

## Correction

### NEUROSCIENCE

Correction for “Transfer of complex regional pain syndrome to mice via human autoantibodies is mediated by interleukin-1–induced mechanisms,” by Zsuzsanna Helyes, Valéria Tékus, Nikolett Szentes, Krisztina Pohóczky, Bálint Botz, Tamás Kiss, Ágnes Kemény, Zsuzsanna Környei, Krisztina Tóth, Nikolett Lénárt, Hajnalka Ábrahám, Emmanuel Pinteaux, Sheila Francis, Serena Sensi, Ádám Dénes, and Andreas Goebel, which was first published June 10, 2019; 10.1073/pnas.1820168116 (*Proc. Natl. Acad. Sci. U.S.A.* **116**, 13067–13076).

The authors note that, due to a printer’s error, the affiliations for Ádám Dénes appeared incorrectly. They should instead appear as Momentum Laboratory of Neuroimmunology, Institute of Experimental Medicine, H-1083, Budapest, Hungary; and Division of Neuroscience and Experimental Psychology, University of Manchester, Manchester M13 9PT, United Kingdom. The corrected author and affiliation lines appear below. The online version has been corrected.

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# Transfer of complex regional pain syndrome to mice via human autoantibodies is mediated by interleukin-1-induced mechanisms

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Neuroimmune interactions may contribute to severe pain and regional inflammatory and autonomic signs in complex regional pain syndrome (CRPS), a posttraumatic pain disorder. Here, we investigated peripheral and central immune mechanisms in a translational passive transfer trauma mouse model of CRPS. Small plantar skin-muscle incision was performed in female C57BL/6 mice treated daily with purified serum immunoglobulin G (IgG) from patients with longstanding CRPS or healthy volunteers followed by assessment of paw edema, hyperalgesia, inflammation, and central glial activation. CRPS IgG significantly increased and prolonged swelling and induced stable hyperalgesia of the incised paw compared with IgG from healthy controls. After a short-lasting paw inflammatory response in all groups, CRPS IgG-injected mice displayed sustained, profound microglia and astrocyte activation in the dorsal horn of the spinal cord and pain-related brain regions, indicating central sensitization. Genetic deletion of interleukin-1 (IL-1) using IL-1 $\alpha$  knockout (KO) mice and perioperative IL-1 receptor type 1 (IL-1R1) blockade with the drug anakinra, but not treatment with the glucocorticoid prednisolone, prevented these changes. Anakinra treatment also reversed the established sensitization phenotype when initiated 8 days after incision. Furthermore, with the generation of an IL-1 $\beta$  floxed (<sup>fl/fl</sup>) mouse line, we demonstrated that CRPS IgG-induced changes are in part mediated by microglia-derived IL-1 $\beta$ , suggesting that both peripheral and central inflammatory mechanisms contribute to the transferred disease phenotype. These results indicate that persistent CRPS is often contributed to by autoantibodies and highlight a potential therapeutic use for clinically licensed antagonists, such as anakinra, to prevent or treat CRPS via blocking IL-1 actions.

CRPS | autoantibody | complex regional pain syndrome | interleukin-1 | anakinra

Complex regional pain syndrome (CRPS), with a prevalence of about 1:2,000, is a chronic pain condition experienced by humans. CRPS is usually triggered by trauma to the distal regions of a limb and is further associated with limb-restricted edema; sensory, vasomotor, sudomotor, motor, and trophic abnormalities; and profound sensory central nervous system (CNS) reorganization (1). In CRPS, typically no or only minimal tissue destruction occurs (2), and although morphological change, such as disuse atrophy, can sometimes be observed, this is not thought to explain the experienced intense pain (3). The underlying pathophysiological mechanisms are poorly understood. Systemic inflammatory markers remain normal in CRPS patients, but regional inflammatory mediators and autoimmunity are suggested to contribute to the manifestation of the symptoms (4). Furthermore, neuroplasticity mechanisms within the spinal cord and the brain are believed to sustain persistent pain (5).

While most patients with CRPS show an improvement within months, either with or without treatment (6), 20% of patients develop persistent pain, often lasting for years or even through their lifetime (7). This type of persistent pain is intrusive and results in among the lowest quality of life scores in medical conditions (8). Among the few drug trials performed to date (9), neither conventional drugs used to relieve pain, (i.e., nonsteroidal antiinflammatory drugs, opioids, antidepressants, or anticonvulsants) nor steroids have shown significant efficacy in persistent CRPS. Implantation of a spinal cord stimulator, which delivers electrical impulses to the dorsal column, can override CRPS pain in about 50% of patients, but the duration of the optimum effect is limited (9). Since many patients cannot be successfully treated, the treatment of CRPS remains an important unresolved problem and is still an unmet medical need (10). Thus, to better understand the peripheral and

## Significance

Complex regional pain syndrome (CRPS) is a poorly understood painful condition, which typically arises after distal limb trauma; 20% of patients may develop lifelong severe intractant pain with few therapeutic options. In this study, we show that immunoglobulin G autoantibodies from patients with severe, persistent CRPS, on transfer to hind paw-injured mice, elicit important features of the clinical condition and profound glial activation in pain-related brain regions. Blockade of the proinflammatory cytokine interleukin-1 (IL-1) both prevents and reverses these changes. Our findings suggest that antibody-mediated autoimmunity contributes to the development of severe CRPS after injury and that blockade of IL-1 actions may be an attractive therapeutic prospect. Investigation of autoantibody contribution to other unexplained chronic pain syndromes seems warranted.

Author contributions: Z.H., V.T., E.P., Á.D., and A.G. designed research; Z.H., V.T., N.S., K.P., B.B., T.K., Á.K., Z.K., K.T., N.L., H.Á., S.F., S.S., Á.D., and A.G. performed research; Z.K., K.T., N.L., E.P., S.F., and Á.D. contributed new reagents/analytic tools; Z.H., V.T., N.S., K.P., B.B., H.Á., E.P., Á.D., and A.G. analyzed data; and Z.H., N.S., K.P., E.P., S.F., Á.D., and A.G. wrote the paper.

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central pathophysiological mechanisms underlying CRPS, reliable and validated animal models are desperately needed (2).

We have recently shown that passive transfer of serum immunoglobulin G (IgG) from CRPS patients to hind paw-injured rodents elicits key features (unilateral hyperalgesia and edema) of the clinical condition (11). This suggests a “two-hit” process, where circulating IgG autoantibodies (i.e., the first hit) are rendered pathogenic in the context of paw injury (the second hit)-related regional or central modifications (2). Although these behavioral results indicated that serum IgG autoantibodies contribute to the disease pathophysiology and thus, provided first evidence for the construct validity of the transfer model, the observed abnormalities were modest and short-lasting, and the mechanisms mediating them have remained unknown.

Using samples available from patients who consented to repeat donation of larger blood volumes or who had received plasma exchange treatment (12), we have now developed an enhanced passive IgG transfer trauma model and have examined its translational validity. We investigated (i) whether and how the transferred behavioral signs in rodents are augmented and sustained with daily human IgG injections and whether there are differences between preparations from different patients; (ii) the degree of regional posttraumatic immune activation in the paw given that mild, transient immune activation in the affected skin is sometimes detected in patients (13) and its correlation to behavioral parameters; (iii) the degree and mechanisms of posttraumatic glial activation in the spinal dorsal horn, since strong CNS reorganization is recognized in the clinical cases (5); and finally, (iv) whether targeting specific inflammatory pathways at the time of or after trauma can prevent or reverse transferred complex regional pain syndrome (tCRPS) to provide a translatable therapeutic approach.

## Results

### Daily Administration of Serum IgG from CRPS Patients Induces Profound and Persistent Postincisional Mechanical Hyperalgesia.

The preoperation mechanonociceptive threshold values of the affected limbs were  $8.65 \pm 0.08$ ,  $8.69 \pm 0.09$ , and  $8.60 \pm 0.07$  in the saline, healthy IgG-, and CRPS IgG-treated mice, respectively (not significantly different) (Fig. 1A). Plantar skin and muscle incision induced a 45–50% relative decrease of the mechanonociceptive threshold in all groups 1 d after the surgery. On daily injections, paw sensitivity quickly recovered to mild hyperalgesia in both saline and healthy IgG-injected animals, with mild, nonsignificantly enhanced values remaining in the healthy IgG group vs. the saline group throughout the experimental period. Injection of IgG from CRPS patients caused significantly augmented hyperalgesia compared with IgG from healthy volunteers, which seemed to be further enhanced toward the end of the experimental period. This effect was evident in the IgG preparations from each individually tested patient ( $n = 7$ ) as well as a preparation pooled from seven separate patients (SI

Appendix, Fig. S1). The observed 15–32% absolute threshold reduction was twofold compared with that seen in our previously published experimental model (injections on days –1, 0, 5, and 6) (11). Contralateral paws retained normal sensitivity in all groups (SI Appendix, Fig. S2).

