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Differential expression patterns of cytokines in complex regional pain syndrome

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Abstract

Complex regional pain syndromes (CRPS) are characterized by persistent and severe pain after trauma or surgery. Neuroimmune alterations are assumed to play a pathophysiological role. Here we set out to investigate whether patients with CRPS have altered systemic pro- and anti-inflammatory cytokine profiles compared to controls on mRNA and protein level. We studied blood cytokine mRNA and protein levels of the pro-inflammatory cytokines tumor necrosis factor-α (TNF), interleukin-2 (IL-2) and IL-8 and the anti-inflammatory cytokines IL-4, IL-10, and transforming growth factor-β1 (TGFβ1) in 40 prospectively recruited patients with CRPS I, two patients with CRPS II, and 34 controls. Quantitative real-time PCR and enzyme linked immunosorbent assay were used. Additionally, the patients underwent quantitative sensory testing and were assessed with the McGill pain questionnaire and the Hospital anxiety and depression scale. Patients with CRPS had higher blood TNF and IL-2 mRNA levels ($p = 0.005$; $p = 0.04$) and lower IL-8 mRNA levels ($p < 0.001$) than controls. The mRNA for the anti-inflammatory cytokines IL-4 and IL-10 was reduced in the patient group ($p = 0.004$; $p = 0.006$), whereas TGFβ1 mRNA levels did not differ between groups. These results were paralleled by serum protein levels, except for TGFβ1, which was reduced in patients with CRPS, and for IL-8, which gave similar protein values in both groups. Sensory testing showed a predominant loss of small fiber-related modalities in the patient group. The shift towards a pro-inflammatory cytokine profile in patients with CRPS suggests a potential pathogenic role in the generation of pain.

Keywords: CRPS; Cytokines; Tumor necrosis factor-α; Interleukins; Chronic pain; Quantitative sensory testing

1. Introduction

Complex regional pain syndrome (CRPS) usually develops after trauma and is characterized by spontaneous pain, allodynia and mechanical hyperalgesia, motor dysfunction and autonomic changes. It has recently been hypothesized that acute stage CRPS is in part caused by exaggerated posttraumatic inflammation (Huygen et al., 2002; Birklein, 2005). The phenotype of acute CRPS indeed mimics the cardinal symptoms of inflammation including increased skin temperature, edema, redness, pain, and loss of function. It was shown that protein levels of the neuropeptide calcitonin-gene related peptide (CGRP) are increased in serum samples of patients with CRPS and neurogenic flare and plasma protein extravasation are enhanced upon transcutaneous electrical C-fiber stimulation (Birklein et al., 2001; Weber et al., 2001), indicating facilitated neurogenic inflammation.

Besides neuropeptides the pro-inflammatory cytokines are implicated in exaggerated pain states after physical

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trauma. These may induce pain and hyperalgesia by direct and indirect mechanisms. Examples for direct mechanisms leading to hyperalgesia are peripheral nociceptors sensitized by pro-inflammatory cytokines (Junger and Sorkin, 2000) and enhanced spinal nociceptive transmission (Cuellar et al., 2004). Furthermore, tumor necrosis factor-α (TNF), a prototypic pro-inflammatory cytokine, increases the production and release of CGRP in in-vitro and in-vivo models (Opree and Kress, 2000b; Schäfers et al., 2003; Bowen et al., 2006). If cytokines play a role in the pathogenesis of CRPS, it might be possible to detect increased systemic levels of pro-inflammatory cytokines in patients with CRPS, or reduced levels of anti-inflammatory cytokines. To test this hypothesis, we investigated 42 patients with CRPS (40 with CRPS I, i.e. CRPS without concomitant nerve injury, and two with CRPS II) and 34 age- and gender-matched healthy volunteers. We found that protein levels and mRNA levels of pro-inflammatory cytokines are elevated in these patients.

2. Patients and methods

2.1. Patients and controls

Patients with CRPS were prospectively recruited at the Department of Neurology, University of Mainz between August 2004 and January 2006. CRPS I was diagnosed if a major peripheral nerve lesion was excluded by neurological examination, CRPS II if a major nerve lesion was present, and if the patients fulfilled the current diagnostic criteria for CRPS research (Burton et al., 2005). By definition, other diagnoses that might cause pain had to be excluded by history, clinical examination and routine blood testing. Forty patients with CRPS I and two patients with CRPS II (patients #3 and #8 in Table 1) were enrolled, 29 women and 13 men with a median age of 53 years (range 28–77 years). The median disease duration was 12 weeks (range 1–70 weeks). The experiments were approved by the Ethics Committee of the University of Mainz. Table 1 gives a synopsis of patient data.

The control group for cytokine measurements (control group 1) consisted of 34 age- and gender-matched healthy volunteers recruited at the Department of Neurology, University of Würzburg. There were 24 women and 10 men, with a median age of 51 years (range 27–76 years). These volunteers had no pain disorder or infectious illness at the time of blood sampling.

The control group for sensory profiling (control group 2) was different from the control group for cytokines. This group consisted of 32 age- and gender-matched healthy volunteers, recruited at the Department of Neurology, University of Mainz. Each control person was investigated in the same body area as the matching CRPS patient. There were 21 women and 11 men, with a median age of 55 years (range 26–75 years).

2.2. Questionnaires

For self-assessment the German counterpart of the McGill pain questionnaire (Stein and Mendl, 1988) and the German version of the Hospital Anxiety and Depression Scale (HADS) (Herrmann et al., 1991) were used. We asked the patients to fill in the McGill questionnaire in order to describe their pain at rest at the time point of blood withdrawal and QST. As a measure of pain the pain rating index (PRI), the sum score of rank values, was calculated. Furthermore, all patients were asked to assess their maximum pain at rest in the last 24 h on a numerical rating scale (NRS) ranging from zero (no pain) to 10 (worst pain imaginable). HADS assesses the patients' mood and thoughts in the last week with 20 questions, HADS-A for the item "anxiety" and HADS-D for the item "depression". A score of 11 and more indicates depression and increased anxiety. Since we started using the HADS when the complete QST protocol (see paragraph below) was introduced into routine examination of CRPS patients, the HADS was obtained from 32 patients only.

2.3. Quantitative sensory testing (QST)

Thirty-one of the 40 patients with CRPS I and one of the two patients with CRPS II (patient #8) underwent quantitative sensory testing (QST) using a standard QST device (Somedic, Sweden) according to a standardized protocol of the DFNS (Deutscher Forschungsverbund Neuropathischer Schmerz) which has been published recently (Rolke et al., 2006). Ten patients were enrolled before the protocol was established and therefore not examined by QST. QST was performed directly after blood was drawn for cytokine analyses. In the patients with CRPS I the most affected painful area was tested; in patient #8 with CRPS II QST was performed on the back of the hand in the ulnar nerve territory. Thermal and mechanical thresholds were determined. Briefly, parameters included cold and warm detection thresholds (CDT, WDT), thermal sensory limen (TSL), paradoxical heat sensation (PHS), cold and heat pain thresholds (CPT, HPT), mechanical detection threshold (MDT), mechanical pain detection threshold (MPT), mechanical pain sensitivity (MPS), dynamic mechanical allodynia (DMA), wind-up ratio (WUR) of 10 painful stimuli, vibration detection threshold (VDT) and pressure pain threshold (PPT). Based on the log transformed raw values for each item, a z-score sensory profile was calculated for the entire group of examined patients. The data of the group of CRPS patients were compared to the data of age- and gender matched healthy controls that were examined in accordant body areas.

