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# Bedeutung der T-Zell-Immunologie bei chronischen Schmerzen

Kumulative Habilitationsschrift

Zur Erlangung des akademischen Grades eines habilitierten Doktors
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vorgelegt von Dr. med. Benjamin Luchting

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## 1. Einleitung

Mit einer Prävalenz von bis zu 30% bei Erwachsenen stellen chronische Schmerzen ein bedeutendes gesundheitliches Problem dar (Wolff, Clar et al. 2011). Seit Jahrzehnten wird das Phänomen des chronischen Schmerzes intensiv beforscht. Aufgrund seiner Komplexität sind hinsichtlich der Pathophysiologie jedoch noch viele Fragen offen. In jüngerer Zeit ergaben sich zunehmend Hinweise darauf, dass das Immunsystem maßgeblich an der Entstehung und Chronifizierung von Schmerzen beteiligt ist (Austin and Moalem-Taylor 2010, Tian, Ma et al. 2012). Die Mehrzahl der dazu publizierten Arbeiten beschreibt einen Zusammenhang zwischen Schmerzen und einer Aktivierung des Immunsystems. Die Erkenntnisse stützen sich dabei vorwiegend auf die Analyse pro- und antiinflammatorischer Zytokine. Während bei der Entstehung akuter Schmerzen insbesondere Zytokine und Granulozyten bedeutsam sind, scheinen bei chronischen Schmerzen die T-Lymphozyten als Hauptbestandteil des adaptiven Immunsystems eine besondere Rolle zu spielen (Perkins and Tracey 2000). In diesem Zusammenhang wurde beispielsweise eine Imbalance der proinflammatorischen TH1 und der antiinflammatorischen TH2 Zellen bei chronischen Blasenschmerzen und dem komplexen regionalen Schmerzsyndrom (CRPS) beschrieben (Liu, Xia et al. 2006, Schinkel, Gaertner et al. 2006). In den letzten Jahren wurde die TH1/TH2 Dichotomie erweitert durch die Identifikation neuer T-Zell Untergruppen, wie beispielsweise der TH17 Zellen und der regulatorischen T-Zellen ($T_{regs}$) (Kleinschnitz, Hofstetter et al. 2006). Während TH17 Zellen normalerweise eine Schlüsselrolle bei diversen Autoimmunerkrankungen spielen, sorgen $T_{regs}$ für eine Kontrolle der adaptiven Immunantwort. Bei neuropathischen Schmerzen wurde ein Zusammenhang zwischen proinflammatorischen TH17 Zellen und einer vermehrten Schmerzempfindlichkeit gesehen, während die antiinflammatorischen Tregs eher an einer endogenen Wiederherstellung und Kontrolle der neuronalen Funktion beteiligt sind (Kleinschnitz, Hofstetter et al. 2006, Austin and Moalem-Taylor 2010). Trotz all dieser neuen vielversprechenden Erkenntnisse gibt es bislang jedoch keine systematische Untersuchung zur Rolle des adaptiven Immunsystems bei unterschiedlichen

Schmerzsyndromen wie Rückenschmerz, neuropathischem Schmerz und dem CRPS. Dies war daher das Ziel der vorliegenden Habilitationsschrift.

## 2. Das adaptive Immunsystem bei unterschiedlichen Schmerzsyndromen

**Veröffentlicht als:**

**B. Luchting**, B. Rachinger-Adam, J. Heyn, L. C. Hinske, S. Kreth, S. C. Azad
Anti-inflammatory T cell shift in neuropathic pain
J Neuroinflammation, 2015

**B. Luchting**, B. Rachinger-Adam, J. Zeitler, L. Egenberger, P. Möhnle, S. Kreth, S. C. Azad
Disrupted TH17/Treg balance in patients with chronic low back pain
PLoS One, 2014

### 2.1. Chronisch unspezifischer Rückenschmerz (CLBP)

Chronische Rückenschmerzen (CLBP) stellen die häufigste chronische Schmerzerkrankung dar und sind einer der größten Kostenverursacher des Gesundheitssystems (Balague, Mannion et al. 2012). Die Genese ist multifaktoriell und die Therapie richtet sich entsprechend nach dem sog. „Bio-Psycho-Sozialen Schmerzmodel“. Der erste Teil des vorliegenden Projektes befasst sich mit der Analyse von T-Zell Funktionen bei Patienten mit CLBP vor und nach erfolgreicher Therapie.

**Methodik:** Bei 37 Patienten mit CLBP und 25 gesunden Probanden wurden die quantitative Verteilung von TH1-, TH2-, TH17-Zellen und $T_{regs}$, sowie T-Zell relevante Zytokine und mRNA-Expression bestimmt. Alle Patienten unterzogen sich einem 4-wöchigen multimodalen Schmerzprogramm. Vor Beginn, am Ende und 6 Monate nach Beendigung des Programms erfolgten Blutentnahmen. Patienten mit einer anhaltenden Schmerzreduktion wurden als „Responder“ gewertet, und deren Ergebnisse im zeitlichen Verlauf analysiert.

**Ergebnisse:** Gegenüber Probanden wiesen CLBP Patienten einen signifikant höheren Anteil an Tregs bei deutlich reduziertem Anteil an TH17-Zellen auf. Entsprechend der durchflusszytometrischen Analysen war die Expression von $T_{reg}$-spezifischem FoxP3 und von TGF-β signifikant erhöht. Die Konzentration T-Zell spezifischer Zytokine war sowohl bei Patienten als auch bei Gesunden

unterhalb bzw. nur knapp oberhalb der detektierbaren Grenzen. 35% der Patienten erlebten eine anhaltende, signifikante (> 50%) Schmerzreduktion durch das multimodale Schmerzprogramm. Trotz der signifikanten klinischen Besserung zeigte sich bei ihnen jedoch weder am Ende des Programms, noch 6 Monate später eine Normalisierung der gestörten TH17/$T_{reg}$-Balance.

**Interpretation:** Patienten mit CLBP weisen ein gestörtes Gleichgewicht zwischen TH17- und $T_{reg}$-Zellen zugunsten einer erhöhten Antiinflammation auf. Diese Verschiebung bleibt trotz suffizienter Therapie der Schmerzen langfristig bestehen. Dies lässt vermuten, dass die Chronifizierung von Rückenschmerzen durch eine vorbestehende Dysbalance von T-Zell Subpopulationen begünstigt wird.

### 2.2. Neuropathischer Schmerz (NeP)

Die suffiziente Behandlung neuropathischer Schmerzen stellt in der klinischen Praxis häufig eine Herausforderung für die behandelnden Ärzte dar (Cohen and Mao 2014). Trotz intensiver Forschung sind hinsichtlich der zugrundeliegenden pathophysiologischen Mechanismen bis heute noch viele Fragen offen. In der unter 2.1. beschriebenen Untersuchung an Patienten mit CLBP wurde ein gestörtes Verhältnis von pro- und antiinflammatorischen T-Zellen (TH17/$T_{reg}$ Balance) beobachtet, welches sich auch nach suffizienter Therapie nicht normalisierte. Nachdem nozizeptive und neuropathische Schmerzen als unterschiedliche Entitäten beschrieben werden, befasste sich die darauffolgende Untersuchung mit der Analyse von T-Zell Funktionen bei Patienten mit NeP.

**Methodik:** Bei 26 Patienten mit NeP und 26 gesunden Probanden wurde durchflusszytometrisch wiederum die quantitative Verteilung von pro- und antiinflammatorischen TH1-, TH2-, TH17-Zellen und $T_{regs}$ bestimmt. Zur Bestätigung der durchflusszytometrischen Daten erfolgte zudem die Analyse der T-Zell spezifischen mRNA-Expression von FoxP3, TGF-β und RORγT

mittels quantitativer Real-Time PCR, sowie die Bestimmung der Plasma-Konzentration T-Zell relevanter Zytokine durch einen Multiplex Immunoassay.

**Ergebnisse:** Ebenso wie CLBP-Patienten, wiesen auch Patienten mit NeP einen signifikant höheren Anteil an antiinflammatorischen $T_{regs}$ bei deutlich reduziertem Anteil an proinflammatorischen TH17-Zellen auf. Bei Ihnen war die Expression von $T_{reg}$-spezifischem FoxP3 und von TGF-β ebenfalls signifikant erhöht. Die Konzentration T-Zell spezifischer Zytokine war sowohl bei Patienten als auch bei Gesunden unterhalb bzw. nur knapp oberhalb der detektierbaren Grenzen.

**Interpretation:** Bei den Patienten mit neuropathischen Schmerzen zeigte sich ein gestörtes Gleichgewicht von TH17- und $T_{reg}$-Zellen zugunsten einer erhöhten Antiinflammation. Interessanterweise entsprach dieses Ergebnis auch dem von Patienten mit CLBP, die keinerlei neuropathische Schmerzkomponente aufwiesen. Dies deutet darauf hin, dass die Veränderungen des adaptiven Immunsystems keinen spezifischen Pathomechanismus des Schmerzsyndroms an sich widerspiegeln, sondern eher im Zusammenhang mit der Gesamterkrankung „chronischer Schmerz“ zu sehen sind.

### 2.3. Komplexes regionales Schmerzsyndrom (CRPS)

Beim CRPS handelt es sich um ein komplexes Syndrom, das durch das gleichzeitige Auftreten von Schmerzen, trophischen Veränderungen und motorischen Störungen gekennzeichnet ist. Die Symptome korrelieren dabei nicht mit der Schwere des auslösenden Ereignisses, u.U, kann das CRPS auch nach Bagatelltraumen oder sogar spontan auftreten. Ein zentraler Bestandteil des CRPS ist eine „neurogene“ Entzündung verbunden mit einer zentralnervösen Störung des sympathischen Nervensystems (Parkitny, McAuley et al. 2013). Das klinische Bild stellt eine Mischform aus nozizeptivem und neuropathischem Schmerz dar.

**Methodik:** Bei 15 Patienten mit CRPS und 15 hinsichtlich Alter und Geschlecht vergleichbaren gesunden Probanden wurden die quantitative Verteilung von T-Zell Untergruppen, T-Zell relevanten Zytokinen und mRNA-Expression bestimmt.

**Ergebnisse:** Gegenüber den gesunden Probanden wiesen CRPS Patienten interessanterweise zwar einen deutlich reduzierten Anteil an TH17-Zellen auf, entgegen der Erwartung aber keine erhöhte Anzahl an $T_{regs}$. Obwohl in der Literatur zahlreiche Arbeiten veränderte Zytokinspiegel beschreiben, zeigten unsere Analysen aus dem Serum keine richtungsweisenden Ergebnisse. Auch hier lag die Konzentration T-Zell spezifischer Zytokine sowohl bei Patienten als auch bei Gesunden unterhalb bzw. nur knapp oberhalb der detektierbaren Grenzen.

**Interpretation:** Patienten mit CRPS weisen eine deutlich erniedrigte Anzahl an proinflammatorischen TH17-Zellen auf, allerdings mit Ausbleiben einer korrespondieren Erhöhung der antiinflammatorischen $T_{regs}$. Damit unterscheidet sich das CRPS zumindest in Bezug auf die Verteilung der T-Zell Untergruppen von den anderen oben beschriebenen Schmerzsyndromen.

## 3. Regulatorische Mechanismen des adaptiven Immunsystems bei unterschiedlichen Schmerzsyndromen

**Veröffentlicht als:**

J. Heyn*, **B. Luchting***, L. Hinske, M. Hübner, SC Azad, S. Kreth
miR-124a and miR-155 enhance differentiation of regulatory T cells in patients with neuropathic pain
J Neuroinflammation, 2016

**B. Luchting**, J. Heyn, L. Hinske, S. C. Azad
Expression of miRNA-124a in CD4 cells reflects response to a multidisciplinary treatment program in patients with chronic low back pain
Spine, 2016

**B. Luchting***, J. Heyn*, T. Wöhrle*, B. Rachinger-Adam, S. Kreth, L. C. Hinske, S. C. Azad
Differential expression of P2X7 receptor and IL-1β in nociceptive and neuropathic pain
J Neuroinflammation, 2016

Trotz grundlegend verschiedener Entitäten von CLBP, NeP und CRPS zeigten unsere T-Zell Analysen teilweise deckungsgleiche Veränderungen in allen drei Schmerzsyndromen. Im zweiten Schritt fokussierten wir uns daher auf mögliche zugrunde liegende Mechanismen. Im Allgemeinen wird eine T-Zell Differenzierung durch eine komplexe Vernetzung von pro- und anti-inflammatorischen Zytokinen reguliert. Nachdem unsere Zytokinbestimmung in keinem der von uns untersuchten Schmerzsyndrome richtungsweisende Befunde aufzeigten, analysierten wir im Folgenden mögliche epigenetische Mechanismen. Als wichtige Regulatormoleküle der posttranskriptionellen Genexpression erlangen MicroRNAs (miRNAs) in den letzten Jahren zunehmende Bedeutung (Bali and Kuner 2014, Descalzi, Ikegami et al. 2015). MiRNAs sind 20-22 Basenpaare lange, nicht-kodierende Einzelstrang-RNAs, welche durch eine spezifische Bindung an komplementäre Bindungssequenzen

eine Degradation von mRNAs bewirken können. Nach Literaturrecherche und aus Vorarbeiten unserer Arbeitsgruppe bezüglich T-Zell relevanter miRNAs bei Entzündungsreaktionen, erfolgte daraufhin die Analyse verschiedener Schmerz- und T-Zell relevanter miRNAs.

### 3.1. Chronisch unspezifischer Rückenschmerz (CLBP)

Bei Patienten mit CLBP wurden insgesamt acht relevante miRNAs untersucht. Dabei zeigte sich, dass lediglich drei (miRNA-150, miRNA-155, miRNA-124a) miRNAs bei Schmerzpatienten im Vergleich zu gesunden Kontrollen signifikant hochreguliert waren. Diese wurden daher bei den Patienten vor und nach Teilnahme an dem 4-wöchigen, teilstationären multimodalen Therapieprogramm bestimmt. Nach Abschluss des multimodalen Programms erfolgte die Unterteilung der Patienten in Responder (≥50% Schmerzlinderung) und Non-Responder (<50% Schmerzlinderung). Es zeigte sich, dass nur die Expression von miRNA-124a durch die Therapie beeinflusst wurde. Interessanterweise war dies jedoch lediglich bei den Respondern der Fall, die eine signifikante Hochregulation der miRNA-124a aufwiesen.

### 3.2. Neuropathischer Schmerz (NeP)

Bei Patienten mit NeP zeigte sich im Vergleich zu Gesunden ebenfalls eine deutliche Hochregulation der miRNA-124a und der miRNA-155. Nachdem die Zytokinspiegel keine hinreichende Erklärung für den antiinflammatorischen T-Zell Shift ergaben, untersuchten wir mögliche miRNA assoziierte T-Zell Mechanismen. In *in vitro* Untersuchungen konnten wir daraufhin eine direkte miR-Zielgen-Interaktion mittels Reportergenassay nachweisen. Hierzu wurde der 3´-untranslatierte Bereich des SIRT1 Gens mittels PCR amplifiziert und in einen Luciferase-Reporter-Vektor kloniert. Das Vektorkonstrukt wurde zusammen mit den jeweiligen pre-miRs (bzw. Kontroll-miR) in HEK293-Zellen transfiziert und Reporteraktivitäten luminometrisch quantifiziert. Unsere Ergebnisse konnten dabei aufzeigen, dass eine erhöhte Expression von miRNA-124a und miRNA-155 bei NeP über eine Hemmung von SIRT1 zu einer gesteigerten Ausdifferenzierung von immunsuppressiven $T_{regs}$ führt.

### 3.3. Komplexes regionales Schmerzsyndrom (CRPS)

Im Gegensatz zu Patienten mit CLBP und NeP wiesen CRPS Patienten keine Erhöhung der antiinflammatorischen $T_{regs}$, interessanterweise jedoch eine signifikant gesteigerte CD39-Expression der $T_{regs}$ auf. Eine CD39-Hochregulation kann die antiinflammatorische Kapazität von $T_{regs}$ verstärken und somit als ein kompensatorischer Mechanismus zur ausbleibenden $T_{reg}$-Erhöhung gewertet werden. Ob die $T_{reg}$-Inhibition möglicherweise durch bestimmte miRNAs erfolgt, ist Gegenstand aktuell laufender Versuche.

### 3.4 Bedeutung des purinergen Systems

P2X7-Rezeptoren (P2X7R) sind Subtypen der Familie von sieben purinergischen P2X-Rezeptoren und werden vorwiegend auf Immunzellen und Microglia exprimiert. Im Zusammenspiel mit dem Zytokin IL-1β scheinen sie eine zentrale Rolle in der Pathophysiologie von Schmerzen zu spielen. Obwohl bereits in zahlreichen tierexperimentellen Studien P2X7R-Inhibitoren als mögliche schmerztherapeutische Ansätze untersucht wurden, gibt es bisher keine Daten über das P2X7R Expressionsmuster auf Immunzellen bei verschiedenen Schmerzsyndromen. Wir analysierten daher durchflusszytometrisch und mittels Real-Time PCR die P2X7R-Expression auf Lymphozyten und Monozyten bei Patienten mit CLBP und NeP und verglichen die Ergebnisse mit denen gesunder Probanden. Darüber hinaus bestimmten wir die Serumspiegel des mit dem P2X7R interagierenden IL-1β. Entgegen der zuvorigen Ergebnisse zeigte sich hier eine signifikante Erhöhung von P2X7R-Protein, P2X7R-mRNA und IL-1β exklusiv bei Patienten mit NeP, nicht aber bei Patienten mit CLBP. Wir mutmaßten daher, daß das Zusammenspiel zwischen P2X7R und IL-1β ein vorrangiges Phänomen bei der Entstehung von NeP darstellt.

## 4. Identifizierung eines potentiellen Biomarkers für Schmerzintensität

**Veröffentlicht als:**

**B. Luchting***, L. Hinske*****, B. Rachinger-Adam, S. Kreth, S. C. Azad
sICAM-1: A Potential Biomarker for Pain Intensity in Chronic Pain Patients
*Biomarkers in Medicine, 2017*

In der klinischen Praxis werden Schmerzen bislang basierend auf den Angaben des Patienten behandelt, wobei die geäußerte Schmerzstärke maßgeblich Art und Umfang der Schmerztherapie bestimmt. Bei Patienten, bei denen diese Erhebung nicht möglich ist, kann sich der behandelnde Arzt nur auf unspezifische Parameter wie erhöhten Blutdruck und Herzfrequenz, Stöhnen oder Tränenfluss stützen. Dass daraus häufig eine unzureichende Schmerztherapie resultiert, konnte beispielsweise bei Patienten in Pflegeheimen oder Intensivstationen eindrucksvoll belegt werden. Bis zu 80% der Patienten in Pflegeeinrichtungen leiden an Schmerzen, werden aber teilweise nicht adäquat therapiert (Barkin, Barkin et al. 2005, Zwakhalen, Hamers et al. 2006). Auch bei der Mehrheit von Patienten auf Intensivstationen ist eine unzureichende Schmerztherapie beschrieben und potentiell mit einer längeren Liegezeit und Beatmungsdauer, höheren Infektionsraten und einem häufigeren Auftreten von posttraumatischen Belastungsstörungen (PTBS) assoziiert (Chanques, Jaber et al. 2006, Payen, Bosson et al. 2009, Joffe, Hallman et al. 2013). Zudem sind viele weitere Patientengruppen davon betroffen, wie z.B. Kleinkinder, Patienten mit Demenz oder anderen Erkrankungen des zentralen Nervensystems, sowie bewusstseinsgetrübte Patienten.

Obwohl im Rahmen unserer Analyse T-Zell relevanter Zytokine im Hinblick auf unsere T-Zell Differenzierung keine wegweisenden Ergebnisse zu verzeichnen waren, konnten wir ein T-Zell unabhängiges Zytokin identifizieren, welches als potentieller Biomarker für Schmerzstärke dienen könnte: sICAM-1 (Interzelluläres Adhäsionsmolekül-1) korrelierte bei 169 Patientenproben hoch signifikant mit der von den Patienten geäußerten Schmerzstärke (r=0.42,

p<0.001). Potentiell verfälschende Einflussgrößen (sog. Confounder und Effektmodifikatoren) wurden daraufhin von uns mittels umfangreicher statistischer Analysen ausgeschlossen. Bei sICAM-1 handelt es sich um ein Protein, welches in geringer Konzentration auf der Oberfläche von Endothelzellen und Immunzellen exprimiert wird. Nach Stimulation durch Schmerzen oder Zytokine kommt es zu einer raschen und starken Hochregulation von sICAM-1, wodurch eine Immunzell-Endothelzell-Interaktion eingeleitet wird. Durch diese Interaktion können Opioid-tragende Immunzellen das Gefäßsystem verlassen und in das schmerzverursachende Gewebe gelangen, um durch die Freisetzung ihrer Opioide die lokale Schmerzreaktion zu reduzieren (Hubbard and Rothlein 2000, Machelska, Mousa et al. 2002). Die erhöhte Freisetzung von sICAM-1 korrelierte in vorliegender Untersuchung hoch signifikant mit der Schmerzstärke, unabhängig vom zugrundeliegenden Schmerzsyndrom. Erhöhte sICAM-1 Werte wurden bereits bei verschiedenen Erkrankungen wie Migräne, Pankreatitis, Subarachnoidalblutungen und koronarer Herzerkrankung beschrieben (Mack, Mocco et al. 2002, Remahl, Bratt et al. 2008, Zhu and Jiang 2012). Diese Erkrankungen sind allesamt schmerzhaft, allerdings hat bisher keine Arbeit den Zusammenhang zwischen sICAM-1 und der Schmerzstärke untersucht, sondern sICAM-1 als unspezifischen Marker für die jeweilige Erkrankung betrachtet. SICAM-1 als Marker für Schmerzintensität könnte im Rahmen eines PoCT-Verfahren (Point-of-Care-Testing) eine gezielte und besser steuerbare Schmerztherapie ermöglichen und dadurch zu einer Verbesserung der Schmerztherapie in Pflegeeinrichtungen und Intensivstationen führen.

Nachdem ein solcher Biomarker Einzug in die klinische Praxis nehmen könnte und somit auch eine patentrelevante Erfindung darstellt, meldeten wir diese Erfindung der Ludwig-Maximilians-Universität München (LMU). Nach positiver Begutachtung durch die Bayerische Patentallianz wurde die Erfindungsmeldung von der LMU in Anspruch genommen und ist seit dem 13.10.2015 als internationale Patentanmeldung hinterlegt. Ein Ethikantrag zur klinischen Überprüfung dieses Biomarkers bei Demenzkranken wird derzeit erstellt.

## 5. Zusammenfassung

Ziel des hier vorgestellten Habilitationsprojekts war es, an Hand von klinisch-experimentellen Untersuchungen immunologische Veränderungen bei Patienten mit verschiedenen Schmerzsyndromen näher zu charakterisieren und zugrundeliegende molekulare Regulationswege aufzuzeigen. Es wurden dabei an drei unterschiedlichen Schmerzsyndromen mit grundlegend unterschiedlichen Entitäten teilweise deckungsgleiche immunologische Veränderungen beobachtet. Wir vermuteten daher, dass hierfür Faktoren verantwortlich sein könnten, die nicht unmittelbar mit der eigentlichen Nozizeption verbunden sind. Entgegen der bisher vorherrschenden Meinung, dass Schmerzen generell eher mit einer Proinflammation assoziiert sind, weisen unsere Untersuchungen bei chronischen Schmerzpatienten aber auf eine Antiinflammation hin.

Diese wurde bislang vor allem im Zusammenhang mit chronischem Stress und Depressionen beschrieben (Irwin and Miller 2007, Hong, Zheng et al. 2013). Da alle Formen chronischer Schmerzen regelhaft mit chronischem Stress und depressiven Verstimmungen assoziiert sind, verglichen wir die Stress-Scores unserer Patienten. Nachdem alle Patientengruppen hierbei vergleichbare, deutlich erhöhte Scores aufwiesen, deuten unsere Ergebnisse darauf hin, dass die immunologischen Veränderungen weniger einen spezifischen pathophysiologischen Mechanismus des jeweiligen Schmerzsyndroms, sondern vielmehr eine Reaktion auf den schmerzassoziierten chronischen Stress widerspiegeln. Diese Interpretation wurde darüber hinaus durch unsere epigenetischen Untersuchungen untermauert: Die Hochregulation der miRNA-124a bei CLBP und NeP sowie die Assoziation zwischen Hochregulation und Therapieerfolg nach multimodaler Therapie weisen auf eine mögliche, schmerzunabhängige Beteiligung hin. MiRNA-124a stellt eine der wichtigsten miRNAs bei psychologischen Erkrankungen und chronischem Stress dar (Dwivedi 2014). Darüber hinaus wird sie auch als „NeurimmirR“ bezeichnet, welche als Schaltzentrale zwischen dem zentralen Nervensystem und dem Immunsystem agieren kann (Soreq and Wolf 2011). Manakov und Kollegen konnten dazu hunderte von Stress-induzierten Transkripten aufzeigen, welche

durch die miRNA-124a beeinflusst werden (Manakov, Morton et al. 2012). Wir vermuteten daher, dass die veränderte miRNA-124a Expression nach multimodaler Therapie eher mit der Verbesserung der psychischen Parameter als mit der Reduzierung des Schmerzes *per se* in Zusammenhang steht.

Zusammenfassend konnte durch die erlangten Erkenntnisse die Komplexität von chronischen Schmerzten weiter untermauert werden. Basierend darauf könnten neue Therapieansätze identifiziert werden. Darüber hinaus stellt der identifizierte potentielle Biomarker sICAM-1 ein vielversprechendes Tool zur besseren Erfassung unterversorgter Schmerzzustände dar. Mittels eines solchen PoCT-Verfahrens wäre daher in der klinischen Praxis eine gezielte und besser steuerbare Schmerztherapie möglich, beispielweise in Pflegeeinrichtungen, Intensivstationen oder bei dementen- und bewusstseinsgetrübten Patienten. Entsprechende klinisch-experimentelle Projekte sind auf den Weg gebracht und sollten in absehbarer Zeit klinische Anwendungsfelder identifizieren.

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# miR-124a and miR-155 enhance differentiation of regulatory T cells in patients with neuropathic pain

Jens Heyn†, Benjamin Luchting†, Ludwig C. Hinske, Max Hübner, Shahnaz C. Azad and Simone Kreth*

## Abstract

**Background:** Accumulating evidence indicates that neuropathic pain is a neuro-immune disorder with enhanced activation of the immune system. Recent data provided proof that neuropathic pain patients exhibit increased numbers of immunosuppressive regulatory T cells (Tregs), which may represent an endogenous attempt to limit inflammation and to reduce pain levels. We here investigate the molecular mechanisms underlying these alterations.

**Methods:** Our experimental approach includes functional analyses of primary human T cells, 3′-UTR reporter assays, and expression analyses of neuropathic pain patients' samples.

