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PAIN

Genome-wide association studies with experimental validation identify a protective role for B lymphocytes against chronic post-surgical pain

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Abstract

Background: Chronic post-surgical pain (CPSP) significantly impacts patients' recovery and quality of life. Although environmental risk factors are well-established, genetic risk remains less understood.

Methods: A meta-analysis of genome-wide association studies followed by partitioned heritability was performed on 1350 individuals across five surgery types: hysterectomy, mastectomy, abdominal, hernia, and knee. In subsequent animal studies, withdrawal thresholds to evoked mechanical stimulation were measured in *Rag1* null mutant and

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wild-type mice after plantar incision and laparotomy. Cell sorting by flow cytometry tracked recruitment of immune cell types.

Results: We discovered 77 genome-wide significant single-nucleotide polymorphism (SNP) hits, distributed among 24 loci and 244 genes. Meta-analysis of all cohorts estimated a SNP-based narrow-sense heritability for CPSP at ~39%, indicating a substantial genetic contribution. Partitioned heritability analysis across a wide variety of tissues revealed enrichment of heritability in immune system-related genes, particularly those associated with B and T cells. *Rag1* null mutant mice lacking both T and B cells exhibited exacerbated and prolonged allodynia up to 42 days after surgery, which was rescued by B-cell transfer. Recruitment patterns of B cells but not T cells differed significantly during the first 7 days after injury in the footpad, lymph nodes, and dorsal root ganglia.

Conclusions: These findings suggest a key protective role for the adaptive immune system in the development of chronic post-surgical pain.

Keywords: B cells; chronic post-surgical pain; genome-wide association study; immune system; lymphocytes

Editor's key points

- Genetic risk factors for development of chronic post-surgical pain (CPSP) are unclear.
- The authors performed a meta-analysis of genome-wide association studies followed by partitioned heritability on 1350 individuals across five surgery types, with subsequent validation in animal models.
- The analysis identified 77 single-nucleotide polymorphisms spread over 24 genomic loci associated with CPSP severity, with heritability of ~39% indicating a substantial genetic contribution.
- Functional analysis strongly suggested a critical contribution of the adaptive immune system in the genetic factors associated with CPSP, and animal studies indicated a particular role for B lymphocytes.
- These underlying immune mechanisms for CPSP identify a new potential approach for treatment and prevention.

Chronic post-surgical pain (CPSP) is a debilitating condition affecting 5–85% of patients undergoing surgery, varying with the type of surgery.[1] Chronic pain is defined as pain persisting for >3 months, and includes both nociceptive (inflammatory) and neuropathic components.[1] CPSP has a large negative impact on the quality of life of those affected.[1–3]

Despite its high prevalence, research on CPSP has been sparse and its underlying mechanisms are only partially understood. Clinical (e.g. type of surgery, surgical skill), demographic, and psychosocial risk factors have been identified to predispose to CPSP,[2,4] but these only partially explain the observed variance. Currently, a basic understanding of the genetic risk factors for CPSP and its molecular aetiology is lacking.[2,4] Studies in other chronic pain syndromes have demonstrated considerable heritability, with estimates ranging 30–70% and a median of 45%.[5–9] Candidate gene-based studies on CPSP have provided conflicting evidence.[10–13] Nonetheless, CPSP has recently been introduced as a distinct disease entity in the *International Classification of Diseases v.11* (ICD-11), which should raise awareness and increase interest in CPSP research.[1]

One way forward to decipher the complex aetiology of CPSP is via genome-wide association studies (GWASs), which provide a hypothesis-free tool for identification of genetic contributors.[5] Thus far, one small GWAS of CPSP has been conducted, but no genome-wide significant risk locus was identified.[14] We performed a GWAS of CPSP in six independent cohorts covering five surgical types (open hernia repair, hysterectomy, abdominal surgery, knee surgery, and mastectomy) in adults of both sexes. Functional annotation of the meta-analysis of the independent cohorts via heritability partitioning identified a possible role of the adaptive immune system in CPSP. Further mouse studies confirmed the involvement of B cells specifically among lymphocytes.

Methods

Human studies

Patients undergoing hysterectomy, mastectomy, abdominal surgery, hernia repair, or knee surgery were recruited and genotyped. Post-surgical pain was assessed 3–6 months after surgery. Genotype data were imputed, then used for input in GWAS by surgery type, with pain intensity as the quantitative trait. All GWASs were then combined in a meta-analysis. The results were analysed using partitioned heritability. Details can be found in the 'Extended Materials and Methods' section of the Supplementary material.