In all groups, injured paws developed about 30% relative paw swelling (defined as edema) on day 1, but there were no changes in contralateral paws (SI Appendix, Fig. S3). Edema resolved in healthy IgG and saline groups, but CRPS IgG injection significantly slowed edema resolution (Fig. 1B). While the pattern of transferred hyperalgesia was uniform, there was important variability in the degree and pattern of transferred swelling between different patient preparations, with no correlation between these two parameters (SI Appendix, Figs. S2 and S3). Postsurgery minimal weight loss was observed compared with baseline for a few days, and the weights of the animal then fully recovered without significant differences between groups (SI Appendix, Fig. S4). We observed no spontaneous nocifensive behaviors, such as paw biting, lifting, or licking.

### CRPS IgG Does Not Alter Vascular Plasma Leakage but Increases Neutrophil Myeloperoxidase Activity Early After Paw Incision.

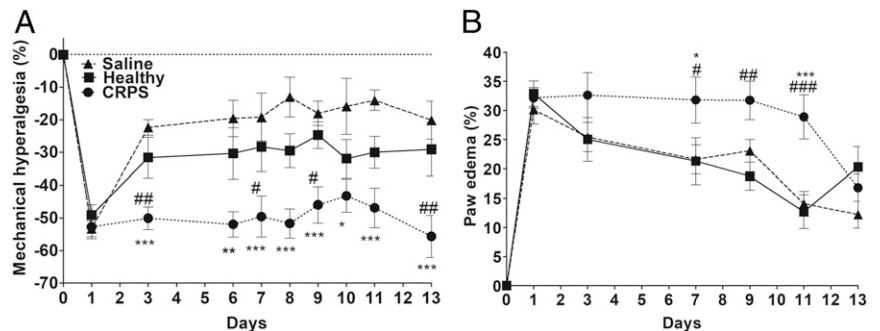
Indocyanin green (ICG)-derived fluorescence detecting vascular leakage showed a nonsignificant trend to increase in the injured paws in all groups 2 d after paw incision; CRPS IgG did not specifically affect plasma extravasation [saline injured:  $1.52 \times 10^9 \pm 1.11 \times 10^8$ ; healthy IgG injured:  $1.42 \times 10^9 \pm 1.54 \times 10^8$ ; CRPS IgG injured:  $1.70 \times 10^9 \pm 2.2 \times 10^8$ ; fluorescence intensity: (photons per second per  $1 \text{ cm}^2$  per steradian) per microwatt per  $1 \text{ cm}^2$ ].

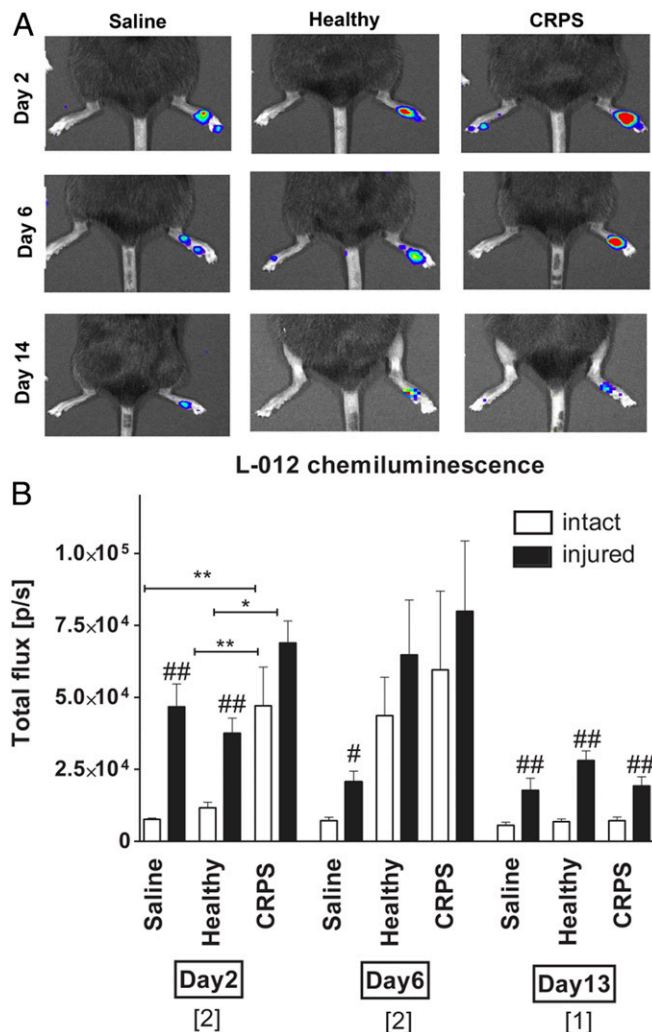
As expected, in vivo imaging of 8-amino-5-chloro-7-phenylpyrido[3,4-d]pyridazine-1,4(2H,3H) dione (L-012)-derived bioluminescence, used as a sensitive marker of inflammatory cell activity [most prominently neutrophil-derived myeloperoxidase (MPO)], showed increased signal in response to limb trauma alone (Fig. 2). We found a significant increase in neutrophil MPO activity in the CRPS IgG-treated animals on the affected side 2 d after the incision compared with in the control groups. Differences in MPO activity in CRPS IgG vs. healthy IgG groups had disappeared by day 6, although significant increases in the injured paw were still present compared with the intact side on day 13. Importantly, while strong variability was observed between different IgG preparations in influencing MPO activity (SI Appendix, Fig. S5 and Table S2), bioluminescence measured on day 2 or 6 did not correlate with either maximal paw swelling or hyperalgesia on day 6. This suggests that altered MPO activity in the injured paw of tCRPS mice is an unrelated IgG effect that is unlikely to explain the marked impact of CRPS IgG on paw hypersensitivity.

### CRPS IgG Does Not Promote Inflammation or Neuropathy in the Paw.

We further examined whether the tCRPS behavioral signs were related to locally augmented inflammatory responses or to neuropathic changes. In successive experiments, animals were

**Fig. 1.** Effect of intraperitoneal injection of serum IgG derived from CRPS patients or healthy controls on plantar incision-induced mechanical hyperalgesia (A) and swelling (B) of the injured mouse hind paw. IgG was administered daily starting on day 0. The right hind paws were incised on day 0 about 6 h after Ig injection. Shown are pooled results from all three long-term experiments to either day 10 or 13 with three different IgG preparations (2–4) (individual results are in Fig. 2, and patients details are in SI Appendix, Table S1). Data are means  $\pm$  SEM. Two-way ANOVA was followed by Bonferroni's multiple comparison test. Healthy indicates the healthy control IgG-injected group, and CRPS indicates the CRPS IgG-injected group. \* $P < 0.05$  (vs. saline-treated control mice); \*\* $P < 0.01$  (vs. saline-treated control mice); \*\*\* $P < 0.001$  (vs. saline-treated control mice); # $P < 0.05$  (vs. healthy IgG-treated mice); ## $P < 0.01$  (vs. healthy IgG-treated mice); ### $P < 0.001$  (vs. healthy IgG-treated mice).





**Fig. 2.** Imaging ROS demonstrates the development of inflammation in the injured hind paws of mice. In vivo images of L-012–derived bioluminescence were obtained during general anesthesia on days 2, 6, and 13 after paw incision. Typical images, with red color indicating strong bioluminescence, are shown in *A*, and quantification of the bioluminescence intensity is in *B*. Data at each time point represent the pooled results from experiments conducted with separate CRPS/healthy control IgG preparations (numbers of preparations per time point are in brackets) (details are in *SI Appendix, Fig. S4 and Table S2*) and are shown as means  $\pm$  SEM of  $n = 6–18$  mice per group. One-way ANOVA was followed by Bonferroni's multiple comparison test. Healthy indicates the healthy control IgG-injected group, and CRPS indicates the CRPS IgG-injected group. \* $P < 0.05$  vs. respective control groups; \*\* $P < 0.01$  vs. respective control groups; # $P < 0.05$  vs. respective intact side; ## $P < 0.01$  vs. respective intact side.

killed between experimental days 1 and 13, and paw tissues were harvested to assess various inflammatory changes (animal numbers and preparations are in *SI Appendix, Table S3*).