**Results:** We demonstrate that microRNAs (miRNAs) are involved in the differentiation of Tregs in neuropathic pain. We identify miR-124a and miR-155 as direct repressors of the histone deacetylase sirtuin1 (SIRT1) in primary human $CD4^+$ cells. Targeting of SIRT1 by either specific siRNA or by these two miRNAs results in an increase of Foxp3 expression and, consecutively, of anti-inflammatory Tregs (siRNA: $1.7 \pm 0.4$; miR-124a: $1.5 \pm 0.4$; miR-155: $1.6 \pm 0.4$; $p < 0.01$). As compared to healthy volunteers, neuropathic pain patients exhibited an increased expression of miR-124a ($2.5 \pm 0.7$, $p < 0.05$) and miR-155 ($1.3 \pm 0.3$; $p < 0.05$) as well as a reduced expression of SIRT1 ($0.5 \pm 0.2$; $p < 0.01$). Moreover, the expression of these two miRNAs was inversely correlated with SIRT1 transcript levels.

**Conclusions:** Our findings suggest that in neuropathic pain, enhanced targeting of SIRT1 by miR-124a and miR-155 induces a bias of $CD4^+$ T cell differentiation towards Tregs, thereby limiting pain-evoking inflammation. Deciphering miRNA-target interactions that influence inflammatory pathways in neuropathic pain may contribute to the discovery of new roads towards pain amelioration.

**Trial registration:** German Clinical Trial Register DRKS00005954

**Keywords:** Neuropathic pain, miRNA, Regulatory T cells, Histone deacetylase sirtuin1, Analgesia

## Background

Neuropathic pain is caused by impairment of somatosensory functions in both the peripheral and central nervous system [1]. It is often associated with spontaneous pain, dysesthesia, paraesthesia, and hyperalgesia (increased pain caused by painful stimuli) and allodynia (increased pain caused by non-painful stimuli) [2, 3]. The treatment of neuropathic pain is ambitious, and outcomes often are unsatisfactory [4]. Despite intensive analgesic treatment, significant attenuation of pain is only achieved in a limited number of patients [5].

There is emerging evidence that aberrant responses of the immune system substantially contribute to the development of neuropathic pain [6]. Immune cells respond to nerve injury by migration into the nervous system at the side of injury, thereby releasing mediators, which affect intercellular signaling [7]. Although the precise role of immune cells in neuropathic pain remains unclear, adoptive transfer of immune cells producing pro-inflammatory cytokines significantly increase pain sensitivity, whereas transfer of cells producing anti-inflammatory cytokines decrease pain sensitivity in nerve-injured rats [8]. Recent data investigating neuropathic pain in humans published by

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our group point into the same direction. We showed that patients exhibit altered ratios of peripheral T-helper cell subsets. Specifically, increased numbers of immunosuppressive regulatory T cells (Tregs) have been found [9, 10]. This could reflect an endogenous strategy to limit inflammation and to reduce pain levels in neuropathic pain, which is of interest with respect to future treatment approaches.

The current study aims to investigate the molecular mechanisms underlying these alterations. We focused on the expression of the histone deacetylase sirtuin1 (SIRT1), which is supposed to play a significant role in the development and function of Tregs [11, 12]. SIRT1 controls transcription factor forkheadbox-p3 (Foxp3), the master regulator of Treg differentiation [12]. Treatment with SIRT1 inhibitors increased Foxp3 gene expression with consecutive increase of Treg differentiation in mice [13]. We thus hypothesized that these mechanisms might also be involved in the Treg alterations observed in neuropathic pain patients.

## Methods

### Patients

Patients appearing with neuropathic pain in our Department of Pain Medicine were verified for fulfilling the inclusion criteria and asked about their agreement to participate in the study. Neuropathic pain was defined as "pain caused by a lesion or disease of the somatosensory nervous system" such as polyneuropathy, postherpetic neuralgia, or trigeminal neuralgia/neuropathy using the PainDETECT-questionnaire [14]. Additionally, quantitative sensory testing was performed to all patients, according to the protocol of the German Research Group on neuropathic pain [15]. Patients suffering from low back pain with radiculopathy (even if the radicular component was clearly predominant) or patients with autoimmune, chronic, inflammatory, neoplastic, or psychiatric diseases were excluded. None of the patients had been treated with corticosteroids or had received known immunomodulatory agents currently or in the past. Acute inflammation was excluded by determination of C-reactive protein (CRP), total- and differential leucocyte, and measurement of body temperature. Eleven patients fulfilled the inclusion criteria. Blood samples of these patients were obtained as well as from 9 healthy volunteers after written consents were obtained. Additionally, patients were asked to quote their average pain intensity using an 11-point numerical rating scale (NRS) with 0 representing "no pain" and 10 "worst pain imaginable". For patients' characteristics, see Table 1.

The prospective study protocol followed the principles of the Declaration of Helsinki and was reviewed and approved by the Ethics Committee of the LMU Munich and registered on German Clinical Trial Register (Registration Trial DRKS00005954). Patients included in this study have also been part of a recently published study by Luchting et al. [9] showing an anti-inflammatory T cell shift in patients suffering from neuropathic pain.

**Table 1** Patient characteristics

| Item | Healthy | Neuropathic pain | *p* value |
|---|---|---|---|
| Numbers (*n*) | 9 | 11 | |
| Age | 36 ± 9 | 54 ± 12 | < 0.05 |
| Female | 55 % | 64 % | n.s. |
| BMI | 22.9 ± 2.9 | 25.3 ± 3.4 | n.s. |
| NRS (rest) | 0.0 ± 0.0 | 4.8 ± 2.3 | < 0.05 |
| NRS (motion) | 0.0 ± 0.0 | 7.6 ± 1.7 | < 0.05 |
| KAB | 1.5 ± 0.4 | 3.4 ± 0.7 | < 0.05 |

Results are expressed as mean ± standard deviation (SD)
*BMI* body mass index, *NRS (rest/motion)* numeric rating scale (0 to 10) of pain, 0: "no pain," 10: "worst pain imaginable," *KAB* questionnaire for self-perceived stress ranging (1–6), 1: "no stress," 6: "maximum stress," *n.s.* not significant

### miRNA selection and target prediction

In the current manuscript, we focused on the evaluation of miR-124a and miR-155. These microRNAs (miRNAs) were selected as follows: We first sought to identify miRNAs that have been found to be differentially expressed in pain- and inflammation-related syndromes [16, 17]. Of these, only miR-124a and miR-155 were predicted to target SIRT1. These predictions were based on the established target prediction algorithm TargetScan [18].

### RNA isolation and cDNA synthesis

Total RNA was isolated using either the RNAqueous® Micro Kit or the mirVana miRNA Isolation Kit followed by subsequent DNase treatment (Turbo DNase, Ambion) according to the manufacturer's instructions. Quantity and purity of the isolated RNA were measured using a NanoDrop 2000 spectrophotometer (Thermo Scientific). Complementary DNA (cDNA) was synthesized from 1 μg of total RNA using SuperScript III First Strand Synthesis System (Invitrogen), as per manufacturer's instructions.

### Quantitative RT-PCR

cDNA was synthesized from equal amounts of total RNA using Superscript III reverse transcriptase (Invitrogen) and oligo(dT) and random hexamer primers following the supplier's instructions. Quantitative analyses of messenger RNA (mRNA) levels were performed in duplicates on a Light Cycler 480 (Roche Diagnostics) using either UPL probes and specific primers or specific single assays (Table 2, Roche Diagnostics, Penzberg). The cycling conditions comprised an initial denaturation phase at 95 °C for 5 min, followed by 45 cycles at 95 °C for 10 s, 60 °C for

**Table 2** Primer sequences for real-time PCR

| | Sequence/assay ID |
|---|---|
| Foxp3 | Roche RealTime Ready Single Assay ID 113503 |
| SIRT1 | for 5′-TGT ACG ACG AAG ACG ACG AC-3′ (UPL probe #63) rev 5′-TTC ATC ACC GAA CAG AAG GTT-3′ (UPL probe #63) |
| TBP | for 5′-GAACATCATGGATCAGAACAACA-3′ (UPL probe #87) rev 5′-ATAGGGATTCCGGGAGTCAT-3′ (UPL probe #87) |
| SDHA | for 5′-GAGGCAGGGTTTAATACAGCA-3′ (UPL probe #80) rev 5′-CCAGTTGTCCTCCTCCATGT-3′ (UPL probe #80) |

30 s, and 72 °C for 15 s. Data were normalized to the reference genes SDHA and TBP [19].

#### *Quantification of miRNA expression*

Expression of miR-124a, miR-155, and U47 (endogenous control) was quantified using TaqMan miRNA assays (Applied Biosystems) following the manufacturer's protocol. In brief, equal amounts of RNA (10 ng) were reverse transcribed using miRNA-specific stem-loop primers and the TaqMan MicroRNA Reverse Transcription Kit (Applied Biosystems). Real-time PCR (RT-PCR) was performed in duplicate using LightCycler 480 Probes Master on the LightCycler 480 instrument applying the following cycling conditions: denaturing at 95 °C for 10 min, 45 cycles of 95 °C for 15 s, and 60 °C for 60 s. U47 RNA was used for normalization of miRNA expression data.

### Western blot analysis

Thirty-five micrograms of total protein extracts was electrophoresed in an 8 % SDS–PAGE and subsequently electroblotted onto PVDF membranes. Non-specific binding sites on the membrane were blocked using 5 % non-fat dry milk in TBS-Tween. SIRT1 antibody (Cell Signaling Technology, Danvers, MA) was diluted in PBST supplemented with 1 % non-fat dry milk (dilution factor 1:2000). β-actine (Cell Signaling Technology, dilution factor 1:40,000) served as a loading control. Immunoreactive bands were visualized using horseradish peroxidase-labeled goat anti-mouse or goat anti-rabbit antibodies and the Signal Fire ECL Substrate (Cell Signaling Technology, Danvers, MA).

### Purification of peripheral human $CD4^+$ T cells

$CD4^+$ T cells were isolated from peripheral blood mononuclear cells (PBMCs) by magnetic separation with Whole Blood CD4 MicroBeads (MACS Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer's instructions. Viability and cell number were ascertained by ViCell analyzer (Beckman Coulter, Fullerton, CA).

### Cell cultures and stimulation conditions

Primary $CD4^+$ T cells were cultured in six-well plates in RPMI 1640 medium (Sigma-Aldrich, St. Louis, MO) supplemented with 10 % heat-inactivated fetal calf serum (Biochrom, Berlin, Germany), penicillin (100 IU/mL), streptomycin (100 μg/mL), sodium pyruvate, and L-glutamine (Gibco, Life Technologies, Darmstadt, Germany) at 37 °C in a humidified atmosphere of 5 % $CO_2$ in air.

For differentiation into Tregs, $CD4^+$ T cells ($7 \times 10^5$/mL) were cultured under Treg differentiating conditions (anti-CD3/CD28 Dynabeads (Invitrogen, Carlsbad, Germany) for 36 h, rhIL-2 and TGF-ß for four additional days.) To evaluate the effect of miR-124a, miR-155, or siSIRT1 on Treg differentiation, $CD4^+$ T cells were transfected with these miRNAs, siRNA, or negative control 6 h before stimulation of $CD4^+$ T cells was initiated.

### Flow cytometric staining and analysis

For identification and quantification of Tregs, multicolor flow cytometry was used after surface staining of peripheral blood mononuclear cells with specific antibodies. These antibodies include anti-human CD4 and Foxp3. To quantify the number of Tregs after transfection of $CD4^+$ T cells and incubation under Treg skewing conditions, Tregs were identified by surface staining with anti-human CD4+ and intracellular staining with FoxP3 antibody (Biolegend, San Diego, CA, USA). The amount of Tregs was expressed as a ratio of $CD4^+Foxp3^+$ T cells as a percentage of $CD4^+$ T cells. Tregs in patients and healthy volunteers were identified after surface staining of PBMCs with monoclonal antibodies specific for anti-human CD4, CD25, and CD127 and intracellular staining with an anti-human Foxp3 antibody. CD4+CD25highCD127lowFoxp3 + cells were defined as Tregs.

### Cloning and mutagenesis of vector constructs

The psiCHECK-2 Target Expression Vector (Promega, Madison, WI, USA) was used for generation of 3′-untranslated region (3′-UTR) reporter constructs as described before [20]. Briefly, the 3′-UTR of SIRT1 containing the predicted target sites of miRNA-124a and miRNA-155 were amplified by PCR from human genomic DNA (100 ng) with the primers given in Table 3 (synthesized by Metabion, Martinsried, Germany). Cycling conditions were as follows: 95 °C for 3 min denaturing; 30 cycles of 95 °C for 30 s, 61.2 °C for 30 s, and 72 °C for 30 s; and a final extension at 72 °C for 5 min. PCR products were cloned into the *PmeI* and *XhoI* restriction sites of the psiCHECK-2 plasmid. Site-directed mutagenesis [20] of the putative miR-124a or the three miR-155 binding sites was performed using the QuickChange Lightning Mutagenesis Kit

**Table 3** Primer sequences for the 3′-UTR of SIRT1

| Primer | Sequence | Restriction site |
|---|---|---|
| SIRT1-3′UTR for | 5′-*CTCGAG*CTGTGAAACAGGAAGTAACAGACA-3′ | *XhoI* |
| SIRT1-3′UTR rev | 5′-*GTTTAAA***C**TGGCAGTAATGGTCCTAGCTG-3′ | *PmeI* |

Restriction enzymes and the cutting sides of these enzymes are italicized

(Stratagene) with the primers given in Table 4. All plasmids were verified by sequence analysis (MWG Biotech, Ebersberg, Germany).

### Cell transfections and luciferase assay

Cell transfections were performed by electroporation using the Neon™ transfection system (Invitrogen, Life Technologies, Darmstadt, Germany). $CD4^+$ T cells were transfected with 50 nM pre-miR-124a, pre-miR-155, or negative control. For luciferase assay, HEK-293 cells were co-transfected with 1 μg of psiCheck-2 dual luciferase reporter plasmids containing the 3′-UTR of SIRT1 and either pre-miR-124a, pre-miR-155, or negative control (Ambion, Austin, TX, USA) at a final concentration of 50 nM. HEK-293 cells (European Collection of Cell Cultures) were grown in Dulbecco's modified Eagle medium (DMEM—Lonza, Walkersville, MD) supplemented with 10 % heat-inactivated fetal bovine serum (FBS), 1 % penicillin/streptomycin/glutamine, and 1 % NEAA at 37 °C in a humidified atmosphere of 5 % $CO_2$ in air. Forty-eight hours after transfection, cells were lysed and analyzed for firefly and renilla luciferase activity using the Dual-Glo-Luciferase Assay System (Promega), and *Renilla* luciferase activities were normalized to *Firefly* activities. All experiments were performed in triplicates.

### Statistical analyses

All statistical analyses were performed using SigmaStat 12.0 (Systat Software, Chicago, USA). Every statistical analysis was started with testing for normal distribution using the Shapiro-Wilk Test. Further analyses were performed with Student's $t$ test for all data with normal distribution and the nonparametric Mann-Whitney rank-sum test for all data without normal distribution. Values are expressed as mean ± standard deviation (SD). $p$ values <0.05 were considered as statistically significant.

## Results

### SIRT1 mRNA expression is decreased in neuropathic pain

First, to confirm our previous findings, we determined the Foxp3/$CD4^+$ cell ratio in neuropathic pain patients as compared to healthy volunteers. As shown in Fig. 1a, neuropathic pain patients exhibited significantly elevated Foxp3/CD4+ ratios (1.6 ± 0.9 in neuropathic pain vs. 0.8 ± 0.5 in healthy controls; $p < 0.05$). Noteworthy, number of Tregs was not correlated with age (Additional file 1: Figure S1A). Next, we investigated the mRNA expression of SIRT1 in $CD4^+$ T cells obtained from patients suffering from neuropathic pain as compared to healthy volunteers. As shown in Fig. 1b, neuropathic pain patients exhibited a markedly reduced SIRT1 mRNA expression (0.5 ± 0.2 in neuropathic pain vs. 1.0 ± 0.4 in healthy controls; $p < 0.01$, Fig. 1b).

### Knockdown of SIRT1 in human $CD4^+$ T cells induces Treg differentiation in vitro

SIRT1 is known as an important negative regulator of Foxp3 expression in murine T cells. To gain insight into its functions in human T lymphocytes, we next analyzed the impact of SIRT1 knockdown in primary human $CD4^+$ T cells on Foxp3 expression and Treg differentiation. Transfection of human $CD4^+$ T cells with SIRT1 siRNA significantly reduced both SIRT1 mRNA and protein expression (mRNA 0.5 ± 0.1, $n = 6$; $p < 0.01$, Fig. 2a) as compared to normal control (NC). After incubation of transfected and stimulated cells under Treg skewing conditions for 4 days, an increase of Foxp3 mRNA expression by approximately 30 % was found (1.3 ± 0.1, $n = 3$; $p < 0.01$, Fig. 2b). Accordingly, Treg differentiation was clearly enhanced (1.7 ± 0.4, $n = 3$; $p < 0.01$, Fig. 2c). These findings imply that a decreased

**Table 4** Primers for mutagenesis

| Primer | Sequence | Position |
|---|---|---|
| SIRT mut 124a for | 5′-TATTTAAAAGCTTAGCCTGG*A*TTAAAACTAGAGATCAACTTTCTCAGA-3′ | 1211–1217 |
| SIRT mut 124a rev | 5′-GCTGAGAAAGTTGATCTCTAGTTTTAA*T*CCAGGCTAAGCTTTTAAATA-3′ | |
| SIRT mut 155_1 for | 5′-CAGGAATTGTTCCACCAG*GG*TTAGGAACTTTAGCATGTC-3′ | 36–42 |
| SIRT mut 155_1 rev | 5′-GACATGCTAAAGTTCCTAA*C*CCTGGTGGAACAATTCCTG-3′ | |
| SIRT mut 155_2 for | 5′-TTGATCTTTTCCACAAG*GG*TTAAACTGCCAAAATGTG-3′ | 929–935 |
| SIRT mut 155_2 rev | 5′-CACATTTTGGCAGTTTAA*C*CCTTGTGGAAAAGATCAA-3′ | |
| SIRT mut 155_3 for | 5′-GAAATTGCACAGTAAG*GG*TTTATTTTTCAGACCATT-3′ | 1408–1414 |
| SIRT mut 155_3 rev | 5′-AATGGTCTGAAAAATAAA*C*CCTTACTGTGCAATTTC-3′ | |

Sequenzes of the mutagenesis are italicized

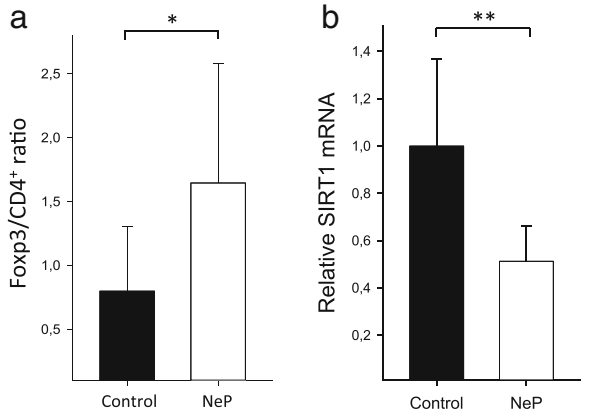


**Fig. 1** SIRT1 is down-regulated in $CD4^+$ T cells of patients with neuropathic pain. **a** T cells obtained from patients with neuropathic pain were characterized by surface staining with monoclonal antibodies specific for anti-human CD4, CD25, and CD127 and intracellular staining with an anti-human Foxp3 antibody followed by flow cytometric analysis. The Foxp3/$CD4^+$ ratio found in neuropathic pain patients (NeP, $n = 11$) and in healthy controls (control, $n = 9$) is shown. **b** Total RNA was extracted from purified $CD4^+$ T cells of patients with neuropathic pain (NeP, $n = 11$) as well as from $CD4^+$ T cells of healthy volunteers (control, $n = 9$). Relative expression of SIRT1 was quantified by quantitative PCR (qPCR) using SDHA and TBP as reference genes. The results indicate fold reduction of SIRT1 in patients with neuropathic pain vs. healthy volunteers. Data are given as means ± SD; $*p < 0.05$, $**p < 0.01$

SIRT1 expression may significantly contribute to the increase of Treg cells in neuropathic pain.

### miR-124a and miR-155 are potential candidates of SIRT1 regulation in neuropathic pain

We next hypothesized that regulation by specific miRNAs may influence SIRT1 expression and may thus account for the observed alterations of SIRT1 mRNA expression in neuropathic pain. To this end, we combined results of published microarray data in patients with chronic pain syndromes with target prediction in silico. These analyses revealed miR-124a and miR-155 as potential candidates involved in the regulation of SIRT1 in neuropathic pain. Target prediction tools suggested three specific binding sites for miR-155 and a single specific binding side for miR-124a with high probability within the 3′-UTR of the SIRT1 transcript (Fig. 3a).

### SIRT1 expression is directly regulated by miR-124a and miR-155

To provide an experimental proof of a direct interaction between miR-124a and/or miR-155 with the SIRT1 3′-UTR, we performed luciferase reporter assay on a psiCheck-2 plasmid containing a Renilla luciferase gene upstream of the SIRT1 3′-UTR. HEK293 cells were transiently co-transfected with the reporter vector construct and either pre-miR-124a or pre-miR-155 or NC, and luciferase activity was measured. As shown in Fig. 3b, reporter activity was significantly reduced by both miRNAs (miR-124a 72 ± 7 %, miR-155 68 ± 13 %, $n = 8$; $p < 0.01$), as compared to NC.

Site-directed mutagenesis of either the miR-124a or the three miR-155 binding sites within the 3′-UTR of SIRT1 strongly diminished the inhibitory effect of the respective miRNA (Fig. 3c). These data demonstrate that both miRNAs regulate SIRT1 expression by direct targeting of specific binding sites within the 3′-UTR of SIRT1.

Next, we validated the impact of miR-124a and miR-155 on the expression of SIRT1. We assessed SIRT1 mRNA levels after transfection of human $CD4^+$ T cells with either miR-124a or miR-155 mimics or with negative control. As depicted in Fig. 3d, SIRT1 mRNA (miR-124a 0.75 ± 0.1, miR-155 0.72 ± 0.1; $n = 6$; $p < 0.01$) and protein expression significantly decreased after transient transfection of both miRNAs as compared to control.

Taken together, we provide evidence that SIRT1 mRNA expression in primary human T cells is directly regulated by miR-124a and miR-155.

### miR-124a and miR-155 control SIRT expression in neuropathic pain

We next determined the expression of miR-124a and miR-155 in $CD4^+$ T cells obtained from patients with neuropathic pain and from healthy volunteers. Expression of both miRNAs was significantly higher in patients with neuropathic pain as compared to healthy volunteers (miR-124 2.5 ± 0.7, $p < 0.05$, Fig. 2a, miR-155 1.3 ± 0.3; $p < 0.05$, Fig. 4a). Correlation analyses in human $CD4^+$ T cells revealed for both miRNAs a significant inverse correlation with SIRT1 transcript levels (miR-124a: $r = -0.75$, $p < 0.001$, $n = 20$, miR-155: $r = -0.6$, $p = 0.006$, $n = 20$, Fig. 4b, c), which strongly points to an important role of both miRNAs as regulators of SIRT1 in vivo. There was no significant correlation between age and either miR-124a, miR-155, or SIRT1 mRNA expression (Additional file 1: Figure S1B-D).

### miR-124a and miR-155 increase Treg differentiation

To investigate the impact of both miRNAs on Treg differentiation, we transfected human $CD4^+$ T cells with either pre-miR-124a or pre-miR-155 followed by culturing under Treg skewing conditions for 4 days. As shown in Fig. 5a, Foxp3 mRNA expression was significantly increased in miRNA-transfected cells as compared to controls (miR-124a 1.5 ± 0.4; $n = 6$; $p < 0.01$; miR-155 1.5 ± 0.4; $n = 6$; $p < 0.01$; Fig. 5a). Additionally, expression of the Treg signature molecules EOS, CTLA4, and IL2RA [21] was also elevated (Additional file 1: Figure S2). Accordingly, an enhancement of Treg

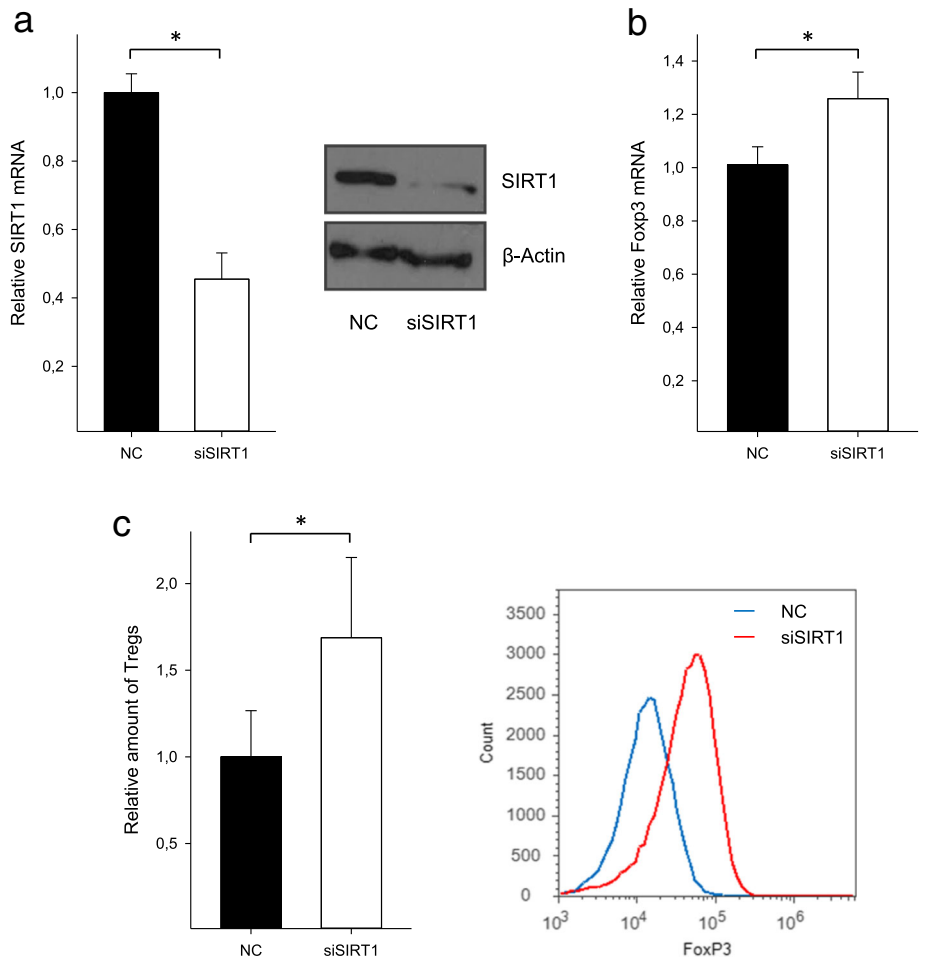


**Fig. 2** SIRT1 is a negative regulator of Foxp3 expression in human T cells. SIRT1 knockdown in primary human $CD4^+$ T cells of healthy volunteers was performed by specific siRNA, and Foxp3 mRNA expression and Treg differentiation was analyzed after incubation under Treg skewing conditions for 4 days. **a** Successful transfection of siRNA was confirmed by qPCR (*left panel*) and Western Blot analysis (*right panel*). One blot is representative of $n = 3$. **b** Relative Foxp3 mRNA expression after transfection and Treg differentiation as measured by qPCR. **c** Treg subpopulation as determined by FACS analysis (*left panel*), a histogram representative of $n = 6$ individual experiments performed in duplicates is shown in the right panel. Data are given as means ± SD; $^*p < 0.01$, $n = 6$

differentiation was found (miR-124a 1.5 ± 0.4; $n = 6$; $p < 0.01$; miR-155 1.6 ± 0.4; $n = 6$; $p < 0.01$; Fig. 5b, c). In accordance with these in vitro findings, we found a significant correlation between both miR-124a and miR-155 expression and Foxp3 mRNA expression in human $CD4^+$ T cells (Additional file 1: Figure S3). These findings demonstrate an impact of miR-124a and miR-155 on Treg differentiation via targeting of SIRT1.

