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# Supplementary Materials for

## Acute inflammatory response via neutrophil activation protects against the development of chronic pain

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**The PDF file includes:**



**Other Supplementary Material for this manuscript includes the following:**

## SUPPLEMENTARY MATERIALS AND METHODS

### HUMAN LBP COHORT

#### Blood collection and RNA extraction

An RNA standard operating procedure was developed and validated to achieve uniformity and provide details in each sample (*50*). Whole peripheral blood was collected at both visits, $t_0$ and $t_1$, at the Pain Service of University Hospital of Parma. Tempus blood RNA tubes were used (Applied Biosystems, n. 4342792, Beverly, MA, USA), shaken vigorously for 10–15 sec immediately after collection for RNA stabilization, and stored at -20 °C. Total RNA was isolated using Maxwell® 16 LEV simplyRNA Blood Kit (Promega, Madison, WI, USA) according to the manufacturer's instructions. After, total RNA was quantified using the NanoDrop ND-1000 (Thermo Scientific, Waltham, MA, USA), and optical purity of RNA was defined according to the 260/280 ratio not less than 1.8 and 260/230 ratio between 1.8–2.2 of the isolated RNA. Then, the RNA integrity was assessed with the Agilent 4200 TapeStation (Agilent Technologies, Santa Clara, CA, USA) using the RNA Screen Tape assay (Agilent Technologies, n. 5067-5576). An average of 8 µg of total RNA was recovered from frozen whole blood, displaying RIN values between 6.5–8.4. Purified RNA was stored at -80 °C in 25-µl aliquots.

#### Whole transcriptome sequencing (RNA-Seq)

All RNA-sequencing was performed by the Genome Quebec Innovation Centre (McGill University, Montreal, Canada). Transcriptome libraries were generated from 1 µg of total RNA using the Kapa RNA-stranded Sample Prep Kit (KK8400, KAPABiosystems, Wilmington, MA, USA) following the manufacturer's protocols. Briefly, poly-A mRNA was purified using poly-T

oligo-attached magnetic beads using two rounds of purification. During the second elution of the poly-A RNA, the RNA was fragmented and primed for cDNA synthesis. During cDNA synthesis, dUTP was incorporated in the second-strand synthesis, and subsequently the dUTP-containing strand was selectively degraded. Adenylation of the 3' ends and ligation of adapters were done following the manufacturer's protocol. Enrichment of DNA fragments with adapter molecules on both ends was performed using 10 cycles of PCR amplification using the KAPA PCR mix and Illumina-adapted primers cocktail. Paired-end 2x100 nucleotides sequencing was performed using the Illumina HiSeq2000 machine running TruSeq v3 chemistry at Genome Québec.

**Processing of RNA-Seq data**

Deep-sequencing reads were aligned on the human genome version hg19/GRCh37 using STAR aligner version 2.5.1b (*51*). Gene-level counting was performed using featureCounts version 1.6.0 (*52*), using RefSeq gene boundaries (*53*), Gene-centric outlier expression levels detected using an iterative leave-one-out method (*54*).

The quality of sequencing was confirmed by the RNA integrity numbers (RIN; mean=7.9, SD=0.8), total average number of sequenced paired-end reads (mean=135.8M, SD=26.6M, min=49.0M), sequencing mapping rates or properly paired mapped reads to total paired reads in sequencing (mean=95.8%, SD=0.8%, min=93.8%) (Table S2A), and by the stability of results from DESeq2 after removing samples with highest gene expression outliers (*54*). Following principal component analyses of the transcriptomes, we established that the variables associated with the first few principal components considered in differential gene

expression analyses were RIN, age, sex, and smoking status (Table S2B). In total, 196 time-paired RNA-Seq samples for 98 individuals were obtained.

Finally, to enable comparisons between different contrasts and to correct for multiple testing from gene pools of the same sizes, we transformed the counts on genes into units of transcripts per million (tpM) for each sample; we required that all samples featured tpM levels of at least 0.1, hence only 12081 genes out of the 27937 total (43.2%) were considered for further analyses. Resulting DESeq2 P-values obtained using counts (not tpM) were re-corrected for multiple testing with FDR, this time from the shortened list of genes.

**Pathway analyses**

A pathway enrichment score was computed using the R package 'fgsea' (*48*) with gene sets from Gene Ontology's biological processes (*55*, *56*). The pathways' definition files were taken from the URL http://download.baderlab.org/EM_Genesets/December_01_2019/Human/symbol/GO/; December 2019 version. The assignment of activation or repression of a pathway was based on the accumulated evidence of concordant fold-change direction of genes that are part of the pathway (*57*); a positive (negative) value indicates that many or most genes of the pathways were regulated in an up (down)-ward fashion. The genes with the greatest difference in expression in a pathway were defined as "leading edge" genes, as they determined in which direction the pathway was anticipated to be regulated. Here, a differentially expressed pathway could reach statistical significance even though few or none of the associated genes individually reached statistical significance for differential expression in a transcriptome-wide analysis.

**Blood cell type population fraction estimates**

Estimates of blood cell type population fractions from RNA-Seq data were obtained using CIBERSORT (*22*). Input data for CIBERSORT were gene expression levels, in tpM units. tpM were derived from featureCounts (*52*), counts on genes normalized to gene lengths provided by RefSeq (*53*). Estimates for cell type fractions were used as co-variables for some analyses of differential expression of genes, accounting for different populations of cell types for those with fractions ≥1%.

**Blood cell type gene expression**

Gene lists for blood cell types were extracted from the LM22 gene expression matrix of CIBERSORT (*22*). A gene was retained in a cell's list only if its expression level was greater than the average across all other cell types. We calculated the gene overlap between four selected cell types using the online tool to draw a 4-way Venn diagram at interactivenn.net (*58*).