Mouse studies

Rag1 null mutant and wild-type mice of both sexes were submitted to plantar incision and laparotomy surgical assays, supplemented with B cells, T cells, or both. Withdrawal responses to mechanical stimuli were determined using calibrated von Frey filaments. Cell sorting by flow cytometry was performed to monitor immune cell infiltration and recruitment, in the footpad/skin, dorsal root ganglia (DRG), and inguinal lymph nodes. Experimental details can be found in the 'Extended Materials and Methods' section of the Supplementary material.

Statistical analyses

For GWAS, linear regression was used to assess the association between allelic dosage and pain intensity. We complemented PLINK's linear regression model with other models tested with the R statistical package (R Foundation for Statistical Computing, Vienna, Austria), including Poisson, quasi-Poisson, and negative binomial, and found that the linear

Table 1 Summary of genome-wide association study results per surgery type. Genome-wide significance (GWS) threshold was set at 5×10^{-8}, whereas nominal significance (NOMS) was set at 5×10^{-7}. λGC, genomic control's lambda; GWS, genome-wide significance; NOMS, nominal significance; P_{PVE}, associated *P*-value; PVE, percent variance explained of the phenotype by genetics.

Surgery	GWS	Loci	NOMS	λ GC	PVE (%)	P_{PVE}
Hysterectomy	24	12	107	1.03	99	0.07
Abdominal	3	2	21	1.03	99	0.33
Mastectomy	0	0	0	1.02	83	0.14
Hernia	44	6	69	1.02	64	0.36
Knee (meta)	1	1	1	1.02	12	0.41
Meta-analysis	5	3	22	1.00	39	0.02
Total	77	24	220			

regression provided the most-flat, least-skewed distribution of association *P*-values (data not shown), which ideally should be uniformly distributed (given a fair null model of association; e.g. linear regression of the phenotype to the additive genetic inheritance burden model).

Mouse data were analysed using GraphPad Prism, v.7.04 (GraphPad Software, Inc., La Jolla, CA, USA). Normality and homoscedasticity of all data sets were confirmed using Shapiro–Wilk and Levene tests, respectively. Data were analysed using repeated measures analysis of variance (ANOVA), followed by *post hoc* testing as appropriate. A criterion $\alpha=0.05$ was adopted for statistical significance.

For each statistical test, the threshold for significance and the methods used for correction for multiple testing are indicated.

Results

Genome-wide association studies

Data from a total of 1350 individuals with simultaneous genotype and phenotype information were used for association studies at the genome-wide scale, first as per surgery type, followed by a meta-analysis of all cohorts (Table 1). Genome-wide association results are displayed in Manhattan plots (Fig. 1) for single-nucleotide polymorphism (SNP)-, gene-, and pathway-wise results. We observed many statistically significant hits at all levels of analysis (Bonferroni's genome-wide 5×10^{-8} threshold for SNPs, false discovery rate [FDR] 20% for genes and pathways; Supplementary Table S5). Significant SNP hits were grouped in loci in which the lead SNP was highlighted along genes residing immediately before, within, and immediately after these hits (Table 2). QQ plots indicated statistical significance at SNP, gene, and pathway levels guarding against FDRs of up to 20% (Fig. 1; Supplementary Table S5).

A total of 77 genome-wide significant SNP hits were uncovered scattered across 24 loci, 244 genes, and 64 pathways (Supplementary Table S5). Only the mastectomy GWAS failed to yield nominally significant SNP hits ($P<10^{-7}$). Among the 24 significant genetic loci, one locus spanning *ZNF594-DT*, *RABEP1*, and *NUP88* genes was significant in both the hernia GWAS and in the meta-analysis of all cohorts. We did not specifically test for effect differences in sex in cohorts featuring both sexes because of a relatively small sample size of each cohort. As sex was used as a co-variable in cohorts with both sexes for all analyses, we expected to observe mostly sex-independent effects. All point estimates for percent phenotypic variance explained by genetics, also known as narrow-sense heritability, were found to be non-significant ($P>0.05$), except for the overall meta-analysis with 39% variance of the CPSP phenotype explained by genetic effects ($P=0.02$).