## Discussion

The pathophysiology of neuropathic pain is not fully understood. Recent studies have established proof that aberrant responses of the adaptive immune system substantially contribute to the development of this clinical disorder. Underlying mechanisms, however, are largely unknown. In this study, we show an involvement of miRNAs in the regulation of inflammatory processes in neuropathic pain. We identify miR-124a and miR-155 as direct repressors of the deacetylase SIRT1. Targeting of SIRT1 by these miRNAs results in an increase of Foxp3 expression and, consecutively, of anti-inflammatory Tregs. We here show that in patients suffering from neuropathic pain as compared to healthy volunteers, an increased expression of miR-124a and miR-155 inhibits SIRT1 expression, which enhances $CD4^+$ T cell differentiation towards Tregs.

Peripheral nerve injury leads to the release of factors that recruit and activate immune cells from the circulation. These cells secrete pro-inflammatory mediators that contribute to the development of pain symptoms. In particular, the T cell response is considered an important contributor to the development of neuropathic

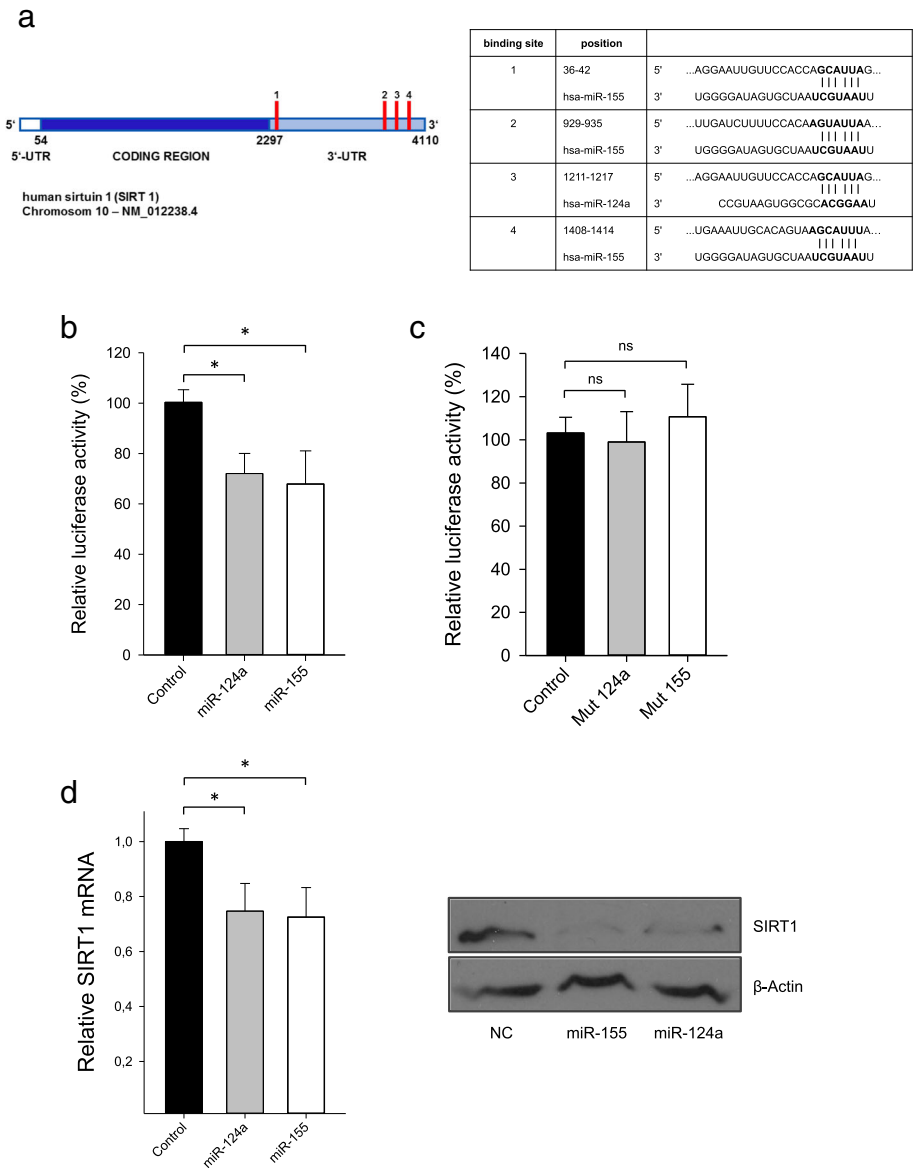


**Fig. 3** SIRT1 is a novel miR-124a and miR-155 target. Depiction of the genomic structure of the human SIRT1 gene on chromosome 10 and location of the putative miR-124a and miR-155 binding sites within its 3′-UTR (**a**). Target prediction algorithm identified a putative miR-124a binding site and three putative miR-155 binding sites, indicated by the *red bars*. Positions and seed sequences of the putative binding sites are listed in the adjacent table (**a**). A reporter vector containing the SIRT1 3′-UTR was co-transfected with pre-miR-124a or pre-miR-155 into HEK-293 cells, and hRLuc reporter activity was determined relative to a vector construct containing the SIRT1 3′-UTR co-transfected with pre-miR-scrambled control (**b**). Control constructs lacking either the miR-124a (Mut 124a) or the miR-155 binding sites (Mut 155) were generated by site-directed mutagenesis. Both mutant vectors were co-transfected with the respective miRNA or with scrambled control into HEK-293 cells, and hRLuc reporter activity was determined; luciferase activity relative to scrambled control is given. Data are means ± SD; *ns* not significant, $*p < 0.01$, $n = 8$. **c** $CD4^+$ T cells of healthy donors were transiently transfected with miR-124a, miR-155, or scrambled control, respectively, and stimulated with anti-CD3/CD28 Dynabeads for 36 h. Relative SIRT1 mRNA was detected by qPCR, $n = 6$, $*p < 0{,}01$ (**d**, *left panel*), and SIRT1 protein expression was determined by Western Blot analysis (**d**, *right panel*). One blot is representative of $n = 3$

pain. In animal models of peripheral nerve injury, pain sensitivity of T cell deficient animals was significantly attenuated, which could be restored by adoptive transfer of pro-inflammatory cytokine producing Th1 cells [8]. On the other hand, expansion of Tregs, which limit immune responses of pro-inflammatory T cells, led to a significant reduction of pain hypersensitivity [22] while depletion of Tregs promoted pain hypersensitivity by inducing altered systemic concentrations of cytokines in mice [6]. The latter findings point towards a possible role of Tregs in the limitation of pain promoting inflammatory responses. In a very recently published

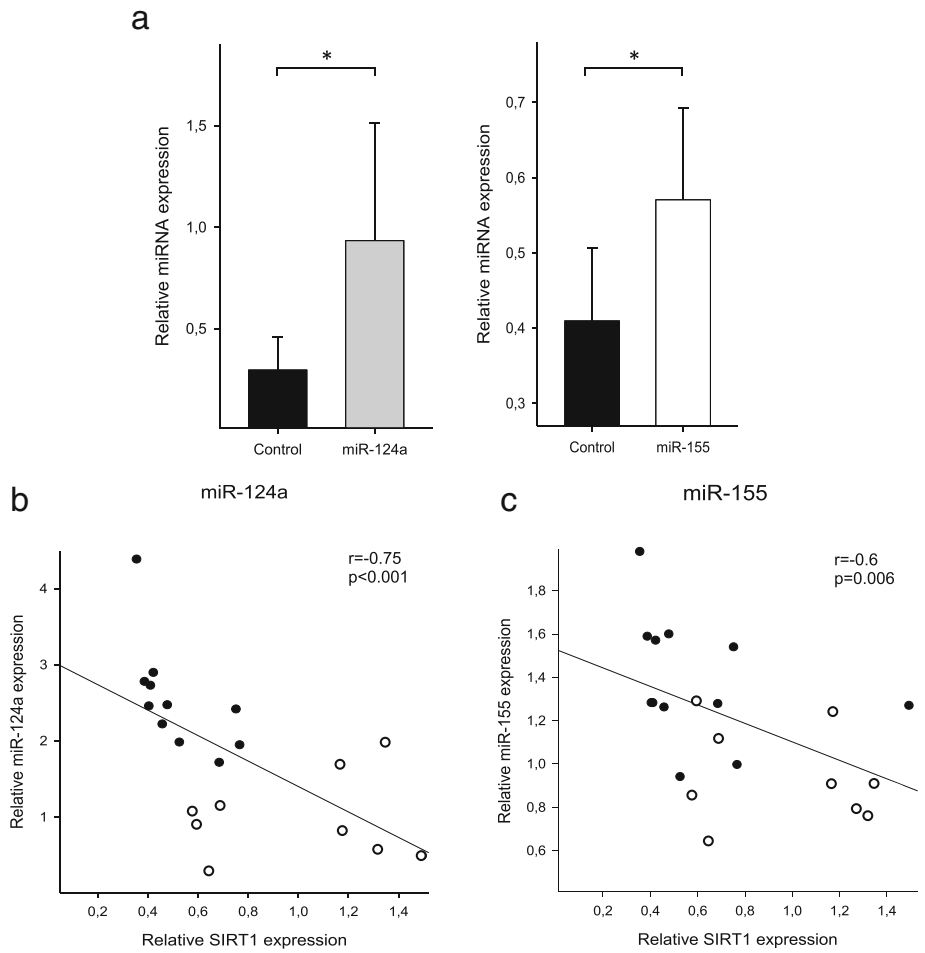


**Fig. 4** miR-124a and miR-155 are upregulated in $CD4^+$ T cells of patients with neuropathic pain. **a** Total RNA was extracted from purified $CD4^+$ T cells of patients suffering from neuropathic pain ($n = 11$) as well as from those of healthy volunteers ($n = 9$, control). Relative expression of endogenous miR-124a and miR-155 was quantified by qPCR using U47 RNA as normalizing control. Expression of miR-124a (**a**, left panel) and miR-155 (**a**, right panel) in patients with neuropathic pain as compared to healthy volunteers is shown. Data are given as means ± SD; $^*p < 0.05$. Correlation of miR-124a expression (**b**) and miR-155 expression (**c**) and SIRT1 transcript levels in human $CD4^+$ T cells

study, we reported an increase of the Treg subpopulation in the peripheral blood of patients suffering from neuropathic pain, which also points into that direction. The pathways leading to the observed Treg induction, however, have not been addressed yet.

We here suggest a decreased expression of the histone-deacetylase SIRT1 as a possible underlying mechanism. SIRT1 is known to control Treg differentiation and function (i) by promoting Foxp3 gene expression and (ii) by Foxp3 lysine ε-aminodeacetylation leading to ubiquitination and proteasomal degradation. Here, we show that targeting of SIRT1 by specific siRNA promotes Treg differentiation of human $CD4^+$ T cells in vitro. Similar results have been found in a recently published study, which reported an increased differentiation of naive T cells to Tregs after treatment with SIRT1 inhibitors in mice. Our findings strongly suggest that a decrease of SIRT1 expression contributes to the observed increase of Treg cells in neuropathic pain patients.

SIRT1 is subject to regulation on a transcriptional and posttranscriptional level [23, 24]. Particularly in tumors and endothelial cells, miRNAs have been shown to influence SIRT1 expression (e.g., miR-29c [23], miR-141 [23], miR-200 [24], miR-204 [25]). Based on the assumption that alterations of miRNA profiles might also be involved in the regulation of SIRT1 in neuropathic pain, we focused on miRNAs as potential suppressors of SIRT1 in this context. We identified miRNA-124a and miRNA-155 as potential candidates binding to the SIRT1-3′-UTR with high probability in silico. miR-155 is expressed in multiple types of immune cells and has been proposed to affect a wide range of immunological

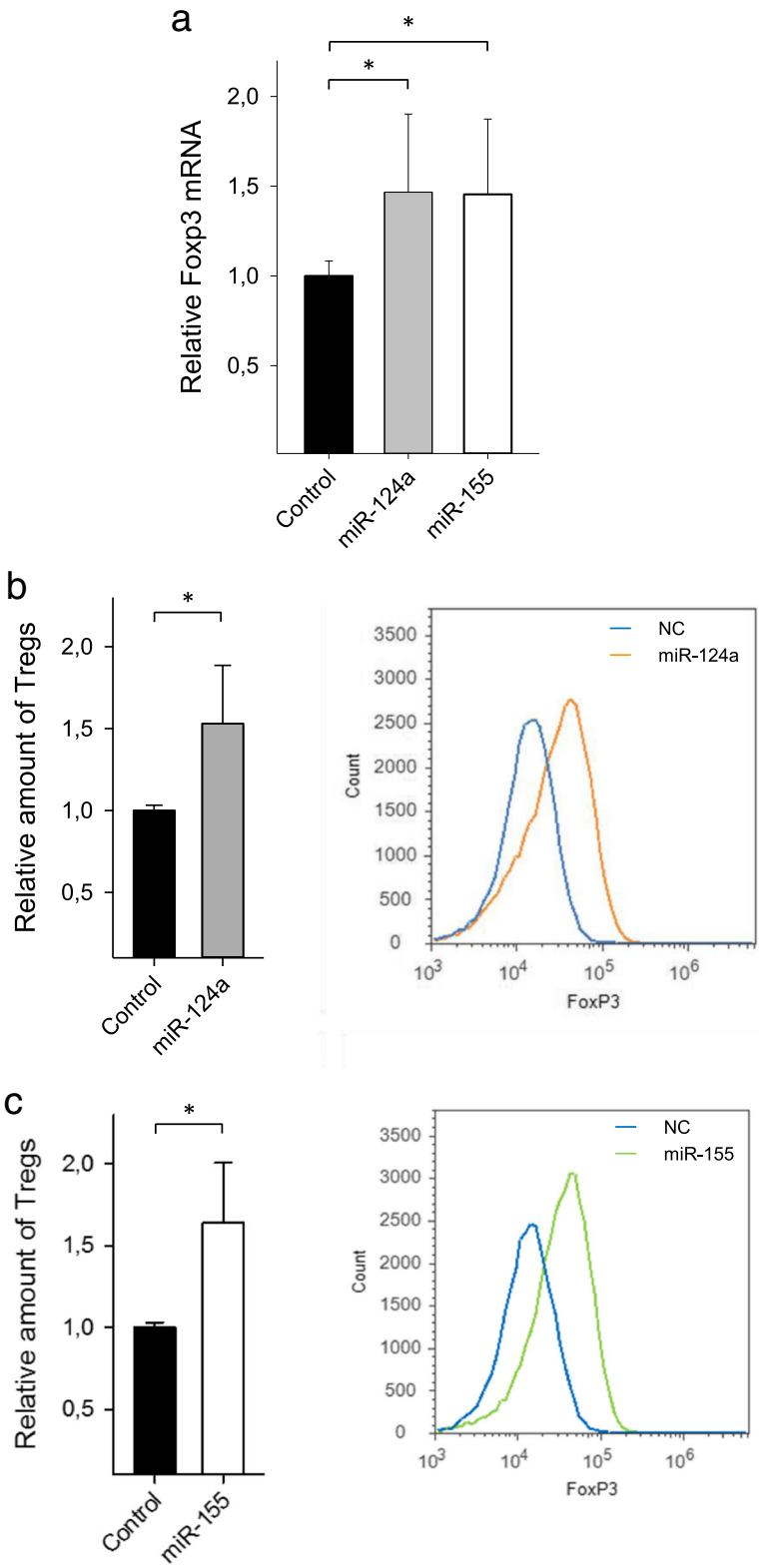


**Fig. 5** miR-124a and miR-155 enhance Treg differentiation. Human $CD4^+$ T cells were transfected with either pre-miR-124a, pre-miR-155, or scrambled control, followed by culturing under Treg skewing conditions for 4 days. Relative Foxp3 mRNA expression was detected by qPCR (**a**). The number of Tregs was determined by FACS analysis (**b**, **c**). Additionally, representative histograms of the FACS analysis are shown in **b** and **c** (*right panels*). Data are given as means ± SD; *$p < 0.01$, $n = 6$

processes under physiologic conditions as well as in the course of immune responses [26–31]. In mice studies, it has been shown that the expression of miR-155 in Treg cells is required to maintain normal Treg numbers and function, which was in part attributed to miR-155-mediated SOCS1 repression [32, 33]. In a rat model of neuropathic pain, inhibition of miR-155 was shown to reduce cytokine production of microglial cells via SOCS1 repression, thereby attenuating pain symptoms [34].

miR-124a is predominantly expressed in the central nervous system (CNS). There, it displays specific temporal and spatial expression profiles in various cell types and affects a variety of biological functions. Dysregulation of miR-124 has been linked to several pathologic conditions of the CNS, such as brain tumors, neurodegeneration, epilepsy, and neuroimmune disorders. Furthermore, miR-124a is involved in macrophage polarization, which impacts a variety of diseases. For example, in animal models of pain, intrathecal application of miR-124a resulted in a decrease of pro-inflammatory cytokines secreted by microglia/macrophages, which led to a reduction of persistent hyperalgesia [35, 36].

Roads of miRNA regulation are redundant and highly dependent on the cellular and physiological context. Here, we reveal a new function of miR-124a and miR-155 in T cells in neuropathic pain: Our experiments show that both miRNAs suppress SIRT1 mRNA expression by direct targeting of specific binding sites. Accordingly, overexpression of miR-124a and miR-155 in human $CD4^+$ T cells in vitro suppressed SIRT1 and, in accordance with our in vitro results obtained by transfection of SIRT1 siRNA, induced a bias towards Treg differentiation.

Clinical data also support this hypothesis: In T cells of neuropathic pain patients, we detected an increased expression of miR-124a and miR-155. Moreover, the expression of these two miRNAs was inversely correlated with SIRT1 transcript levels, which strongly supports the hypothesis that the Treg shift observed in neuropathic pain, indeed, is at least partially driven by a miRNA-mediated mechanism.

## Conclusions

Increasing peripheral Treg numbers may be an endogenous attempt to limit inflammation, thus reducing pain levels in neuropathic pain. We here demonstrate that lymphocytic miRNAs significantly contribute to these adaptive processes. Deciphering miRNA-target interactions that influence inflammatory pathways in neuropathic pain may thus help to develop new approaches of pain amelioration.

## Additional file

**Additional file 1: Figure S1.** Correlation analysis of age and (A) Treg numbers, (B) SIRT1 mRNA, (C) miRNA-124 expression, and (D) miR-155 expression. Analyses revealed no significant correlations. Black dots: Neuropathic pain patients, white dots: Healthy volunteers. Figure S2 Human CD4 + T cells were transfected with either pre-miR-124a, pre-miR-155, or scrambled control, followed by culturing under Treg skewing conditions for 4 days. Relative mRNA expression the Treg signature molecules EOS, CTLA4, and IL2RA was detected by qPCR; $*p < 0.05$, $**p < 0.01$, $n = 5$. Figure S3. Correlation analysis of either miR-124a (A) or miR-155 (B) and Foxp3 mRNA expression. Black dots: Neuropathic pain patients, white dots: Healthy volunteers. (PPTX 227 kb)

### Abbreviations

Tregs: Regulatory T cells; SIRT1: Histone deacetylase sirtuin1; Foxp3: Transcription factor forkheadbox-p3; NeP: Neuropathic pain; CRP: C-reactive protein; NRS: Numerical rating scale; PBMCs: Peripheral blood mononuclear cells; qPCR: Quantitative real-time PCR (RT-PCR); FACS: Fluorescent-activated cell sorting; KAB: Kurzfragebogen zur aktuellen Beanspruchung; SDHA: Succinate dehydrogenase complex subunit A; TBP: TATA box binding protein; SD: Standard deviation; CNS: Central nervous system; BMI: Body mass index

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### Availability of data and materials

All material used in this manuscript will be made available to researchers subject to confidentiality.

### Authors' contributions

JH, BL, and MH performed the experiments; JH, BL, LCH, and SK wrote the manuscript; BL and SCA recruited the patients and prepared the blood samples; all authors analyzed the data; JH, BL, and SK designed the experiments. All authors read and approved the final manuscript.

### Competing interests

The authors declare that they have no competing interests.

### Consent for publication

Not applicable.

### Ethics approval and consent to participate

The prospective study protocol followed the principles of the Declaration of Helsinki and was reviewed and approved by the Ethics Committee of the LMU Munich (ethical approval number: 331–10) and registered on German Clinical Trial Register (Registration Trial DRKS00005954).

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Journal of Neuroinflammation

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# Differential expression of P2X7 receptor and IL-1β in nociceptive and neuropathic pain

Benjamin Luchting†, Jens Heyn*†, Tobias Woehrle†, Banafscheh Rachinger-Adam, Simone Kreth, Ludwig C Hinske and Shahnaz C Azad

## Abstract

**Background:** Despite substantial progress, pathogenesis and therapy of chronic pain are still the focus of many investigations. The ATP-gated P2X7 receptor (P2X7R) has previously been shown to play a central role in animal models of nociceptive inflammatory and neuropathic pain. Recently, we found that the adaptive immune system is involved in the pathophysiology of chronic nociceptive and neuropathic pain in humans. So far, data regarding P2X7R expression patterns on cells of the adaptive immune system of pain patients are scarce. We therefore analyzed the P2X7R expression on peripheral blood lymphocytes and monocytes, as well as serum levels of IL-1β in patients suffering from chronic nociceptive and neuropathic pain in comparison to healthy volunteers in order to identify individuals who might benefit from a P2X7R modulating therapy.

**Methods:** P2X7R messenger RNA (mRNA) and protein expression were determined in patients with either chronic nociceptive low back pain (CLBP) or neuropathic pain (NeP), and in healthy volunteers by quantitative real-time PCR (qPCR) and by fluorescence-assisted cell-sorting (FACS), respectively. IL-1β serum levels were measured with a multiplex cytokine assay.

**Results:** Compared to healthy volunteers, P2X7R mRNA (1.6-fold, $p = 0.038$) and protein levels were significantly increased on monocytes (NeP: $24.6 \pm 6.2$, healthy volunteers: $17.0 \pm 5.4$; $p = 0.002$) and lymphocytes (NeP: $21.8 \pm 6.5$, healthy volunteers: $15.6 \pm 5.2$; $p = 0.009$) of patients with NeP, but not in patients with CLBP. Similarly, IL-1β serum concentrations were significantly elevated only in NeP patients (1.4-fold, $p = 0.04$).

**Conclusions:** A significant upregulation of P2X7R and increased IL-1β release seems to be a particular phenomenon in patients with NeP. P2X7R inhibitors may therefore represent a potential option for the treatment of this frequently intractable type of pain.
German Clinical Trial Register (DRKS): Registration Trial DRKS00005954.

**Keywords:** Neuropathic pain, Chronic low back pain, T cells, TH17, Treg, Il-1β, Neuroinflammation

## Background

Purinergic P2X receptors (P2XRs) are ATP-gated cation channels, divided into seven subtypes (P2XR1-P2XR7). They are predominantly expressed on cells of the hematopoietic lineage including macrophages, microglia, and lymphocyte subtypes [1].

The subtype receptor P2X7R has been found to play a major role in central nervous system (CNS) processes, including neurodegeneration, traumatic brain injuries, psychiatric disorders, and pain. The exceptional role of P2X7R in pain syndromes has been shown in various animal models of nociceptive inflammatory and neuropathic pain [2–5]. In humans, P2X7R messenger RNA (mRNA) expression was shown to be increased in leukocytes of patients with chronic fatigue syndrome [6]. Results of experimental P2X7R inhibition in a rat model of inflammatory arthritis, induced by injection of Freund's

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complete adjuvant into one hind paw, were promising [7]. Furthermore, P2X7R antagonists have been tested in clinical trials in patients with rheumatoid arthritis [8, 9]. P2X7R activation leads to a rapid increase in intracellular calcium concentrations and triggers the release of the pro-inflammatory and pro-nociceptive cytokine IL-1β [10]. IL-1β is known to be a key mediator in neurodegeneration, chronic inflammation, and chronic pain by affecting neuronal cell death [1, 10]. Pharmacological inhibition of IL-1β attenuates hyperalgesia induced by spinal cord inflammation in rats [11]. Moreover, higher serum levels of IL-1β have been implicated in the pathogenesis of depression [12, 13]. Accordingly, P2X7R inhibitors displayed an antidepressant activity in mice [14]. P2X7R has been found to be readily expressed on the cell surface of both microglia and immune cells, suggesting a link between the CNS and the immune system [1, 15]. Since the adaptive immune system is critically involved in the pathophysiology of chronic pain [16], we systematically investigated the expression of P2X7R on lymphocytes and monocytes of patients suffering from either chronic low back pain (CLBP) or chronic neuropathic pain (NeP) in comparison to healthy volunteers. We analyzed P2X7R protein by flow cytometry (FACS) and determined P2RX7 mRNA expression by real-time PCR (qPCR). Furthermore, we analyzed the expression of the cytokine IL-1β.

## Methods

### Ethics statement

The study followed the principles of the Declaration of Helsinki, was approved by the Ethics Committee of the LMU Munich, and has been registered by the German Clinical Trial Register (Registration Trial DRKS00005954).

### Subjects

Subject recruitment was estimated for 2 years. Patients presenting to our Department of Pain Medicine with chronic pain who met the inclusion criteria, as well as healthy volunteers, were enrolled after written informed consent.