**TEMPOROMANDIBULAR DISORDERS (TMD) REPLICATION COHORT**

Reproducibility of the LBP results was investigated in two replication cohorts from a study of painful temporomandibular disorder (TMD). The cohorts were community-based volunteers from four U.S. study sites recruited into the "Orofacial Pain Prospective Evaluation and Risk Assessment" (OPPERA) study that began in 2006 (*59*). The first replication cohort comprised adults who had first-onset, acute TMD when enrolled from 2013-2016, and who were followed for a six months period to identify those who had persistent TMD. The cohort was selected during screening interviews of 166,062 phone numbers between 2013 through 2016, which identified 327 subjects who reported ≥5 days of TMD pain in the preceding 30 days, but no TMD symptoms at any time in their life before that period. During a baseline ($t_0$) study visit, 162

of them has examiner-classified TMD based on the DC-TMD criteria (*60*). Six months later ($t_1$), 118 were again examined to classify their clinical TMD status. We defined the TMD subjects who no longer met TMD criteria at 6 months as the resolved pain group (R), and otherwise as the persistent pain group (P). In total, 64 prospective samples were analyzed in that prospective cohort. The second TMD cohort was from another OPPERA study protocol that enrolled adults into a cross-sectional from 2014-2016 (*61*). This analysis used samples from 86 adults with examiner-classified chronic TMD based on DC-TMD criteria and 65 TMD-free controls.

At each visit, participants donated blood, which was collected in PaxGene tubes and stored at -80°C, and RNA isolated using Qiacube column-based separation or Chemagic magnetic bead separation. Approximately 250ng Total RNA was used for mRNA-Seq library preparation by following the Illumina TruSeq stranded mRNA sample preparation guide (Illumina Inc, San Diego, CA). The first step in the workflow involved purifying the poly-A containing mRNA molecules using poly-T oligo-attached magnetic beads. Following purification, the mRNA was fragmented into small pieces using divalent cautions under elevated temperature. The cleaved RNA fragments were copied into first strand cDNA using reverse transcriptase and random primers. This was followed by second strand cDNA synthesis using DNA Polymerase I and RNase H. Strand specificity was achieved by replacing dTTP with dUTP in the Second Strand Marking Mix (SMM), as the incorporation of dUTP in second strand synthesis effectively quenched the second strand during amplification. Further specificity was achieved by addition of Actinomycin D to the First Strand Master Mix Act D (FSA), which prevented spurious DNA dependent synthesis during first strand synthesis, while allowing RNA dependent synthesis. The resulting cDNA fragments then went through an end repair process, the addition of a single ‘A’ base, and then ligation of the adapters. The products were then purified

and enriched with PCR to create the final RNA-Seq library. After RNA-Seq libraries were subjected to quantification process, pooled for cBot amplification and subsequent sequencing run with Illumina HiSeq 3000 platform with 50bp single read sequencing. After the sequencing run, demultiplexing with Bcl2fastq2 was employed to generate the fastq file for each sample.

In Total, 65 healthy controls samples, 86 samples from chronic TMD cases, and 64 prospective samples were analyzed, in which 42 samples were from subjects with acute pain at $t_0$, from whose we had 14 follow up samples from subjects with persistent pain and 8 from subjects who resolved their pain at $t_1$.

**MOUSE EXPERIMENTS**

To assess how the inhibiting the inflammatory response would impact the resolution of pain from an acute injury we performed a series of mouse experiments using neuropathic and inflammatory assays, and a variety of anti-inflammatory and non-anti-inflammatory analgesics. In addition, the relevance of neutrophils was assessed directly. All mouse studies were performed by an experimenter blinded to drug condition, and subjects were randomized to drug groups. Power analyses were not performed because effect sizes could not be anticipated in advance. Instead, we used sample sizes typical for these assays (*65*).

*Mice*: CD-1 male and female mice aged 6–12 weeks (ICR:Crl, Charles River, St. Constant, QC) were used in these experiments. All mice were housed in standard shoebox cages with 2–4 (same-sex) per cage in a light (14:10 h, lights on at 07:00 h) and temperature-controlled (20±1 °C) environment with ad libidum access to food (Harlan Teklad 8604) and tap water. Mice were acclimated to the vivarium for 7 days post-arrival and before testing. Each mouse was used

in a single drug experiment. Equal numbers of male and female mice were included in each cohort.

*Drugs*: Dexamethasone (PromoCell, Heidelberg, Germany) was administered via daily subcutaneous injections at a dosage of 0.5 mg/kg/day. Diclofenac, morphine, and gabapentin were administered via intraperitoneal (i.p.) injection at a dose of 25 mg/kg/day, 5 mg/kg/day, and 100 mg/kg/day, respectively. Lidocaine was administered via subcutaneous (s.c.) injection of 20 µl of 2% lidocaine into the plantar hind paw. All drugs other than dexamethasone were purchased from Sigma-Aldrich (St. Louis, MO, USA). Anti-Ly6G (BioX Cell [InVivoPlus anti-mouse Ly6G/Ly6C (Gr-1)]) and its isotype control (BioX Cell [InVivoPlus rat IgG2b isotype control, anti-keyhole limpet hemocyanin]) were injected at a dose of 125 µg/day. S100A8 and S100A9 were injected (10 µl) into the plantar surface of the inflamed (see below) hind paw at a dose of 1 µg/day.

*Neutrophils*: Neutrophils were isolated using the EasySep Mouse Neutrophil Enrichment kit (Stem Cell Technologies, Vancouver, Canada) as per manufacturer's instructions. Briefly, blood was collected by cardiac puncture in a 21-gauge needle coated with 10% EDTA and pooled from 2–3 mice in PBS/2 mM EDTA. Red blood cells were lysed after incubation in 0.83% $NH_4Cl$ for 10 min at 4 °C, with the reaction stopped by adding PBS+10% FBS. Cells were centrifuged at 300 *g* for 5 min and resuspended in PBS+10% FBS. Cells were then incubated with rat serum and enrichment cocktail for 15 min at 4 °C and centrifuged at 300 *g* for 10 min. Cells were then resuspended in biotin selection cocktail for 15 min at 4 °C, followed by magnetic beads for an additional 10 min, and placed into the separation magnet for 3 min. Non-adherent cells were removed, and adherent cells washed off of the walls of the tube and

resuspended in PBS for injection. Cells ($5x10^6$, in 10 μl) were injected into the plantar surface of the inflamed hind paw (see below) of mice within 30 min of isolation.

