution was done with the Shapiro–Wilk Test. Verification of differences between groups was accomplished by the *t* test or nonparametric Mann–Whitney rank sum test. The Benjamini–Hochberg correction was used due to multiple analyses. p values ≤ 0.05 were considered statistically significant. All results were expressed as mean ± standard deviation.

Results

Characteristics of patients with CRPS

Patients with CRPS had an age of 38.9 ± 17.1 years, a BMI of 22.8 ± 4.0 kg/m^2, a NRS (rest) of 4.3 ± 1.7, a NRS (motion) of 7.3 ± 1.5, and a duration of symptoms of 7.3 ± 3.8 months. The percentage of females was 83%. 83% of patients with CRPS showed an edema, 50% presented allodynia, and 33% an increased skin temperature of the affected area.

Patients with CRPS presented a decreased proportion of Th17 cells

Using flow cytometry, we found a significant decrease in the percentage of Th17 cells in the peripheral blood of patients suffering from CRPS (CRPS: 0.6 ± 0.3% vs. healthy volunteers: 1.6 ± 1.1%, $p < 0.05$; Fig. 1b, left panel). Real-time PCR experiments confirmed this result. The relative mRNA expression of Th17-specific transcription factor RORγT was significantly decreased in CRPS (CRPS: 1.3 ± 0.9 vs. healthy volunteers: 2.7 ± 1.0, $p < 0.01$; Fig. 1b, right panel).

Recent research suggests an involvement of Tregs in the development and maintenance of CLBP and NeP [6, 9, 10]. We, therefore, analyzed the proportion of Tregs in CRPS. Surprisingly, patients with CRPS did not exhibit altered Treg levels when compared to healthy volunteers (CRPS: 2.3 ± 1.1% vs. healthy volunteers: 1.8 ± 0.8%, n.s.; Fig. 1c, left panel). This result was confirmed by qPCR analyzing Treg-specific transcription factor Foxp3 (CRPS: 0.12 ± 0.06 vs. healthy volunteers: 0.13 ± 0.04, n.s.; Fig. 1c, right panel).

Patients with CRPS presented an altered Th17/Treg balance

Since the identification of Th17 cells and Tregs, the ratio of Th17/Treg is used to characterize immune response. Due to the described reduction of Th17 cells, the Th17/Treg ratio was significantly lower in the peripheral blood of patients with CRPS when compared to healthy volunteers (Fig. 1d) resulting in an anti-inflammatory T-cell shift in these patients.

Patients with CRPS show an increased amount of $CD39^+$ Tregs

It has been reported, that $CD39^+$ Tregs have the ability to suppress the development of Th17 cells [11]. In our analyses, we found reduced amounts of Th17 cells in CRPS. Therefore, we assumed that the expression of CD39 on Tregs is increased in CRPS. Indeed, we found enhanced amounts of $CD39^+$ Tregs in patients suffering from CRPS when compared to healthy volunteers (Fig. 1e).