Tissues or cell types with enriched heritability

We completed a functional analysis of the genetic summary statistics to take advantage of the large number of identified genes to reveal the pathophysiology underpinning CPSP by testing tissues and cell types whose uniquely expressed genes would carry excess heritability when contrasted against other, non-tissue-specific genes. To do so, we performed partitioned heritability analysis, with partitions based on genes expressed in cell types or tissues pertinent to pain states. The selected gene expression database contained many nervous tissues, including various brain regions and the spinal cord and peripheral nervous system ganglia (in particular, dorsal root and trigeminal), various immune cell types, musculoskeletal tissues, and other tissues and cell types.[15]

Overall, 126 tissues and cell types were tested, grouped into eight broad categories (Fig. 2). We found enriched heritability at the FDR 20% level in immune cells, more specifically in B- and T-cell subtypes, and in CD34+CD38− haematopoietic progenitor stem cells (Fig. 2a; Supplementary Table S6A). To reduce the impact of between-study differences in surgical sites, of surgery types, and of mean age, we performed a random effects meta-analysis, followed by a sensitivity analysis testing the robustness of the meta-analysed results under fixed- and random-effects models. The results of partitioned heritability were largely the same and reinforced the enriched heritability for CPSP in both B and T cells (Fig. 2b and c; Supplementary Table S6B). No evidence was found to support contributions from neutrophils, macrophages, central or peripheral nervous system tissues, or musculoskeletal tissues. The findings were replicated in another gene expression database itself,[16] also containing brain regions and immune cell types (Supplementary Table S6C). There, B- and T-cell subtypes were also significantly enriched among eight cell and tissue types (with enrichment $P\leq0.05$). Further support for contributions of B and T cells came from another dataset[17] with H3K9ac and H3K27ac chromatin markers ($P\leq0.05$), where acetylated H3K9 turns on gene expression and acetylated H3K27 enhances gene transcription (Supplementary Table S6D). In this dataset, among 20 cell types with evidence for enrichment at $P<0.05$, 11 were different subsets of T cells and one was primary B cells. Although it is unclear whether specific B- or T-cell subtypes are most important in post-surgical pain chronicity given the datasets available, our results point to the adaptive immune