2.4. Blood withdrawal

Blood was obtained by venipuncture from all CRPS patients for routine analysis including whole blood cell counts, electrolyte studies, renal and liver function tests, blood glucose, and C-reactive protein (CRP) levels. Another 30 ml of venous blood was collected between 8.00 and 9.00 am to avoid physiological diurnal alterations in cytokine expression. To prevent influences on the systemic cytokine levels the following exclusion criteria were defined: heavy physical activity in the prior three days, food intake within 60 min before blood sampling, alcohol consumption on the previous day, current infectious diseases or fever. For quantitative real-time PCR (qRT-PCR) analysis 20 ml blood was withdrawn into tubes containing ethylenediaminetetraacetic acid (EDTA) and stored at −80 °C after aliquotting into 2 ml Eppendorf caps

(500 μl each). For enzyme linked immunosorbent assay (ELISA) studies 10 ml blood was centrifuged at room temperature with 3300 *g* for 5 min. Immediately afterwards the supernatant serum was collected in 2 ml Eppendorf caps and stored at −80 °C before further processing.

2.5. mRNA extraction

mRNA was extracted from frozen blood samples as described previously (Kruse et al., 1997). In brief, 500 μl of frozen blood was thawed on ice and incubated with 500 μl guanidine thiocyanate (Roti Quick Kit®, Carl Roth, Karlsruhe, Germany) and 650 μl phenol. After centrifugation at 3300 *g* and 4 °C for 15 min the supernatant was mixed with 600 μl of isopropanol and incubated for 40 min. After another centrifugation step total mRNA was washed with 70% ethanol and dissolved in diethylpyrocarbonate (DEPC) treated water. The optical density values of the extracted mRNA were measured with an Eppendorf photometer (Eppendorf, Hamburg, Germany).

2.6. Reverse transcription PCR

For reverse transcription of 750 ng of the extracted mRNA to cDNA TaqMan Reverse Transcription Reagents® (Applied Biosystems, Darmstadt, Germany) were used. The reaction was carried out at a final volume of 100 μl. Five microliters of random hexamers were added to 750 ng RNA and the mixture was filled up with RNAse free water to an end volume of 37.8 μl. After heat denaturation (85 °C, 3 min), 2 μl of Oligo-dt, 10 μl of 10× PCR-buffer, 6.25 μl Multiscribe reverse transcriptase, 2 μl RNAse inhibitor, 22 μl $MgCl_2$ and 20 μl dNTPs were added. The cycler conditions (ABI PRISM 7700 Cycler, Applied Biosystems, Darmstadt, Germany) were as follows: annealing at 25 °C for 10 min, reverse transcription at 48 °C for 60 min and enzyme inactivation at 95 °C for 5 min.

2.7. qRT-PCR

Five microliters of cDNA was used for qRT-PCR, which was performed in the GeneAmp 7700 sequence detection system® (Applied Biosystems, Darmstadt, Germany) capable of fluorescence using TaqMan Universal Master Mix® (Applied Biosystems, Darmstadt, Germany). Gene specific oligonucleotide primers and probes for human TNF (Assay-ID: Hs00174128_m1), IL-2 (Assay-ID: Hs00174114_m1), IL-8 (Assay-ID: Hs00174103_m1), IL-4 (Assay-ID: Hs00174122_m1), IL-10 (Assay-ID: Hs00174086_m1), and TGFβ1 (Assay-ID: Hs99999918_m1), as well as the endogenous control 18sRNA, were obtained as TaqMan Gene Expression Assays® (Applied Biosystems, Darmstadt, Germany). The reaction mixture contained 12.5 μl TaqMan Universal Master Mix® and 1.25 μl of the specific primer in a final volume of 25 μl. The cycler conditions were as follows: incubation for 2 min at 50 °C followed by another incubation step at 95 °C for 10 min, afterwards 45 cycles with 15 s at 95 °C and 1 min at 60 °C. Each qRT-PCR plate contained a calibrator sample, which was the blood sample of the control person in the control group 1 whose threshold cyclus (Ct)-values were next to the calculated mean of all control blood samples specific for each primer. In order to guarantee primer specificity and to exclude genomic contamination, negative controls without cDNA template were run on each qRT-PCR well plate. All samples were measured as triplicates, while the very stable 18s-values were measured as duplicates. The evaluation of the obtained data was performed with the comparative $\Delta\Delta$Ct-method as described by the manufacturer (User bulletin #2 Applied Biosystems, P/N 4303859, 1997) and elsewhere (Winer et al., 1999).

2.8. ELISA

Commercial ELISA kits were used to determine serum cytokine levels in patients and healthy controls. The pro-inflammatory cytokines TNF, IL-2, and IL-8 and the anti-inflammatory cytokines IL-4, IL-10, and TGFβ1 were examined with the following kits: human TNF Immunoassay kit, sensitivity: 1.7 pg/ml (Biosource International, Nivelles, Belgium); human IL-2 enzyme amplified sensitivity immunoassay (EASIA®), sensitivity: <0.1 IU/ml (Biosource, Nivelles, Belgium); human IL-8 ultrasensitive immunoassay kit, sensitivity: <100 fg/ml (Biosource, Nivelles, Belgium); human IL-4 ultrasensitive immunoassay kit, sensitivity: <0.27 pg/ml (Biosource, Nivelles, Belgium); human IL-10 immunoassay (Quantikine®HS), sensitivity: <0.5 pg/ml (R&D Systems, Wiesbaden, Germany); human TGFβ1 immunoassay (Quantikine®), sensitivity: <7 pg/ml (R&D Systems, Wiesbaden, Germany). All assays were measured in duplicate on 96-well plates and the results are given as pg/ml, IU/ml, or fg/ml. Since in all but two controls no IL-2 protein, and in 27 patients no IL-10 protein was measurable these samples were arbitrarily estimated as 0.01 IU/ml or 0.01 pg/ml, respectively, to be able to perform statistical analyses.

2.9. Statistical analysis

All data were analyzed using SPSS software (Version 14.0, Munich, Germany). Since our qRT-PCR and ELISA data did not display normal distribution in the Kolmogorov–Smirnov-test, we used the Mann–Whitney *U*-test for pairwise comparison. The QST data were log transformed and the *z*-score of each item was calculated. Afterwards one-way ANOVA was performed for group comparison. The Spearman's rank correlation coefficient was used to calculate correlations between numerical cytokine mRNA and protein data and the QST data. For the questionnaire data compared with the cytokine mRNA values an explorative analysis was used. *P*-values of <0.05 were considered statistically significant.