*Assays*: The chronic constriction injury (CCI) procedure (*62*) was performed under isoflurane anesthesia, and consisted of an incision made below the mouse's left hip bone followed by exposure of the sciatic nerve and the application of three ligatures with 4/0 silk thread loosely tied around the sciatic nerve proximal to its trifurcation. The incision was then closed in layers. Mice were given 6 days of recovery before behavioral testing commenced. This assay is a well-known model of neuropathic pain.

A recently developed mouse assay of myofascial low back pain was used in one experiment (*63*). Nerve growth factor (NGF; Sigma; 0.75 μg dissolved in a 30 μl volume of phosphate-buffered saline) was injected twice, 4 days apart. Mice were lightly anesthetized under isoflurane/oxygen anesthesia and placed in a prone position. After shaving the low back skin, the L5 spinous process was located using the iliac crest as a landmark. Injections were made 1.5 mm lateral to the L5 spinous process using a 30-gauge needle attached to a 1-ml syringe into the muscle (1 mm up from the bone).

In most experiments, complete Freud's adjuvant (50% CFA, 20 μl volume) was injected into the plantar surface of the mouse's left hind paw as a model of inflammatory pain.

Before and after all injuries, mechanical paw-withdrawal threshold was measured with von Frey filaments using the up-down staircase method of Dixon (*64*), as previously described (*65*). In every experiment, a baseline threshold determination was made on Day -1. Drugs or cells were administered daily from Day 0 to Day 6 (dexamethasone, diclofenac, gabapentin, lidocaine), on Day 3 and Day 5 (S100A8, S100A9, and neutrophils), or every other day from Day 0 to Day 20 (anti-Ly6G). von Frey testing on Day 6 occurred 1 hour after the drug injection

on that day. Testing continued until both vehicle and drug groups had returned to their baseline statistically (with the exception of CFA+diclofenac), even though not all individual mice had necessarily recovered. Thus, in an attempt to minimize the duration that mice remained in pain, the length of experiments were different depending on the assay used and the purpose of the experiment, from 40 days post-drug to 120 days post-drug.

*Data analysis*: Time course data of von Frey thresholds are shown graphically. To quantify the acute effects of drugs on mechanical thresholds during drug exposure (on Day 4–6 after CCI or CFA), the percentage of maximum possible allodynia was quantified as: % allodynia = [(baseline threshold – post-drug threshold)/baseline threshold] x 100. A reduction of % allodynia would indicate acute analgesic and/or anti-inflammatory action of the treatment. To quantify the duration of the entire CCI- or CFA-induced allodynic episode, the day-by-day mechanical thresholds of each subject was considered. The Days to Return quantification provided consisted of the first of two consecutive days that a subject's threshold had returned to within 0.5 SD (via group means) of its baseline threshold. The use of 0.5 SD is, of course, arbitrary, but the use of other values yielded highly similar conclusions.

**DRUG USE IN A HUMAN STUDY POPULATION**

We drew on the UK Biobank (UKB) study population of 502,494 study subjects who provided consent without withdrawing as assessed on May 1, 2020 (*66*, *67*). We considered associations between analgesic medication use at baseline ($v_0$) by medication category on development of chronic back pain at the first two of subsequent visits ($v_1$ and $v_2$). Subjects were selected into the study if they reported back pain at v0 based on the touchscreen questionnaire question "In the last month have you experienced any of the following that interfered with your usual activities?

(You can select more than one answer)" if back pain was selected at $v_0$ (field 6159), amounting to 130,084 individuals. We excluded subjects who answered “Yes” to “Have you had back pain for more than 3 months (field 3571), leaving 40,531 subjects. We then only included subjects who answered the verbal interview question on number of medications entered (field 137), and who answered the field 3571 back pain question data at $v_1$ or $v_2$ (435 subjects were present for $v_1$ and $v_2$), leaving a final set for inclusion of 2,624 subjects. Family relatedness among the 2,624 subjects was considered and only two pairs of individuals were found to be related; one individual per pair was selected at random for inclusion. The final set of controls who reported acute back pain at v0 but did not develop chronic back pain at $v_1$ or $v_2$ was 2,163 subjects (answering no to the back pain question (field 3517) and 461 cases developed chronic back pain at $v_1$ or $v_2$ (answering yes to the back pain question (field 3517)). The mean span between $v_0$ and $v_1$ was 4.4 years (SD=0.91) for cases and 4.4 years (SD=0.91) for controls, and between $v_0$ and $v_2$ was 7.7 years (SD=1.4) for cases and 7.7 years (SD=1.4) for controls.

Based on field 137 from the verbal interview at v0, we considered medications with known analgesic effects. In order to classify individual medication use, specific drug names reported for each individual (trade name or generic) as mentioned in DF:20003.0 (baseline, http://biobank.ctsu.ox.ac.uk/crystal/field.cgi?id=20003) were considered according to the code assigned for the particular drug in the UKB database. This code was used to match with the WHO ATC code (https://www.whocc.no/atc_ddd_index/) for each drug. The first three levels of the ATC code were used to assign class. In cases where drugs fell under more than one class, the analgesic medication class was used. Drugs taken by fewer than 10 individuals who reported acute back pain were not considered. As only systemic but not topical corticosteroids are used for treating low back pain, we considered only those drugs for our analysis. Final analgesic drug

categories analyzed were: non-steroid anti-inflammatory (NSAIDs), paracetamol (a.k.a. acetaminophen) and anti-depressants. Baseline differential leukocyte percentages were accessed from field 30200 for neutrophils.

The white blood cell counts (WBCs) of the eligible participants were measured on fresh samples as an absolute number per unit volume, and their component leukocytes (lymphocytes, monocytes, neutrophils, eosinophils, and basophils) as absolute measures and proportions of the overall WBC; all using an automated, clinically validated, Coulter LH 750. Calibration and quality control were performed in line with the manufacturer's recommendations. Further details of these measurements can be found in the UK Biobank online showcase and protocol (http://www.ukbiobank.ac.uk).