Substance P (SP) levels increased in the injured paw, with higher levels in the CRPS IgG group on day 6 (Fig. 3*A*), whereas calcitonin gene-related peptide (CGRP) levels were not significantly altered (Fig. 3*B*), consistent with earlier findings (11). Increased levels of inflammatory mediators were seen in the injured paws, with gradual decrease over time [shown in Fig. 3 *C–F* for interleukin-6 (IL-6), tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), monocyte chemoattractant protein-1 (MCP-1), and IL-1 $\beta$  and in *SI Appendix, Fig. S6* for additional mediators]. We detected no differences in the levels of inflammatory mediators between the CRPS and healthy IgG groups (Fig. 3 *C–F* and *SI Appendix, Fig. S6*) at any

time point, except for a mild CRPS IgG-induced MCP-1 increase at day 13 (Fig. 3*E*). Notably, at the time of maximum hyperalgesia (13 d postinjury), most mediators were undetectable. There were no significant changes in plasma concentrations of any cytokines after correction for multiple testing (*SI Appendix, Fig. S7*).

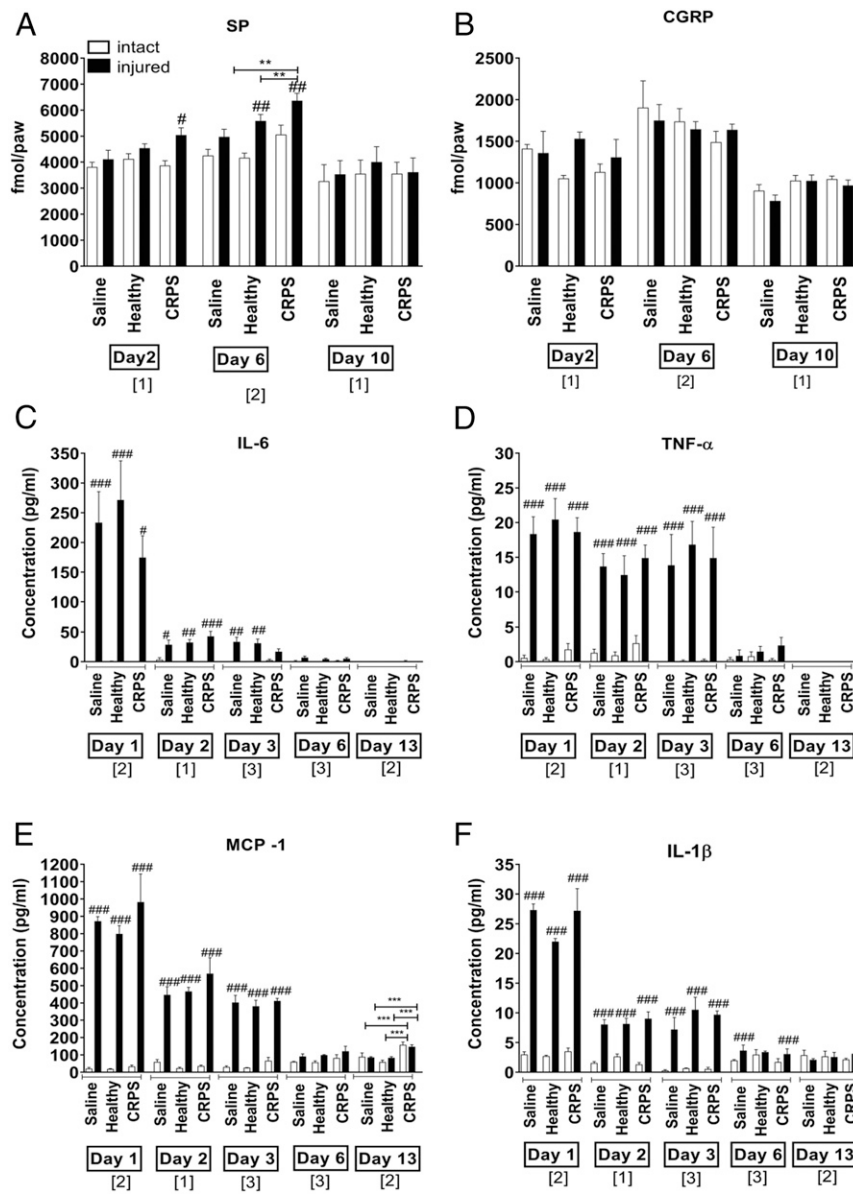
Histological examination revealed moderate infiltration of inflammatory cells into areas immediately adjacent to the incision early after surgery, with no obvious difference between groups; there was no evidence of infiltration by inflammatory cells on day 13 in any experimental group. Since some patients with persistent CRPS exhibit mild small fiber neuropathy (14), we also examined mouse paw biopsies from CRPS IgG-injected animals for any evidence of structural changes to small skin nerves with both light and electron microscopy. The morphology of the axons in the right (injured) and left (intact) paws as well as the ultrastructure of nonmyelinated and thin-myelinated axons in the dermis appeared very similar on aspect, and there were no significant differences between sides on quantification of axon numbers and diameters (*SI Appendix, Table S4*).

**CRPS IgG Facilitates Sustained Microglia Activation in the CNS.** We next investigated whether altered activity of microglia or astrocytes in pain-related circuits (15) would reflect the marked effects of CRPS IgG on pain sensitivity responses. In CNS areas receiving input from the injured paw, CRPS IgG induced remarkable increases in both astrocyte and microglia cell activities compared with both saline and healthy IgG groups. In the dorsal horn, this response was sometimes much stronger than the increases representing the incision trauma (Fig. 4). Astrocyte reactivity in the CRPS IgG animals was augmented in all CNS areas at early time points (days 3 and 6), whereas microglia staining was enhanced throughout the experimental period (including day 13) (Fig. 4 and *SI Appendix, Fig. S9*), indicating that increased mechanical hypersensitivity in this model is associated with transient astrocyte and persistent microglial activation in the CNS.

#### Early IL-1 Receptor Blockade with Anakinra Prevents the Development of tCRPS, While Delayed Anakinra Treatment Reverses Established tCRPS and Reduces Glial Activation.

Since both microglia and astrocytes are important sources of proinflammatory cytokines that are known to contribute to pain hypersensitivity responses (16, 17) and IL-1 is a key mediator that influences neuronal activity (18, 19), we investigated the effects of glucocorticoid (prednisolone) treatment or interleukin-1 receptor (IL-1R) antagonist (anakinra) treatment on CRPS IgG-induced behavioral signs and inflammatory changes. Prednisolone (4 mg/kg) or anakinra (10 mg/kg) was daily administered intraperitoneally, starting 5 h before surgery (day 0) and extending throughout the experimental period. One day after surgery, mechanical hyperalgesia developed equally in all groups (Fig. 5*A* and *B*). Glucocorticoid treatment transiently reduced CRPS IgG-induced mechanical hyperalgesia (between days 2 and 3), but this effect was lost by day 7. In contrast, anakinra prevented all CRPS IgG-induced effects throughout the experimental period (Fig. 5*B*). Anakinra, but not prednisolone treatment, almost completely reversed glia cell activation in the ipsilateral dorsal horn on day 6 (Fig. 5*D* and *E*). Notably, anakinra treatment also significantly reduced paw MCP-1 levels on day 3; however, there were no other differential effects on levels of peripheral mediators between these two treatments (*SI Appendix, Fig. S10*). Furthermore, delayed administration of anakinra starting from day 8 onward reversed the established tCRPS phenotype (Fig. 5*C*) and completely reversed the associated increased dorsal horn microglia activation on day 13 (Fig. 5*F*).