3. Results

3.1. Patients' characteristics

The patients' characteristics including demographic data, inciting event, disease duration, current medication, signs of CRPS in addition to pain and hyperalgesia, chronic pain history, score on a numerical rating scale for mean pain at rest in the last 24 h and the results

Table 1
Patient characteristics with individual McGill, HADS-A, and HADS-D score

Patient, age [years], gender	Inciting event	Time since symptom onset [weeks]	Current analgesic medication	Location of symptoms	Signs present in addition to pain and hyperalgesia from the deep tissue[a]	Chronic pain in history [0 = no, 1 = yes]	McGill pain rating index (PRI)	NRS (at rest, last 24 h)	HADS-A/ HADS-D score
1/53/F	Fracture of the right radial bone	7	Prednisolone, pregabalin, tramadol	Right hand	1, 2, 3, 4	0	42	3	10/6
2/36/F	Minimal trauma of the left foot	7	None	Left foot	2, 3	0	27	1	9/8
3/45/F	Nerve reconstruction in interosseus anterior syndrome at left arm	3	Diclofenac, oxycodon, amitriptyline	Left arm	1, 2, 3, 4	1	28	4	0/3
4/53/F	Surgery of right knee	7	Oxycodon, pregabalin, venlafaxin, prednisolone	Right foot to knee	1, 2, 3	1	45	9	7/5
5/47/M	Crush injury of right finger	16	Methylprednisolone, pregabalin, duloxetine	Right finger	1, 2, 3, 4	0	40	3	12/7
6/49/F	Fracture of the left radial bone	12	Tramadol, ibuprofen	Left hand	1, 2, 3, 4	0	32	8	6/6
7/62/M	Fracture of the left hand	16	Alendronic acid, diclofenac	Left hand	1, 2, 3, 4	0	16	10	2/4
8/44/M	Surgery in carpal tunnel syndrome on the right side	24	Pregabalin, oxcarbazepine, amitriptyline	Right hand	1, 3	0	36	7	9/1
9/61/M	None	26	Alendronic acid	Right foot to knee	1, 3	0	2	6	7/6
10/55/M	Dupuytren surgery of right hand	16	Diclofenac, celecoxib	Right hand	1, 2, 3, 4	1	36	2	16/17
11/37/F	Distorsion of left hand	70	Metamizole, acetyl salicyl acid, tramadol as needed	Left hand	1, 2, 3	1	38	8	4/3
12/69/M	Tendon disruption right hand	16	None	Right hand	1, 3	1	0	0	12/13
13/70/F	Fracture of left forearm	8	Pregabalin	Left forearm	1, 2, 3	0	9	2	13/12
14/58/F	Fracture of left metatarsal bones	29	Pregabalin	Left foot	1, 2, 3	0	4	2	None
15/52/M	Tendon disruption left foot	20	Pregabalin	Left foot	1, 2, 3	0	6	5	0/0
16/53/F	Fracture of right radial bone	6	Diclofenac	Right hand	1, 2, 3, 4	1	5	4	8/5
17/63/M	Fracture of left metacarpal bones	13	None	Left hand	1, 2, 3, 4	0	5	6	9/6
18/43/F	Tendon disruption left foot	8	Prednisolone, pregabalin, paroxetine	Left foot	3, 4	0	32	1	13/13
19/63/F	Surgery of left hand	28	None	Left hand	1, 2, 3	1	3	2	None
20/56/M	Fracture of left tibial bone and metatarsal bones	8	Pregabalin	Left foot	1, 3	1	7	5	6/7
21/53/F	Fracture of right talocalcanean joint	56	Tilidine + naloxone, pregabalin, amitriptyline	Right foot	1, 2, 3, 4	1	language problems	5	None

22/52/F	Fracture of right hand dig. V	25	Ibuprofen as needed	Right hand dig. V	1, 3	1	7	5	None
23/63/F	Fracture of the right dig. V	12	Metamizole	Right hand	1, 2, 3, 4	1	58	8	7/3
24/29/F	Surgery in carpal tunnel syndrome on the right	26	Pregabalin	Right hand	1, 2, 3	0	17	7	10/6
25/38/M	Burning of right forearm	62	None	Right forearm	1, 2, 3, 4	1	60	9	None
26/65/F	Fracture of left radial bone	10	None	Left hand	1, 2, 3	0	3	9	1/4
27/61/F	Distorsion of right toe	70	Diclofenac	Right foot	2, 3, 4	1	5	2	8/5
28/53/F	Fracture of left forearm and left wrist	6	Ibuprofen, pregabalin	Left hand	1, 2, 3	0	3	7	None
29/35/M	Fracture of metacarpal bone V right hand	10	Pregabalin, amitriptyline	Right hand	1, 2, 3, 4	1	30	5	10/6
30/37/F	Fracture of metatarsal bones left foot	12	None	Left foot	1, 3	1	28	5	None
31/47/F	Right wirts distorsion	20	None	Right hand	1, 3	0	38	6	None
32/30/F	Contusion of right hand	20	Diclofenac	Right hand	1, 2, 3	0	17	4	0/0
33/53/F	Fracture of right radial bone	10	None	Right hand	1, 2, 3	1	42	4	3/5
34/54/F	Fracture of right radial bone	10	Pregabalin	Right hand	1, 3, 4	0	Language problems	6	6/7
35/77/F	Fracture of right radial bone	9	Pregabalin	Right hand	1, 2, 3	0	17	5	None
36/58/F	Fracture of right radial bone	16	None	Right hand	1, 2, 3	0	16	1	None
37/55/F	Torn ligament left foot	36	Oxcarbazepine	Left foot	2, 3	1	34	2	3/3
38/55/M	Fracture of left humerus	11	None	Left hand	1, 2, 3, 4	0	4	2	7/4
39/69/F	Surgery of left hallux	6	Diclofenac	Left hallux	1, 3	1	21	8	9/9
40/50/F	Distorsion of right hand	3	None	Right hand	1, 3	0	3	1	0/2
41/69/F	Fracture of left radial bone	13	Metamizole, alendronic acid, methylprednisolone	Left hand to forearm	1, 2, 3, 4	0	9	4	9/7
42/28/M	Crush injury of left foot	8	Non-steroidal antirheumatics	Left foot	1, 2, 3, 4	0	36	8	12/8

Dig.=digit;NRS=Numerical rating scale (0 = no pain; 10 = worst pain imaginable); F=female; M=male;wks=weeks.

[a] All patients reported symptoms in all of the four categories of the current IASP diagnostic criteria (abnormalities in pain processing; skin color/temperature changes; vasomotor/sudomotor changes or edema; motor dysfunction and trophic changes). In addition, all patients presented at least two signs out of these categories, which are listed in the table above. These signs are coded as follows: (1) motor symptoms (weakness, tremor, dystonia or myoclonic jerks) (2) sensory symptoms (hypaesthesia, hyperesthesia, hypalgesia, pin-prick hyperlagesia, allodynia, dysesthesia) (3) autonomic symptoms (skin color changes, skin temperature differences >1 °C, sweating abnormalities) (4) trophic changes (increased or decreased hair or nail growth).

of the McGill pain and HADS-A/HADS-D questionnaires are summarized in Table 1.