### Inclusion criteria

Patients suffering from chronic non-specific low back pain (CLBP) without any signs of neuropathic pain components and patients suffering from neuropathic pain (NeP) were included. CLBP was defined as persistent low back pain not attributable to a detectable pathology (e.g. infection, tumor, osteoporosis, trauma, inflammatory disorder, or radicular syndrome). NeP was diagnosed according to its international definition ("pain caused by a lesion or disease of the somatosensory nervous system"). All patients were assessed by detailed pain history, physical examination, and the PainDETECT-questionnaire. This questionnaire consists of several items related to neuropathic pain symptoms (burning sensations, tingling or prickling sensations, shooting/lancinating, hyperalgesia, dysesthesia, allodynia, or paresthesia) with excellent sensitivity (85 %) and specificity (80 %) [17]. Healthy volunteers were evaluated for any history of pain. If no history of pain was detected in these individuals, they were included in the study.

### Exclusion criteria

Exclusion criteria were autoimmune, chronic systemic, inflammatory, neoplastic, or psychiatric diseases, drug abuse, and pregnancy. Patients on irregular medication with opioids, non-opioids, or co-analgesics were excluded. None of the patients had been treated with corticosteroids or had received immunomodulatory agents before or during the study. Any acute inflammatory process was ruled out by laboratory testing including serum concentration of C-reactive protein (CRP) and total and differential leukocyte count as well as measurement of the body temperature. Patients with mixed pain (nociceptive and neuropathic components), e.g., low back pain with radiculopathy, were excluded.

### Assessment of pain, stress, and depressive symptomatology

Patients rated their recalled average pain intensity both at rest and while moving using an 11-point numerical rating scale (NRS): 0 meaning "no pain" and 10 meaning "worst pain imaginable." Self-perceived stress was evaluated using the German version of the Questionnaire for Actual Demands ("KAB": "Kurzfragebogen zur aktuellen Beanspruchung") in patients and in healthy volunteers. The KAB was designed to repeatedly quantify the individual's level of acute or chronic stress. It is highly sensitive to short-term or situational changes during a stressful experience [18]. The rating on a scale ranging from 1 to 6 is based on normalized adjectives. Higher KAB values indicate the perception of increased stress levels. The center for epidemiologic studies depression scale (CES-D scale) which measures depressive symptomatology in the general population was used to assess depressive symptomatology in patients and in healthy volunteers. This scale contains 20 items to explore feelings or experiences during the past week. These 20 items belong to 4 main categories: depressed affect, positive affect, somatic complaints/retarded activity, and interpersonal experiences. Response options range from 0 to 3 for each item (0 = rarely or never, 1 = some or little of the time, 2 = moderately or much of the time, 3 = most or almost all of the time). Results range from 0 to 60, with higher values representing more depressive symptoms. Values >25 are considered pathological [19].

### Leukocyte count and cytokine assessment

Peripheral blood was collected from patients and healthy volunteers between 9:00 and 9:30 AM. Samples were assessed for differential leukocyte count by routine laboratory testing. For cytokine assessment, blood was centrifuged at 2000×*g* for 10 min to obtain cell-free serum. After centrifugation, supernatants were harvested and frozen at −80 °C until further use. IL-1β serum concentrations were determined using a human cytokine immunoassay (Myriad Rules-Based Medicine Inc., Austin, Texas, USA). The microbead assay is based on a Luminex technology and quantifies protein in a similar manner to standard sandwich ELISA techniques, with comparable sensitivity and range [20].

### Flow cytometric staining and analysis

Peripheral blood mononuclear cells (PBMCs) from heparinized venous blood samples were separated by Ficoll density gradient centrifugation (Sigma Aldrich, Taufkirchen, Germany). PBMCs were then cryopreserved in RPMI freezing media containing 10 % FCS and 10 % DMSO, frozen at −30 °C for 24 h, and then stored at −196 °C [21]. For FACS analyses, samples were thawed rapidly and washed twice with ice-cold FACS buffer (HBSS containing 1 % BSA and 0.1 % $NaN_3$) to eliminate any remaining DMSO. For extracellular staining, cells were co-incubated with PerCP-labeled antihuman CD4 antibody (1:50, Biolegend, San Diego, CA, USA) and FITC-labeled antihuman P2X7R antibody (1:100, Alomone Labs, Jerusalem, Israel) at room temperature for 1 hour. Again, cells were washed twice with FACS buffer (400×*g*, 5 min, 4 °C) to remove excessive antibodies. P2X7R expression was then analyzed with an Attune Acoustic Focusing Cytometer (Life Technologies, Carlsbad, USA) as described by Gudipaty et al. [22]. Representative density plots and gating strategy are displayed in Fig. 1; representative histograms for evaluation of $CD4^+/CD4^-$ cells and for analyses of mean fluorescence intensity (MFI) are shown in Fig. 2.

### Quantitative RT-PCR

$CD4^+$ cells were isolated from PBMCs by magnetic bead separation with the Whole Blood CD4 MicroBeads kit (MACS Miltenyi Biotec, Auburn, CA, USA) according to the manufacturer's recommendations. Subsequently, total RNA was isolated with the mirVana miRNA Isolation Kit, followed by a DNase digest with the Turbo DNA-free kit (Ambion). Quantity and purity of the isolated RNA were measured with a NanoDrop ND-1000 spectrophotometer (Peqlab). After the amplification of total RNA using the TargetAmp 1-Round aRNA Amplification Kit (Epicentre Biotechnologies, Madison, WI, USA) and purification using an RNeasy Mini Kit (Qiagen), cDNA synthesis was performed with the SuperScript III First Strand Synthesis System (Invitrogen) and oligo-dT and random hexamer primers (Qiagen). Quantitative RT-PCR was performed in duplicates with a LightCycler 480 instrument (Roche Diagnostics, Mannheim, Germany) using LightCycler 480 Probes Master and RealTime Ready Single Assays (Roche Diagnostics). Cycling conditions were as follows: 95 °C for 10 min, 45 cycles at 95 °C for 10 s, 60 °C for 30 s, and 72 °C for 15 s. Relative mRNA expression of P2RX7 was calculated by the Relative Quantification Software (Roche Diagnostics) using an efficiency-corrected algorithm with standard curves and reference gene normalization against the reference genes succinate dehydrogenase complex subunit A (SDHA) and TATA box binding protein (TBP) as described previously [23]. Primer sequences and assay characteristics are given in Table 1.

### Data analyses

Statistical analyses were performed with SigmaStat 12.0 (Systat Software, Chicago, USA). Every statistical analysis was started with testing for normal distribution using the Shapiro Wilk Test. Differences between groups were

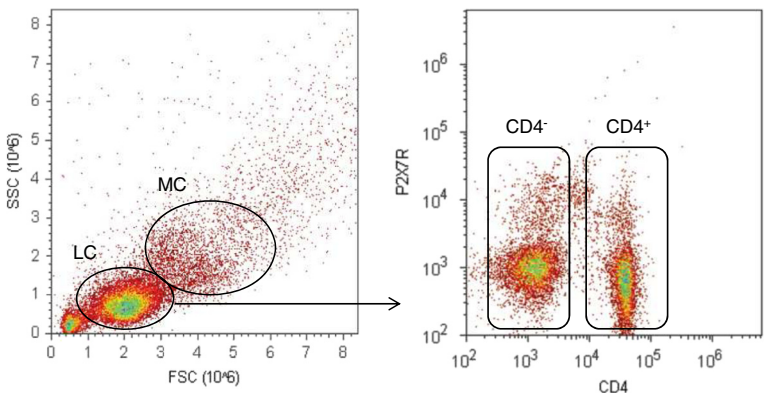


**Fig. 1** Gating strategy for the detection of lymphocytes, monocytes, $CD4^+$ and $CD4^-$ cells, lymphocytes (LC), and monocytes (MC) was gated according to forward scatter (FSC) and side scatter (SSC) characteristics; PerCP-labeled antihuman CD4 antibody was used to separate $CD4^+/CD4^-$ cells

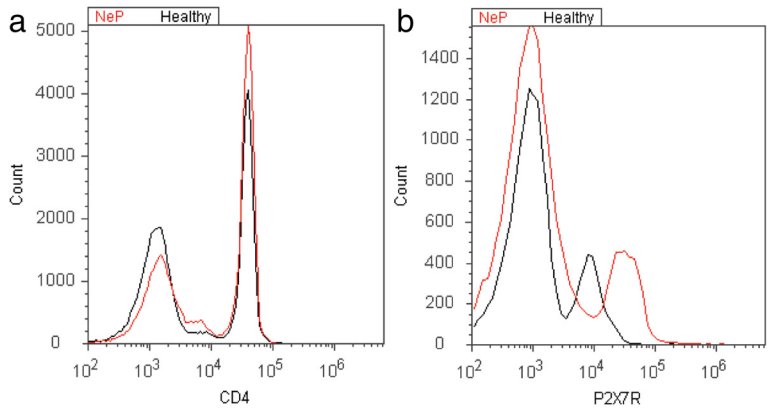


**Fig. 2** Exemplary illustration of P2X7 receptor expression. For quantitative analysis of the proportion of $CD4^+$ cells (**a**) and P2X7R protein expression (**b**), cells were stained with PerCP-labeled antihuman CD4 antibody and FITC-labeled antihuman P2X7R antibody. We separately analyzed each subgroup by the mean fluorescence intensity (MFI) of P2X7R expression. Overlay histograms of representative results of P2X7R expression of one patient/healthy volunteer are displayed in histograms

tested with the *t* test for results with normal distribution and the nonparametric Mann-Whitney Rank Sum Test for all data without normal distribution. Discrete variables were compared with the Fisher's exact test. In order to determine significant differences between pain syndromes, we used a one-way ANOVA tests and multiple comparisons versus a control group (Holm-Sidak method). *p* values <0.05 were considered statistically significant. All results are expressed as mean ± standard deviation (SD).

## Results

### Subjects

Within 2 years of recruitment, 19 patients suffering from CLBP and 19 patients suffering from NeP who met the inclusion criteria as well as 19 pain-free volunteers were enrolled. As shown in Table 2, both groups of patients significantly differed from healthy volunteers in terms of stress level and depressive symptomatology. No significant differences were detected between the patient groups regarding pain levels at rest and during motion, as well as duration of pain (Table 2). Furthermore, statistical analysis of the number of patients receiving analgesic and coanalgesic medication revealed no significant difference between the two groups (Table 3).

**Table 1** RT-PCR assay characteristics and primer sequences

| Gene | Primer sequence |
|---|---|
| TBP-87 | for 5′ GAACATCATGGATCAGAACAACA 3′ |
| | rev 5′ ATAGGGATTCCGGGAGTCAT 3′ |
| SDHA-132 | for 5′ GAGGCAGGGTTTAATACAGCA 3′ |
| | rev 5′ CCAGTTGTCCTCCTCCATGT 3′ |
| P2RX7 | Roche RealTime Ready Single Assay ID 106724 |

### Differential blood count and quantification of $CD4^+$ cells

We quantified the number of neutrophil granulocytes, representing an essential part of the innate immune system, as well as total lymphocytes and $CD4^+$ T cells as key players of the adaptive immune response. As shown in Fig. 3, numbers of polymorphonuclear leukocytes (CLBP: 57.1 ± 8.7 %, NeP: 58.4 ± 9.1 %, healthy volunteers: 55.2 ± 9.0; n.s.), total lymphocytes (CLBP: 33.2 ± 6.9 %, NeP: 29.9 ± 7.6 %, healthy volunteers: 34.4 ± 7.2; n.s.), and $CD4^+$ T cells (CLBP: 44.1 ± 11.4 %, NeP: 41.7 ± 11.3 %, healthy volunteers: 44.5 ± 10.5; n.s.) did not differ between patients suffering from CLBP, NeP, or healthy volunteers.

**Table 2** Patients characteristics

| | Healthy | CLBP | NeP |
|---|---|---|---|
| Numbers | 19 | 19 | 19 |
| Age (years) | 40 ± 11 | 47 ± 13 | 58 ± 13* |
| Female (%) | 58 % | 79 % | 68 % |
| BMI (kg/m$^2$) | 23.6 ± 2.9 | 23.9 ± 3.1 | 24.6 ± 3.8 |
| Duration of pain (years) | 0.0 ± 0.0 | 5.9 ± 4.2* | 4.5 ± 2.8* |
| NRS (rest) | 0.0 ± 0.0 | 3.5 ± 2.2* | 5.1 ± 2.2* |
| NRS (motion) | 0.0 ± 0.0 | 4.4 ± 2.1* | 6.9 ± 2.1* |
| KAB | 1.7 ± 0.7 | 3.4 ± 0.9* | 3.3 ± 0.9* |
| CES-D | 2.4 ± 2.2 | 21.8 ± 7.3* | 21.0 ± 9.6* |

Patients with either CLBP or NeP significantly differed from healthy volunteers in terms of duration of pain, NRS, KAB, and CES-D. However, no significant differences were detected between CLBP and NeP patients for any of the parameters. Data are presented as mean ± SD, $n = 19$, $*p < 0.05$ versus healthy in paired Student's *t* test (NeP) and Mann-Whitney Rank Sum Test (CLBP)
*BMI* body mass index; *NRS* (rest/motion) numeric rating scale (0–10) of pain, 0: "no pain," 10: "worst pain imaginable," *KAB* questionnaire for self-perceived stress ranging from 1 (no stress) to 6 (max. stress), *CES-D* center for epidemiologic studies depression scale, *CLBP* chronic low back pain, *NeP* neuropathic pain (symmetrical polyneuropathy, peripheral mononeuropathy, postherpetic neuralgia, orofacial pain)

**Table 3** Patients' medication at the beginning of the study

| Medication at beginning of the study | CLBP ($n = 19$) | NeP ($n = 19$) |
|---|---|---|
| Ibuprofen (no. (%)) | 3 (16) | 2 (11) |
| Diclofenac (no. (%)) | 2 (11) | 3 (16) |
| Paracetamol (no. (%)) | 3 (16) | 2 (11) |
| Metamizole (no. (%)) | 1 (5) | 1 (5) |
| Opioids (no. (%)) | 2 (11) | 3 (16) |
| Pregabalin (no. (%)) | 0 (0) | 1 (5) |
| Duloxetine (no. (%)) | 0 (0) | 1 (5) |

There is no significant difference in the intake of analgesics or coanalgesics in patients with CLBP and NeP. Data are presented as mean ± SD, $n = 19$, statistical testing was performed using Fisher's exact test
*CLBP* chronic low back pain; *NeP* neuropathic pain (symmetrical polyneuropathy, peripheral mononeuropathy, postherpetic neuralgia, orofacial pain)

### P2RX7 mRNA expression is increased in patients with NeP, but not in CLBP

The relative expression of P2RX7 mRNA was determined by qPCR. Compared to healthy volunteers, significantly elevated mRNA levels (1.6-fold) were detected in patients with NeP (NeP: 1.6 ± 0.6, healthy volunteers 1.0 ± 0.3, $p < 0.05$; Fig. 4), while only a mild increase of P2RX7 mRNA expression (1.1-fold) was found in patients with CLBP (CLBP: 1.1 ± 0.6, healthy volunteers 1.0 ± 0.3, n.s.; Fig. 4).

### P2X7R protein expression is significantly increased on lymphocytes and monocytes of patients with NeP

Consistent with the results on mRNA expression, FACS results (MFI) revealed that compared to healthy volunteers, P2X7R protein expression levels on lymphocytes (NeP: 21.8 ± 6.5, healthy volunteers: 15.6 ± 5.2; $p = 0.009$; Fig. 5a) and monocytes (NeP: 24.6 ± 6.2, healthy volunteers: 17.0 ± 5.4; $p = 0.002$; Fig. 5d) were significantly enhanced in patients with NeP, but not in patients with CLBP. This P2X7R upregulation was detected on both $CD4^+$ monocyte (NeP: 21.0 ± 6.4, healthy volunteers: 13.2 ± 4.8; $p < 0.001$; Fig. 5b) and $CD4^-$ monocyte (NeP: 21.5 ± 6.5, healthy volunteers: 16.6 ± 4.9; $p = 0.039$; Fig. 5d) cells.

### Differential IL-1β levels in patients with neuropathic pain and CLBP

In a number of preclinical studies, IL-1β has been shown to be mainly involved in neurodegeneration, inflammation, and pain [1, 10] and to be a key mediator of the P2X7R-pain interplay. In order to find out whether the observed differences in P2X7R expression between patients with CLBP and NeP are also reflected by IL-1β, we analyzed serum levels of this pro-inflammatory and pro-nociceptive cytokine by multiplex enzyme-linked immunoassay. Concomitant with the P2X7R elevation, we found significantly increased serum levels of IL-1β (1.4-fold) only in the peripheral blood of patients suffering from neuropathic pain (Fig. 6).

### Confounding analyses

Since P2X7R expression has been associated with various diseases, we tested a set of potentially confounding variables for significant differences in distribution between healthy volunteers and patients [24, 25]. As elevated receptor expression is associated with depression and anxiety, we tested the CES-D depression scores and KAB values. Healthy volunteers showed significantly lower depression and stress scores, but no differences were

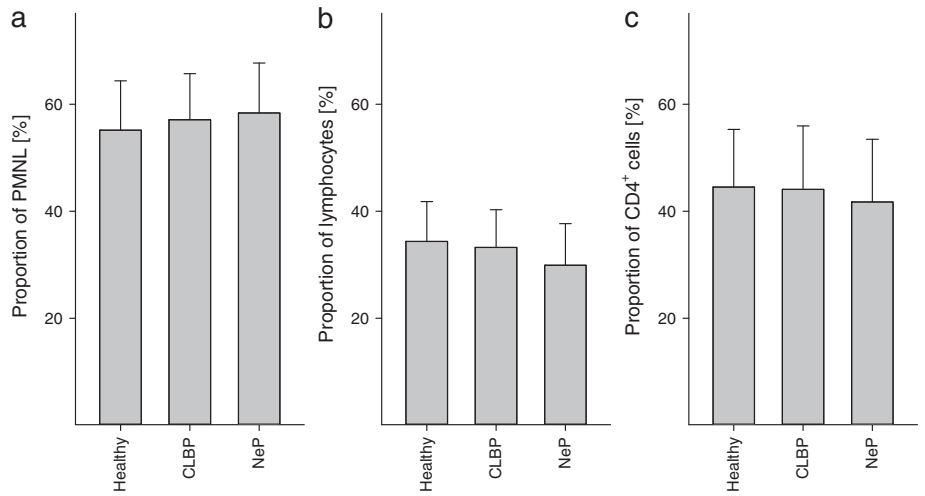


**Fig. 3** Differential blood count and quantification of $CD4^+$ cells. In order to avoid misinterpretation of potentially elevated P2X7R protein expression based on different cell counts, we quantified numbers of polymorphonuclear leukocytes (**a**), lymphocytes (**b**), and $CD4^+$ cells (**c**). No differences were found between patients suffering from CLBP, NeP, and healthy volunteers. Data are presented as mean ± SD, $n = 19$, $^*p < 0.05$ in one-way ANOVA with multiple comparisons versus healthy volunteers (Holm-Sidak method)

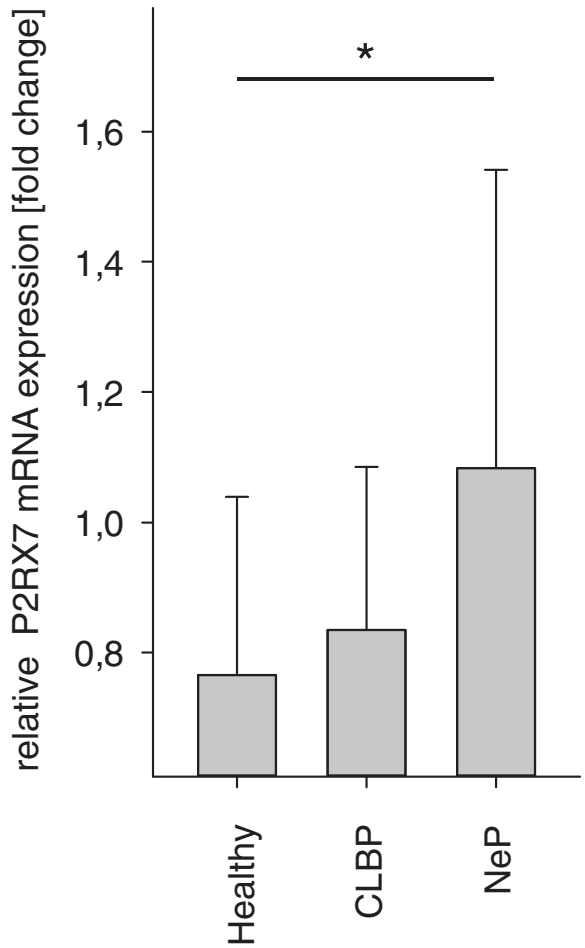


**Fig. 4** P2RX7 mRNA expression is exclusively increased in patients with neuropathic pain. To confirm the flow cytometric observations with elevated P2X7R protein expression, predominantly on $CD4^+$ cells and only in NeP, we determined the relative P2RX7 mRNA expression of $CD4^+$ cells by qPCR. Affirmatively, increased mRNA levels were consistent with the flow cytometric analyses and exclusively elevated in patients with NeP. ($p = 0.038$). *$p < 0.05$. Data are presented as mean ± SD, $n = 19$, *$p < 0.05$ in one-way ANOVA with multiple comparisons versus healthy volunteers (Holm-Sidak method)

found between patients with nociceptive and neuropathic pain. Age differed significantly between patients and healthy volunteers. However, a potential confounding effect was excluded by investigating the correlation between age and P2X7R levels (no significant correlation for both, Pearson's and Spearman's correlation test; $r = 0.18$, $p = 0.16$ and rho = 0.17, $p = 0.18$, respectively), as well as checking P2X7R levels in age-matched subgroups that yielded similar results as the main analysis. Moreover, no correlation was found regarding gender and receptor expression. Subgroup analysis of neuropathic pain syndromes revealed no differences in P2X7R expression between peripheral polyneuropathy/mononeuropathy, postherpetic neuralgia or orofacial pain.

## Discussion

In this study, we found a significantly increased expression of P2X7R mRNA and protein in lymphocytes and monocytes as well as higher IL-1β serum levels in patients suffering from NeP, but not in those with nociceptive CLBP. These results might point to an important role of P2X7R and IL-1β in the pathogenesis and maintenance of NeP.

Chronic pain is a global health problem, affecting up to 60 % of the population [26]. Over the last years, significant effort has been made to investigate endogenous pain-modulating factors [27]. In various animal models of nociceptive, inflammatory, and neuropathic pain, the endogenous receptor P2X7 was the focus of interest [2–5]. Recent data indicate that nociceptive information from the periphery to the CNS is transmitted through various ion channels and receptor pathways [28, 29]. The ATP-sensitive P2X7R, which is particularly localized on immune and microglial cells, is part of this reporter system [29]. In response to inflammation or cellular damage, ATP activates P2X7R, which represents an important step in the transmission of sensory information to the central nervous system [4, 30]. Recent studies suggest that P2X7R is involved in the pathogenesis of neurological disorders such as epilepsy, stroke, neuralgia, multiple sclerosis, Alzheimer's disease, Parkinson's disease, and Huntington's disease [31]. Moreover, the P2X7R is associated with mood disorders like major depression or anxiety [31, 32]. Upon activation, P2X7R triggers a series of physiological events that culminate in the posttranscriptional processing and release of IL-1β from monocytes [10]. IL-1β is a pro-inflammatory and pro-nociceptive cytokine which was shown to be a key mediator in chronic pain [33]. In addition, there is increasing evidence that enhanced release of IL-1β after P2X7R activation antagonizes morphine analgesia and accounts for the development of morphine tolerance, which may partly explain the insufficient effect of opioids in a considerable number of NeP patients [34]. IL-1β induces the transcription of cyclo-oxygenase 2 (COX-2) and nitric oxide synthase (iNOS), which play a central role in the generation and maintenance of pain [35, 36]. Within clinical settings, the efficacy of agents to treat neuropathic pain is variable. While COX-2 inhibitors are particularly effective against the inflammatory component of neuropathic pain, their effect on the intensity of pain is generally not satisfactory [37]. Contrary, antiepileptic drugs and antidepressants are useful to modulate the intensity of pain, but rather inefficient to treat the inflammatory component [38]. P2X7R and IL-1β are known to modulate inflammation and nociception, which recently led to the discovery of pharmacological agents selectively blocking P2X7R [30]. Genetic modulation or pharmacological blockade of P2X7R induces a

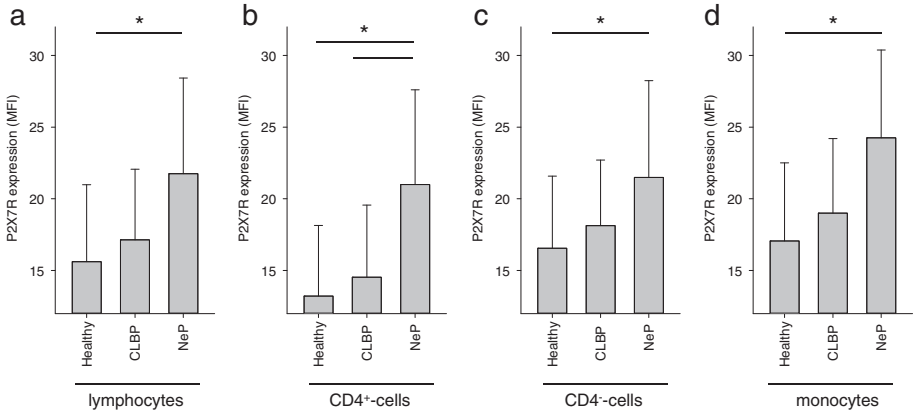


**Fig. 5** P2X7R expression on lymphocytes (**a**), $CD4^+$ (**b**) and $CD4^-$ cells (**c**), and monocytes (**d**) is elevated in patients with neuropathic pain. PBMCs were stained with PerCP-labeled antihuman CD4 antibody and FITC-labeled antihuman P2X7R antibody and analyzed by FACS. The results show a significantly elevated P2X7R expression only in patients suffering from neuropathic pain on the analyzed cell type (lymphocytes ($p = 0.009$), $CD4^+$ cells ($p < 0.001$), $CD4^-$ cells ($p = 0.039$), and monocytes ($p = 0.002$)). Data are presented as mean ± SD, $n = 19$, $^*p < 0.05$ in one-way ANOVA with multiple comparisons versus healthy volunteers (Holm-Sidak method)

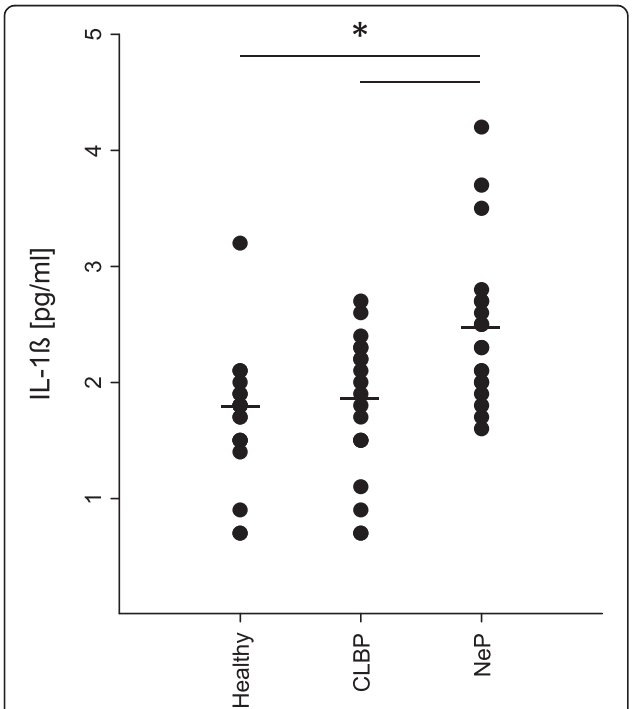


**Fig. 6** IL-1β levels are elevated in patients with neuropathic pain. As IL-1β was shown to be the key mediator in P2X7R-pain interplay, we analyzed serum levels of this pro-inflammatory and pro-nociceptive cytokine by multiplex immune assay. Affirmatively to the results with elevated P2X7Rs, we solely found significant increased serum levels in the peripheral blood of patients with NeP $^*p < 0.05$. Data are presented as mean ± SD, $n = 19$, $^*p < 0.05$ in one-way ANOVA with multiple comparisons versus healthy volunteers (Holm-Sidak method)

regression of symptoms in animal models of neurological disorders and reduces the intensity of inflammatory and neuropathic pain in mice [4, 39, 40].