We fit logistic regression models to test for association between each of these categories and development of chronic back pain at $v_1$ or $v_2$. Preliminary data analysis identified potential confounders—age at baseline, sex and ethnicity—these variables were used as covariates in all logistic regression models. Models were fit one-by-one for each medication exposure variable. A full model was fit including all medication categories together. Logistic regression modeling was conducted in R v. 4.0.2 using the function glm with the binomial (logit) family specified for estimation of odds ratios and Wald tests were conducted and corresponding P-values computed for each explanatory variable.

We then considered further potential confounders for the development of chronic pain. Higher pain levels and higher psychological distress at the acute stage are two factors that have been shown repeatedly to be associated with the development of chronic pain. Thus, we added two additional covariates: a quantitative measure of count of painful sites reported in the last month (out of a total possible of seven anatomical sites) and a composite variable representing

depression and/or anxiety/panic attacks, taken from the UK Biobank self-reported conditions field 20002, codes 1286 and 1287 respectively in a similar manner as described previously (*68*). The number of painful sites has been used is a proxy for severity (and therefore intensity) of pain, also as described previously (*69*).

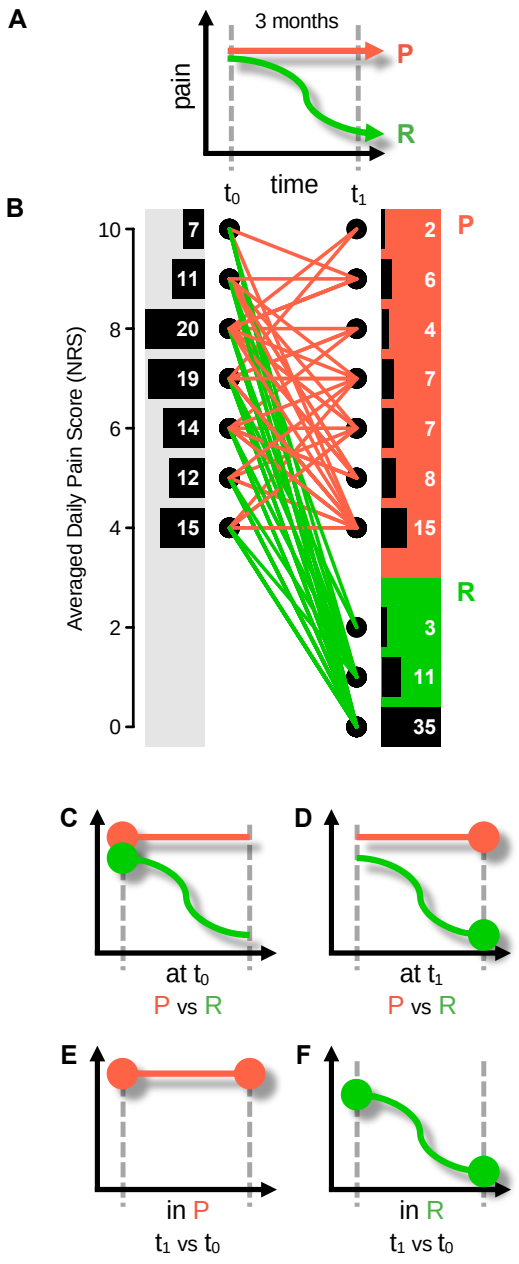



**Figure S1. Low Back Pain Transcriptomics Study Design. (A)** Study design. 98 patients reporting substantial back pain at enrolment ($t_0$) were assessed three months later at follow-up ($t_1$). At $t_1$, half of participants self-reported averaged daily pain scores of 4 and above, or persistent pain (P), whereas the other half - below 4, or resolved pain (R). Spontaneous resolution of pain is depicted in R (green), whereas persistency in P (orange). **(B)** Pain score

trajectories between visits. Trajectories colored by pain outcome: resolved pain in green, whereas persistent pain in orange. Numbers indicate participant counts in each pain score slots. **(C-F)** Transcriptomics analysis contrasts enabled by the study design. A total of four contrasts have been made in which the transcriptomes. Large dots indicate the conditions that are compared. Study design pictograms are: **(C)** at $t_0$, between persistent and resolved pain outcomes, **(D)** at $t_1$, between persistent and resolved, **(E)** in those with persistent pain, between clinical visits $t_1$ and $t_0$. **(F)** in those with resolved pain, between $t_1$ and $t_0$.

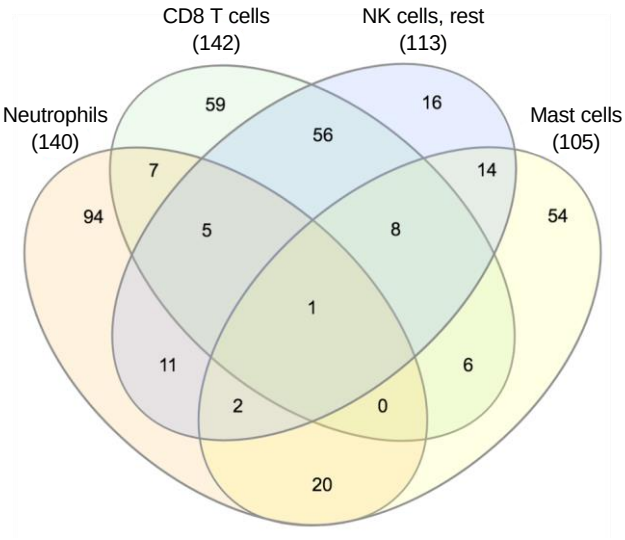



**Figure S2. Overlap of Highly Expressed Genes in Selected Blood Cell Types.** The overlap is quantified using a 4-way Venn diagram. Cell type specific gene expression from LM22 expression matrix of CIBERSORT. Figure drawn with the online tool interactivenn.net.