3.2. Routine blood analysis

Of the 42 patients, one had an elevated CRP value of 12 mg/l (normal range 0–5 mg/l) and one patient had leukocytosis of 17.400/μl (normal range 5.000–10.000/μl). None of the patients had a combination of elevated CRP, leukocytosis and fever as a sign for acute inflammation.

3.3. Questionnaire data

Forty of the 42 patients completed the McGill pain questionnaire, two patients failed to answer the questions because of language problems. The McGill pain rating index (PRI) was calculated. The median value at time of investigation was 17 (range 0–70). Table 1 gives the individual PRI scores of the patients.

Twenty-nine of 32 patients completed the HADS questionnaire. One had language problems, two refused. For the item "anxiety" six patients and for the item "depression" four patients had a score of 11 or higher indicating clinically significant anxiety and depression. The median score was 7.5 (range 0–16).

3.4. QST

Thirty-one CRPS I patients and one CRPS II patient were examined with QST. Fig. 1 shows the *z*-profile of all CRPS patients compared to the age- and gender matched healthy control group. As a group, patients with CRPS had significantly impaired sensory perception with increased CDT ($p < 0.0001$), WDT ($p = 0.004$), TSL ($p < 0.0001$), and VDT ($p < 0.0001$) compared to controls. MPT slightly failed to be significant ($p = 0.054$). Patients with CRPS also had reduced PPT ($p < 0.0001$).

3.5. Cytokine mRNA

Patients with CRPS had significantly higher blood TNF and IL-2 relative mRNA levels than controls ($p = 0.01$ for IL-2; $p = 0.04$ for TNF, Fig. 2a and b). IL-8 relative mRNA levels were higher in controls ($p < 0.001$, Fig. 2c). IL-4 and IL-10 relative mRNA levels were significantly lower in patients with CRPS ($p = 0.004$ for IL-4; $p = 0.01$ for IL-10) (Fig. 2c and d) compared to the control group, whereas TGFβ1 relative mRNA levels did not differ between groups (Fig. 2e). Since disease duration can alter systemic cytokine levels

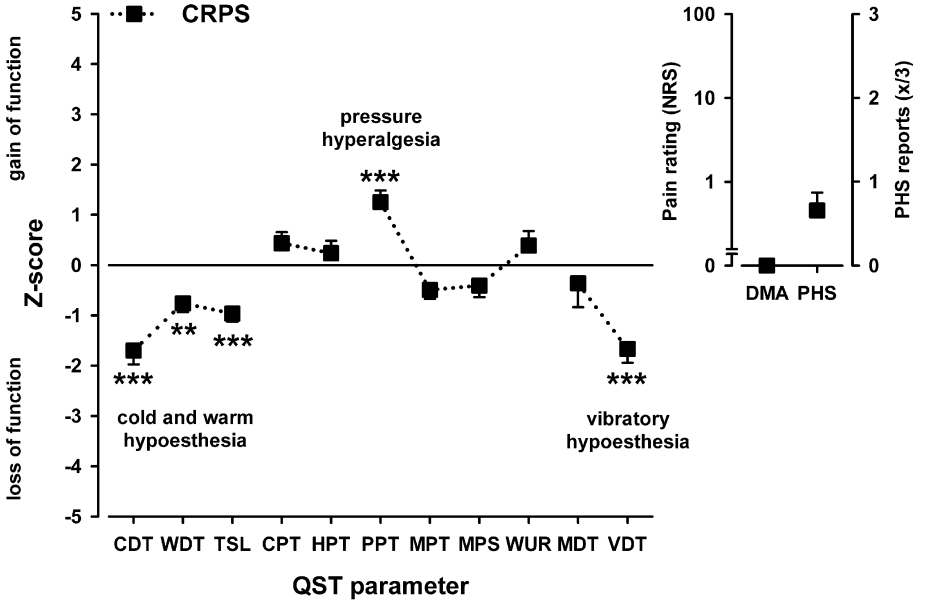

Fig. 1. The *z*-score sensory profile of 31 CRPS I patients and one CRPS II patient is shown for all tested QST parameters over the affected limb. The *z*-score profile of CRPS patients was compared with that of healthy control subjects as represented by a *z*-score "zero". The sensory profile of the affected limbs shows a predominant loss of sensory function in terms of cold hypoesthesia (CDT), warm hypoesthesia (WDT), an increased thermal sensory limen (TSL; temperature difference between alternating warm and cold stimuli) with an increased number of paradoxical heat sensations (PHS), and vibratory hypoesthesia (VDT). In contrast to a decreased cutaneous sensitivity across C-fiber, A-δ- and A-β-fiber mediated stimuli, deep pain sensitivity to blunt pressure (PPT) was increased. This sensory profile is consistent with deafferentation in combination with peripheral sensitization of the nociceptive system in this subgroup of CRPS patients. *z*-score: Numbers of standard deviations between patient data and group-specific mean values of healthy control subjects. CDT, cold detection threshold; WDT, warm detection threshold; TSL, thermal sensory limen; PHS, paradoxical heat sensation; CPT, cold pain threshold; HPT, heat pain threshold; PPT, pressure pain threshold; MPT, mechanical pain threshold; MPS, mechanical pain sensitivity; WUR, wind-up ratio; MDT, mechanical detection threshold; VDT, vibration detection threshold; DMA, dynamic mechanical allodynia. Shown data represent mean *z*-scores ± SEM. For DMA and PHS raw data are shown (means ± SEM). Asterisks denote the level of significance: $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$ (one-way ANOVA).