Cytokine levels are not altered in CRPS

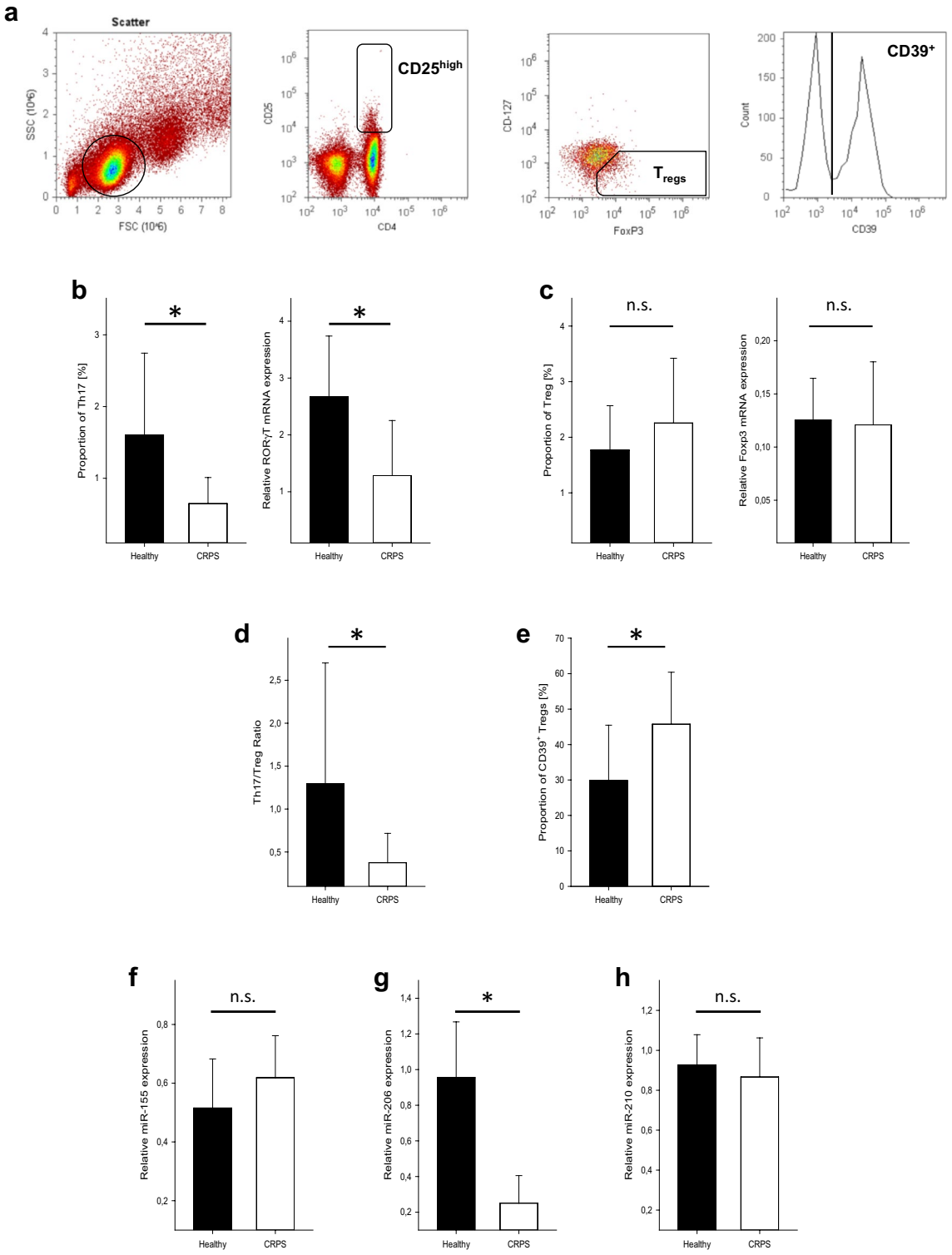
Cytokines are well-known regulators of T-cell differentiation and CD39 expression. We, therefore, analyzed the blood levels IL-1, IL-4, IL-6, and IL-23 as well as the mRNA expression of TGF-ß and IFN-γ. Overall, the cytokine blood levels and mRNA expression did not differ significantly between both study groups. Our findings suggest that the systemic levels of the analyzed cytokines are not responsible for the altered Th17/Treg balance in CRPS.

MiR-206 expression is significantly reduced in CRPS

Recent studies identified miR-155, miR-206 and miR-210 as a direct regulator of Th17 differentiation or rather found an inverse correlation of miRNA expression and proportion of Th17 cells [12, 13]. The expression of miR-155 and miR-210 in $CD4^+$ cells of patients with CRPS did not differ significantly as compared to healthy volunteers (Fig. 1f, h). However, the expression of miR-206 was significantly decreased in $CD4^+$ cells of patients with CRPS (Fig. 1g). Based on these results, miR-206 seems to play an important role in the regulation of Th17 differentiation in CRPS.