These findings are consistent with our results demonstrating an increased expression of lymphocyte and monocyte P2X7R and IL-1β in patients suffering from NeP. It is not surprising, that only slight elevations of P2X7R protein, P2RX7 mRNA expression and of IL-1β levels were found in patients with CLBP, as CLBP is usually not associated with significant immune activation. These findings might point to a minor role of the P2X7R/IL-1β interplay in the pathophysiology of CLBP. This assumption is supported by recent research, showing a communication link between the immune system and the CNS [1, 15]. Lesion of a peripheral nerve leads to both a transition of microglia to the side of damage and an infiltration of immune cells in the vicinity of the synapse between primary afferent fibers and nociceptive neurons in the dorsal horn of the spinal cord [41, 42]. These activated immune cells release many pro-inflammatory mediators, such as IL-1β which cross the blood-brain barrier [15] and modulate pain intensity [40, 43]. A crucial regulator of IL-1β release is P2X7R [10]. Peripheral knock-down of P2X7R in mice leads to a significant decrease of IL-1β release and reduction of pain intensity [40].

Since elevated P2X7R expression has also been associated with mood disorders such as depression and anxiety, we tested the CES-D depression scores and KAB values as potentially confounding variables. Healthy volunteers had significantly lower depression and stress scores than both patient subgroups, but no differences were found between patients suffering from CLBP or NeP. In order to exclude further factors being responsible for the different expression of P2X7R/IL-1β in pain syndromes, we performed

confounder-analyses. Although age differed between patients and healthy volunteers, a potential confounding effect could be excluded. Furthermore, we found that gender did not correlate with the expression of P2X7R or IL-1β either. Regarding subgroups of NeP, no differences with respect to the P2X7R expression were found between patients suffering from peripheral polyneuropathy/mononeuropathy, postherpetic neuralgia or orofacial pain. One limitation of our study is that the analysis of P2X7R protein and mRNA expression was performed on lymphocytes, whereas IL-1β levels were determined in the peripheral blood. Since P2X7R is also expressed on the surface of other immune cells such as macrophages, which are also a major source of IL-1β production, further studies are needed to clearly define the source of elevated IL1β levels. Furthermore, it would be interesting to take the monocytes followed by LPS priming and ATP challenge to demonstrate different IL-1β release between groups.

## Conclusions

In conclusion, we here report that, in patients with NeP, P2X7R expression is significantly elevated. Activation of P2X7 has been shown to result in IL-1β release. Thus, based on our data, we propose the hypothesis that increased P2X7R expression leads to increased IL-1β blood levels, which may either predispose or maintain neuropathic pain. This P2X7R-driven inflammatory component seems to be absent in patients with CLBP. Our results suggest a major role of the purinergic-receptor/cytokine-interplay in NeP and may help identify patients who might benefit from P2X7R modulating treatment approaches.

#### Abbreviations

BMI: body mass index; CES-D scale: center for epidemiologic studies depression scale; CLBP: chronic low back pain; CNS: central nervous system; COX-2: cyclo-oxygenase 2; CRP: C-reactive protein; FACS: fluorescent-activated cell sorting; FSC: forward scatter; IL: interleukine; iNOS: nitric oxide synthase; KAB: Kurzfragebogen zur aktuellen Beanspruchung; LC: lymphocytes; MC: monocytes; MFI: mean fluorescence intensity; NeP: neuropathic pain; NRS: numerical rating scale; P2XR: purinergic P2X receptor; PBMC: peripheral blood mononuclear cell; RT-PCR: quantitative real-time PCR (qPCR); SD: standard deviation; SDHA: succinate dehydrogenase complex subunit A; SSC: side scatter; TBP: TATA box binding protein; Treg: regulatory T cell.

#### Competing interests

The authors declare that they have no competing interests.

#### Authors' contributions

All authors read and approved the final manuscript; BL performed all experiments; JH, BL, TW, and SCA wrote the manuscript; BL and BRA recruited the patients and prepared the blood samples; SK and TW gave conceptual advice and provided methodology; LCH, BL, SCA, and JH analyzed the data; BL, JH, and SCA designed the experiments.

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# Anti-inflammatory T-cell shift in neuropathic pain

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## Abstract

**Background:** The classification of pain into nociceptive and neuropathic pain is based on characteristic symptoms and different pathophysiological mechanisms. In a recent investigation, we found a disrupted TH17/Treg balance in patients suffering from chronic unspecific low back pain (CLBP). These patients did not show any signs of neuropathy. There is evidence for a considerable impact of the immune system also in neuropathic pain. However, the role of the adaptive immune system is still unclear. In the present study, we investigated systemic T-cell subset responses and T-cell related cytokine profiles in patients with chronic neuropathic pain.

**Methods:** We analyzed T-cell subsets, mRNA expression and T-cell-related cytokine profiles in 26 patients suffering from neuropathic pain in comparison to 26 healthy controls. Using multicolor flow cytometry (FACS), we quantified the number of T helper cells 1 (TH1), TH2, TH17 and regulatory T-cells (Tregs). Forkhead-Box-Protein 3 (FoxP3), Transforming growth factor-β (TGF-β) and RAR-related orphan receptor-γT (ROR-γT) mRNA expression was determined by quantitative real-time PCR (qPCR) and levels of pain-related cytokines were measured by Human Cytokine Multiplex Immunoassay (Macrophage inflammatory protein-1α (MIP-1α), Tumor necrosis factor-α (TNF-α), Interferon-γ (IFN-γ), Interleukin (IL) -4, IL-6, IL-10, IL-17, and IL-23).

**Results:** We found a TH17/Treg imbalance with significantly increased anti-inflammatory Tregs and decreased pro-inflammatory TH17 cells in patients with neuropathic pain as compared to healthy controls. These results were confirmed on mRNA level: Treg-related FoxP3 and TGF-β mRNA expression was elevated, whereas expression of TH17-related RORγT was reduced. Cytokine analyses revealed only marginal changes.

**Conclusions:** Our investigation revealed a clear shift of T-cell subsets towards anti-inflammation in patients with neuropathic pain. Interestingly, this is quite similar to our previous findings in CLBP patients, but even more pronounced. Therefore, it remains to be elucidated in future investigations whether the immune changes represent an underlying pathophysiological mechanism or an epiphenomenon induced by ongoing pain and stress.

**German Clinical Trial Register (DRKS):** Trial registration number: DRKS00005954

**Keywords:** Neuropathic pain, T-cells, TH17, Treg, Cytokines, Neuroinflammation

## Background

Neuropathic pain represents a major problem in clinical practice. In contrast to nociceptive pain, which is caused by damage or potential damage to tissue, neuropathic pain occurs due to a lesion or disease of the peripheral or central nervous system. It is characterized by burning and lancinating pain sensations and further somatosensory disturbances like hypo- and hypersensitivity. Very often, neuropathic pain is more difficult to treat and more refractory to common analgesics, including non-steroidal anti-inflammatory drugs and opioids, than nociceptive pain [1,2]. Despite extensive research, the underlying pathophysiological mechanisms of neuropathic pain are still not fully understood. In recent years, increasing evidence indicates a pivotal role of the immune system in neuropathic pain [3,4]. The majority of previously published data link pain syndromes with higher levels of pro-inflammatory cytokines. Due to these findings, attempts were made in numerous studies to reduce neuropathic pain by blocking pro-inflammatory or enhancing anti-inflammatory immune cells and cytokines [3]. For example, in animal models of neuropathy, tumor necrosis factor-α (TNF-α), Interleukin (IL) -6, IL-17 and

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Makrophage inflammatory protein1-α (MIP1-α) blockers reduced pain hypersensitivity [5-9]. Comparable results were obtained by increasing the anti-inflammatory cytokines IL-4, IL-10 or Transforming growth factor-β (TGF-β) [10-12]. Nevertheless, despite those promising experimental findings, there are no pharmacological agents available for the specific immunological therapy of neuropathic pain until now.

Cytokines and neutrophils are important during the early stages of acute pain, whereas T-lymphocytes seem to play a central role in chronic neuropathic pain [13]. Regarding T-cells as key players of the adaptive immune system, a TH1/TH2 imbalance has already been shown in patients with complex regional pain syndrome (CRPS) and chronic pelvic pain [14,15]. In recent years, TH1/TH2 dichotomy has been extended by the identification of two other $CD4^+$ T-cell lineages: TH17 and regulatory T-cells (Tregs) [16]. TH17 cells appear to be the key effector T-cells in a variety of human autoimmune diseases and Tregs play a vital role in controlling adaptive immune responses. In neuropathy, TH17 has been linked to increased pain sensitivity and destructive effects promoting persistent pain [16], while Tregs were found to be mainly involved in the endogenous recovery [17]. Recently published data showed an increased proportion of Tregs in patients with postherpetic neuralgia [18]. Assuming a beneficial role for an anti-inflammatory T-cell shift, a phase one trial was carried out with an anti-CD28 antibody, preferentially expanding TH2 cells and Tregs in human volunteers. Despite promising results in several animal models, the clinical trial had to be cancelled because of severe side effects [19]. In patients with neuropathic pain, the role of T-cell subsets has not yet been investigated.

In a recent study, we found that patients suffering from nociceptive, non-specific chronic low back pain (CLBP), without any signs of neuropathic pain components, display a clear disruption of the TH17/Treg balance as compared to healthy volunteers [20]. Since clinical and pathophysiological mechanisms differ considerably between nociceptive and neuropathic pain, we aimed to detail changes of pro- and anti-inflammatory T-cell subsets and the respective relative mRNA expression, as well as pain-related cytokine levels in patients with chronic neuropathic pain in comparison to pain free controls. While the cytokine measurement did not reveal any relevant results, we found a distinct anti-inflammatory shift of the T-cell subsets and their respective mRNA expression.

## Methods

### Ethics statement

The study followed the principles of the Declaration of Helsinki and was approved by the Ethics Committee of the Ludwig Maximilians University Munich (Ethical approval number: 331–10). This study was registered on German Clinical Trial Register (Trial registration: DRKS00005954).

### Subjects

Patient recruitment of our prospective study was estimated to last for two years. All patients presented to our Department of Anesthesiology and Pain Medicine, Ludwig-Maximilians University Munich with neuropathic pain for at least six months were assessed for fulfillment of the inclusion criteria and asked for their consent to participate in the study. In addition, healthy pain-free volunteers without any signs or history of pain were asked for their participation. Neuropathic pain was diagnosed according to its international definition: 'pain caused by a lesion or disease of the somatosensory nervous system' [2], by pain history, physical examination and the PainDETECT questionnaire.

This questionnaire consists of several items related to neuropathic symptoms (burning sensations, tingling or prickling sensations, shooting or lancinating, hyperalgesia, dysesthesia, allodynia or paresthesia) with excellent sensitivity (85%) and specificity (80%) [21]. Additionally, quantitative sensory testing (QST) was performed in all patients according to the protocol of the German Research Group on neuropathic pain [22]. Patients with mixed pain (nociceptive and neuropathic components) like complex regional pain syndrome (CRPS) and low back pain with radiculopathy were excluded. Further exclusion criteria were autoimmune, chronic systemic, inflammatory, neoplastic or psychiatric diseases, as well as drug abuse and pregnancy. Patients taking any current medication with opioids, non-opioids or co-analgesics were excluded. None of the patients had been treated with corticosteroids or had received immunomodulatory agents currently or in the past. Any signs of acute inflammatory disease were disclosed by laboratory examination, including plasma concentration of C-reactive protein (CRP), total and differential leucocyte count, as well as measurement of the body temperature. Patients rated their recalled average pain intensity using an 11-point numerical rating scale (NRS): 0 meaning 'no pain' and 10 meaning 'worst pain imaginable'.

Self-perceived stress was evaluated using the German version of the Questionnaire for Actual Demands (KAB: *Kurzfragebogen zur aktuellen Beanspruchung*) in patients and healthy volunteers. The KAB was designed to repeatedly quantify an individual's acute or chronic stress. It is highly sensitive to short-term or situational changes during a stressful experience. The rating is based on a six-point scale ranging from one to six based on normalized adjectives. Higher KAB values indicate increased perceived levels of stress [23].

### Cytokine assessment

Samples of peripheral blood from all patients and healthy controls were collected between 9:00 and 9:30 am, centrifuged at 2000 × g/10 min and stored in polypropylene aliquot tubes at −80°C. Samples were then assessed for levels of T-cell-related cytokines using a human cytokine multiplex immunoassay (Myriad Rules-Based Medicine Inc., Austin, Texas, United States). The multiplex microbead assay is based on Luminex technology and measures proteins in a similar manner to standard sandwich ELISA, with comparable sensitivity and range. Regarding the detection limits, the lower limit of quantitation (LLOQ) for the cytokines were: MiP1-α: 42.0 pg/ml, TNF-α: 23.0 pg/ml, IFN-γ: 1.5 pg/ml, IL-4: 29.0 pg/ml, IL-6: 11.0 pg/ml, IL-10: 6.9 pg/ml, IL-17: 4.0 pg/ml, and IL-23: 0.59 pg/ml. The LLOQ is the lowest concentration of an analyte in a sample that can be reliably detected and at which the total error meets the laboratory's requirements for accuracy [24].

### Flow cytometric staining and analysis

Peripheral blood mononuclear cells (PBMCs) were separated by density gradient preparation over Ficoll-Uropoline (Sigma Aldrich, Taufkirchen, Germany) of all heparinized venous blood samples. Then, PBMCs were cryopreserved in Roswell Park Memorial Institute medium (RPMI) freezing media (Sigma Aldrich, Taufkirchen, Germany), containing 10% Fetal calf serum (FCS), (Sigma Aldrich, Taufkirchen, Germany) and 10% Dimethyl sulfoxide (DMSO), (Sigma Aldrich, Taufkirchen, Germany) [25], and stored at −30°C for 24 hours, and then at −196°C until measurement. After storage, samples were thawed rapidly and washed twice to eliminate DMSO. For TH1, TH2 and TH17 analysis, cells were stimulated for five hours with cell stimulation cocktail, including protein transport inhibitors Phorbol 12-myristate 13-acetate (PMA), ionomycin, Brefeldin A and monensin (eBioscience, San Diego, California, United States), according to the manufacturer's protocol. Subsequently, cells were extracellularly stained with anti-human CD4 antibody and consecutively fixed and permeabilized (Fix-Perm-Solutions A and B, Life Technologies, Darmstadt, Germany) for intracellular staining with anti-human Interferon-γ, Interleukin (IL) -4 and IL-17 antibody (Biolegend, San Diego, California, United States). T-cell distribution was measured by fluorescent-activated cell sorting (FACS) analysis with the Attune Acoustic Focusing Cytometer (Life Technologies, Carlsbad, United States), and exemplary pictures of the gating strategy for TH17 cells are displayed in Figure 1 and Additional files 1 and 2. Tregs were identified and quantified after surface staining of PBMCs with monoclonal antibodies (mAbs) specific for anti-human CD4, CD25 and CD127 and intracellular staining with an anti-human FoxP3 antibody (Biolegend, San Diego, California, United States). The frequencies of $CD4^+CD25^{high}$ T-cells and $CD4^+CD25^{high}CD127^{low}FoxP3^+$ T-cells were expressed as percentage of total $CD4^+$ T-cells by sequential gating on lymphocytes. Exemplary pictures of the gating strategy for Tregs are displayed in Figure 2 and Additional files 1 and 2. Isotype controls (Biolegend, San Diego, California, United States) were given for compensation and confirmation of antibody specificity.

### Quantitative real-time PCR (qPCR)

$CD4^+$ cells were isolated from PBMCs by magnetic separation with Whole Blood CD4 MicroBeads (MACS Miltenyi Biotec, Auburn, California, United States) according to the manufacturer's recommendations. Subsequently, total RNA was isolated using the mirVana miRNA Isolation Kit followed by a DNase-digest with Turbo DNA-free Kit (Ambion, Darmstadt, Germany). Quantity and purity of the isolated RNA were measured using a NanoDrop ND-1000 spectrophotometer (Peqlab, Erlangen, Germany). After amplification of total RNA using TargetAmp 1-Round aRNA Amplification Kit (Epicentre Biotechnologies, Madison, Wisconsin, United States) and purification using RNeasy Mini Kit (Qiagen, Hilden, Germany), cDNA synthesis was performed with SuperScript III First Strand Synthesis System (Invitrogen, Darmstadt, Germany) and random hexamers (Qiagen, Hilden, Germany). Quantitative RT-PCR was performed in duplicates with the LightCycler 480 instrument (Roche Diagnostics, Mannheim, Germany) using LightCycler 480 Probes Master and RealTime ready single assays (Roche Diagnostics, Mannheim, Germany) and UniversalProbeLibrary (UPL) probes. The Real-Time ready single assays contain target-specific primers and a UPL-LNA probe (Roche Diagnostics, Mannheim, Germany). Primer sequences and qPCR characteristics are given in Table 1. The cycling conditions comprised an initial denaturation phase at 95°C for 10 minutes, followed by 45 cycles at 95°C for 10 seconds, 60°C for 30 seconds and 72°C for one second. Relative mRNA expression of FoxP3, TGF-β and RORγT was calculated by Relative Quantification Software (Roche Diagnostics, Mannheim, Germany) using an efficiency-corrected algorithm with standard curves and reference gene normalization against the reference genes succinate dehydrogenase complex subunit A (SDHA) and TATA box binding protein (TBP) as described in Ledderose *et al.* [26].

### Statistical analyses

Statistical analyses were performed using SigmaStat 12.0 (Systat Software, Chicago, United States). Every statistical analysis was started with testing for normal distribution using the Shapiro-Wilk test. Testing for differences between groups was accomplished by the *T*-test for all data with normal distribution and the nonparametric Mann–Whitney rank sum test for all data without normal distribution. Family-wise error rate was controlled at a

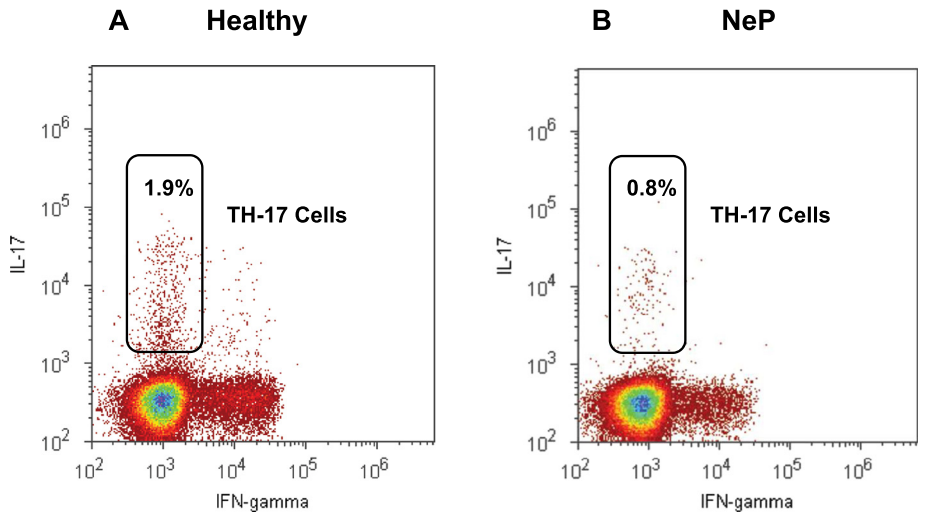


**Figure 1 Gating strategy for the detection of pro-inflammatory TH17 cells.** PBMCs were stained with Brilliant Violet (BV421)-labeled anti-human IL-17 antibody. Lymphocyte population was gated from PBMCs according to forward scatter (FSC) characteristics and side scatter (SSC) characteristics (see Figure 2) and then separated in IL-17$^+$ TH17 cells. Representative results of a healthy control with a higher number of TH17 cells **(A)** and a patient suffering from neuropathic pain with less TH17 cells **(B)** are shown. TH, T helper cells; PBMC, Peripheral blood mononuclear cells; IL, Interleukin.

false discovery level of q <0.05, and *P* values were adjusted accordingly following the Benjamini-Hochberg algorithm. *P* <0.05 were considered to be statistically significant. Results are expressed as mean ± standard deviation (SD) in the text.

## Results

### Subjects

Within two years of recruitment, 26 patients fulfilling the inclusion criteria and 26 healthy controls were enrolled. The characteristics of the participating patients and pain syndromes are given in Table 2.

### Granulocytes and lymphocytes were only slightly changed in neuropathic pain

Venous blood was drawn between 9:00 and 9:30 am into vacutainers containing Ethylenediaminetetraacetic acid (EDTA) for routine laboratory studies. Upon analyzing the number of neutrophil granulocytes, representing an essential part of the innate immune system, as well as lymphocytes, we found only slight alterations in patients with neuropathic pain (neutrophils: 55.4 ± 9.1% in controls versus 58.6 ± 9.3% in neuropathic pain, $P = 0.268$; lymphocytes: 33.8 ± 8.1% in controls versus 29.2 ± 8.2% in neuropathic pain, $P = 0.069$, Table 3).

### Cytokine measurement did not reveal relevant results

The specific functions of T-cell subsets are based on their respective cytokine release. TH1 cells produce predominantly pro-inflammatory cytokines such as IFN-γ and TNF-α, which support cellular immunity, whereas TH2 cells release anti-inflammatory cytokines, including IL-4 and IL-10, which mediate humoral immunity. TH17 cells particularly produce the potent pro-inflammatory cytokine IL-17, which is involved in many inflammatory conditions. IL-23 is a key cytokine in the control of inflammation in peripheral tissues, which stimulates naïve CD4 T-cells to differentiate into TH17 cells, in conjunction with IL-6 and TGF-β. Tregs have an anti-inflammatory role by releasing anti-inflammatory cytokines like IL-10 and TGF-β. However, it seems likely, that TH1 and TH17 cytokines are central to increased pain sensitivity, whereas TH2 and Treg derived cytokines may be protective.

Therefore, we analyzed blood levels of pain-associated and T-cell-related cytokines using human cytokine multiplex immunoassay (MIP-1α, TNF-α, IFN-γ, IL-4, IL-6, IL-10, IL-17, and IL-23). Serum levels of IL-4, TNF-α and IFN-γ were neither detectable in the peripheral blood of patients nor in healthy controls. No differences between patients and healthy controls were found analyzing IL-6, IL-10 and IL-17. In accordance with numerous described studies, serum levels of pro-inflammatory cytokines MIP-1α and IL-23 were significantly higher in the peripheral blood of patients suffering from neuropathic pain. However, it has to be noted that IL-23 was the only cytokine with values above the so-called LLOQ, the lowest concentration that can be reliably detected (see Methods section). (IL-6: 1.2 ± 0.8 pg/ml in controls versus 2.5 ± 2.4 pg/ml in neuropathic pain, $P = 0.064$; IL-10: 3.56 ± 2.45 pg/ml in controls versus 3.69 ± 2.40 pg/ml in neuropathic pain, $P = 0.84$; IL-17: 3.53 ± 2.11 pg/ml in controls versus 4.29 ± 2.02 pg/ml in neuropathic pain, $P = 0.23$; MIP-1α: 17.2 ± 11.2 pg/ml in controls versus 28.4 ± 16.4 pg/ml in

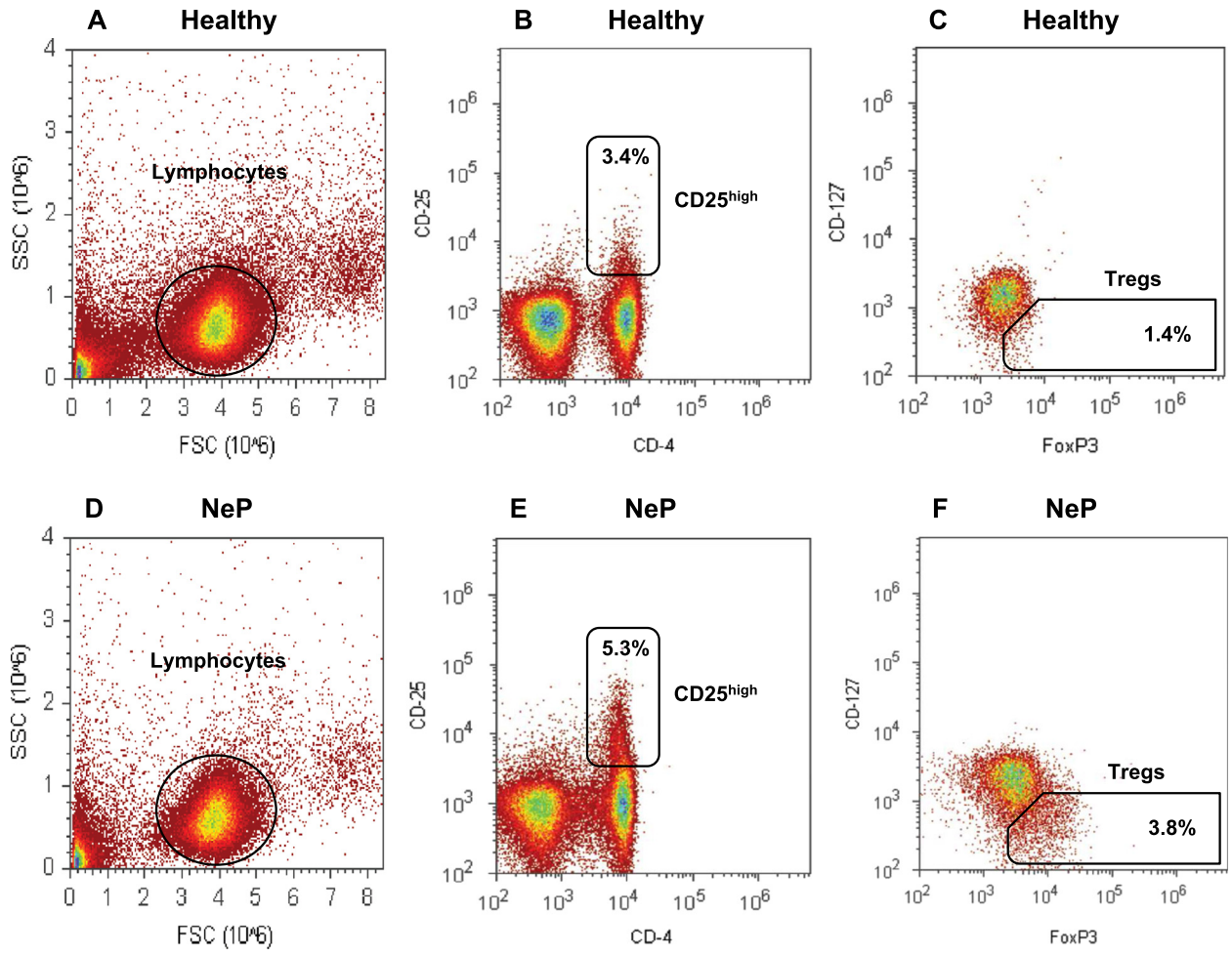


**Figure 2 Gating strategy for the detection of the investigated Tregs.** PBMCs were extracellularly stained with PerCP-labeled anti-human CD4-, PE-labeled anti-CD25 antibody, Brilliant Violet (BV570)-labeled anti-CD127 antibody and intracellularly stained with Alexa Fluor (AF488)-labeled anti-human FoxP3 antibody. Lymphocyte population was gated according to forward scatter (FSC) characteristics and side scatter (SSC) characteristics (A + D). Gated lymphocytes were then separated in $CD4^+CD25^{high}$ cells (B + E) and $CD4^+CD25^{high}CD127^{low}FoxP3^+$ cells (C + F, named Treg). Representative results of a healthy control **(A-C)** and a patient with neuropathic pain **(D-F)** are shown. Regulatory T-cells, Tregs; PBMC, Peripheral blood mononuclear cells; Forkhead-Box-Protein 3, FoxP3.