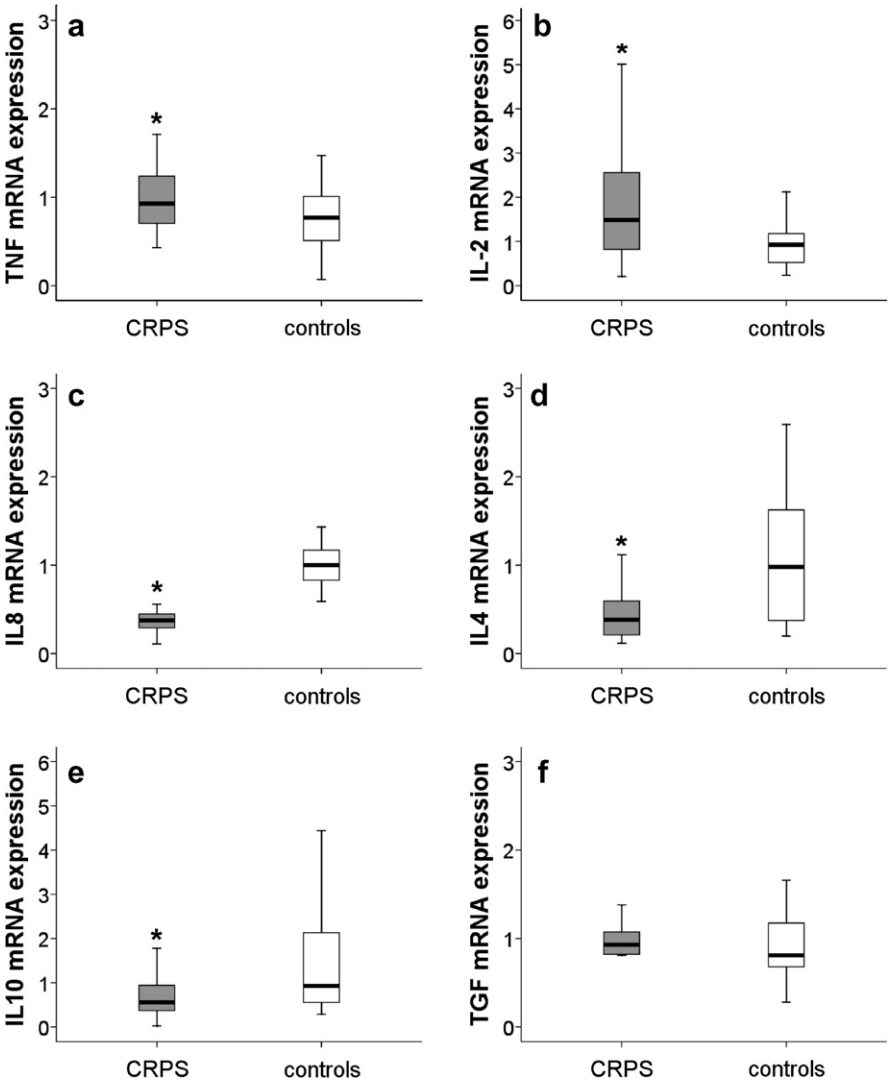

Fig. 2. Boxplots illustrating blood cytokine mRNA levels in patients with CRPS I compared to control group 1. The box is limited by the first and the third quartile, the fat horizontal line in the box marks the median value, and the whiskers indicate the upper and lower extreme values. Patients with CRPS I had higher TNF (a, $^{*}p = 0.04$) and IL-2 (b, $^{*}p = 0.005$). mRNA levels than controls. Levels of IL-8 (c, $^{*}p < 0.001$), IL-4 (d, $^{*}p = 0.004$), and IL-10 mRNA (e, $^{*}p = 0.006$) were lower in patients CRPS I. TGFβ1 mRNA levels did not differ between groups (f).

in patients with CRPS (Munnikes et al., 2005), we investigated subgroups of patients according to the time from the onset of symptoms (1–4 weeks, 5–8 weeks, 9–12 weeks, 13–16 weeks, 20–30 weeks, >30 weeks). No significant difference was found between groups with different disease durations (data not shown). The two patients with CRPS II had cytokine values close to the median of the values for CRPS I.

3.6. Cytokine protein levels

TNF blood protein levels were not significantly higher in patients with CRPS than in controls (Fig. 3a). Patients had significantly increased IL-2 protein levels than controls (Fig. 3b). Serum IL-8 protein levels show a trend to be higher in patients (n.s., Fig. 3c). IL-4 protein levels did not differ between groups (Fig. 3d). Patients with CRPS had significantly lower IL-10 and TGFβ1 protein levels than healthy controls (Fig. 3e and f). Values for the two patients with CRPS II were in the range of the values of patients with CRPS I.

3.7. Cytokines and clinical symptoms

In an explorative subgroup analysis cytokine values were compared to different patients' characteristics. Chronic pain other than CRPS (e.g. headache, low back pain) was present in the medical history of 18 patients (Table 1). Their cytokine values did not differ from patients without chronic pain in their history. There was also no difference comparing the cytokine mRNA and protein levels of the patients with pain scores as assessed by the McGill PRI and with mood and anxiety

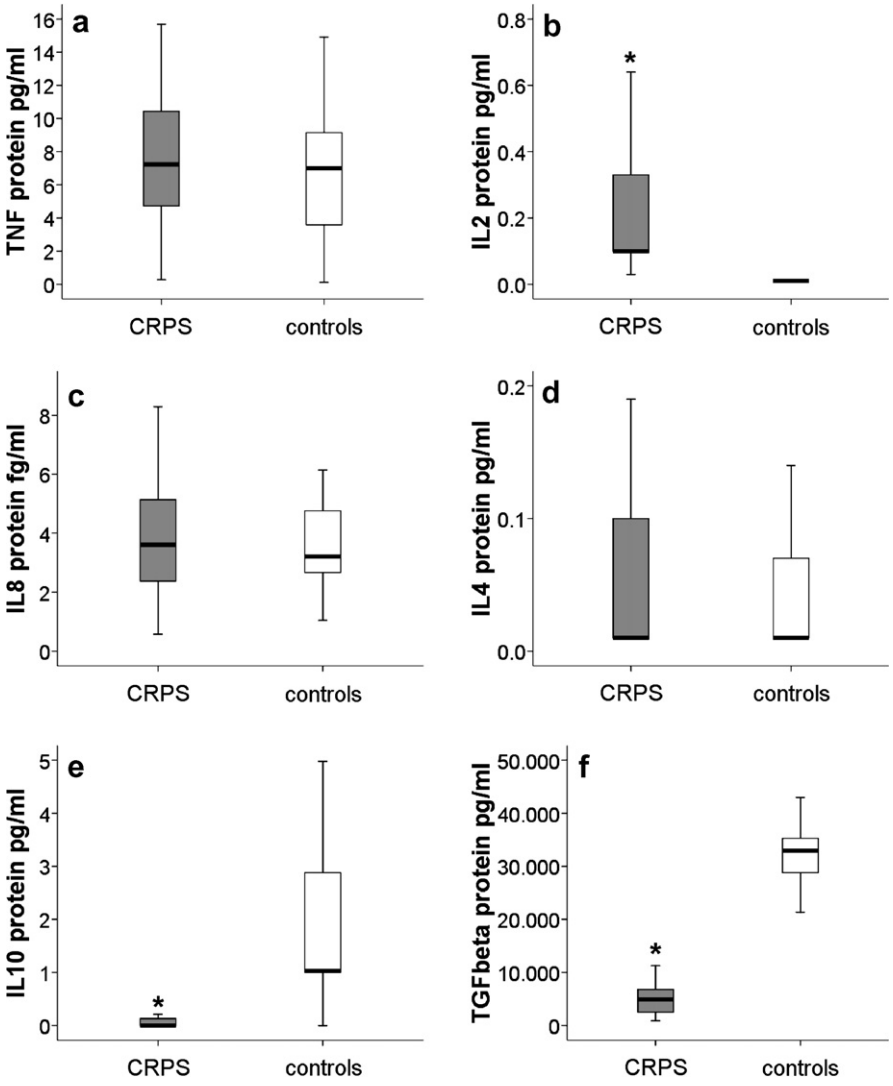

Fig. 3. Boxplots illustrating serum cytokine protein levels in patients with CRPS I compared to control group 1. The box is limited by the first and the third quartile, the fat horizontal line in the box marks the median value, and the whiskers indicate the upper and lower extreme values. TNF protein levels were not different in patients with CRPS I compared to controls (a, n.s.). IL-2 protein levels were higher in patients with CRPS I compared to controls (b, $^{*}p < 0.001$). IL-8 (c) and IL-4 (d) protein levels were not different from controls (n.s.). IL-10 (e, $^{*}p < 0.001$) and TGFβ1 (f, $^{*}p < 0.001$) protein levels were lower in patients with CRPS I compared to controls.