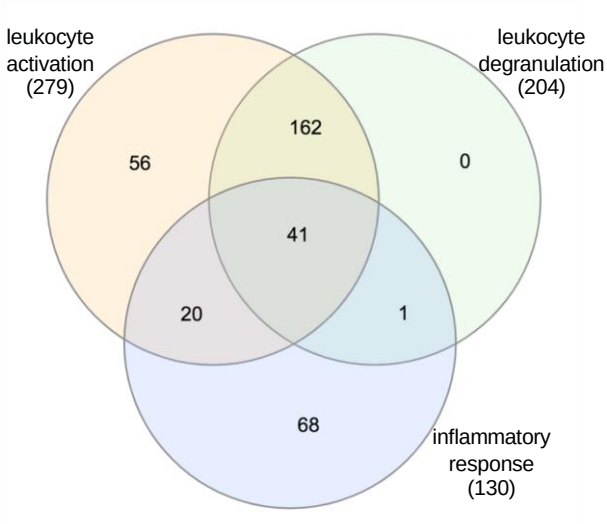



**Figure S3. Overlap Between Leading-Edge Genes in the Leukocyte Activation, Leukocyte Degranulation and Inflammatory Response Pathways.** The overlap is quantified using a 3-way Venn diagram. Figure drawn with the online tool www.interactivenn.net.

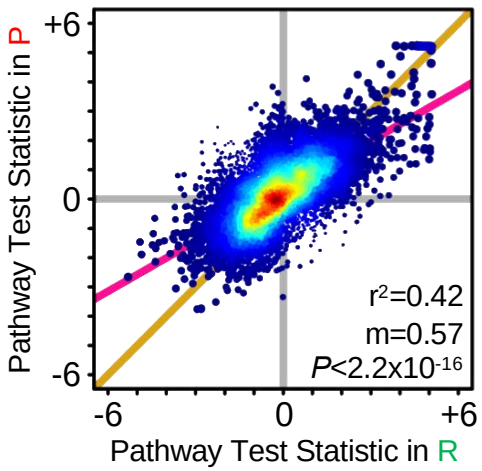
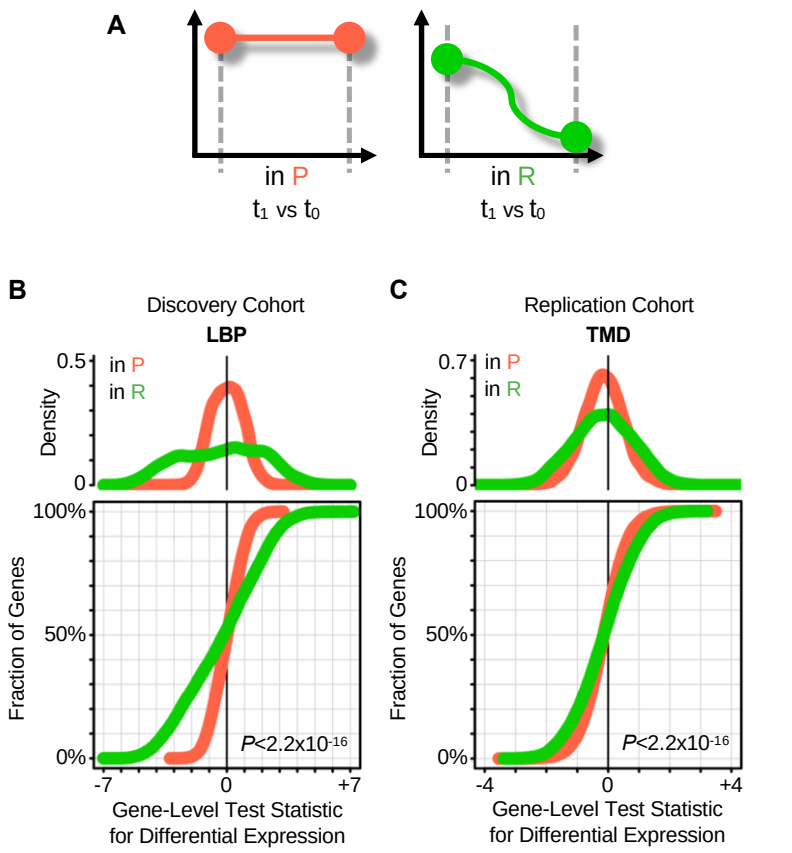



**Figure S4. Concerted Pathway Trajectories in Time Between the Two Pain Groups.** Each dot is a pathway. Pathway coordinates are in test statistic space, obtained from pathway analyses by fgsea. Color coding of dots suggest pathway density, from minimum (blue) to maximum (red). The slanted pink line was obtained from a linear regression of the data, whereas the slanted gold line from theoretical expectation of equal trajectories. Percent variance explained (Pearson's $r^2$), slope (m), and P-value of regression are reported.




**D**

| | | LBP | | TMD | | META | | |
|---|---|---|---|---|---|---|---|---|
| fixed | variable | ES | pval | ES | pval | Z | pval | repl? |
| **GO:0006954** | **inflammatory response** | | | | | | | |
| in P | t1 vs t0 | -0.21 | 9.32E-02 | -0.21 | 6.95E-01 | -1.66 | 9.68E-02 | N.A. |
| in R | t1 vs t0 | -0.39 | 1.07E-07 | -0.45 | 1.11E-07 | -7.21 | 5.54E-13 | yes |
| at t0 | P vs R | -0.42 | 1.12E-07 | -0.47 | 1.39E-07 | -7.18 | 6.75E-13 | yes |
| **GO:0042119** | **neutrophil activation** | | | | | | | |
| in P | t1 vs t0 | -0.26 | 8.76E-05 | -3.92 | 8.78E-01 | -3.51 | 4.42E-04 | N.A. |
| in R | t1 vs t0 | -0.42 | 1.04E-07 | -5.32 | 1.07E-07 | -7.22 | 5.21E-13 | yes |
| at t0 | P vs R | -0.45 | 1.08E-07 | -5.31 | 1.31E-07 | -7.20 | 6.24E-13 | yes |
| **GO:0043312** | **neutrophil degranulation** | | | | | | | |
| in P | t1 vs t0 | -0.26 | 2.03E-04 | -0.18 | 8.92E-01 | -3.32 | 8.89E-04 | N.A. |
| in R | t1 vs t0 | -0.42 | 1.04E-07 | -0.60 | 1.07E-07 | -7.22 | 5.23E-13 | yes |
| at t0 | P vs R | -0.45 | 1.08E-07 | -0.59 | 1.32E-07 | -7.19 | 6.27E-13 | yes |