**Selective Deletion of Microglial IL-1 $\beta$  Ameliorates While Ubiquitous Deletion of IL-1 $\alpha$  Completely Prevents tCRPS.** Given that CRPS IgG caused significant dorsal horn glia cell activation without influencing paw IL-1 levels and that blockade of IL-1 prevented



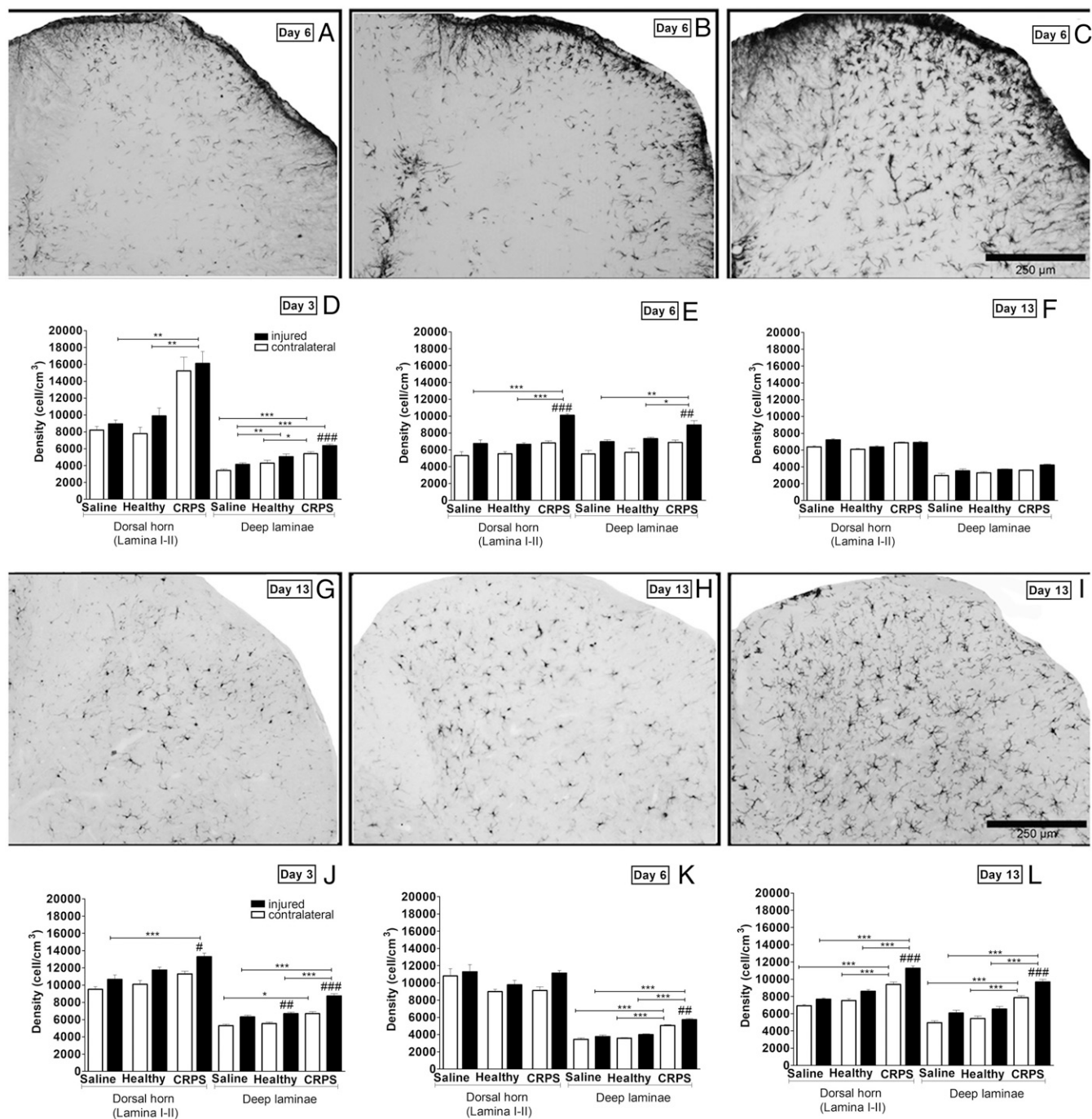
**Fig. 3.** Effects of human IgG transfer on sensory neuropeptide and inflammatory cytokine concentrations in the hind paws. Concentrations of (A) SP and (B) CGRP were measured by RIA in hind paw homogenates excised after they were killed. Concentrations of (C) IL-6, (D) TNF- $\alpha$ , (E) MCP-1, and (F) IL-1 $\beta$  were measured by cytometric bead array from the same samples. Data are from one to three experiments per time point (brackets below x axes) each with different patient preparations. Shown are means  $\pm$  SEM. One-way ANOVA was followed by Bonferroni's multiple comparison test. Healthy indicates the healthy control IgG-injected group, and CRPS indicates the CRPS IgG-injected group. \*\* $P < 0.01$  vs. respective control groups; # $P < 0.05$  vs. respective intact side; ## $P < 0.01$  vs. respective intact side; ### $P < 0.001$  vs. respective intact side;

the tCRPS phenotype, we then investigated whether tCRPS was associated with enhanced glial IL-1 production in the dorsal horn. Immunofluorescence revealed increased microglial IL-1 $\beta$  production in lumbar (L)4/L5 dorsal horn microglia cells only in the tCRPS group (Fig. 6A), while IL-1 $\alpha$  was not detected. We then examined whether the tCRPS phenotype would be altered by genetic knockout (KO) of IL-1. We found that CRPS IgG-injected IL-1 $\alpha\beta$  KO mice failed to develop enhanced hyperalgesia and showed even less posttraumatic paw swelling (Fig. 6B and C) than mice treated with healthy IgG. To investigate whether increased microglial IL-1 $\beta$  production is sufficient to mediate the effects of CRPS IgG on increased mechanical hyperalgesia, we generated an IL-1 $\beta$  floxed<sup>(fl/fl)</sup> mouse line. Exons 4 and 5 of the *IL-1B* gene were flanked with loxP sites, resulting in the generation of IL-1 $\beta$ <sup>fl/fl</sup> allele (SI Appendix, Fig. S11). IL-1 $\beta$ <sup>fl/fl</sup> mice were crossed with Cx3cr1<sup>CreER</sup> mice (20), resulting in microglial deletion of IL-1 $\beta$  on tamoxifen administration (M-IL-1 $\beta$  KO), while most peripheral Cx3cr1-positive cells recovered IL-1 $\beta$  production due to their higher turnover as shown by using other cre-dependent reporter lines previously (20). In fact, IL-1 $\beta$  protein levels were markedly reduced in IL-1 $\beta$

KO microglia after repeated intraperitoneal injections of bacterial lipopolysaccharide compared with wild-type (WT) microglia, but no changes were seen in splenic macrophages derived from tamoxifen-treated Cx3cr1<sup>CreER</sup>  $\times$  IL-1 $\beta$ <sup>fl/fl</sup> mice compared with controls (SI Appendix, Fig. S12). Elimination of microglial IL-1 $\beta$  significantly reduced mechanical hyperalgesia and paw edema in mice treated with CRPS IgG, although this effect was smaller than in the case of IL-1 $\alpha\beta$  KO mice (Fig. 6B and C). Total numbers of microglia were reduced in IL-1 $\alpha\beta$  KO mice but were not altered in response to microglial IL-1 $\beta$  deletion in the CRPS group (Fig. 6D), suggesting that, while microglial IL-1 $\beta$  is an important driver of chronic neuroinflammation contributing to persistent pain, other IL-1 $\beta$ -producing cells or actions mediated by IL-1 $\alpha$  could also contribute to CRPS symptoms in mice.