scores as assessed by HADS in the upper and lower quintile of the entire patient group (data not shown). According to QST measurements, in 23 of 32 patients sensory minus signs (loss of function) and in 9 of 32 patients sensory plus signs (gain of function, e.g. hyperalgesia) were predominant. Cytokine mRNA and protein levels were not different between these two patient groups (data not shown). Finally we analyzed the difference of cytokine mRNA and protein levels between patients with acute (<12 weeks disease duration, $n = 20$) and chronic disease (>12 weeks, $n = 20$), and between subjects with clinically severe CRPS (all four signs present, see Table 1, $n = 15$) compared to patients with clinically less severe CRPS (less than four signs present, $n = 27$). Again no significant differences could be found.

4. Discussion

We found elevated levels of pro-inflammatory cytokines and reduced levels of anti-inflammatory cytokines in patients with CRPS. Specifically, mRNA levels of the pro-inflammatory cytokines TNF and IL-2 and serum IL-2 protein levels were elevated, and mRNA levels of the anti-inflammatory cytokines IL-4 and IL-10 were reduced. TGFβ1 protein levels were also lower in patients with CRPS. Taken together, these findings show a pro-inflammatory cytokine profile in our patients with CRPS.

Animal studies and clinical trials have provided evidence for the pathophysiological role of cytokines in the induction and maintenance of pain. Pro-inflammatory cytokines are predominantly algesic, while anti-

inflammatory cytokines show mostly analgesic effects (Marchand et al., 2005; Üçeyler and Sommer, 2007). For instance, the pro-inflammatory cytokine TNF induces pain after local application and is upregulated in neuropathic pain models, while IL-4 and IL-10 are analgesic (Hao et al., 2006; Milligan et al., 2006). We thus hypothesize that the pro-inflammatory profile observed may be a crucial factor in the pathogenesis of CRPS.

The pathophysiology of CRPS is still incompletely understood. Recent data point to an exaggerated neurogenic inflammation after trauma as an igniting factor. In particular, patients with CRPS have elevated serum levels of CGRP and enhanced neurogenic flare upon transcutaneous electrical stimulation (Weber et al., 2001). Pro-inflammatory cytokines have been shown to increase the release of CGRP from sensory nerves (Opree and Kress, 2000a). Levels of TNF and other pro-inflammatory cytokines are rapidly upregulated after bone fracture and with cast immobilization (Guo et al., 2004). Thus, an increase in cytokine levels and neuropeptide release can be regarded as physiological processes after trauma. Patients who develop CRPS may have a delayed resolution of this inflammatory cascade. It may be speculated that a systemic pro-inflammatory cytokine profile, as shown in the present study, may be one of the crucial predisposing factors for the development of CRPS. The fact that exaggerated neurogenic inflammation is not confined to the affected limb but can also be found to a lesser degree on the unaffected side long after resolution of CRPS symptoms (Leis et al., 2004) further supports this assumption. However, whether elevated local or systemic cytokine levels have a greater impact on pain development in patients with CRPS remains to be elucidated.

The patients in our study were heterogeneous according to CRPS duration, pain levels, history of chronic pain, and severity of symptoms other than pain. Such variations are not uncommon and are often described in clinical studies on CRPS (e.g. (Veldman et al., 1993; Birklein et al., 2000)). Subgroup analysis, however, did not show differences in cytokine RNA expression or plasma cytokine protein concentrations. Moreover, a subgroup of our patients underwent detailed QST in order to investigate possible correlations between cytokine expression and distinct psychophysical findings. Again, subgroups with prominent 'minus signs' or 'plus signs' did not differ in cytokine expression.

The most prominent finding on QST was an increase in detection thresholds for temperature, vibratory stimuli and tendentially for pin-prick. There are several possible explanations for these minus signs. It has been recently shown that although CRPS I by definition does not include a major nerve lesion, minor nerve damage commonly occurs in the course of the disease (Albrecht et al., 2006; Oaklander et al., 2006). Although minor nerve damage might explain reduced temperature and pin-prick sensation, it cannot explain the reduction of vibration perception, which is rather mediated plurisegmentally via muscle and bone afferent nerves. Another mechanism may be central inhibition of non-noxious mechanical input from the painful limb, similarly to what has been shown during experimental heat-induced pain (Apkarian et al., 1994) and during painful electrical muscle stimulation (Kosek and Hansson, 2002). This central inhibition might occur at the spinal level (Magerl and Treede, 2004) or through the inhibition of perception discrimination in the primary somatosensory cortex by sustained C-fiber excitation (Pleger et al., 2005). Finally, we did not find a correlation between the QST data and cytokine mRNA and protein values. This underlines the notion that a pro-inflammatory cytokine profile may be a predisposing trait rather than a causative factor for specific symptoms and signs, and this profile may increase the susceptibility of a person to develop CRPS upon traumatic injury, rather being the consequence of trauma itself.

The concept of a pathophysiological role of cytokines in CRPS is further supported by reports of successful anti-TNF treatment of patients with CRPS. One example is the TNF receptor antibody infliximab, which was used to treat two patients (Huygen et al., 2004). In both patients pain relief was observed, and the TNF and IL-6 content in blister fluids from the affected limb was reduced. Other studies gave encouraging results using thalidomide, which can inhibit TNF production (Sampaio et al., 1991; Barnes et al., 1992), degrade TNF mRNA (Moreira et al., 1993) and recruit IL-10 producing macrophages (George et al., 2000). In a patient with CRPS and concomitant Behçet's disease, significant pain reduction was achieved by thalidomide treatment (Ching et al., 2003). Another patient who suffered from CRPS and multiple myeloma experienced complete resolution of CRPS after thalidomide medication (Rajkumar et al., 2001). In a study with 42 patients suffering from CRPS seven patients had dramatic pain relief and six patients had modest pain relief under thalidomide treatment (Schwartzman et al., 2003). The less toxic analogue of thalidomide, lenalidomide, was effective in patients with CRPS in an open-label trial (Manning, 2006).

We could detect some discrepancies between mRNA and protein data. While TNF mRNA was elevated in patients with CRPS, TNF protein was not. This may be due to the low stability of the TNF molecule (Seko et al., 2006), which may hinder reliable detection in ELISA. Also, TNF is posttranslationally regulated, such that discrepancies in mRNA and protein levels may indeed occur (Moss et al., 1997). In a previous study this problem was addressed by analyzing the soluble TNF-α receptor 1 (sTNF-R1), which is more stable than the TNF protein, and these data supported a role of TNF in CRPS pathophysiology (Maihöfner et al., 2005a). Similarly, IL-8 was reduced

in patients with CRPS at the mRNA level, but not at the protein level. In contrast, TGFβ1 mRNA was not different between groups, but TGFβ1 protein was lower in CRPS patients than in controls. These findings might again be due to differential posttranslational regulation.