**Figure S5. Replication of the Time Evolution of Genes and Pathways, in Subjects With Persistent or Resolved Pain. (A)** Study design pictograms, showing contrasts in time in those with persistent pain (P, orange), and in those with resolved pain (R, green). **(B,C)** Differential gene expression in time. The differential gene expression is tracked in those with resolved pain (green) and with persistent pain (orange) separately, with magnitude of the differential

expression measured by the test statistic of the difference at gene-level. Top panels show normalized histograms or density plots, whereas bottom panels show cumulative fractions of the same data. Kolmogorov-Smirnov test P-value for difference between P and R groups indicated at the bottom right corners. **(B)** Data from the discovery cohort for low back pain (LBP). **(C)** Data from the replication cohort for temporomandibular disorders (TMD). **(D)** Differential pathway expression in time. fgsea's enrichment scores (ES) and corresponding enrichment P-values (pval) are shown for the discovery (LBP; blue) and replication (TMD; gold) cohorts. Meta-analysis (meta; green) of combined Z-scores (Z) and corresponding P-values (pval) are also shown. Far right column indicates if the pathway has been replicated. N.A. stands for not applicable.

| | | -= TMD =- | |
|---|---|---|---|
| fixed | variable | ES | pval |
| **GO:0006954** | **inflammatory response** | | |
| healthy | R @ t0 | 0.34 | 4.60E-07 |
| healthy | R @ t1 | -0.31 | 8.45E-04 |
| healthy | P @ t0 | -0.46 | 1.31E-07 |
| healthy | P @ t1 | -0.43 | 1.11E-07 |
| healthy | chronic | -0.13 | 9.98E-01 |
| **GO:0042119** | **neutrophil activation** | | |
| healthy | R @ t0 | 0.49 | 2.37E-07 |
| healthy | R @ t1 | -0.35 | 1.09E-07 |
| healthy | P @ t0 | -0.54 | 1.24E-07 |
| healthy | P @ t1 | -0.50 | 1.07E-07 |
| healthy | chronic | 0.19 | 1.91E-02 |
| **GO:0043312** | **neutrophil degranulation** | | |
| healthy | R @ t0 | 0.49 | 2.37E-07 |
| healthy | R @ t1 | -0.34 | 2.18E-07 |
| healthy | P @ t0 | -0.54 | 1.24E-07 |
| healthy | P @ t1 | -0.50 | 1.07E-07 |
| healthy | chronic | 0.19 | 3.25E-02 |

**Figure S6. Contrasts of Pathways Between Healthy Subjects and Those With Pain.** Contrast shown for selected pathways in the OPPERA TMD cohort; inflammatory response (GO:0006954), neutrophil activation (GO:0042119) and degranulation (GO:0043312). fgsea's enrichment score (ES) and associated P-value (pval) shown for each one of the four study design end points and with those with chronic pain (variable) against healthy controls (fixed).

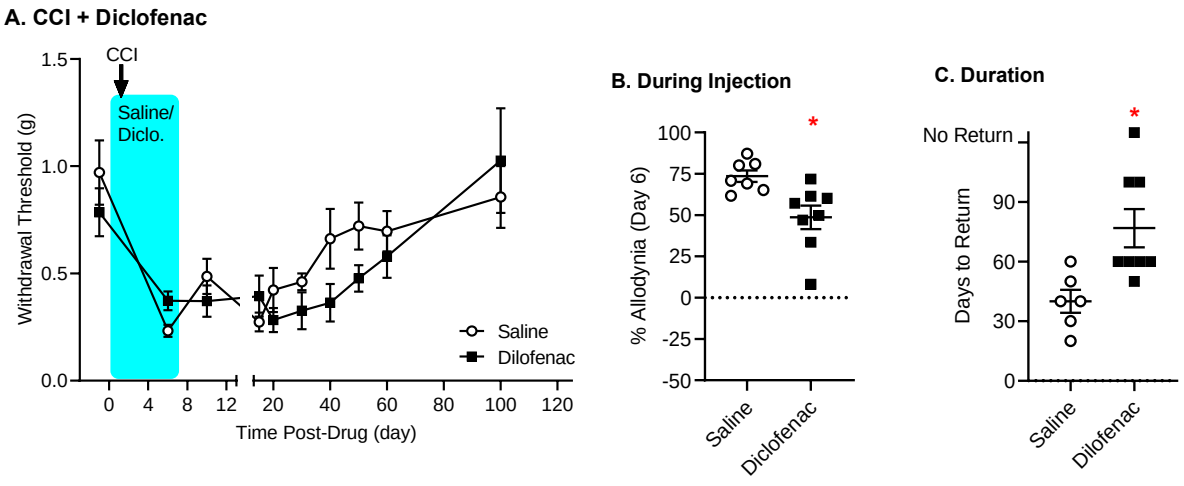



**Figure S7. Prolongation of CCI-induced Allodynia by Diclofenac. (A)** Mechanical pain thresholds before and after chronic constriction injury (CCI) of the sciatic nerve in mice treated from day 0-6 with saline or diclofenac (Diclo.). Symbols represent mean ± SEM hind paw withdrawal threshold (g). **(B)** Percentage of maximum possible allodynia (% allodynia) on day 6 post-drug; see Methods for calculation details. Error bars represent SEM. **(C)** Days required to return to baseline thresholds; see Methods. Error bars represent SEM. Mice not returning to baseline by day 100 were assigned a value of 120. *$P$<0.05 compared to saline.