Discussion

The etiology of CRPS is still a matter of debate; several studies in animals and humans showed an involvement of the immune system and inflammatory proteins [5, 11, 14]. In this study, we found changes in T-cell subsets in



the pathogenesis of CRPS: Th17 cell percentages were decreased in CRPS, whereas the percentage of Tregs did not significantly change. However, the percentage of $CD39^+$ Tregs, a known suppressor of Th17 differentiation [8], was significantly increased in CRPS. Furthermore, the altered expression of miR-206 (another modulator of Th17 differentiation) was decreased in these patients. Therefore, changed numbers of $CD39^+$ Tregs and miRNA-206 expression may explain at least in part the decrease of Th17 cells in CRPS.

◂**Fig. 1** **a** Gating strategy for the detection of $CD39^+$ Tregs. The lymphocyte population was gated from PBMCs according to forward (FSC) and side scatter (SSC) characteristics (left). Gated lymphocytes were then separated in $CD4^+CD25^{high}$ cells (middle left, named $CD25^{high}$) and $CD4^+CD25^{high}CD127^{low}FOXP3^+$ cells (middle right, named Treg). Finally, Tregs were separated into $CD39^-$ and $CD39^+$ cells (right). **b** Patients with CRPS show decreased proportions of Th17 cells as well as RORγT mRNA expression in $CD4^+$ cells. The amount of Tregs and the transcription factor Foxp3 is barely changed in CRPS (**c**). The ratio of Th17/Treg was significantly decreased in CRPS (**d**). Patients with CRPS show an increased number of $CD39^+$ Tregs a known inhibitor of Th17 cell differentiation (**e**). MiR-155, miR-206, and miR-210 are also associated with the differentiation of Th17 cells. Patients with CRPS present a decreased miR-206 expression in $CD4^+$ cells (**g**), whereas the expression of miR-155 (**f**) and miR-210 (**h**) did not differ significantly when compared to healthy volunteers. Data are given as means ± SD; $*p < 0.05$

Patients with CRPS had a decrease in the percentage of Th17 cells without any significant changes in the amount of Tregs. These results were surprising since we found previously an increase of Tregs and a decrease in Th17 cells in patients with NeP or CLBP [6, 9, 10]. The differences in the T-cell subsets observed in CRPS patients and those seen in NeP and CLBP patients support the thesis that immune responses in CRPS patients are unique and differ from those observed in other chronic pain conditions.

MiRNAs are evolutionarily conserved short, approximately 18–22-nucleotide-long, noncoding single-stranded RNA molecules which regulate gene expression [15]. Recent studies showed an involvement of miRNAs in the differentiation of immune cells—furthermore, miRNA profiles have been proposed as diagnostic tools in CRPS [16]. Recent research demonstrates a regulation of Th17 cell differentiation by miR-155 and miR-210 [17, 18]. However, analyzing the expression of miR-155 and miR-210 did not show a significant difference in patients with CRPS. Therefore, these miRNAs seem to be not involved in the regulation of Th17 cells in CRPS.

Haas et al. detected an elevated expression of miR-206 in in vitro differentiated Th17 cells and in IL-17 producing innate lymphocytes [12]. Overexpression of miR-206 results in an increase of pro-inflammatory cytokines in immune cells [13]. In line with these findings, we found a decrease of miR-206 in $CD4^+$ peripheral blood mononuclear cells and a reduced number of Th17 cells in patients suffering from CRPS. This finding supports the hypothesis that the decrease of miR-206 expression could be responsible for the reduced numbers of Th17 cells in CRPS.

The cell-surface protein CD39 is a known inhibitor of Th17 differentiation [8]. It catalyzes the conversion of ATP into AMP which will be further processed to adenosine [7, 8]. Conforming to these results, we found an increased proportion of $CD39^+$ Tregs and as consequence a reduction of Th17 cells in patients suffering from CRPS. These changes might be a physiological mechanism to limit the inflammatory response in CRPS.

Taken together, we found an altered Th17/Treg balance in patients with CRPS as a consequence of decreased pro-inflammatory Th17 cells. We suggest that the decrease in Th17 cells is regulated by an increase of $CD39^+$ Tregs and a decreased expression of miR-206 in $CD4^+$ cells. The alteration of CD39 and miR-206 expression may be a possible mechanism to control the inflammatory process in CRPS.

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Compliance with ethical standards

Conflict of interest The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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