Previous investigators used different methods of cytokine analysis and reported partially conflicting results. In most studies, pro-inflammatory cytokines were increased. TNF and TNF receptor 1 were found elevated in CRPS (Maihöfner et al., 2005b). In the cerebrospinal fluid, patients with CRPS had elevated levels of IL-1β and IL-6 and reduced levels of IL-4 and IL-10 (Alexander et al., 2005; Alexander et al., 2006). Others used more local approaches, related to the site of CRPS. IL-8 protein levels were found elevated in blood samples taken from the affected extremity compared to the unaffected extremity and to controls (Schinkel et al., 2006). Like in another previous study (van de Beek et al., 2001) we did not find differences in systemic serum IL-8 protein. This discrepancy concerning IL-8 may indeed indicate increased local release at the symptomatic side. Concordantly, other studies showed elevated TNF and IL-6 protein levels in suction blister fluids in the affected limb of patients with CRPS (Huygen et al., 2002; Munnikes et al., 2005; Groeneweg et al., 2006). In a recent study the local cytokine expression in affected skin of patients with CRPS was examined with a multiplex bead array in blister fluid. Of the investigated cytokines, IL-6, IL-8, and TNF were increased in the affected skin of patients with CRPS, whereas IL-10 levels did not differ from skin samples of healthy subjects (Heijmans-Antonissen et al., 2006).

There are some limitations to our study. (1) Several factors are potential determinators of cytokine levels, which we tried to control as much as possible but could not exclude completely. We standardized time of blood withdrawal and instructed patients for instance not to drink alcohol or perform physical activity on the day before blood withdrawal. Patients with clinical or laboratory signs of systemic inflammation were excluded. (2) Concomitant medication could also influence systemic cytokine levels. This is particularly known for antidepressants (Weizman et al., 1994; Kubera et al., 2000). In our study only four of the investigated 42 patients were under tricyclic antidepressants, therefore we assume no effect on the entire patient group results. Sixteen of our patients were on pregabalin. Systemic cytokine mRNA and protein levels did not differ between patients who were on pregabalin compared to patients who were not (data not shown). (3) Our patient group was inhomogeneous in disease duration, which could have been another variable affecting cytokine expression patterns (Munnikes et al., 2005). The subgroup analysis however did not reveal differences between cytokine expression at different time points. The question remains if systemic cytokine levels differ between patients after CRPS resolution and patients with chronic CRPS.

In conclusion, this study adds to the growing evidence of a neuro-immune disturbance in patients with CRPS. These findings may have important implications for the treatment of these patients.

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References

Albrecht PJ, Hines S, Eisenberg E, Pud D, Finlay DR, Connolly MK, et al. Pathologic alterations of cutaneous innervation and vasculature in affected limbs from patients with complex regional pain syndrome. Pain 2006;120:244–66.

Alexander GM, Perreault MJ, Reichenberger ER, Schwartzman RJ. Changes in immune and glial markers in the CSF of patients with Complex Regional Pain Syndrome. Brain Behav Immun 2006.

Alexander GM, van Rijn MA, van Hilten JJ, Perreault MJ, Schwartzman RJ. Changes in cerebrospinal fluid levels of pro-inflammatory cytokines in CRPS. Pain 2005;116:213–9.

Apkarian AV, Stea RA, Bolanowski SJ. Heat-induced pain diminishes vibrotactile perception: a touch gate. Somatosens Mot Res 1994;11:259–67.

Barnes PF, Chatterjee D, Brennan PJ, Rea TH, Modlin RL. Tumor necrosis factor production in patients with leprosy. Infect Immun 1992;60:1441–6.

Birklein F. Complex regional pain syndrome. J Neurol 2005;252:131–8.

Birklein F, Riedl B, Sieweke N, Weber M, Neundorfer B. Neurological findings in complex regional pain syndromes –analysis of 145 cases. Acta Neurol Scand 2000;101:262–9.

Birklein F, Schmelz M, Schifter S, Weber M. The important role of neuropeptides in complex regional pain syndrome. Neurology 2001;57:2179–84.

Bowen EJ, Schmidt TW, Firm CS, Russo AF, Durham PL. Tumor necrosis factor-alpha stimulation of calcitonin gene-related peptide expression and secretion from rat trigeminal ganglion neurons. J Neurochem 2006;96:65–77.

Burton AW, Bruehl S, Harden RN. Current diagnosis and therapy of complex regional pain syndrome: refining diagnostic criteria and therapeutic options. Expert Rev Neurother 2005;5:643–51.

Ching DW, McClintock A, Beswick F. Successful treatment with low-dose thalidomide in a patient with both Behcet's disease and complex regional pain syndrome type i: case report. J Clin Rheumatol 2003;9:96–8.

Cuellar JM, Montesano PX, Carstens E. Role of TNF-alpha in sensitization of nociceptive dorsal horn neurons induced by application of nucleus pulposus to L5 dorsal root ganglion in rats. Pain 2004;110:578–87.

George A, Marziniak M, Schäfers M, Toyka KV, Sommer C. Thalidomide treatment in chronic constrictive neuropathy

decreases endoneurial tumor necrosis factor-alpha, increases interleukin-10 and has long-term effects on spinal cord dorsal horn met-enkephalin. Pain 2000;88:267–75.

Groeneweg JG, Huygen FJ, Heijmans-Antonissen C, Niehof S, Zijlstra FJ. Increased endothelin-1 and diminished nitric oxide levels in blister fluids of patients with intermediate cold type complex regional pain syndrome type 1. BMC Musculoskelet Disord 2006;7:91.

Guo TZ, Offley SC, Boyd EA, Jacobs CR, Kingery WS. Substance P signaling contributes to the vascular and nociceptive abnormalities observed in a tibial fracture rat model of complex regional pain syndrome type I. Pain 2004;108:95–107.

Hao S, Mata M, Glorioso JC, Fink DJ. HSV-mediated expression of interleukin-4 in dorsal root ganglion neurons reduces neuropathic pain. Mol Pain 2006;2:6.

Heijmans-Antonissen C, Wesseldijk F, Munnikes RJ, Huygen FJ, van der Meijden P, Hop WC, et al. Multiplex bead array assay for detection of 25 soluble cytokines in blister fluid of patients with complex regional pain syndrome type 1. Mediators Inflamm 2006;2006:28398.

Herrmann C, Scholz KH, Kreuzer H. Psychologic screening of patients of a cardiologic acute care clinic with the German version of the Hospital Anxiety and Depression Scale. Psychother Psychosom Med Psychol 1991;41:83–92.