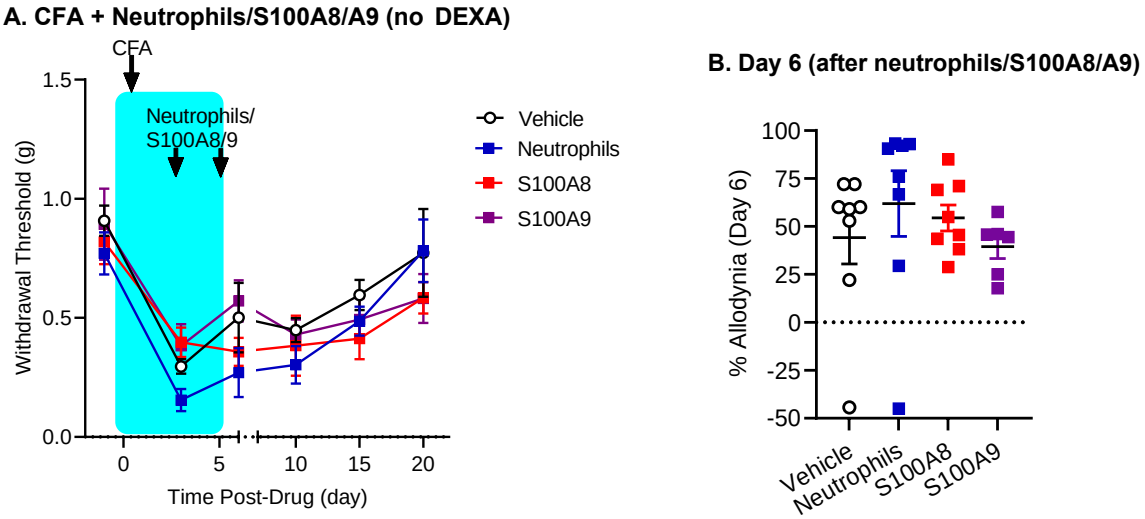



**Figure S8. No Effect on CFA-Induced Mechanical Allodynia of Neutrophils and S100A8/A9 in the Absence of Dexamethasone. (A)** Mechanical pain thresholds before and after injection of complete Freund's adjuvant (CFA) in mice treated on day 3 and day 5 with neutrophils, S100A8, or S100A9, but (in contrast to Figure 5C) in the absence of dexamethasone (DEXA). Symbols represent mean ± SEM hind paw withdrawal threshold (g). **(B)** Percentage of maximum possible allodynia (% allodynia) on day 6 post-drug; see Methods for calculation details. Error bars represent SEM. Neither the repeated measures ANOVA nor comparisons at day 6 (or any other time point) showed differences compared to vehicle.

| | Pain Outcome @ $t_1$ | | P vs R | Sex | | M vs F |
|---|---|---|---|---|---|---|
| Attribute | (R)esolving | (P)ersistent | P-value | (M)ales | (F)emales | P-value |
| # Samples | 49 | 49 | N/A | 57 | 41 | N/A |
| Sex | 16 F, 33 M | 25 F, 24 M | 0.10 | N/A | N/A | N/A |
| Outcome | N/A | N/A | N/A | 33 R, 24 P | 16 R, 25 P | 0.10 |
| Age (yrs) | 62.6 (15.2) | 63.2 (17.3) | 0.84 | 60.3 (15.3) | 66.5 (17.0) | 0.07 |
| BMI | 26.4 ( 3.6) | 27.9 ( 3.7) | 0.04 | 27.9 ( 3.7) | 26.2 ( 3.5) | 0.02 |
| NRS @ $t_0$ | 6.6 ( 2.0) | 7.0 ( 1.6) | 0.32 | 6.4 ( 1.8) | 7.3 ( 1.8) | 0.02 |
| NRS @ $t_1$ | 0.3 ( 0.6) | 6.1 ( 1.9) | $8x10^{-36}$ | 2.5 ( 2.9) | 4.2 ( 3.4) | 0.01 |
| TSPD @ $t_0$ | 9.7 ( 5.9) | 14.6 ( 7.8) | $7x10^{-04}$ | 11.0 ( 7.2) | 13.9 ( 7.3) | 0.05 |
| Smoker | 19 y, 30 n | 14 y, 35 n | 0.29 | 21 y, 36 n | 12 y, 29 n | 0.57 |
| Medication - Treatment | | | | | | |
| Paracetamol | 19 y, 30 n | 10 y, 39 n | 0.08 | 19 y, 38 n | 10 y, 31 n | 0.38 |
| * prophy | 4 y, 15 n | 4 y, 6 n | 0.39 | 4 y, 15 n | 4 y, 6 n | 0.39 |
| Opioids | 18 y, 31 n | 27 y, 22 n | 0.10 | 27 y, 30 n | 18 y, 23 n | 0.84 |
| * prophy | 12 y, 6 n | 15 y, 12 n | 0.54 | 16 y, 11 n | 11 y, 7 n | 1.00 |
| NSAID | 33 y, 16 n | 28 y, 21 n | 0.40 | 32 y, 25 n | 29 y, 12 n | 0.20 |
| * prophy | 6 y, 27 n | 3 y, 25 n | 0.49 | 4 y, 28 n | 5 y, 24 n | 0.72 |
| CSAID | 17 y, 32 n | 14 y, 35 n | 0.66 | 20 y, 37 n | 11 y, 30 n | 0.51 |
| * prophy | 8 y, 9 n | 3 y, 11 n | 0.26 | 8 y, 12 n | 3 y, 8 n | 0.70 |
| Any AID | 36 y, 13 n | 33 y, 16 n | 0.66 | 38 y, 19 n | 31 y, 10 n | 0.38 |
| * prophy | 13 y, 23 n | 5 y, 28 n | 0.06 | 11 y, 27 n | 7 y, 24 n | 0.59 |
| Both AID | 14 y, 13 n | 9 y, 16 n | 0.28 | 14 y, 19 n | 9 y, 10 n | 0.78 |
| * prophy | 8 y, 6 n | 2 y, 7 n | 0.20 | 7 y, 7 n | 3 y, 6 n | 0.67 |

**Table S1. Epidemiological Data Related to the Pain Outcome at the Second Visit and Sex.** Values in parentheses are standard deviations. Abbreviations are: resolving pain group, R; persistent pain group, P; male, M; female, F; first visit, $t_0$; second visit, $t_1$; yes, y; no, n; body mass index, BMI; numeric rating scale, NRS; total score pain detect, TSPD; anti-inflammatory drug, AID; nonsteroidal AID, NSAID; corticosteroid AID, CSAID; total score pain detect, TSPD; not applicable, N/A. Among those taking medication, those taking medication in a prophylaxis fashion before the first visit, prophy.