**Table 1 RT-PCR assay characteristics and primer sequences**

| Gene | Primer sequence |
|---|---|
| SDHA | Roche RealTime Ready Single Assay ID 102136 |
| TBP | Roche RealTime Ready Single Assay ID 101145 |
| FoxP3 | Roche RealTime Ready Single Assay ID 113503 |
| TGF-β | for 5'ACTACTACGCCAAGGAGGTCAC 3' |
| | rev 5'TGCTTGAACTTGTCATAGATTTCG 3', UPL probe #31 |
| RORγT | for 5'CAGCGCTCCAACATCTTCT 3' |
| | rev 5'CCACATCTCCCACATGGAC 3', UPL probe #69 |

**Table 2 Patient characteristics**

| Item | Healthy | NeP (all) | PNeP | PHN | OFP |
|---|---|---|---|---|---|
| **Numbers (n)** | 26 | 26 | 13 | 7 | 6 |
| **Age** | 39 ± 11 | 56 ± 14* | 49 ± 14* | 71 ± 6* | 53 ± 10* |
| **Female** | 52% | 73% | 69% | 71% | 83% |
| **BMI** | 23.4 ± 2.8 | 24.0 ± 3.4 | 25.1 ± 3.2 | 22.4 ± 3.4 | 23.6 ± 3.8 |
| **NRS (rest)** | 0.0 ± 0.0 | 4.5 ± 2.3* | 4.3 ± 2.5* | 3.7 ± 1.6* | 5.6 ± 2.1* |
| **NRS (motion)** | 0.0 ± 0.0 | 6.2 ± 2.7* | 6.0 ± 2.3* | 4.7 ± 3.9* | 6.6 ± 3.3* |
| **KAB** | 1.9 ± 0.7 | 3.2 ± 0.8* | 3.3 ± 0.7* | 2.9 ± 1.1* | 3.1 ± 0.8* |

NeP (all): all patients suffering from neuropathic pain; PNeP: Peripheral neuropathic pain (symmetrical polyneuropathy/peripheral mononeuropathy); PHN: postherpetic neuralgia; OFP: orofacial pain; NRS (rest/motion): Numeric rating scale (0 to 10) of pain, 0: 'no pain', 10: 'worst pain imaginable'; KAB: Questionnaire for self-perceived stress ranging from one (no stress) to six (maximum stress). Results are expressed as mean ± standard deviation (SD), *$P$ <0.05 versus healthy controls.

**Table 3 Differential blood count, flow cytometric and RT-PCR results of patient subgroups**

| Item | Healthy | NeP (all) | PNeP | PHN | OFP |
|---|---|---|---|---|---|
| Neutrophils (%) | 55.4 ± 9.1 | 58.6 ± 9.3 | 57.5 ± 10.1 | 57.3 ± 9.0 | 62.3 ± 8.5 |
| Lymphocytes (%) | 33.8 ± 8.1 | 29.2 ± 8.2 | 30.5 ± 7.9 | 28.3 ± 9.6 | 27.2 ± 8.1 |
| $CD4^+$ Counts × 1000 | 28.7 ± 7.3 | 33.5 ± 15.4 | 36.8 ± 18.8 | 30.3 ± 15.3 | 30.8 ± 2.9 |
| $CD4^+$ (%) | 43.4 ± 9.9 | 47.5 ± 11.9 | 49.9 ± 12.6 | 44.3 ± 13.8 | 46.6 ± 8.0 |
| TH1 (%) | 9.7 ± 4.7 | 9.6 ± 4.1 | 10.9 ± 4.7 | 7.2 ± 3.1 | 9.3 ± 2.7 |
| TH2 (%) | 1.3 ± 1.2 | 1.5 ± 0.8 | 1.7 ± 0.7 | 0.9 ± 0.5 | 2.0 ± 0.9 |
| TH17 (%) | 1.3 ± 1.0 | 0.7 ± 0.4* | 0.9 ± 0.4 | 0.4 ± 0.1* | 0.8 ± 0.5 |
| RORγT | 2.7 ± 1.4 | 1.9 ± 1.0 | 1.8 ± 0.6* | 1.9 ± 1.2* | 2.2 ± 1.5 |
| $CD4^+CD25^{high}$ (%) | 3.7 ± 0.7 | 5.4 ± 1.5* | 5.1 ± 1.8* | 5.0 ± 0.7* | 6.4 ± 0.9* |
| Tregs (%) | 2.0 ± 1.0 | 3.9 ± 1.3* | 3.5 ± 1.1* | 4.0 ± 1.9* | 4.4 ± 0.9* |
| FoxP3 | 0.6 ± 0.2 | 1.2 ± 0.8* | 1.0 ± 0.7* | 1.3 ± 0.3* | 1.4 ± 1.3* |

NeP (all): all patients suffering from neuropathic pain; PNeP: Peripheral neuropathic pain (symmetrical polyneuropathy/peripheral mononeuropathy); PHN: postherpetic neuralgia; OFP: orofacial pain; Results are expressed as mean ± standard deviation (SD), *$P$ <0.05 versus healthy controls.

neuropathic pain, $P = 0.022$; IL-23: 0.9 ± 0.3 pg/ml in controls versus 1.2 ± 0.4 pg/ml in neuropathic pain, $P = 0.022$).

**TH17 frequency was distinctly decreased in neuropathic pain**

Although many studies have analyzed the role of TH17 cells in human autoimmune diseases, there are very limited data on the role of TH17 cells in patients with neuropathic pain. TH17 cells act as an important pro-inflammatory component and have been shown to promote inflammation in a number of diseases. The proportion of TH17 cells is expressed as percentage of all T-cells. As shown in Figure 3, the frequency of TH17 cells was evidently decreased in the peripheral blood of patients suffering from neuropathic pain. Affirming these results, the relative mRNA expression of the TH17 cell-specific transcription factor RORγT was reduced, but did not reach significance (TH17 cells: 1.3 ± 1.0% in controls versus 0.7 ± 0.4% in neuropathic pain, $P = 0.046$; relative RORγT mRNA expression: 2.7 ± 1.4 in controls versus 1.9 ± 1.0 in neuropathic pain, $P = 0.064$; Figure 3).

**Treg frequency was distinctly increased in neuropathic pain**

Human regulatory T-cells play a vital role in controlling the adaptive immune response and in maintaining self-

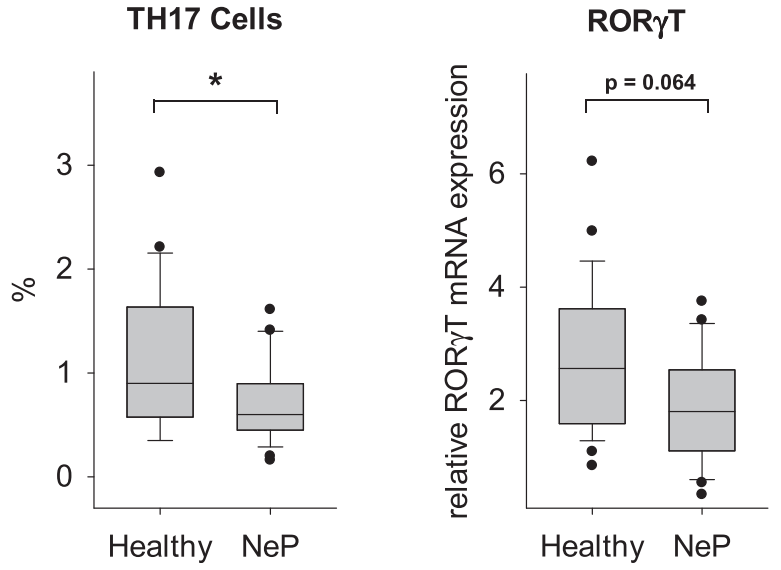


**Figure 3 Reduced TH17 frequency in patients with neuropathic pain.** Using a multicolor flow cytometer, PBMCs were intracellularly stained with anti-human IL-17 antibody after five hours of stimulation. The results show a significantly decreased frequency of TH17 cells in the peripheral blood of patients suffering from neuropathic pain. In accordance with these results, the relative mRNA expression of the TH17 cell-specific transcription factor RORγT was reduced, but did not reach significance. T helper cells, TH; Peripheral blood mononuclear cells, PBMC; Interleukin, IL; RAR-related orphan receptor-γT, ROR-γT; *P <0.05 versus healthy controls.

tolerance. Tregs have been shown to prevent autoimmune diseases and to limit chronic inflammatory and nervous system disturbances. On the other hand, the strong Treg-induced immune suppression also impairs beneficial responses such as anti-tumor immunity [27,28]. However, there were limited data analyzing the functional role of Tregs in neuropathic pain, which therefore remained to be investigated. We analyzed the number of Tregs by flow cytometry, using two staining procedures; classic extracellular staining with $CD4^+CD25^{high}$ and the more specific intra- and extracellular staining procedure with $CD4^+CD25^{high}CD127^{low}FoxP3^+$. We defined $CD4^+CD25^{high}CD127^{low}FoxP3^+$ T-cells as Tregs. The prevalence of Tregs was expressed as a ratio of $CD4^+CD25^{high}CD127^{low}FoxP3^+$ T cells as a percentage of $CD4^+$ T-cells. Figure 3 shows a significantly increased frequency of Tregs in patients with neuropathic pain as compared to controls. To confirm the quantitative observations of the Treg frequency we also determined the relative mRNA expression of the specific transcription factor FoxP3 and TGF-β by quantitative real-time PCR (qPCR). As shown on Figure 4, significantly increased mRNA levels of FoxP3 and TGF-β were observed in patients with neuropathic pain. These results were consistent with the flow cytometric analyses (Tregs: 2.0 ± 1.0% in controls versus 3.9 ± 1.3% in neuropathic pain, $P = 0.007$; relative FoxP3 mRNA expression: 0.6 ± 0.2 in controls versus 1.2 ± 0.8 in neuropathic pain, $P = 0.028$; relative TGF-β mRNA expression: 0.15 ± 0.06 in controls versus 0.25 ± 0.15 in neuropathic pain, $P = 0.009$; Figure 4).

### TH1/TH2 balance was only slightly altered in neuropathic pain

In previous investigations, the ratio of TH1 and TH2 cells was used to characterize immune responses in different diseases. In the present study, a trend towards a decreased TH1/TH2 ratio was observed, which, however, did not reach significance (TH1/TH2: 16.1 ± 17.4 in controls versus 10.1 ± 10.0 in neuropathic pain, $P = 0.56$; Figure 5).

### TH17/Treg balance was markedly disrupted in neuropathic pain

TH17 cells play an important pro-inflammatory role whereas Tregs are strong immune suppressors. Therefore, the balance between TH17 cells and Tregs, along with TH1/TH2 balance, is an important factor in analyzing the immune response. Our results regarding T-cell subsets alone demonstrated markedly reduced pro-inflammatory TH17 cells with simultaneous elevated anti-inflammatory Tregs. Conclusively, as shown in Figure 5, the TH17/Treg ratio was significantly lower in the peripheral blood of patients compared to healthy controls. These results indicate a clear anti-inflammatory T-cell shift in neuropathic pain (TH17/Treg: 0.9 ± 1.1 in controls versus 0.2 ± 0.1 in neuropathic pain, $P < 0.007$; Figure 5).

## Discussion

Neuropathic pain is a severe and frequent condition which affects up to 18% of the population [29]. The pathophysiological mechanisms leading to chronification of neuropathic pain are a major focus of interest, but are

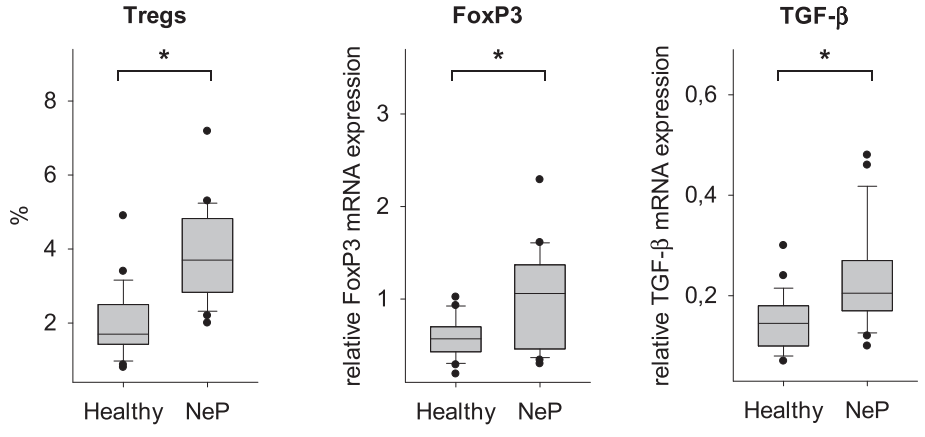


**Figure 4 Increased Treg frequency in patients with neuropathic pain.** In addition to the TH17 cell quantification, the number of Tregs was analyzed after intra- and extracellular staining procedure. $CD4^+CD25^{high}CD127^{low}FoxP3^+$ were defined as Tregs. The results show a significantly increased frequency of Tregs in patients with neuropathic pain as compared to healthy controls. To confirm the quantitative observations of the Treg frequency, the relative mRNA expression of the Treg-specific transcription factor FoxP3 as well as TGF-β was determined by quantitative real-time PCR (qPCR). Affirmatively, increased mRNA levels of FoxP3 and TGF-β were consistent with the flow cytometric analyses. Regulatory T-cell, Treg; T helper cell, TH; Forkhead-box-protein 3, FoxP3; Transforming growth factor-β, TGF-β; *P <0.05 versus healthy controls.

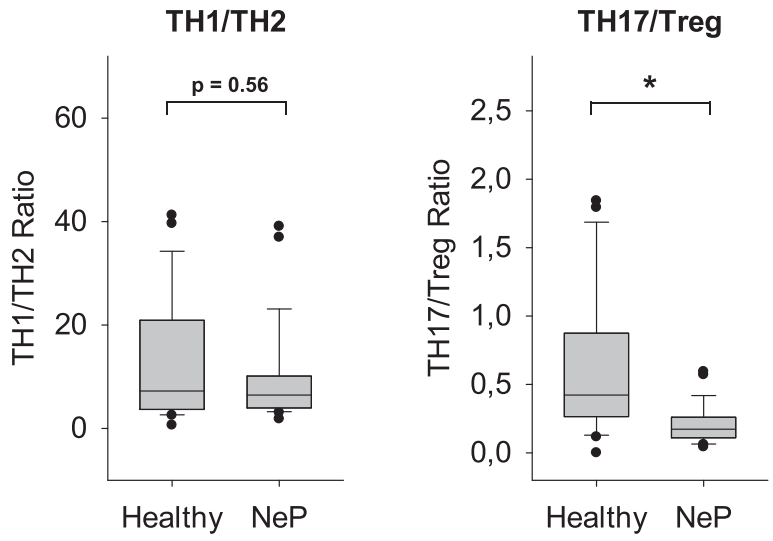


**Figure 5 Altered TH1/TH2 and TH17/Treg balances in patients with neuropathic pain.** While the TH1/TH2 ratio was only slightly decreased, the TH17/Treg balance was significantly enhanced in patients as compared to healthy controls. These results indicate a clear anti-inflammatory T-cell shift in neuropathic pain. T helper cell, TH; Regulatory T-cell, Treg; *P <0.05 versus healthy controls.

not yet completely elucidated. Recent data indicate a critical involvement of the innate and adaptive immune system in the pathophysiology of chronification. Several types of immune cells have been implicated in the pathogenesis of neuropathic pain [3]. The innate immune system has been shown to be important during the early stages of acute pain, represented particularly by neutrophils [13]. Regarding chronification, T-lymphocytes, as key players of the adaptive immune system, seem to be of major importance [30,31]. Traditionally, it has been suggested that neuropathic pain is associated with a pro-inflammatory immune response. Therefore, mainly anti-inflammatory treatments targeting cytokines and immune cells have been evaluated in several animal models of neuropathic pain [3]. In a recent study, neuropathic pain induced by experimental autoimmune neuritis was successfully attenuated by expanding Tregs [17]. In humans, the first Treg-expanding tests were stopped because of life-threatening side effects [19]. Nevertheless, the modulation of T-cells is still the focus of intense research [32].

In the present study, we analyzed the pain-related cytokines MIP-1α, TNF-α, IFN-γ, IL-4, IL-6, IL-10, IL-17 and IL-23 in the peripheral blood of 26 patients with neuropathic pain and compared the results with those of 26 healthy controls. We found that the serum levels of IL-4, TNF-α and IFN-γ were below the detectable limit and no differences were found regarding IL-6, IL-10 and IL-17. Only the pro-inflammatory cytokines MIP-1α and IL-23 were significantly higher in neuropathic pain. It has to be noted though, that except for IL-23, values of all cytokines measured were below the lowest concentration of an analyte in a sample that can be reliably detected. This shortcoming of serum cytokine measurements in pain syndromes has also been described by other authors [33]. Taken together, our results indicated that serum cytokine levels alone are not sufficient to monitor the adaptive immune response in neuropathic pain and led us to analyze the cellular compartment.

By routine laboratory studies regarding the differential leucocyte count, we found only an unchanged number of neutrophil granulocytes and a slightly reduced number of lymphocytes in the peripheral blood of patients with neuropathic pain. Using multicolor flow cytometry, we subsequently quantified the numbers of TH1, TH2, TH17 and Treg cells in the peripheral blood of our patients. Contrary to our initial assumption, we found clear indications for an anti-inflammatory T-cell shift: Pro-inflammatory TH17 cells were significantly decreased, whereas anti-inflammatory Tregs were significantly increased. Consequently, the corresponding TH17/Treg ratio was distinctly shifted towards an anti-inflammatory immune response. To confirm the quantitative observations of the TH17 and Treg frequency, we also determined the relative mRNA expression of the TH17 cell-specific transcription factor RORγT, as well as TGF-β and the Treg-specific transcription factor FoxP3 by quantitative real-time PCR. A diminished RORγT mRNA expression was in line with the reduced number of TH17 cells, while a notably elevated FoxP3 and TGF-β mRNA expression confirmed the increased Treg frequency. Regarding patient subgroups, we found no differences in respect of the anti-inflammatory T-cell shift and mRNA expressions between patients suffering from orofacial pain, postherpetic

neuralgia and other types of peripheral neuropathies (Table 3). The question arises whether the observed changes are of clinical relevance, particularly in view of the overall low portion of the specific T-cell subsets. Furthermore, it would be interesting and relevant to investigate whether the immune changes can also be found in the affected tissue.

Our results are, at first glance, unexpected since the majority of previously published data describes the association between pain and 'immune activation' based on investigations of TH1 and TH2 cells, as well as cytokines. This previous TH1/TH2 paradigm has, however, been revised and updated with the discovery of TH17 cells and the more specific detection of Tregs. Our findings, together with recently published data regarding various T-cell subsets, point to a strong association between pain and 'immune suppression'. Interestingly, the T-cell response in the present study is comparable with our recent findings in patients with CLBP, who also presented with high pain and stress levels, but had no signs of neuropathic pain [20]. There is a general consensus that neuropathic and nociceptive pain are distinct entities, although basic research clearly reveals a huge overlap of underlying pathophysiological mechanisms, including neurotransmitters and cytokines [2]. Our results show for the first time that in both neuropathic and nociceptive pain the adaptive immune system is altered in the same anti-inflammatory way. The context of chronic stress and immune suppression has been described for many years, although not extensively with regard to TH17 cells and Tregs [34]. An anti-inflammatory T-cell shift has been found in patients with chronic mild depression or chronic fatigue syndrome [35-37], and both disorders are frequently associated with all types of chronic pain.

Concerning the cellular mechanisms, T-cell differentiation mainly depends on the cytokine milieu of the microenvironment, but other pathways have also been shown to be involved. For example, the hypothalamic-pituitary-adrenal axis mediates immune regulation through binding of stress hormones like adrenocorticotropic hormone or cortisol to their cognate receptors at the surface of T-cells. Furthermore, the sympathetic nervous system is known to induce immune dysregulation via adrenaline and noradrenaline [38]. These processes in turn play an important role in negative emotional states, such as stress and depression. Our patients with neuropathic pain also suffered from stress and psychological burden as revealed by the enhanced KAB values. We therefore hypothesize that the altered immune responses in both of our studies might reflect a particular chronic pain-related stress reaction

## Conclusions

In summary, we found a TH17/Treg imbalance with increased anti-inflammatory Tregs and decreased pro-inflammatory TH17 cells in patients with neuropathic pain. These results are quite similar to our previous findings in patients with nociceptive CLBP who did not show any signs of neuropathy, but similar pain and stress levels. Therefore, it remains to be clarified in future studies whether the immune changes represent an underlying pathophysiological mechanism or an epiphenomenon induced by ongoing pain and stress.

## Additional files

**Additional file 1: Figure S1.** Exemplary density plots of 10 healthy controls and 10 patients with neuropathic pain showing pro-inflammatory TH17 cells.

**Additional file 2: Figure S2.** Exemplary density plots of 10 healthy controls and 10 patients with neuropathic pain showing anti-inflammatory Tregs.

#### Abbreviations

CLBP: Chronic low back pain; FACS: Fluorescent-activated cell sorting; RT-PCR: Quantitative real-time PCR (qPCR); TH: T-helper cell; Treg: Regulatory T-cell; FoxP3: Forkhead-Box-Protein P3; TGF-β: Transforming growth factor-β; RORγT: RAR-related orphan receptorγT; MIP1-α: Macrophage inflammatory protein1-α; TNF-α: Tumor necrosis factor-α; IFN: Interferon; IL: Interleukin; KAB: German version of the Questionnaire for Actual Demands "*Kurzfragebogen zur aktuellen Beanspruchung*".

#### Competing interests

The authors declare that they have no competing interests.

#### Authors' contributions

All authors read and approved the final manuscript. BL performed all experiments and wrote the manuscript. BL and BRA recruited the patients and prepared the blood samples. BL, BRA, JH, SK and SCA analyzed the data. BL, BRA and SCA designed the experiments.

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# Disrupted TH17/Treg Balance in Patients with Chronic Low Back Pain

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## Abstract

Chronic low back pain (CLBP) is a leading cause of disability and costs in health care systems worldwide. Despite extensive research, the exact pathogenesis of CLBP, particularly the individual risk of chronification remains unclear. To investigate a possible role of the adaptive immune system in the pathophysiology of CLBP, we analyzed T cell related cytokine profiles, T cell related mRNA expression patterns and the distribution of T cell subsets in 37 patients suffering from nonspecific CLBP before and after multimodal therapy in comparison to 25 healthy controls. Serum patterns of marker cytokines were analyzed by Luminex technology, mRNA expression of cytokines and specific transcription factors was measured by real-time PCR, and distribution of TH1-, TH2-, TH17- and regulatory T cell (Tregs) subsets was determined by multicolor flow cytometry. We found that CLBP patients exhibit an increased number of anti-inflammatory Tregs, while pro-inflammatory TH17 cells are decreased, resulting in an altered TH17/Treg ratio. Accordingly, FoxP3 and TGF-β-mRNA expression was elevated, while expression of IL-23 was reduced. Serum cytokine analyses proved to be unsuitable to monitor the adaptive immune response in CLBP patients. We further show that even after successful therapy with lasting reduction of pain, T cell subset patterns remained altered after a follow-up period of 6 months. These findings suggest an involvement of TH17/Treg cells in the pathogenesis of CLBP and emphasize the importance of these cells in the crosstalk of pain and immune response.

***Trial Registration:*** German Clinical Trial Register: Registration Trial DRKS00005954.

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**Competing Interests:** The authors have declared that no competing interests exist.

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## Introduction

Low back pain (LBP) is a common condition with a lifetime prevalence of nearly 84%. Although most patients recover completely within 4–8 weeks, a subset of patients is prone to develop chronic low back pain (CLBP). CLBP has become a major challenge for public health care systems worldwide [1]. The prevalence of CLBP is about 23%; around 12% of the afflicted patients are severely disabled [2,3]. Still, mechanisms driving the chronification of low back pain syndromes remain largely elusive. Pathological physical conditions such as microtraumata, incorrect posture and degenerative processes as well as psychological factors such as overtaxing, emotional distress and inadequate coping have been described to contribute to the pathogenesis of CLBP [4,5]. Increasing evidence indicates a pivotal role of the immune system in acute and chronic pain [6].

Recent studies have reported enhanced serum levels of pro-inflammatory cytokines in various pain syndromes [7,8,9,10]. In the pathogenesis of CLBP, a possible impact of TNF-α was suggested [11]. Moreover, an increased expression of Il-17 in herniated and degenerated lumbar intervertebral discs has been reported, indicating a possible role of this cytokine in the chronification of pain [9].