## Discussion

Here, we show in an enhanced passive transfer trauma model that daily administration of IgG from patients with persistent CRPS to mice elicits intense, unilateral static mechanical hyperalgesia after hind paw injury, which remains stable through the experimental period. This is associated with increased paw edema

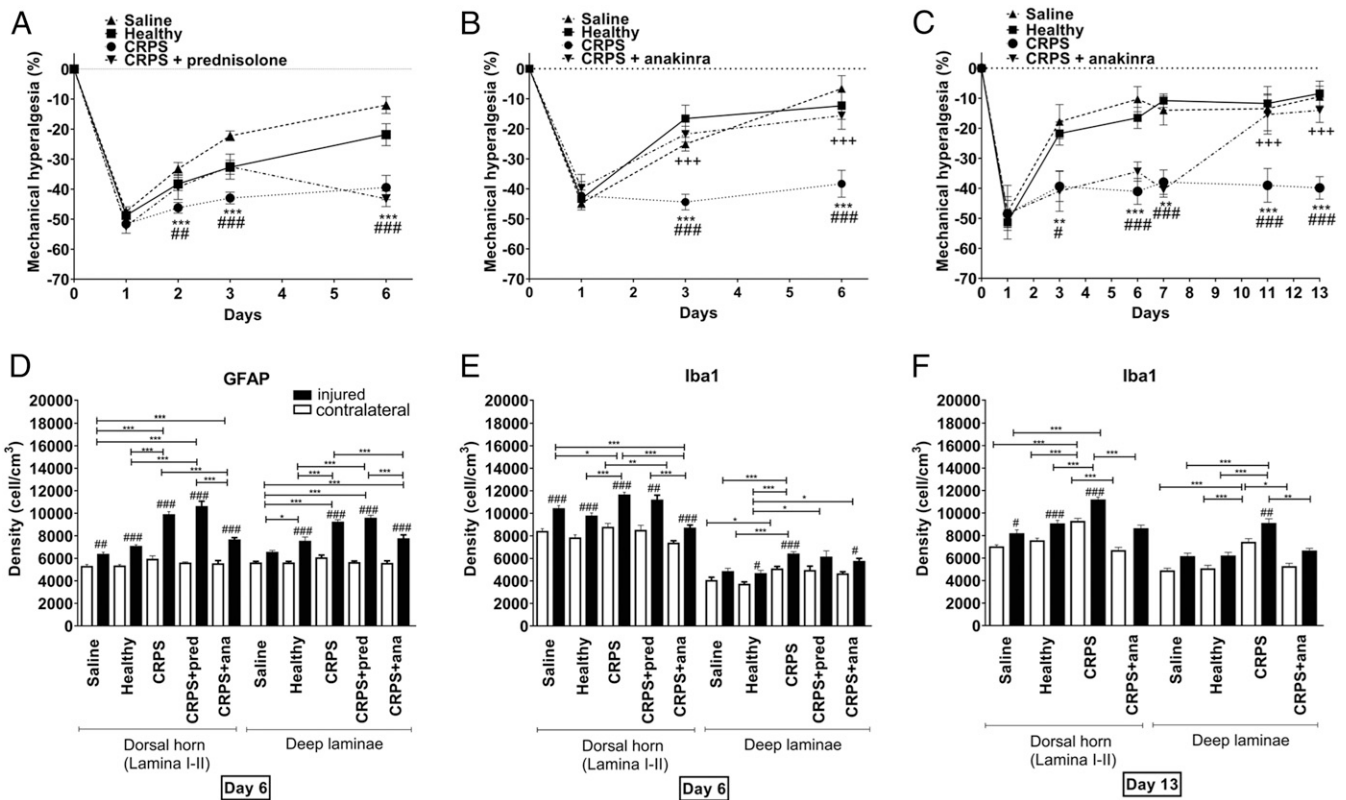


**Fig. 4.** Glial activation in the L5 spinal cord dorsal horn ipsilateral to the paw injury. A–C show GFAP immunopositivity marking astrocytes, and G–I show Iba1 immunopositivity marking microglia cells, with (A and G) saline, (B and H) healthy control IgG, and (C and I) CRPS IgG injections. The GFAP-immunopositive sections shown are from day 6, and Iba1 sections are from day 13 after paw incision. Quantification of astrocyte reactivity (D–F) and microglia staining (J–L) in lamina I–II dorsal horn of the L4–L6 spinal cord and deeper laminae at 3, 6, and 13 d after hind paw incision. Each panel represents the pooled results from two experiments with two different samples (3 and 4). Shown are means  $\pm$  SEM of six to seven mice per group. One-way ANOVA was followed by Bonferroni's modified post hoc test. Healthy indicates the healthy control IgG-injected group, and CRPS indicates the CRPS IgG-injected group. \* $P < 0.05$  vs. respective control groups; \*\* $P < 0.01$  vs. respective control groups; \*\*\* $P < 0.001$  vs. respective control groups; # $P < 0.05$  vs. respective contralateral side; ## $P < 0.01$  vs. respective contralateral side; ### $P < 0.001$  vs. respective contralateral side.

that resolves over time. Collectively, these features resemble the course and pathophysiology of the clinical disease (1). In these extensive integrative studies, a uniform pattern of transferred static mechanical hyperalgesia was seen with all patient preparations, highlighting that seronegativity is unlikely to be common in patients with severe, persistent CRPS. The results emphasize

the translational validity of the model and the importance of autoimmune mechanisms underpinning the pathophysiology of both clinical CRPS and experimental transferred tCRPS.

We found that the normal posttraumatic inflammatory response in the incised paws rapidly declined and fully settled by days 6–13 postincision; the paw inflammation did not correlate



**Fig. 5.** Effects of prophylactic steroid or anakinra treatment (days 0–6) and delayed (therapeutic) anakinra treatment on CRPS IgG-induced mechanical hyperalgesia and glial activation in the spinal cord. *A* and *B* show mechanical hyperalgesia in groups of animals injected intraperitoneally first with human IgG or saline and 3 h later with 4 mg/kg prednisolone, 10 mg/kg anakinra, or saline vehicle on each day between days 0 and 6. *D* and *E* show dorsal horn glia cell activation in these mice on day 6: (*D*) GFAP (astrocyte) and (*E*) Iba-1 (microglia). Results represent the average values derived from two independent experiments with different preparations for each treatment, with four experiments in total; saline, healthy control IgG, and CRPS IgG outcomes are pooled from these experiments. (*C* and *F*) Late anakinra treatment starting on day 8: (*C*) behavioral outcome and (*F*) dorsal horn microglia cell count on day 13. Data are shown as means  $\pm$  SEM. Two-way ANOVA was followed by Bonferroni's multiple comparison test. One-way ANOVA was followed by Bonferroni's modified post hoc test. Healthy indicates the healthy control IgG-injected group, and CRPS indicates the CRPS IgG-injected group. Significance symbols for the behavioral data as follows: \* $P < 0.05$  (CRPS IgG vs. saline-treated control mice); \*\* $P < 0.01$  (CRPS IgG vs. saline-treated control mice); \*\*\* $P < 0.001$  (CRPS IgG vs. saline-treated control mice); # $P < 0.05$  (CRPS IgG vs. healthy control IgG-treated mice); ### $P < 0.001$  (CRPS IgG vs. healthy control IgG-treated mice); +++ $P < 0.001$  (anakinra plus CRPS IgG vs. CRPS IgG-injected mice). Significance immunohistochemistry data: \* $P < 0.05$  vs. respective control groups; \*\* $P < 0.01$  vs. respective control groups; \*\*\* $P < 0.001$  vs. respective control groups; # $P < 0.05$  vs. respective contralateral side; ## $P < 0.01$  vs. respective contralateral side; ### $P < 0.001$  vs. respective contralateral side.

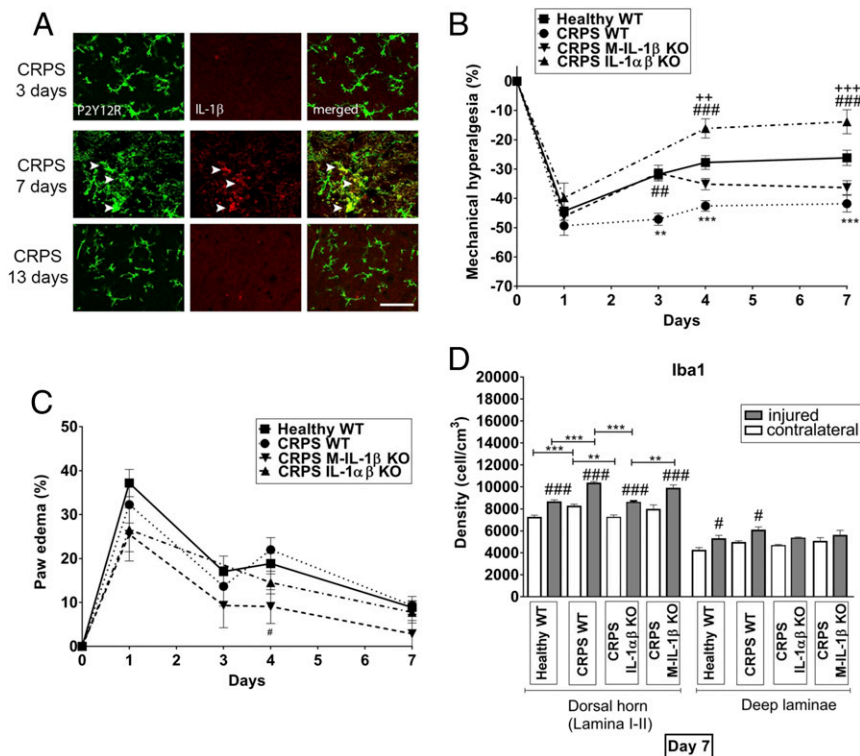
with the degree of CRPS IgG-induced paw hyperalgesia. We found little evidence for any CRPS IgG-related enhanced paw inflammation. Our results thus demonstrate that static mechanical hyperalgesia may not depend on persistent inflammatory mediator release, highlighting that tCRPS is not a model of enhanced posttraumatic inflammatory pain. There was, however, some evidence for abnormal production of two specific mediators in the CRPS group: SP production was increased at early time points, in line with some clinical observations (21) and our earlier results (11), and there was also a mild but significant bilateral increase in MCP-1 on day 13, the role of which will require additional investigations. Although we measured common mediators of inflammation in the injured paws and these were normal, the involvement of additional mediators cannot be excluded.