**Table S2. Transcriptome Quality Control and Principal Component Analyses for All Subjects at Two Time Points (provided as separate Excel file). (A)** RNA-Seq quality control,

showing RNA integrity number (RIN), total number of sequenced reads (total), and total reads mapped to the human genome (mapped). Also shown are the number of outlier genes (column nbX), detected from outlier expression levels. **(B)** Principal component analyses, showing 1-term linear regression P-values for association between selected clinical variables (age, sex, etc) and principal components 1 to 5. The "pct" rows show the percent variance explained by each component.

**Table S3. Differential Expression of Genes, Uncorrected for Blood Cell Type Fractions (provided as separate Excel file).** The differential expression is reported in subjects with resolved (R) and persistent (P) pain at two time points ($t_1$ and $t_0$). **(A)** at $t_0$, P vs R. If log2FoldChange > 0 (column C), then the gene is more expressed in P than in R, otherwise more expressed in R. **(B)** at $t_1$, P vs R. **(C)** in P, $t_1$ vs $t_0$. If log2FoldChange > 0 (column C), then the gene is more expressed at $t_1$ than at $t_0$, otherwise more expressed at $t_0$. **(D)** in R, $t_1$ vs $t_0$.

**Table S4. Differential Expression of Genes, Corrected for Blood Cell Type Fractions (provided as separate Excel file).** The differential expression is reported in subjects with resolved (R) and persistent (P) pain at two time points ($t_1$ and $t_0$). **(A)** at $t_0$, P vs R. If log2FoldChange > 0 (column C), then the gene is more expressed in P than in R, otherwise more expressed in R. **(B)** at $t_1$, P vs R. **(C)** in P, $t_1$ vs $t_0$. If log2FoldChange > 0 (column C), then the gene is more expressed at $t_1$ than at $t_0$, otherwise more expressed at $t_0$. **(D)** in R, $t_1$ vs $t_0$.

**Table S5. CIBERSORT Association Tests of Blood Cell Type Populations with Pain Outcomes (provided as separate Excel file).** The association results are presented for subjects

with resolved (R) and persistent (P) pain at two time points ($t_1$ and $t_0$). **(A)** Test at $t_0$, P vs R. **(B)** Test at $t_1$, P vs R. **(C)** Test in P, $t_1$ vs $t_0$. **(D)** Test in R, $t_1$ vs $t_0$.

**Table S6. Differential Expression of Pathways (provided as separate Excel file).** Pathways are reported in subjects with resolved (R) and persistent (P) pain at two time points ($t_1$ and $t_0$). The differential expression of genes was uncorrected for blood cell type fractions. Tested pathways are from Gene Ontology's (GO) biological processes. **(A)** at $t_0$, P vs R. If enrichment score ES > 0 (column E), then every leading-edge gene (column I) is more expressed in P than in R, otherwise more expressed in R. **(B)** at $t_0$, P vs R, FDR-corrected selected subsets. **(C)** at $t_1$, P vs R. **(D)** at $t_1$, P vs R, FDR-corrected selected subsets. **(E)** in P, $t_1$ vs $t_0$. If enrichment score ES > 0 (column E), then every leading-edge gene (column I) is more expressed at $t_1$ than at $t_0$, otherwise more expressed at $t_0$. **(F)** in P, $t_1$ vs $t_0$, FDR-corrected selected subsets. **(G)** in R, $t_1$ vs $t_0$. **(H)** in R, $t_1$ vs $t_0$, FDR-corrected selected subsets.

**Table S7. Identification of Genes Contributing to the Dynamic Regulation of Cellular Responses (provided as separate Excel file).** The desirable dynamic response of a gene was defined as up-regulation in the R group compared to the P group at $t_0$ (columns B-F; fgsea's differential gene expression analysis), then by down-regulation in the R group at $t_1$ compared to $t_0$ (columns G-K). Genes ranked by decreasing value of multiplication of 1) fold-change test statistics at $t_0$ between P and R groups, 2) fold-change test statistics for the R group between $t_1$ and $t_0$, 3) base mean expression at $t_0$ (column L). **(A)** Genes in the neutrophil degranulation pathway (GO:0043312). **(B)** Genes in the neutrophil activation pathway (GO:0042119). **(C)** Genes in the inflammatory response pathway (GO:0006954).

**Table S8. Replication of Findings in the TMD Cohort (provided as separate Excel file).** Findings reported in subjects with resolved (R) and persistent (P) pain at two time points ($t_1$ and $t_0$). **(A)** Differential expression of genes in P, between $t_0$ and $t_1$, in the TMD cohort. **(B)** Differential expression of genes in R, between $t_0$ and $t_1$, in the TMD cohort. **(C)** Meta-analysis of gene set enrichment analyses in both LBP and TMD cohorts at $t_0$, between P and R. Abbreviations are: meta-analysis, meta; enrichment score, ES; Z-score, Z; P-value, pval. **(D)** Meta-analysis in P, between $_{t0}$ and $t_1$. **(E)** Meta-analysis in R, between $t_0$ and $t_1$. **(F)** Replication of selected pathways. **(G)** Gene expression fold changes in the LBP and TMD cohorts at $t_0$ for the top genes most expressed in neutrophils. **(H)** Gene expression fold changes in the LBP and TMD cohorts in the R pain group for the top genes most expressed in neutrophils. **(I)** Comparisons of the healthy control group with the P and R pain groups as well as with the long-term chronic pain group in the TMD cohort.

**Table S9. Impact of Drug Class on the Development of Chronic Pain (provided as separate Excel file).** Selected subjects from the UK Biobank project reported acute back pain at first visit but chronic back pain at subsequent visits. Shown are odds ratios (OR) between the development of chronic pain and the use of analgesic medication classes. NSAID stands for non-steroidal anti-inflammatory drug; P, Wald test P-value. Additional potential confounding factors are: time interval between visits, depression and anxiety as psychological distress, and number (#) of painful sites.