While the innate immune system has been found to play an important role in acute pain [12], T-Lymphocytes as key players of the adaptive immune system are supposed to be of major importance [13,14] in the pathogenesis of chronic pain. In patients with complex regional pain syndrome (CRPS) and in those suffering from abacterial chronic pelvic pain [15,16], a TH1/TH2 imbalance with increased numbers of TH1 cells has been shown. In recent years, TH1/TH2 dichotomy has been expanded by two further $CD4^+$ T cell lineages, Th17 and regulatory T cells (Tregs). These two T-cell subsets play prominent roles in immune functions: Th17 cells exerting pro-inflammatory effects are key players in the pathogenesis of autoimmune diseases and protection against bacterial infections, while Tregs function to restrain excessive effector T-cell responses. The role of both T cell subsets has extensively been analyzed in tumor growth and in the development of inflammatory and autoimmune diseases [17,18,19,20,21]. Recently published data also indicate an involvement of both T cell subsets in the development of chronic pain [22,23,24,25]. For example, in patients with postherpetic neuralgia (PHN), increased Treg numbers have been found [26]. In addition, there is evidence that these cells play a central role in endogenous recovery from neuropathic pain [27]. Due to the antagonistic functions of TH17 and Treg cells, and in analogy to

the well-known TH1/TH2 paradigm, the ratio between TH17 and Tregs is increasingly used to characterize immune responses.

In CLBP, however, specific alterations in the adaptive immune system have not conclusively been analyzed, yet.

In the current study, we investigated cytokine profiles and T helper cell subset compositions in CLBP patients and healthy controls. Our results indicate that CLBP is associated with characteristic alterations of T helper cell subsets: The TH17/Treg ratio was significantly decreased. We further provide evidence that these alterations persist even in those patients exhibiting significant pain reduction after participation in a standardized multimodal therapy program.

## Materials and Methods

### Ethics statement

The study followed the principles of the Declaration of Helsinki and was approved by the Ethics Committee of the LMU Munich.

### Subjects

During a prospective recruitment period of two years (September 2011 until September 2013), all patients seeking treatment for nonspecific CLBP at our pain clinic were assessed for study specific inclusion and exclusion criteria. Inclusion criteria were CLBP defined as low back pain persisting longer than two month, not attributable to a recognized specific pathological condition (e.g., disc herniation, any type of radiculopathy or other neuropathic pain, infection, tumor, osteoporosis, trauma, structural deformity or inflammatory disorders) and planned participation in a specific 4 week multimodal outpatient program (see Therapy). Exclusion criteria were concomitant autoimmune, chronic, inflammatory, neoplastic-, and psychiatric diseases, drug abuse and pregnancy as well as any preexisting long-term medication with opioids, non-opioid analgesics or co-analgesics. Healthy pain free volunteers without any signs or history of CLBP and concomitant diseases were asked for their participation in the study as controls. In total, 37 patients and 25 healthy controls matching the criteria listed above provided written informed consent and were enrolled in the study. None of the enrolled individuals had been treated with corticosteroids or had received immunomodulatory agents currently or in the past. Acute inflammatory diseases at the time of blood sampling were ruled out by measurement of body temperature and laboratory assessment of C-reactive-Protein (CRP) as wells as total- and differential leucocyte count. This study is registered on German Clinical Trial Register (Registration Trial DRKS00005954), but was not registered before enrollment of participants since all patients received only standard treatment and no further study-related interventions. The authors confirm that all ongoing and related trials for this drug/intervention are registered.

### Therapy

The multimodal outpatient program (MRIP, "Muenchner Ruecken Intensiv Programm") performed at the University of Munich is a clinically established outpatient program for patients with chronic low back pain. In line with specific recommendations for the treatment of chronic disabling low back pain [2,3,28], the program follows a bio-psycho-social approach and comprises medical (examination, education), physical (exercise), work-related and behavioral therapy components. The program is conducted by specialists from at least four professional groups with different backgrounds (e.g. physicians, physiotherapists, psychotherapists, occupational therapists). The group size is limited to 10 patients. The duration of the program is 4 weeks, 5 days a week and 8 hours a day.

### Outcome assessment

Pain and stress levels were routinely evaluated by standardized questionnaires before treatment, at the end of the program and six months after completion of the program (follow-up). Patients were asked to rate their recalled average pain intensity using an 11-point numerical rating scale (NRS): 0 means "no pain" and 10 means "worst pain imaginable". Self-perceived stress was evaluated using the Short Questionnaire on Current Burden (KAB, "Kurzfragebogen zur aktuellen Beanspruchung"). The KAB is able to repeatedly determine an individual's psychological state under the conditions of acute or chronic stress and is highly sensitive to short-term or situational changes during a stressful experience [29]. The rating is based on a 6-point scale ranging from 1 to 6 for all six matched adjectives. Higher KAB values indicate increased perceived stress levels. Responders were defined as patients with improvements in NRS by $\geq$50% due to the treatment program. Healthy controls were asked to fill out questionnaires once.

### Blood sampling

Blood samples were taken from all patients before treatment, at the end of the program and at the follow-up six months after completion of the program. Blood samples from healthy volunteers were taken once at enrollment.

**Cytokine Assessment.** Blood samples were collected, centrifuged and stored in polypropylene aliquot tubes at $-80^{\circ}$C. Samples were then assessed for levels of T cell related cytokines using a human cytokine multiplex immunoassay by Myriad Rules-Based Medicine Inc., Austin, Texas, USA. The multiplex microbead assay is based on Luminex technology and measures proteins in a similar manner to standard sandwich ELISA, with comparable sensitivity and range. Regarding the detection limits, the LLOQ (Lower Limit of Quantitation) for the cytokines were: TNF-$\alpha$: 23.0 pg/ml, IFN-$\gamma$: 1.5 pg/ml, IL-4: 29.0 pg/ml, IL-6: 11.0 pg/ml, IL-10: 6.9 pg/ml, IL-17: 4.0 pg/ml, IL-23: 0.59 pg/ml. The LLOQ is the lowest concentration of an analyte in a sample that can be reliably detected and at which the total error meets the laboratory's requirements for accuracy [30].

### Flow cytometric staining and analysis

After collection of heparinized venous blood samples, peripheral blood mononuclear cells (PBMCs) were separated by density gradient preparation over Ficoll-Uropoline (Sigma Aldrich, Taufkirchen, Germany). Hereupon, PBMCs were cryopreserved in RPMI freezing media containing 10% FCS and 10% DMSO [31] and stored at $-30^{\circ}$C for 24 h and then at $-196^{\circ}$C until measurement. After storage, samples were thawed rapidly in a water bath at 37°C and washed twice to eliminate DMSO. For TH1, TH2 and TH17 analysis, cells were stimulated 5 h with cell stimulation cocktail including protein transport inhibitors (Phorbol 12-myristate 13-acetate (PMA), ionomycin, brefeldin A and monensin, eBioscience, San Diego, CA, USA) according to the manufacturer's protocol. Subsequently, cells were extracellulary stained with anti-human CD4 antibody and consecutively fixed and permeabilized (Fix-Perm-Solutions A and B, Life Technologies, Darmstadt, Germany) for intracellular staining with anti-human INF-$\gamma$ (detection of TH1 cells), IL-4 (detection of TH2 cells) and IL-17 antibody (detection of TH17 cells, all Biolegend, San Diego, CA, USA). T cell distribution was measured by FACS analysis with the Attune Acoustic Focusing Cytometer (Life Technologies, Carlsbad, USA). Tregs were identified and quan-

tified using multicolor flow cytometry after surface staining of PBMCs with mAbs specific for anti-human CD4, CD25 and CD127 and intracellular staining with an anti-human FoxP3 antibody. The frequencies of $CD4^+CD25^{high}$ and $CD4^+CD25^{high}CD127^{low}FoxP3^+$ T cells were expressed as percentage of total $CD4^+$ T cells by sequential gating on lymphocytes. Isotype controls (Biolegend, San Diego, CA, USA) were given for compensation and confirmation of antibody specificity.

### RNA isolation and synthesis of cDNA

$CD4^+$ cells were isolated from PBMCs by magnetic separation with Whole Blood CD4 MicroBeads (MACS Miltenyi Biotec, Auburn, CA, USA). Subsequently, total RNA was isolated using the mirVana miRNA Isolation Kit followed by a DNase-digest with Turbo DNA-free Kit (Ambion). Quantity and purity of the isolated RNA were measured using a NanoDrop ND-1000 spectrophotometer (Peqlab). After amplification of total RNA using TargetAmp 1-Round aRNA Amplification Kit (Epicentre Biotechnologies, Madison, WI, USA) and purification using RNeasy Mini Kit (Qiagen), cDNA synthesis was performed with SuperScript III First Strand Synthesis System (Invitrogen) and random hexamers (Qiagen).

### Quantitative real-time PCR (qPCR)

Quantitative RT-PCR was performed in duplicates with the LightCycler 480 instrument (Roche Diagnostics, Mannheim, Germany) using LightCycler 480 Probes Master and RealTime ready single assays (Roche Diagnostics) and UPL probes. The RealTime ready single assays contain target specific primers and a Universal ProbeLibrary LNA probe. Primer sequences and qPCR characteristics are given in Table 1. The cycling conditions comprised an initial denaturation phase at 95°C for 10 min, followed by 45 cycles at 95°C for 10 s, 60°C for 30 s and 72°C for 1 s. Relative mRNA expression was calculated by Relative Quantification Software (Roche Diagnostics) using an efficiency-corrected algorithm with standard curves and reference gene normalization against the reference genes succinate dehydrogenase complex subunit A (SDHA) and TATA box binding protein (TBP) as described in [32].

### Statistical analyses

All statistical analyses were performed using SigmaStat 12.0 (Systat Software, Chicago, USA). Every statistical analysis was started with testing for normal distribution using the Shapiro Wilk Test. Testing for differences between groups was accomplished by the T-Test for all data with normal distribution (IL-17, IL-23, TGF-β-mRNA, $CD25^+CD25^{high}$) and the nonparametric Mann-Whitney Rank Sum Test for all data without normal distribution (IL-6, IL-10, IL-23-mRNA, IFN-γ-mRNA, FoxP3-mRNA, RORγT-mRNA, Tregs, TH17 cells, TH17/Treg Ratio, TH1/TH2 Ratio). Values are expressed as mean ± standard deviation (SD) in the text and figures and p-values≤0.05 were considered statistically significant.

## Results

### Subjects and treatment variables

23 female and 14 male patients were enrolled. The median age of the patients at inclusion was 44.5 (range 21–73) years. The control group consisted of 25 (13 female/12 male) healthy pain free individuals aged 43.0 (range 24–54) years.

At inclusion, the average pain intensity of the patients was NRS 3.37 (±2.4) at rest and NRS 4.18 (±2.5) during movement. The average pain duration was 70.1 (±78.3) months. Using the KAB to evaluate the intensity of self-perceived stress, patients rated average KAB values at inclusion with 3.31 (±0.83). The average KAB of healthy controls was 1.80 (±0.64).

Upon therapy, 13 of 37 patients (35%) showed a significant reduction of pain scores (NRS) within 4 weeks, as defined by a decrease of pain ratings of ≥50%. They were therefore defined as therapy responders. Follow-up responders were defined as patients with persisting favorable effects according to the aforementioned criteria after 6 months.

### Serum cytokine profiles

Serum protein levels of TNF-α, IFN-γ and IL-4 were neither detectable in the peripheral blood of CLBP patients nor in blood samples of healthy controls. Generally, for both CLBP patients and healthy controls, the serum levels of IL-6, IL-10, Il-17 and IL-23 were found to be just marginally above the detection thresholds. No differences were found for IL-6, IL-10 and IL-17 (Figs. 1A, 1B, 1C). However, only levels of the pro-inflammatory cytokine IL-23 were found to be significantly higher in patients with CLBP before initiation of therapy as compared to healthy controls. (IL-23: 0.94±0.29 pg/ml in healthy controls vs. 1.21±0.43 pg/ml in CLBP patients; $p = 0.009$; Fig. 1D).

### mRNA expression of T cell cytokines

As measurements of specific cytokine profiles in serum turned out to be not conclusive, we determined the mRNA expression of cytokines and T cell specific transcription factors directly in the compartment of $CD4^+$ cells of CLBP patients and healthy volunteers. By means of qPCR, we evaluated the mRNA expression of the TH1 cytokines TNF-α and IFN-γ, the TH2 cytokines IL-4 and IL-10, FoxP3 and TGF-β indicative for Tregs, and IL-6, IL-17, IL 23 and the transcription factor RORγT specific for TH17 cells.

The expression of the TH1 specific cytokine IFN-γ did not exhibit significant differences in CLBP patients as compared to healthy controls (IFN-γ: 4.19±3.54 in CLBP patients vs. 3.60±2.20 in healthy controls, n.s., Fig. 2A). Expression levels of TNF-α, IL-4 and IL-10 were neither detectable in $CD4^+$ T cells of CLBP patients nor in healthy controls. As shown in Fig. 2B, the expression of IL-23 in $CD4^+$ T cell samples of CLBP patients was found to be significantly decreased compared to healthy controls (IL-23: 4.88±2.44 in CLBP patients vs. 7.73±3.77 in healthy controls, $p = 0.006$). The expression of both TGF-β and the transcription factor FoxP3 was significantly increased in $CD4^+$ cells of CLBP patients compared to healthy controls, thereby implying an increased Treg abundance (TGF-β: 0.21±0.07 in CLBP patients vs. 0.14±0.05 in healthy controls, $p = 0.014$, Fig. 3A, FoxP3: 0.21±0.14 in CLBP patients vs. 0.14±0.06 in healthy controls, $p = 0.009$, Fig. 3B). Regarding factors specific for TH17 cells, the expression of IL-6 and IL-17 was neither detectable in $CD4^+$ samples of CLBP patients nor in healthy controls. Expression levels of RORγT did not differ in CLBP patients and healthy controls (RORγT: 0.028±0.02 in CLBP patients vs. 0.025±0.01 in healthy controls, n.s., Fig. 3C). Taken together, qPCR results promoted the hypothesis that CLBP patients may exhibit altered distribution patterns of Treg and TH17 subsets whereas TH1/TH2 balance appeared to be unchanged.

**Table 1.** RT-PCR Assay Characteristics and Primer Sequences.

| Gene | Primer Sequence |
|---|---|
| SDHA | Roche RealTime Ready Single Assay ID 102136 |
| TBP | Roche RealTime Ready Single Assay ID 101145 |
| FoxP3 | Roche RealTime Ready Single Assay ID 113503 |
| IL-4 | for 5′TGCCTCACATTGTCACTGC 3′ |
| | rev 5′GCACATGCTAGCAGGAAGAAC 3′, UPL probe #38 |
| IL-6 | for 5′GATGAGTACAAAAGTCCTGATCCA 3′ |
| | rev 5′CTGCAGCCACTGGTTCTGT 3′, UPL probe #40 |
| IL-10 | for 5′TGCCTTCAGCAGAGTGAAGA 3′ |
| | rev 5′GCAACCCAGGTAACCCTTAAA 3′, UPL probe #67 |
| IL-17 | for 5′TGGGAAGACCTCATTGGTGT 3′ |
| | rev 5′GGATTTCGTGGGATTGTGAT 3′, UPL probe #8 |
| IL-23 | for 5′CAGCTTCATGCCTCCCTACT 3′ |
| | rev 5′GACTGAGGCTTGGAATCTGC 3′, UPL probe #14 |
| TGF-β | for 5′ACTACTACGCCAAGGAGGTCAC 3′ |
| | rev 5′TGCTTGAACTTGTCATAGATTTCG 3′, UPL probe #31 |
| TNF-α | for 5′CAGCCTCTTCTCCTTCCTGAT 3′ |
| | rev 5′GCCAGAGGGCTGATTAGAGA 3′, UPL probe #29 |
| IFN-γ | for 5′GGCATTTTGAAGAATTGGAAAG 3′ |
| | rev 5′TTTGGATGCTCTGGTCATCTT 3′, UPL probe #21 |
| RoRγT | for 5′CAGCGCTCCAACATCTTCT 3′ |
| | rev 5′CCACATCTCCCACATGGAC 3′, UPL probe #69 |

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## CLBP patients exhibit an increased Treg frequency while the TH17 frequency is decreased

To test this hypothesis, we next evaluated the distribution of T cell subsets in blood samples of patients and healthy volunteers by flow cytometric analyses. The relative number of Tregs was assessed by using two different staining protocols: First, with antibodies specific for $CD4^{+}CD25^{high}$ cells and second, specific for $CD4^{+}CD25^{high}CD127^{low}FoxP3^{+}$ cells (Gating strategy is displayed on Fig. 4). TH17 cells were identified by intracellular staining with anti-human IL-17 antibody (gating strategy is displayed on Fig. 5).

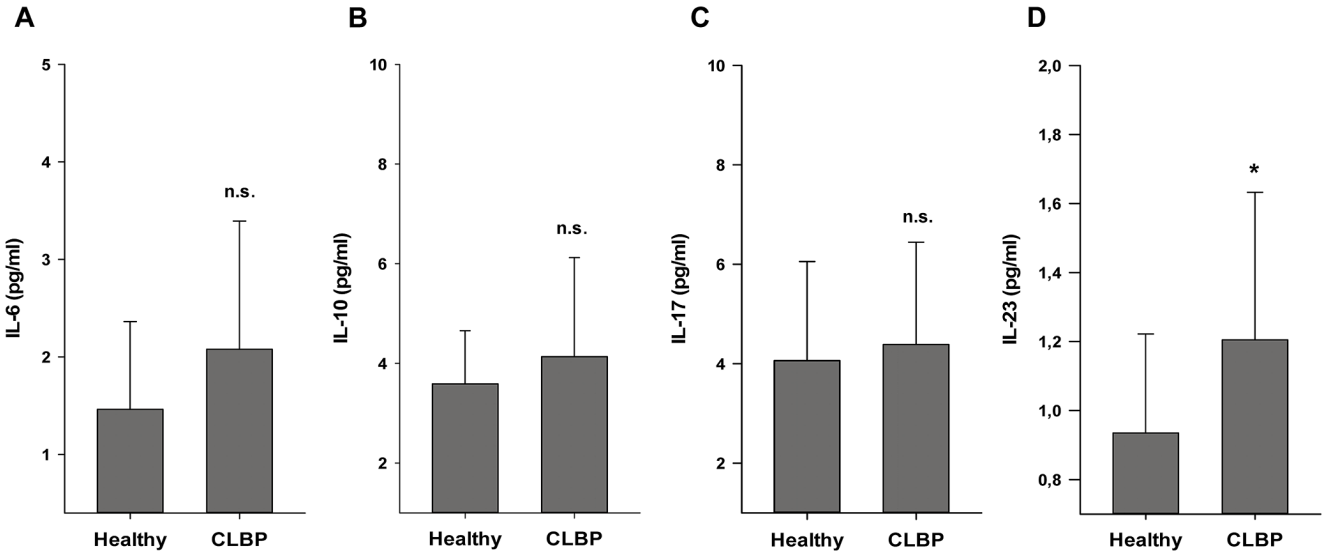


**Figure 1. Concentrations of serum cytokines, determined by using a Human Cytokine multiplex immunoassay.** No differences are found analyzing serum protein levels of IL-6, IL-10 and IL-17 between patients and healthy controls (Fig. 1A, 1B, 1C). Protein levels of pro-inflammatory cytokine IL-23 are significantly higher in the peripheral blood of patients with CLBP compared to healthy controls. Values are expressed as mean ± standard deviation. (IL-23: 0.94±0.29 pg/ml in healthy controls vs. 1.21±0.43 pg/ml in CLBP patients; p = 0.009; Fig. 1D).
doi:10.1371/journal.pone.0104883.g001

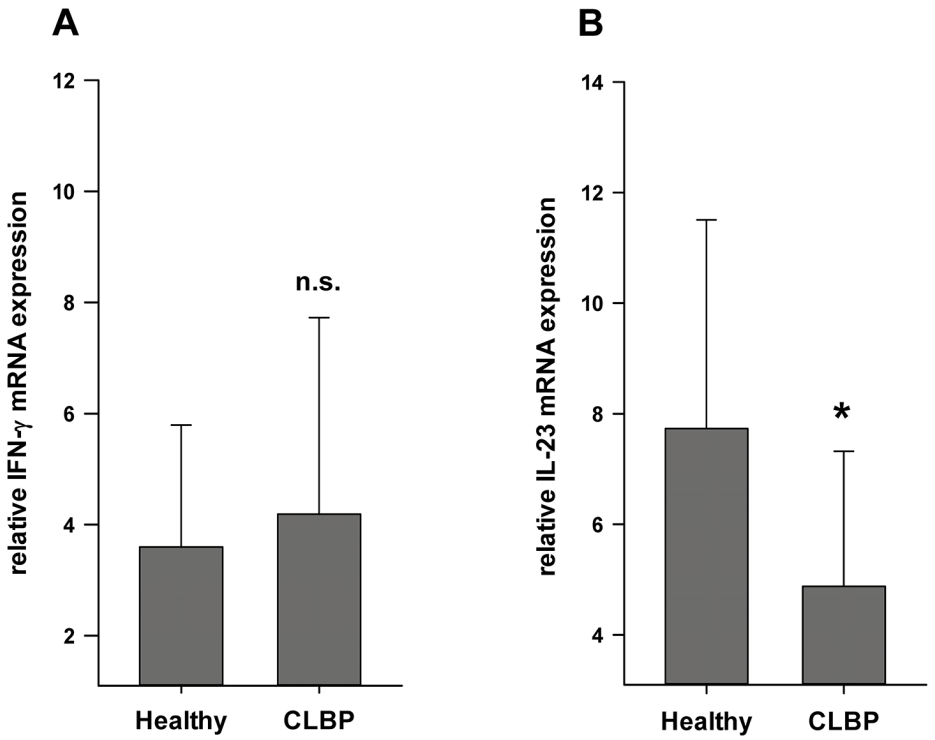


**Figure 2. Expression levels of T cell related cytokine mRNA measured by qPCR.** TNF-α, IL-4 and IL-10 were neither detectable in $CD4^+$ T cells of CLBP patients nor in healthy controls. The expression of IFN-γ did not exhibit significant differences in CLBP patients as compared to healthy controls (IFN-γ: 4.19±3.54 in CLBP patients vs. 3.60±2.20 in healthy controls, n.s.; Fig. 2A) whereas IL-23 expression of in $CD4^+$ T cell samples of CLBP patients was found to be significantly decreased (4.88±2.44 in CLBP patients vs. 7.73±3.77 in healthy controls, p = 0.006; Fig. 2B).
doi:10.1371/journal.pone.0104883.g002

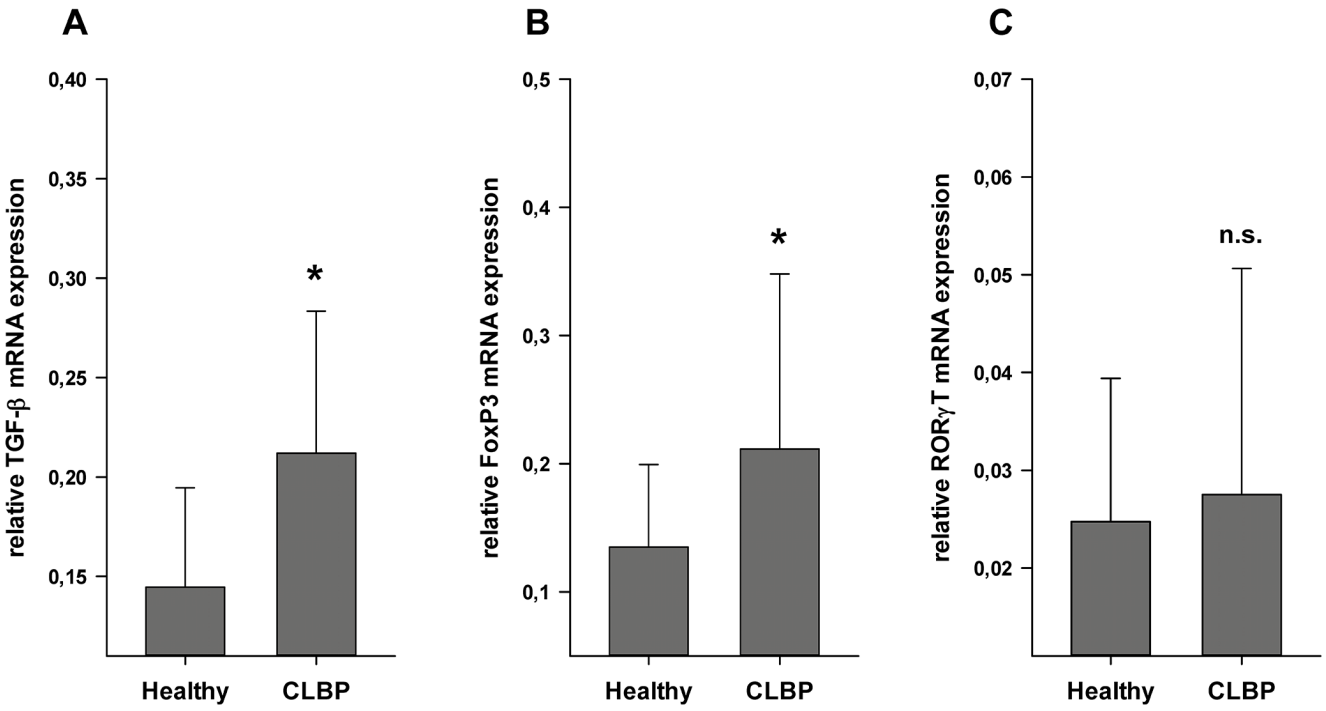


**Figure 3. Expression levels of T cell subset related mRNA measured by qPCR.** TGF-β and FoxP3 mRNA expression, specific for Tregs, was significantly higher in patients with CLBP than in healthy controls (TGF-β: 0.21±0.07 in CLBP patients vs. 0.14±0.05 in healthy controls, p = 0.014; Fig. 3A), (FoxP3: 0.21±0.14 in CLBP patients vs. 0.14±0.06 in healthy controls, p = 0.009; Fig. 3B). TH17 specific expression of IL-17 was neither detectable in $CD4^+$ samples of CLBP patients nor in healthy controls. Expression levels of RORγT did not differ in CLBP patients and healthy controls (RORγT: 0.028±0.02 in CLBP patients vs. 0.025±0.01 in healthy controls, p = n.s.; Fig. 3C).
doi:10.1371/journal.pone.0104883.g003