In mice, the development of tCRPS remained restricted to the injured paw, consistent with the clinical situation, where about 90% of the cases show symptoms in only one traumatized limb (22). The precise mechanisms through which circulating pathogenic IgG antibodies mediate both the regionally restricted posttraumatic clinical CRPS and tCRPS are presently unclear. Early transient trauma-induced inflammatory changes or regional opening of blood–nerve and blood–brain barriers may play a role by promoting the expression of neoantigens or by

providing IgG access to privileged sites (23). The presence of such facilitating mechanisms is supported by our *in vivo* imaging results, which showed plasma leakage and increased MPO activity in the injured paw.

Interestingly, MPO activity was variably enhanced in the animal groups injected with different CRPS IgG preparations, and there was no correlation with mechanical hyperalgesia or swelling in these same animals. These findings may resemble the strong heterogeneity seen between patients in the extent of their limb swellings, color changes, or temperature changes and may also reflect the observation that these clinical parameters do not necessarily correlate with the patients' perceived pain intensities or recorded skin sensitivities (2).

Since inflammatory changes in the paw did not seem to explain the increased mechanical hyperalgesia in the tCRPS mice, we investigated the potential role of glial responses in pain-related neuronal circuits, which have been suggested to contribute to chronification processes in posttraumatic pain models (15, 24, 25). We found that tCRPS was associated with strong microglia and astrocyte activation at all three tested levels of the nociceptive pathway, the ipsilateral spinal cord dorsal horn, the periaqueductal gray, and the contralateral somatosensory cortex. In the dorsal horn, the increases in glial responses in the tCRPS



**Fig. 6.** Deletion of IL-1 $\beta$  or microglia-derived IL-1 $\beta$  fully or partially prevents development of the CRPS IgG-induced phenotype in mice. (A) A population of microglia identified by immunostaining against P2Y12, a specific microglial marker in the brain (59), displays a morphologically activated phenotype and shows immunopositivity for IL-1 $\beta$  at day 7 in the deep laminae of the L4–L5 spinal cord near the central canal. (Scale bar: 50  $\mu$ m.) (B and C) IL-1 $\alpha\beta$  KO mice are fully protected and M-IL-1 $\beta$  KO mice are partially protected from the development of the CRPS IgG-induced phenotype: (B) paw hyperalgesia and (C) paw edema. Two-way ANOVA was followed by Bonferroni's multiple comparison test. Significance in B and C: \*\* $P$  < 0.01 (CRPS IgG WT vs. healthy IgG WT); \*\*\* $P$  < 0.001 (CRPS IgG WT vs. healthy IgG WT); # $P$  < 0.05 (CRPS IgG M-IL-1 $\beta$  KO vs. CRPS IgG WT); ### $P$  < 0.001 (CRPS IgG M-IL-1 $\beta$  KO vs. CRPS IgG WT); +++ $P$  < 0.001 (CRPS IgG IL-1 $\alpha\beta$  KO vs. CRPS IgG WT); ++ $P$  < 0.01 (CRPS IgG IL-1 $\alpha\beta$  KO vs. CRPS IgG M-IL-1 $\beta$  KO); +++ $P$  < 0.001 (CRPS IgG IL-1 $\alpha\beta$  KO vs. CRPS IgG M-IL-1 $\beta$  KO). (D) CRPS IgG-induced microglia activation is abrogated in IL-1 $\alpha\beta$  KO but not in M-IL-1 $\beta$  KO mice. Data are pooled from two experiments with different CRPS IgG preparations for each mouse type and are shown as means  $\pm$  SEM. Healthy indicates the healthy control IgG-injected group, and CRPS indicates the CRPS IgG-injected group. One-way ANOVA was followed by Bonferroni's modified post hoc test. Significance values in D: \*\* $P$  < 0.01 vs. respective control groups; \*\*\* $P$  < 0.001 vs. respective control groups; # $P$  < 0.05 vs. respective contralateral side; ### $P$  < 0.001 vs. respective contralateral side.

vs. control groups were sometimes severalfold larger than the extent of glia activation caused by the paw incision (Fig. 4), suggesting a powerful central effect of the transferred IgG.

These results raise the question of how the observed profound central glial cell activation is mediated. Possible mechanisms may include (i) modification of nociceptor function after direct antibody binding in the periphery as an "autoimmune channelopathy" (26), (ii) release of yet undetermined peripheral mediators (27), (iii) direct binding in the dorsal horn after temporary post-traumatic opening of the blood spinal cord barrier (28), or (iv) a missing link. Independent of the nature of these upstream mechanisms, glial activation is likely to result in the release of mediators, such as IL-1 $\beta$ , TNF- $\alpha$ , or brain-derived neurotrophic factor, with consequent modification of central pain processing (15, 29).

We hypothesized that perioperative antiinflammatory interventions might be effective, and we initially thought that such interventions might act through the blockade of perioperative regional facilitatory factors required to render circulating CRPS autoantibodies pathogenic (4). To investigate whether such interventions could prevent the disease phenotype, we treated mice peritraumatically with high-dose prednisolone. Prednisolone treatment temporarily interrupted the process of autoantibody-dependent sensitization, but it did not stop it. Systemic glucocorticoids are considered potentially effective in very early CRPS based on the results of one preliminary trial (9). These data suggest that, where patients produce harmful autoantibodies, the peritraumatic application of glucocorticoids is unlikely to stop disease progression. In contrast, perioperative treatment with the IL-1R antagonist anakinra in this CRPS model consistently prevented the tCRPS phenotype. Notably, we found that there were only minor differences in the regional paw inflammatory environment based on the production of cytokines and chemokines after treatment with (ineffective) prednisolone and (effective) anakinra (SI Appendix, Fig. S10); furthermore, even delayed blockade of IL-1 actions with anakinra starting on day 8 after the incision trauma, when trauma-induced peripheral inflammatory responses had largely resolved, was highly effective,

suggesting that the pertinent biological effect of anakinra treatment was not restricted to the injured paw.

IL-1 is a potent activator of astrocytes through actions via IL-1R1, whereas both activated microglia and astrocytes can contribute to painful central sensitization through secreting IL-1 (29, 30). These actions can be effectively blocked by anakinra (31–34). In our model, augmented dorsal horn glia cell activation in the tCRPS group was fully reversed by anakinra. Since IL-1-mediated actions are involved in the cross-talk between neurons, microglia, and astrocytes in promoting neuroinflammation (35, 36), we assessed glial IL-1 $\beta$  and IL-1 $\alpha$  production in the spinal cord and found that microglial IL-1 $\beta$  production was increased on day 7 in the CRPS IgG-treated group. In agreement with these data, we found that IL-1 $\alpha\beta$  KO mice were fully protected from developing the tCRPS phenotype and associated glial activation. To specifically assess the functional role of microglial IL-1 $\beta$  in tCRPS-associated hyperalgesia and swelling, we generated a mouse strain (IL-1 $\beta$ <sup>fl/fl</sup> mice), enabling the deletion of IL-1 $\beta$  from microglia. Consistent with earlier data showing prolonged cre-dependent transgene expression in long-lived microglia but not in peripheral macrophage populations with short turnover (20), tamoxifen treatment of Cx3cr1<sup>CreER</sup>  $\times$  IL-1 $\beta$ <sup>fl/fl</sup> mice resulted in a marked reduction of microglial, but not splenic, IL-1 $\beta$  production (SI Appendix, Fig. S12). As seen in IL-1 $\alpha\beta$  KO mice, the absence of microglial IL-1 $\beta$  production fully protected from tCRPS-associated mechanical hyperalgesia up to day 3, whereas it had a weaker effect thereafter. This highlights the importance of microglial IL-1 $\beta$  in the tCRPS disease process but also, the likely involvement of other cells and/or IL-1 $\alpha$  (35).