Huygen FJ, De Bruijn AG, De Bruin MT, Groeneweg JG, Klein J, Zijistra FJ. Evidence for local inflammation in complex regional pain syndrome type 1. Mediators Inflamm 2002;11:47–51.

Huygen FJ, Niehof S, Zijlstra FJ, van Hagen PM, van Daele PL. Successful treatment of CRPS 1 with anti-TNF. J Pain Symptom Manage 2004;27:101–3.

Junger H, Sorkin LS. Nociceptive and inflammatory effects of subcutaneous TNFalpha. Pain 2000;85:145–51.

Kosek E, Hansson P. The influence of experimental pain intensity in the local and referred pain area on somatosensory perception in the area of referred pain. Eur J Pain 2002;6:413–25.

Kruse N, Pette M, Toyka K, Rieckmann P. Quantification of cytokine mRNA expression by RT PCR in samples of previously frozen blood. J Immunol Methods 1997;210:195–203.

Kubera M, Kenis G, Bosmans E, Jaworska-Feil L, Lason W, Scharpe S, et al. Suppressive effect of TRH and imipramine on human interferon-gamma and interleukin-10 production in vitro. Pol J Pharmacol 2000;52:481–6.

Leis S, Weber M, Schmelz M, Birklein F. Facilitated neurogenic inflammation in unaffected limbs of patients with complex regional pain syndrome. Neurosci Lett 2004;359:163–6.

Magerl W, Treede RD. Secondary tactile hypoesthesia: a novel type of pain-induced somatosensory plasticity in human subjects. Neurosci Lett 2004;361:136–9.

Maihöfner C, Forster C, Birklein F, Neundorfer B, Handwerker HO. Brain processing during mechanical hyperalgesia in complex regional pain syndrome: a functional MRI study. Pain 2005a;114:93–103.

Maihöfner C, Handwerker HO, Neundorfer B, Birklein F. Mechanical hyperalgesia in complex regional pain syndrome: a role for TNF-alpha? Neurology 2005b;65:311–3.

Manning DC. Immunomodulatory agents for neuropathic pain syndromes: from animals to CRPS and beyond. Eur J Pain Suppl 2006;1:S20.

Marchand F, Perretti M, McMahon SB. Role of the immune system in chronic pain. Nat Rev Neurosci 2005;6:521–32.

Milligan ED, Sloane EM, Langer SJ, Hughes TS, Jekich BM, Frank MG, et al. Repeated intrathecal injections of plasmid DNA encoding interleukin-10 produce prolonged reversal of neuropathic pain. Pain 2006.

Moreira AL, Sampaio EP, Zmuidzinas A, Frindt P, Smith KA, Kaplan G. Thalidomide exerts its inhibitory action on tumor necrosis factor alpha by enhancing mRNA degradation. J Exp Med 1993;177:1675–80.

Moss ML, Jin SL, Milla ME, Bickett DM, Burkhart W, Carter HL, et al. Cloning of a disintegrin metalloproteinase that processes precursor tumour-necrosis factor-alpha. Nature 1997;385:733–6.

Munnikes RJ, Muis C, Boersma M, Heijmans-Antonissen C, Zijlstra FJ, Huygen FJ. Intermediate stage complex regional pain syndrome type 1 is unrelated to proinflammatory cytokines. Mediators Inflamm 2005;2005:366–72.

Oaklander AL, Rissmiller JG, Gelman LB, Zheng L, Chang Y, Gott R. Evidence of focal small-fiber axonal degeneration in complex regional pain syndrome-I (reflex sympathetic dystrophy). Pain 2006;120:235–43.

Opree A, Kress M. Involvement of the proinflammatory cytokines tumor necrosis factor-alpha, IL-1 beta, and IL-6 but not IL-8 in the development of heat hyperalgesia: effects on heat-evoked calcitonin gene-related peptide release from rat skin. J Neurosci 2000a;20:6289–93.

Opree A, Kress M. Involvement of the proinflammatory cytokines tumor necrosis factor-alpha, IL-1beta, and IL-6 but not IL-8 in the development of heat hyperalgesia: effects on heat-evoked calcitonin gene-related peptide release from rat skin. J Neurosci 2000b;20:6289–93.

Pleger B, Tegenthoff M, Ragert P, Forster AF, Dinse HR, Schwenkreis P, et al. Sensorimotor retuning [corrected] in complex regional pain syndrome parallels pain reduction. Ann Neurol 2005;57:425–9.

Rajkumar SV, Fonseca R, Witzig TE. Complete resolution of reflex sympathetic dystrophy with thalidomide treatment. Arch Intern Med 2001;161:2502–3.

Rolke R, Baron R, Maier C, Tolle TR, Treede RD, Beyer A, et al. Quantitative sensory testing in the German Research Network on Neuropathic Pain (DFNS): Standardized protocol and reference values. Pain 2006.

Sampaio EP, Sarno EN, Galilly R, Cohn ZA, Kaplan G. Thalidomide selectively inhibits tumor necrosis factor alpha production by stimulated human monocytes. J Exp Med 1991;173:699–703.

Schäfers M, Sorkin LS, Sommer C. Intramuscular injection of tumor necrosis factor-alpha induces muscle hyperalgesia in rats. Pain 2003;104:579–88.

Schinkel C, Gaertner A, Zaspel J, Zedler S, Faist E, Schuermann M. Inflammatory mediators are altered in the acute phase of posttraumatic complex regional pain syndrome. Clin J Pain 2006;22:235–9.

Schwartzman RJ, Chevlen E, Bengtson K. Thalidomide has activity in treating complex regional pain syndrome. Arch Intern Med 2003;163:1487–8. author reply 1488.

Seko Y, Cole S, Kasprzak W, Shapiro BA, Ragheb JA. The role of cytokine mRNA stability in the pathogenesis of autoimmune disease. Autoimmun Rev 2006;5:299–305.

Stein C, Mendl G. The German counterpart to McGill Pain Questionnaire. Pain 1988;32:251–5.

Üçeyler N, Sommer C. Cytokine-induced pain: basic science and clinical implications. Rev Analgesia 2007;9:87–103.

van de Beek WJ, Remarque EJ, Westendorp RG, van Hilten JJ. Innate cytokine profile in patients with complex regional pain syndrome is normal. Pain 2001;91:259–61.

Veldman PH, Reynen HM, Arntz IE, Goris RJ. Signs and symptoms of reflex sympathetic dystrophy: prospective study of 829 patients. Lancet 1993;342:1012–6.

Weber M, Birklein F, Neundorfer B, Schmelz M. Facilitated neurogenic inflammation in complex regional pain syndrome. Pain 2001;91:251–7.

Weizman R, Laor N, Podliszewski E, Notti I, Djaldetti M, Bessler H. Cytokine production in major depressed patients before and after clomipramine treatment. Biol Psychiatry 1994;35:42–7.

Winer J, Jung CK, Shackel I, Williams PM. Development and validation of real-time quantitative reverse transcriptase-polymerase chain reaction for monitoring gene expression in cardiac myocytes in vitro. Anal Biochem 1999;270:41–9.