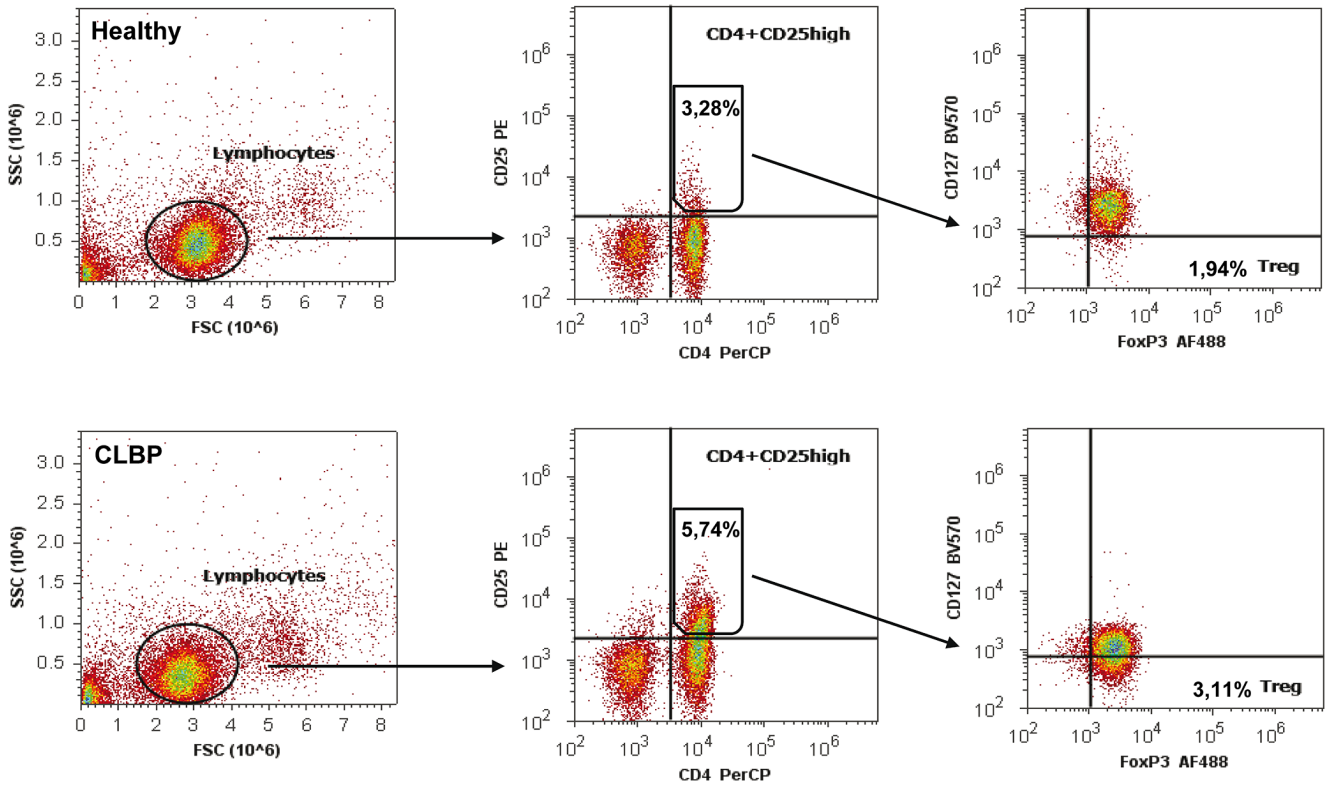


**Figure 4. Gating strategy for the detection of Tregs.** PBMCs extracellular stained with PerCP labeled anti-human CD4-antibody, PE labeled anti CD25-antibody, Brilliant Violet (BV570) labeled anti CD127-antibody and intracellular stained with Alexa Fluor (AF488) labeled anti-human FoxP3-antibody. Lymphocyte population was gated from PBMCs according to forward scatter (FSC) characteristics and side scatter (SSC) characteristics (left). Gated lymphocytes were then separated in $CD4^{+}CD25^{high}$ cells/T cells (middle) and $CD4^{+}CD25^{high}CD127^{low}FOXP3^{+}$ cells/$CD4^{+}$T cells (right, named Treg). Upper row represents the result of a healthy control with less $CD4^{+}CD25^{high}$ T cells (3.28%) and less $CD4^{+}CD25^{high}CD127^{low}FoxP3^{+}$ T cells (1.94%) compared to a patient with CLBP (lower row, 5.74% and 3.11%).
doi:10.1371/journal.pone.0104883.g004

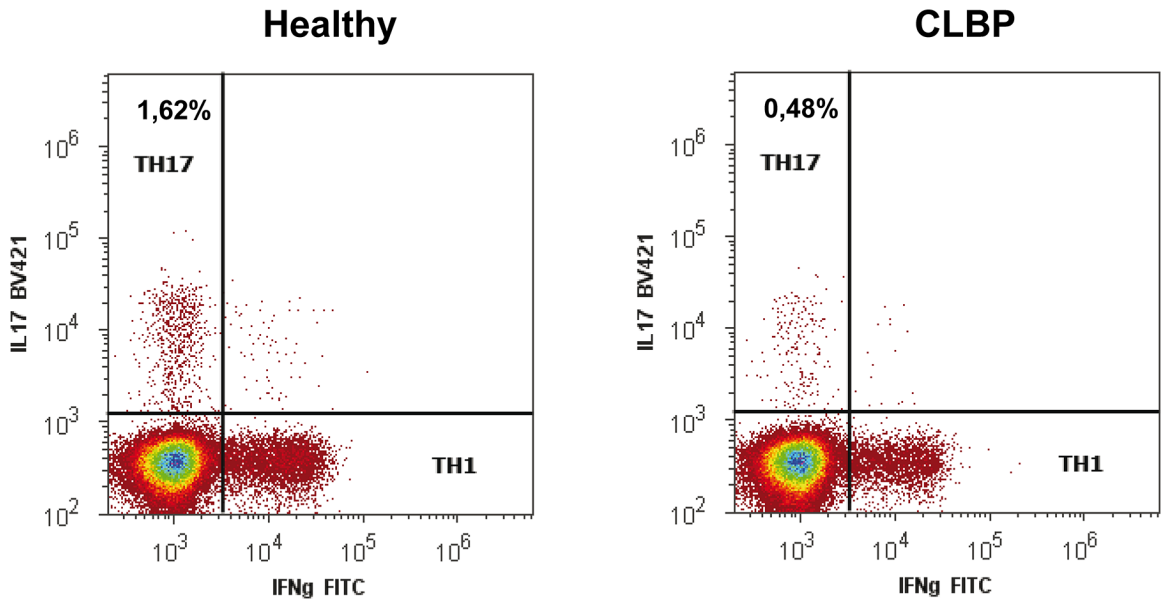


**Figure 5. Gating strategy for the detection of TH1 and TH17 cells.** PBMCs stimulated with cell stimulation cocktail for 5 h followed by intracellular staining with Brilliant Violet (BV421) labeled anti-human IL-17 antibody and FITC labeled anti-human IFN-$\gamma$ antibody.
doi:10.1371/journal.pone.0104883.g005

With both Treg staining protocols, a significantly increased frequency of Tregs was seen in CLBP patients as compared to healthy controls. FACS analysis applying the $CD4^+CD25^{high}$ staining protocol resulted in 4.45±0.88% $CD4^+CD25^{high}$ cells in CLBP patients vs. 3.49±0.5%, $CD4^+CD25^{high}$ cells in healthy controls ($p<0.001$, Figure 6A). $CD4^+CD25^{high}CD127^{low}FoxP3^+$ staining as a more specific staining protocol for Tregs revealed similar results with 2.89±1.07% Tregs in CLBP patients vs. 1.93±0.66% Tregs in healthy controls ($p = 0.001$, Figure 6B). The frequency of TH17 cells, however, was found to be significantly decreased in CLBP patients as compared to healthy volunteers (TH17: 0.46±0.24% in CLBP patients vs. 1.14±0.73% in healthy controls, $p = <0.001$, Figure 6C). Conclusively, ratios of Th17/$CD4^+CD25^{high}$ resp. Th17/$CD4^+CD25^{high}CD127^{low}FoxP3^+$ were significantly decreased in CLBP patients as compared to healthy controls (Th17/$CD4^+CD25^{high}$: 0.12±0.08 in CLBP patients vs. 0.33±0.23 in healthy controls, $p<0.001$, Fig. 7A; Th17/$CD4^+CD25^{high}CD127^{low}FoxP3^+$: 0.23±0.17 in CLBP patients vs. 0.64±0.79 in healthy controls, $p<0.001$, Fig. 7B).

### TH1/TH2 balance is not altered in CLBP patients

As depicted in Fig. 7C, TH1/TH2 balance did not reveal significant differences between CLBP patients and healthy controls; however, a trend towards a decreased TH1/TH2 ratio was observed (TH1/TH2: 9.76±7.27 in CLBP patients vs. 14.72±12.81 in healthy controls, p = n.s.).

### T cell ratios remain altered in CLBP patients after multimodal therapy

To evaluate the impact of therapeutic interventions on the observed T cell subset alterations in CLBP patients, the distribution of TH cells subsets (TH1, TH2, TH17 and Tregs) was analyzed in the group of therapy responders before, immediately after therapy and 6 months later. As depicted in Fig. 8A, these patients showed an ongoing decrease of NRS by ≥ 50% due to the treatment program. The pain reduction was also accompanied by a decrease of the KAB values. However, as shown in Figure 8B, this therapeutic effect was not reflected in any respective adaptation of the T cell subsets.

## Discussion

Pathomechanisms driving the chronification of low back pain still remain largely elusive. While a growing body of evidence suggests a pivotal role of adaptive immune responses in the pathogenesis of chronic pain, these issues have not conclusively been analyzed for CLBP, yet.

Our results indicate that CLBP is associated with characteristic alterations of T helper cell subsets: The ratio between regulatory T cells, playing a vital role in controlling adaptive immune responses, and TH17 cells, one of the key effector T cells mediating autoimmunity [33], was significantly decreased. We further provide evidence that these alterations persist even in these patients exhibiting significant pain reduction after participation in a standardized multimodal therapy program [3].

Assuming that cytokines as central mediators of cellular immunity may mirror immune cell functions, we first analyzed seven T cell related cytokines (TNF-α, IFN-γ, IL-4, IL-6, IL-10, IL-17, IL-23) in serum samples of CLBP patients and healthy controls. TNF-α, IFN-γ and IL-4 were below the detection limits in patients as well as in healthy controls, and the results of the remaining four analytes were only slightly above the detectable limit. Values for the proinflammatory cytokines IL-6 and IL-17 in

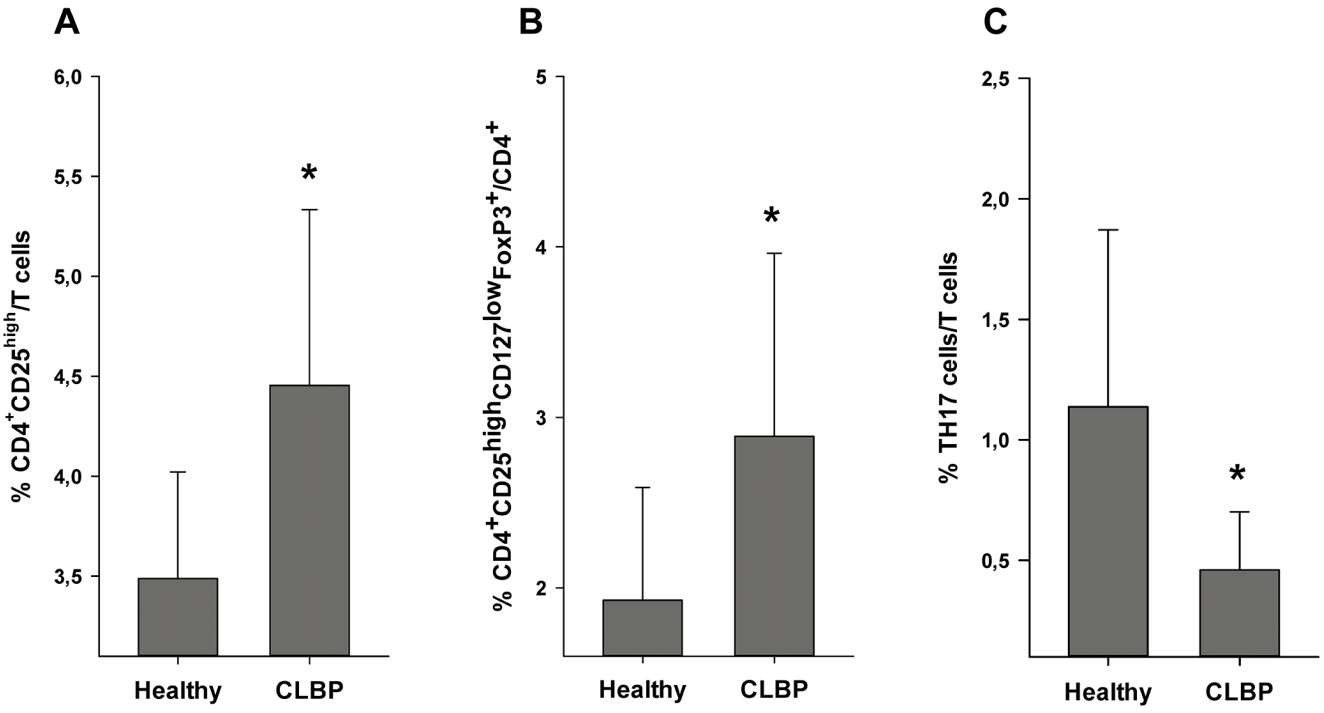


**Figure 6. Flow cytometric quantification of Tregs and TH17 cells.** Results show significantly higher percentage of anti-inflammatory Tregs in patients with CLBP in both staining protocols ($CD4^+CD25^{high}$ cells: 4.45±0.88% in CLBP patients vs. 3.49±0.53% in healthy controls, $p<0.001$; Fig. 6A), ($CD4^+CD25^{high}CD127^{low}FoxP3^+$ cells: 2.89±1.07% in CLBP patients vs. 1.93±0.66% in healthy controls, $p = 0.001$; Fig. 6B). Number of TH17 cells as percentage of T cells in peripheral blood show significantly lower percentage of pro-inflammatory TH17 cells in patients with CLBP (TH17 cells: 0.46±0.24% in CLBP patients vs. 1.14±0.73% in healthy controls, $p<0.001$; Fig. 6C).
doi:10.1371/journal.pone.0104883.g006

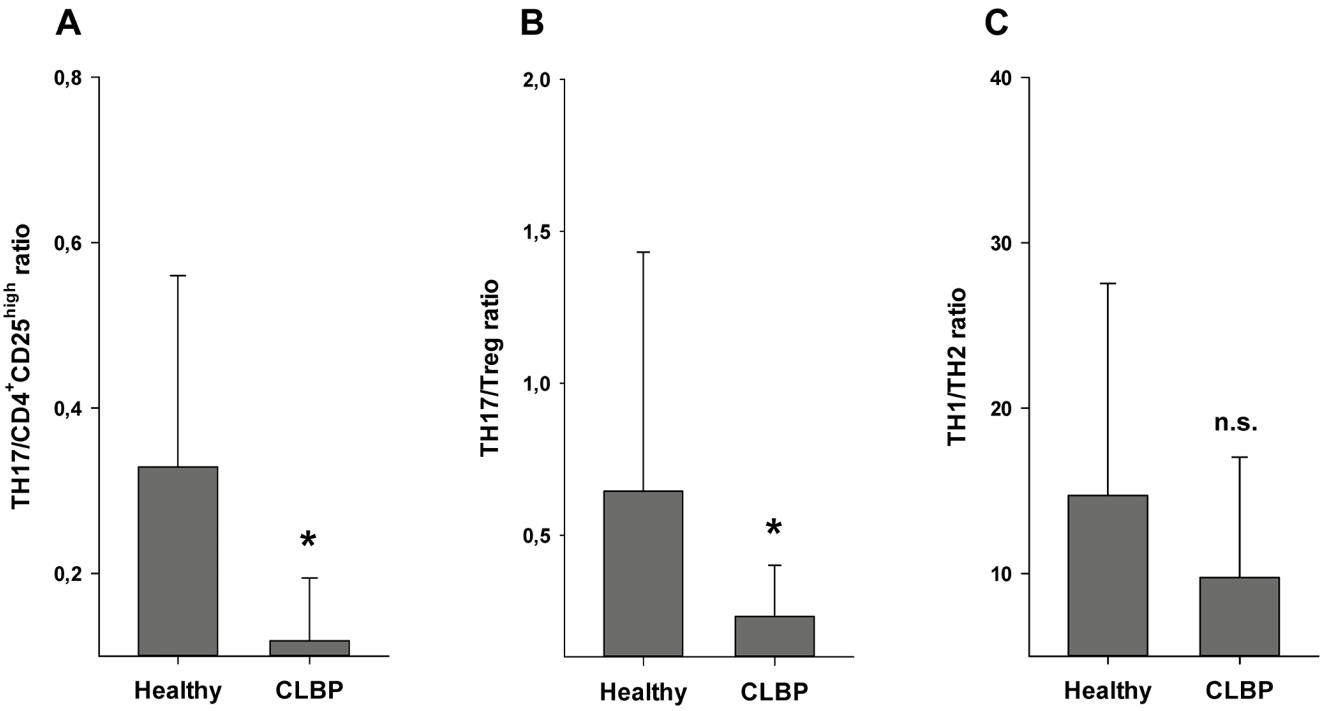


**Figure 7. Ratios of TH17/CD4$^+$CD25$^{high}$, TH17/Tregs and TH1/TH2 cells.** Ratios of Th17/CD4$^+$CD25$^{high}$ and Th17/CD4$^+$CD25$^{high}$CD127$^{low}$-FoxP3$^+$ were significantly decreased in CLBP patients as compared to healthy controls (Th17/CD4$^+$CD25$^{high}$: 0.12±0.08 in CLBP patients vs. 0.33±0.23% in healthy controls, $p<0.001$; Fig. 7A), (Th17/CD4$^+$CD25$^{high}$CD127$^{low}$FoxP3$^+$: 0.23±0.17 in CLBP patients vs. 0.64±0.79 in healthy controls, $p<0.001$; Fig. 7B). Ratio of TH1/TH2 cells in peripheral blood of patients with CLBP and healthy controls were tendencially decreased in patients with CLBP, but did not reach significance (9.76±7.27 in CLBP patients vs. 14.72±12.81 in healthy controls, $p=0.19$, Fig. 7C).
doi:10.1371/journal.pone.0104883.g007

blood samples of CLPB patients were slightly elevated as compared to controls. However, this finding may be of limited clinical relevance as normal plasma concentrations for IL-6 of healthy controls are about 1 pg/ml with immense increases in situations of severe systemic infection ranging up to 10.000-fold. Our results demonstrate only an 1.5-fold increase in IL-6 levels in patients with CLBP, which could even occur after physical activity or in obesity [34]. However, the relevance of cytokine measurements should generally be regarded with caution as serum levels of most cytokines are influenced by a complex interplay of macrophages/monocytes, fibroblasts, endothelial−/epithelial cells and dendritic cells thus complicating the extrapolation from plasma cytokines to immune cell functions. Moreover, ranges of detection exhibits considerable variances between the different assays used [35]. Even different types of Luminex-based platforms exhibit differences in their ability to measure serum levels of cytokines and thus, may be more useful in studies in which relative rather than absolute changes in cytokines are of interest [36,37].

Overall, these data suggest that serum levels of cytokines are not suitable to monitor the adaptive immune response in CLBP and prompted us to analyze the expression of cytokines directly in the compartment of CD4$^+$ cells as central players of the T cell response. While no differences in the expression of TH1 and TH2 cytokines were observed, qPCR results clearly pointed to an increased abundance of Tregs in CLBP patients, as expression of both TGF-β and the transcription factor FoxP3 were significantly increased. Moreover, expression of IL-23 was clearly decreased supporting the assumption that TH17 frequency may be reduced. IL-17 and RORγT, however, did not differ significantly between CLBP and controls which may be due to the fact that the subset of TH17 cells per se is only less than 2% of CD4+ cells. Thus, resolving differences of cytokine expression without prior cell sorting may be difficult. The opposite results of increased IL-23 protein levels and decreased IL23-mRNA-expression is in line with a wide body of literature showing a big discrepancy between mRNA expression and protein levels as a result of control mechanisms. These can affect post-transcriptional, translational and protein degrading processes [38,39].

Our findings encouraged us to investigate T cell subset compositions by flow cytometric analyzes. We used standard staining procedures to identify TH1, TH2, and TH17 cells, whereas for identification of anti-inflammatory Tregs, both classic extracellular staining of CD4$^+$CD25$^{high}$ and a more specific extra- and intracellular staining of CD4$^+$CD25$^{high}$CD127$^{low}$FoxP3$^+$ was applied. As activated human T cells can transiently express FoxP3 and CD25, differentiation of Tregs from activated effector T cells by only using these two markers may suffer from inaccuracies. CD127 is a newly described surface marker that allows distinguishing regulatory T cells from other CD25$^+$ cells [40]. For TH17 identification, we chose two experimental approaches: determination the mRNA expression of the TH17 specific transcription factor RORγT by qPCR, and FACS analyses of IL-17 production, which has revealed as a very reliable method to identify TH17 cells [41]. However, flow cytometry staining protocols combining IL-17 with further markers, e.g. CD161 or CCR6, may further refine these measurements and thus may be implemented in future studies.

Flow cytometry clearly proved the assumed alterations of the TH17/Treg balance, as a significantly increased frequency of Tregs and decreased frequency of TH17 cells was observed in our CLBP patients. Even in flow cytometric analyzes, no differences in the TH1/TH2 ratio were detectable. There are several investi-

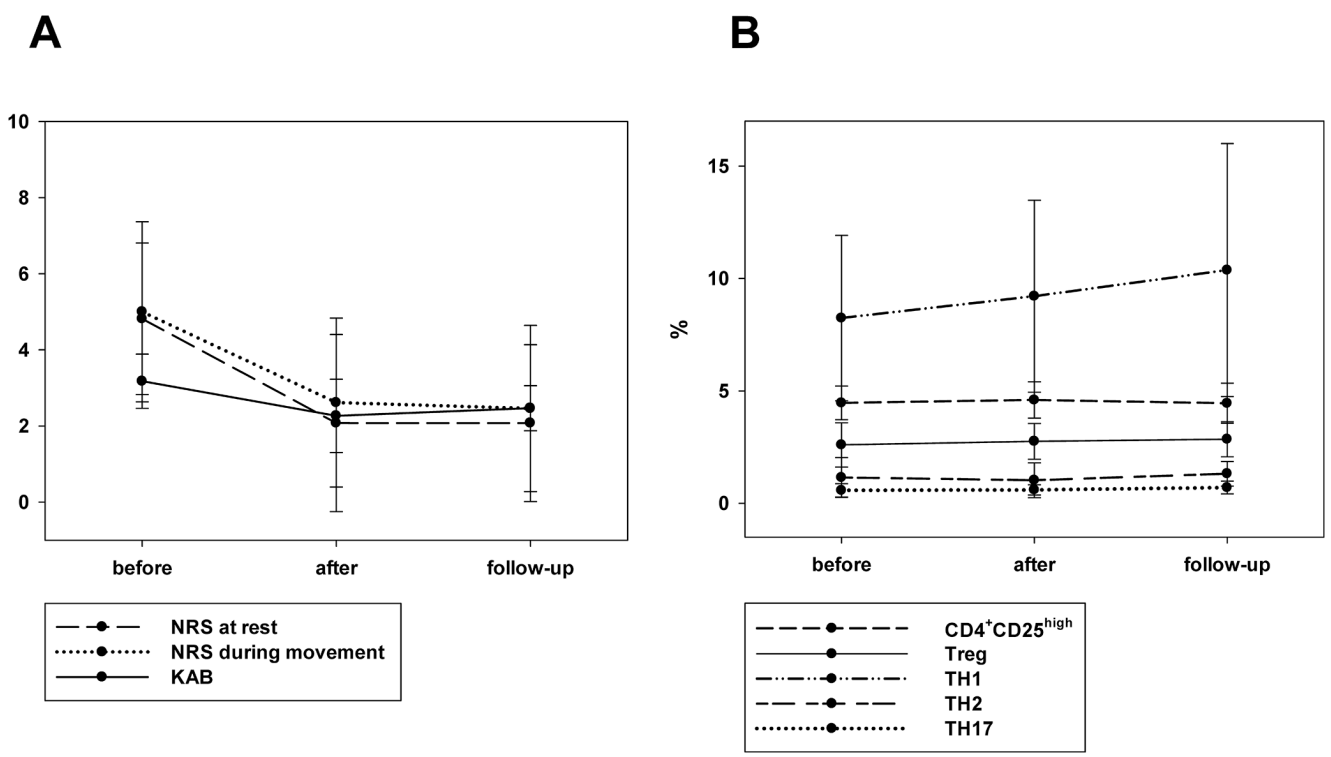


**Figure 8. NRS pain scores, KAB stress scores and T cell subsets before and after treatment.** 35% [n = 13] of all patients benefited by the 4 weeks intensive multimodal therapy with long lasting pain- and stress reduction (Fig. 8A). Even all responders showed a significant pain- and stress reduction of ≥50%, no transformation were observed regarding T cell subsets. None of our analyzed T cell subsets (TH1, TH2, TH17, Tregs) normalized after successful therapy (Fig. 8B). (NRS at rest before/after: p = 0.025, NRS at rest before/follow up: p = 0.003, NRS during movement before/after: p = 0.046, NRS during movement before/follow up: p = 0.012, KAB before/after: p = 0.024, KAB before/follow up: p = 0.019).
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gations which point to a beneficial role of anti-inflammatory cells and cytokines together with a detrimental function of a pro-inflammatory immune response in pain patients [6,7,8,9]. In contrast to these findings, our results showing an anti-inflammatory shift on cellular level are in accordance with other chronic diseases like mild depression or chronic fatigue syndrome [42,43]. A potential explanation for our findings on TH17/Treg balance may therefore be that pain-related, long lasting chronic stress and fatigue induces an ongoing dysregulation of immune cells towards an anti-inflammatory phenotype [44,45,46]. On the other hand, it may also be discussed that dysregulation of the TH17/Treg balance may exist first, thus predisposing the affected individuals to experience chronification of pain symptoms. The latter theory may be supported by our surprising findings that the observed TH17/Treg imbalance persisted despite clinical improvement after multimodal therapy even after a follow-up period of 6 months.

In summary, we found a persisting TH17/Treg imbalance with an increased count of anti-inflammatory Tregs and a decreased number of pro-inflammatory TH17 cells in peripheral blood of CLBP patients pointing to a strong association between chronic pain and immune suppression rather than immune activation. Importantly, these findings are not reflected by serum cytokine concentration, indicating a major role of specific T cell subset measurements in the analysis of pain-related immune responses.

Taken together, the results of the current study suggest an involvement of TH17/Treg in the pathogenesis of CLBP and emphasize the importance of these cells in the crosstalk of pain and immune response.

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## Author Contributions

Conceived and designed the experiments: BL BR SCA. Performed the experiments: BL BR JZ LE. Analyzed the data: BL JZ LE SCA. Contributed reagents/materials/analysis tools: BL JZ LE SCA. Wrote the paper: BL SK SCA. Interpretation of data, making intellectual contributions to the manuscript: PM.

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