Strengths of our study include the robust, multidimensional evaluation of an enhanced CRPS disease transfer model with preparations from patients whose clinical presentations differed using outcomes designed to provide translational validity. Additional strengths are the comprehensive assessment of both peripheral and central markers of immune activation and of several antiinflammatory treatments as well as gene KO strategies, which



have allowed an informed suggestion for clinical studies with a licensed drug not previously reported in this patient group.

Limitations include the upper transfer limit cutoff at 13 d, which was necessary to avoid the adverse effects of serum sickness (37). However, it may be argued that the “chronic phase” in this disease model starts from the second week after incision, when peripheral inflammation and symptoms in the control group resolve. As is often observed with IgG disease transfer in other disease models, tCRPS does not fully match the symptoms of clinical CRPS (38). For example, overt motor dysfunction was not detected, and commonly encountered central features, such as body perception abnormalities, were not revealed. In line with most behavioral studies in animal models of chronic pain (39), we did not demonstrate the presence of spontaneous pain but ascertained nociceptor hypersensitivity by assessing stimulus-evoked pain (mechanical hypersensitivity). Independently, we only transferred serum IgG that was derived from patients suffering from persistent (7) Budapest CRPS with high pain intensities (numeric rating scale > 7); however, these are also the patients who present the most difficult situation in the clinical practice. We cannot rule out the possibility that some patients in this group do not have these antibodies. Since each of the seven individually tested serum IgG preparations induced the abnormal phenotype (*SI Appendix, Fig. S1*), the likelihood that we have missed the absence of such antibodies in more than one-half of patients of a similar population seems very low (<1%) (*Patients, Healthy Controls, and Serum Preparation*); the development of serum diagnostic tests will be required to further detail the proportion of seronegative patients. We did not measure epidermal nerve fiber density or length in the mouse paw skin; therefore, our studies may not fully rule out mild small fiber neuropathy.

In summary, we have devised a robust translational model reproducing pertinent aspects of an “idiopathic,” posttraumatic chronic pain condition. The consistent pathogenicity of various serum IgG preparations indicates that, among patients who have severe forms of this condition, autoantibody contribution is ubiquitous. Since abnormal signs were entirely confined to the injured side, we also established a general principle suggesting that pathogenic circulating autoantibodies can cause regionally restricted disease when triggered by local trauma.

Our results support previous clinical observations that patients with persistent CRPS should respond to immune treatments with a reduction of at least some of their disease features (12). The clinical use of IL-1R antagonists in CRPS has a broad therapeutic potential. Anakinra is clinically licensed both in the United States and in Europe, and short-term use has an acceptable side effect profile (40, 41). Since CRPS regularly develops in the context of elective operations, such as arthroscopy or bunion surgery, prevention of such cases would have very important implications for both patients and procedure-related health care as well as societal costs (42). As our results also suggest that treatment initiated after injury resolution reverts the established transferred phenotype, clinical treatment of patients with persistent CRPS could now be tested in a trial setting.

The cellular and molecular targets of the patient autoantibodies need to be clarified; recent results in other conditions that are not associated with trauma (26, 27) have indicated that pain-sensitizing autoantibodies may directly bind to sensory neurons or indirectly bind to juxtapositioned cells, which then release nerve-sensitizing mediators. The duration of beneficial effects after the drug is withdrawn should be investigated in this experimental model. In addition, identifying the exact cellular targets for IL-1 actions in the pathophysiology of tCRPS could facilitate the development of alternative IL-1 targeting approaches in the prevention or treatment of the clinical disease. The results of this study highlight the important role that auto-

antibodies can play in causing detrimental symptoms, absent tissue destruction and systemic inflammation.

## Materials and Methods

**Patients, Healthy Controls, and Serum Preparation.** NorthWest Ethics Haydock UK approved serum donations. Written informed consent was obtained from all patients. Serum or plasma samples were obtained (*i*) via a dedicated study designed to repeatedly acquire large volumes, (*ii*) from waste plasma after patients’ plasma exchange treatment, and (*iii*) from patients participating in a clinical trial who never had plasma exchange (43)—the latter samples were pooled to minimize animal use. Details are provided in *SI Appendix*. The patients’ baseline characteristics are provided in Table 1. We calculated the probability that we missed an event rate of >50% of patients having no pain-sensitizing autoantibodies by  $[0.5^{(\text{number of experiments} \times \text{fraction of positive experiments})}]$ ;  $0.5^7 = 0.008125$  (i.e., <1%). IgG was prepared from plasma or serum of patients as previously described using Protein G columns for affinity purification followed by elution, buffering, dialysis in normal saline, and concentration to about 8 mg/mL IgG for injection (pooled preparations 12 mg/mL) (11).

**Animals.** The Ethics Committee on Animal Research of the University of Pecs approved experiments involving rodents. Since CRPS affects women two to three times more frequently than men, experiments were carried out on female mice on C57BL/6 background (10–12 wk old, 18–22 g). Breeding, maintenance, and ethical procedures were as previously described (11) (details are in *SI Appendix*).

**Experimental Design.** After acclimatization and conditioning, three control measurements of nociception and paw volume were performed on days –4, –3, and –2. Day 0 was the starting day of intraperitoneal injections (*SI Appendix, Fig. S13*). Mice (five to seven per group) were treated daily to compensate for rapid metabolism of human IgG in the mouse (*SI Appendix, Methods*) with 1 mL of IgG fractions (8 mg/mL) obtained from CRPS patients or healthy volunteers or saline.

Six hours after the injection on day 0, a standardized incision trauma was applied to the right hind paw as described below. All mice removed their stitches within 16 h postsurgery. Measurements (see below) were performed repeatedly starting on day 1 until the respective termination day.

Animals were killed at various time points, and tissues were harvested as previously described (11). In some experiments, animals were perfused transcardially with phosphate buffered saline (PBS) followed by 4% paraformaldehyde as previously described, and the whole brains and spinal cords were excised and prepared for additional immunohistochemistry analyses.

**Plantar Skin and Muscle Incision.** To model a CRPS-triggering injury, we used the hind paw plantar skin muscle incision method under general anesthesia as described earlier (11, 44) and detailed in *SI Appendix*. This model evokes a significant decline of the mechanonociceptive threshold, with a maximum 1 d after surgery, which persists for 7–8 d; the model’s early threshold normalization was considered advantageous, minimizing any animal suffering and allowing for early assessment of IgG-induced changes. All measurements were carried out by two investigators (V.T. and N.S.) who were blinded to treatment assignment. Blinding was performed by the technicians who performed all injections but were otherwise not involved in the study; they differentially coded the animal cages and provided the decoding key after completion of the last measurements.

**Determination of the Mechanosensitivity of the Paw.** Most patients with CRPS have, in addition to their spontaneous pain, pain with the application of pressure to the CRPS-affected limb (“mechanical hyperalgesia”) (45, 46), and all patients included in this study experienced this feature. The corresponding mechanonociceptive threshold of the plantar surface of the mouse hind paw was determined with a dynamic plantar aesthesiometer (Ugo Basile 37400)—a modified electronic von Frey technique—as previously described (11). The blunt-end needle exerting an increasing force to the mouse paw provides a mild but basically painful stimulus activating A $\delta$  and C fibers (47). Threshold decreases are considered as mechanical hyperalgesia and are expressed as percentage decrease of the mechanonociceptive thresholds compared with the baseline values (48, 49).