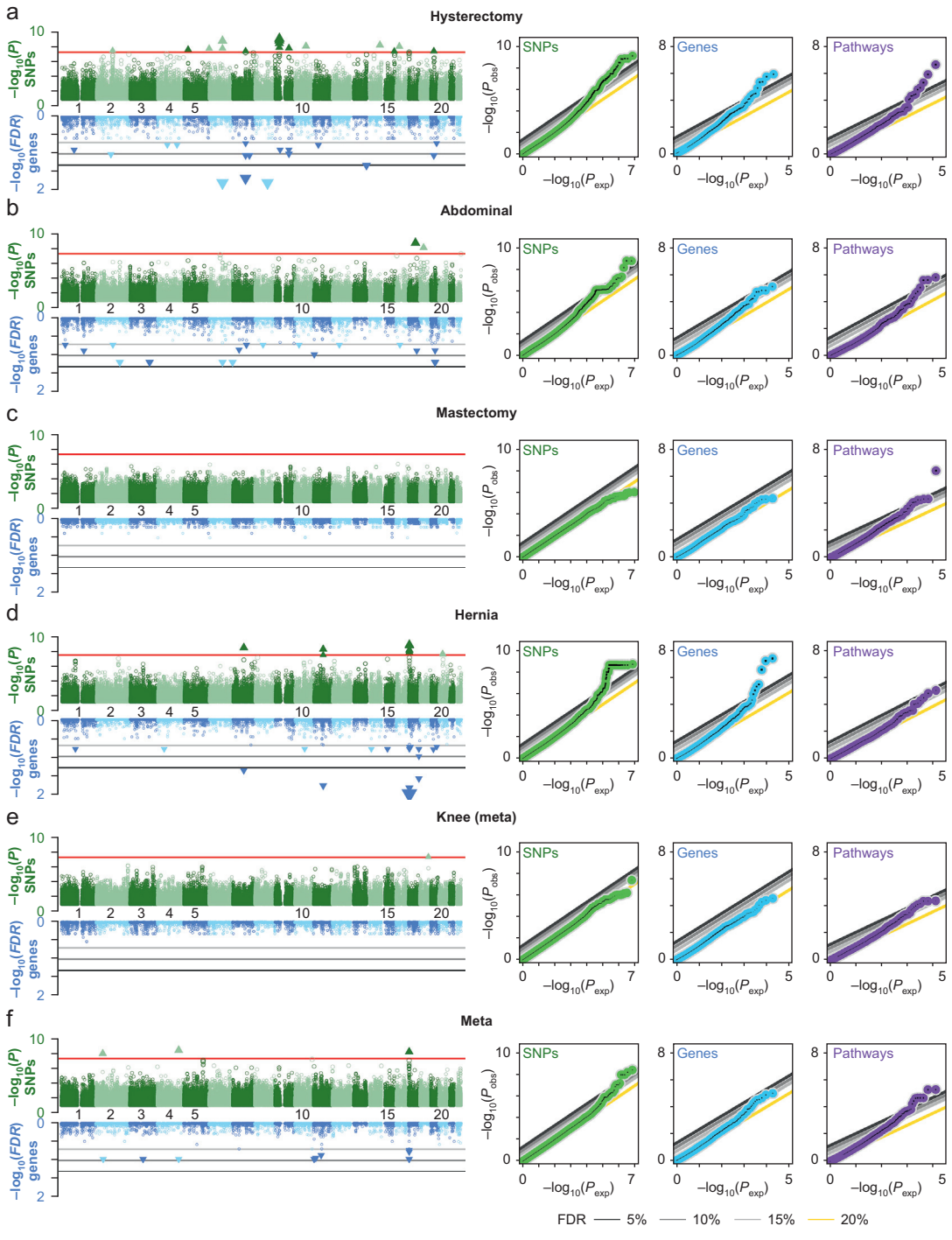


Fig 1. Association summary statistics by surgery types. Summary statistics are shown using: Manhattan plots (left) for single-nucleotide polymorphism (SNP)-level (top, green) and gene-level (bottom, blue) summaries; and QQ plots (right) for SNP-level (green), gene-level (blue), and pathway-level (purple) summaries. Manhattan plots use dark colour hues for odd chromosomes and light colour hues for even ones. SNP-level genome-wide significance indicated using red horizontal bars and gene-level genome-wide significance is indicated using various false discovery rate (FDR) thresholds ranging from 5% (black) to 10% (dark grey) to 20% (light grey). QQ plots track observed P-values (Pobs) as a function of expected ones (Pexp). SNP-level statistics obtained with PLINK or METAL (meta-analysis), whereas gene-level statistics obtained with MAGMA. Surgeries are: (a) hysterectomy, (b) abdominal, (c) mastectomy, (d) hernia, and (e) knee, from a meta-analysis; (f) meta-analysis of all cohorts combined.

Table 2 Summary of genome-wide significant hits. Single-nucleotide polymorphism (SNP) hits are grouped by surgeries (A to E) and by overall meta-analysis (F). Only the lead SNP per locus is shown. *EA*, effect allele; *EAF*, effect allele frequency; *ES*, effect size (beta).

#	Lead SNP	EA	EAF	ES	P-value	Genes
A. Hysterectomy						
1	rs143994530	C	0.022	3.008	2.96E-08	DPP10 | DDX18
2	rs78454748	T	0.026	2.627	2.10E-08	LOC105374704 | CDH6
3	rs74896012	C	0.023	2.975	1.57E-08	LOC101927691 | LINC01622 | FOXQ1
4	rs117244643	T	0.020	3.454	1.45E-09	FHL5 | GPR63 | NDUFAF4
5	rs10488532	T	0.105	1.346	3.38E-08	SAMD9 | SAMD9L | HEPACAM2
6	rs10969869	A	0.018	3.665	7.23E-10	LINGO2 | LINC01242 | LINC01243
7	rs78326996	C	0.020	2.974	1.38E-08	AAED1 | LOC441455
8	rs17882261	G	0.058	1.882	7.36E-09	SFTPA2 | SFTPA1
9	rs76985919	A	0.017	3.569	5.47E-09	SERPINA1 | SERPINA11
10	rs4347600	T	0.028	2.518	3.56E-08	IQGAP1 | CRTC3
11	rs79423608	T	0.115	1.416	8.13E-09	LOC554206 | LINC02175
12	rs140299794	G	0.017	3.365	3.42E-08	LOC100505851 | ZNF254 | HAVCR1P1 | LINC00662
B. Abdominal						
1	rs142578347	G	0.056	2.287	1.79E-09	LINC02089 | C17orf112
2	rs112030385	T	0.127	1.455	7.45E-09	CDH2 | DSC3
C. Mastectomy						
	N/A					
D. Hernia						
1	rs111290518	A	0.080	2.465	4.91E-09	MAGI2 | MAGI2-AS2 | MAGI2-AS3
2	rs7946537	C	0.132	1.810	7.87E-09	LRP5 | PPP6R3 | GAL
3	rs76991866	T	0.071	2.771	1.98E-09	SLC52A1 | ZFP3
4	rs114604537	G	0.063	2.880	2.33E-09	ZNF232 | LOC101928000 | USP6 | ZNF594 | LOC100130950 | SCIMP | RABEP1 | NUP88
5	rs11653414	C	0.041	3.302	1.66E-08	LOC728392 | NLRP1 | LOC339166
6	rs4142128	T	0.049	2.870	3.22E-08	NCOR1P1 | LINC01597
E. Knee						
1	rs1596479	T	0.139	1.464	4.38E-08	LINC01924 | CDH7
F. Meta-analysis						
1	rs138190025	A	0.017	2.766	1.07E-08	NRXN1 | LOC730100 | LINC01867
2	rs114837251	T	0.021	2.638	3.87E-09	MAP9 | GUCY1A1 | GUCY1B1
3	rs3026120	A	0.037	1.625	6.18E-09	LOC100130950 | RABEP1 | NUP88