**Paw Volume Measurement.** Limb swelling is a common feature of CRPS-affected limbs, and all included patients reported intermittent limb swelling. Mouse paw volume was measured using plethysmometry (Ugile Basile

**Table 1. Baseline characteristics of the patient serum donors**

No.	Sex/age	Limb/DD	Pain	Dx	Plex
1	M/62	U/9	10	+II	Y
2	F/37	L/15	7.5	+I	N
3	F/38	L/10	9.5	+I	Y
4	F/36	L/10	7	+I	Y
5	F/40	L/5	7.5	+I	Y
6	F/51	L/8	8	+I	N
7	F/49	L/7	8	+I	Y

Age indicates age in years at the time of plasma/serum acquisition. Limb indicates the affected limb. DD is the disease duration in years. Pain indicates 24-h average pain intensity on a 11-point numeric rating scale (0–10, with 10 = pain as bad as you can imagine). Dx indicates diagnosis, with + indicating that the patient fulfills Budapest research diagnostic criteria (new International Association for the Study of Pain criteria); II denotes trigger injury to a major nerve, and I denotes no trigger injury to a major nerve. Plex indicates plasma derived from plasma exchange. N, no; Y, yes; U, upper limb; L, lower limb.

Plethysmometer 7140). Edema was expressed as a percentage increase compared with the baseline paw volume (48).

**In Vivo Optical Imaging of Plasma Leakage and Neutrophil MPO Activity.** The mechanisms underpinning CRPS IgG-enhanced paw swelling are unknown, but one possibility is augmented plasma extravasation. Intravenous injected ICG, a fluorescent cyanine dye, binds to plasma proteins and remains in the healthy vasculature. Under inflammatory conditions, it can be used to evaluate capillary leakage. ICG (0.5 mg/kg) was dissolved freshly in 5% (wt/vol) aqueous solution of Kolliphor HS 15 and a macrogol-based surfactant and injected intravenously (retrobulbar sinus) under ketamine (100 mg/kg; Calypsol; Gedeon Richter Plc.) and xylazine (10 mg/kg; Sedaxylan; Eurovet Animal Health B.V.) anesthesia 2 d after the paw incision. Fluorescence imaging was performed 20 min postinjection using an IVIS Lumina II in vivo optical imaging system (PerkinElmer; autoacquisition time, f/stop = 1, binning = 2, excitation: 745 nm, emission filter: >800 nm) (50).

A luminol analog chemiluminescent probe, L-012 (Wako Pure Chemical Industries Ltd.) was used for in vivo visualization of reactive oxygen species (ROS)/reactive nitrogen species (RNS) produced by MPO in neutrophils and macrophages; L-012 has a high sensitivity toward ROS/RNS (51). Mouse preparation, injection, imaging, and analysis procedures were conducted as previously described and highlighted in *SI Appendix* (52).

**Measurement of Inflammatory Mediators.** Proinflammatory neuropeptides peripherally released from sensory nerves and inflammatory cytokines released by perineuronal cells are abnormal in some patients with CRPS, and we measured their concentrations in the model.

The preserved frozen paws (see above) were thawed, chopped, and then homogenized in Triton X-100 and Calbiochem Protease Inhibitor Cocktail containing Tris-HCl homogenization buffer at 0 °C. Additional processing details are provided in *SI Appendix*. We measured CGRP- and SP-like immunoreactivities in the paws by a sensitive radioimmunoassay (RIA) technique developed in our laboratory as previously described (11, 53, 54).

Concentrations of IL-1 $\alpha$ , IL-1 $\beta$ , IL-6, TNF- $\alpha$ , KC (CXCL1), MCP-1, G-CSF, RANTES (CCL5), interferon- $\gamma$ , IL-4, and IL-10 in mouse plasma and the paw homogenates were measured by cytometric bead array (BD Biosciences) as previously described (34, 55), and transforming growth factor- $\beta$  and NGF were measured by sandwich enzyme-linked immunosorbent assay according to the manufacturers' instructions (56).

**Paw Skin Light and Electron Microscopy.** After biopsy, samples were immersed into a fixative containing 2.5% glutaraldehyde buffered with phosphate buffer (0.1 M, pH 7.4) overnight at 4 °C, fixed in 1% osmium tetroxide for 35 min, and dehydrated with increasing concentration of ethanol. After complete dehydration, they were transferred to propylene oxide before being placed into aluminum foil boats and then embedded into gelatin capsule containing Durcupan resin (Sigma).

Semithin and ultrathin sections were cut with Leica ultramicrotome. Semithin sections were mounted on glass slides, stained with toluidine blue, and examined under a light microscope. Ultrathin sections were mounted on collodion-coated (Parlodion; Electron Microscopy Sciences) single-slot copper grids contrasted with uranyl acetate and lead citrate, and were examined in a

JEOL 1200EX-II electron microscope. Small nerve fiber quantification methods are detailed in *SI Appendix, Methods*.

**Immunohistochemistry.** Brains and L4–L6 segments of the spinal cord were removed and postfixed for 4 h in 4% paraformaldehyde before being placed into 30% sucrose (Duchefa Biochemie) in 0.1 M PBS overnight at 4 °C. Sections (30  $\mu$ m) were prepared using a freezing microtome (Leica Biosystems Nussloch GmbH) as free-floating sections (57, 58), and they were stained and mounted as described in *SI Appendix*. The sections ( $n = 3$ –4 animal per group) were examined by NeuroLucida software (v07; MBF Bioscience) using a Nikon Eclipse Ni-E bright-field microscope with a computer-controlled stage. A modified unbiased stereology protocol was used for quantification of glial fibrillary acidic protein (GFAP) or Iba1 immunoreactive cells along the nociceptive pathway as previously described (50).

**Immunofluorescence.** Immunofluorescence to detect microglial P2Y<sub>12</sub> (59) and the production of IL-1 $\alpha$  and IL-1 $\beta$  was performed on free-floating brain sections. Images were captured with a Nikon Ni-E C2+ confocal microscope.

**Antiinflammatory Interventions.** We investigated whether early immune suppression would alter the disease course in the model. We administered the synthetic glucocorticoid prednisolone (4 mg/kg intraperitoneally) (60, 61) or the IL-1R antagonist anakinra [10 mg/kg intraperitoneally; Swedish Orphan Biovitrum AB (publ), SE-112 76]. This prednisolone dose corresponds to the highest dose range used in humans [pulse therapy (62)], and the anakinra dose is known to be pharmacologically active in mice and und is comparable with human therapeutic dosages (63–65). Control animals received respective vehicles. The first drug injection was 3 h before the plantar skin and muscle incision and at least 4 h after IgG or control treatment. Drug treatment was then repeated daily through the experimental period. Delayed anakinra treatment was administered daily between days 8 and 13.

**Generation of IL-1 $\beta$  Floxed Mice and Microglial IL-1 $\beta$  KO Mice.** Il1b<sup>tm1a(EUCOMM)Hmgu</sup> embryonic stem cells (66) were purchased from the European Mouse Mutant Cell Repository. Cells from clone HEPD0840-8-E03 were prepared for microinjection according to previously published protocols (67) with minor modifications (*SI Appendix, Methods*) and then microinjected into four- to eight-cell B6N-Tyr<sup>c-Brd</sup>/BrdCrCl embryos. Surviving embryos were surgically implanted into the oviduct of day 0.5 postcoitum pseudopregnant mice. Resulting black/white C57BL/6N chimeras were backcrossed onto C57BL/6N WT mice to assess germline penetrance. Potential founder mice were screened by PCR for LacZ, Neo, and LoxP sites. This line was further crossed with C57BL/6N-Tg(CAG-Flp)1Afst/Mmud mice. The flip recombinase expression by this line resulted in a "conditional ready" (floxed) Il1b<sup>tm1c(EUCOMM)Hmgu</sup> allele where exons 4 and 5 are flanked by loxP sites. To generate microglial IL-1 $\beta$  KO mice, IL-1 $\beta$  floxed mice were crossed with tamoxifen-inducible B6.129P2(C)-CX3CR1<sup>tm2.1(cre/ERT2)Jung/J</sup> mice (JAX stock 020940) (20).

**Statistical Analysis.** Data shown are means  $\pm$  SEM, and two-way repeated measures ANOVA followed by Bonferroni's post hoc test was used for comparison of threshold values between groups at respective timepoints. One-way ANOVA followed by Bonferroni's post hoc test was used for analysis of the immunohistochemistry and cytokine results. A value of  $P < 0.05$  was considered statistically significant.

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