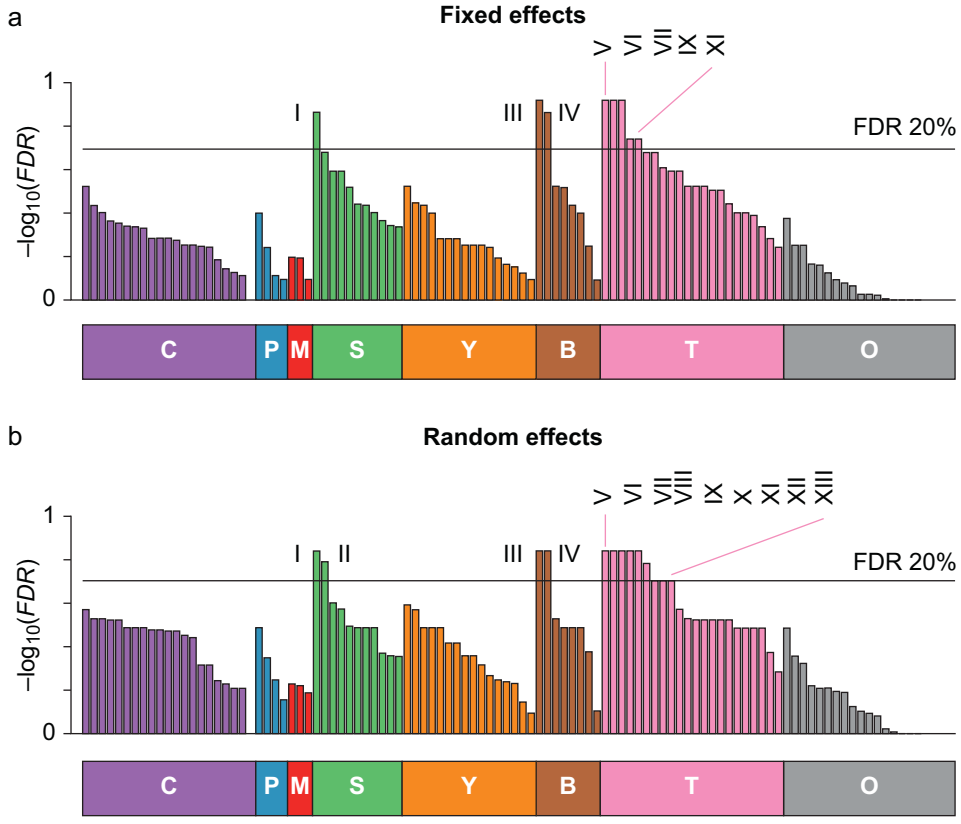
system, but not to the innate immune system or the nervous system, as a key player in CPSP.

Pain behavioural measures in lymphocyte-deficient mice

Based on the results from genetic analyses implicating a contribution of lymphocytes to development of CPSP, we directly tested the role of the adaptive immune system in post-surgical pain using mouse assays. We used plantar incision and laparotomy, two well-characterised preclinical post-surgical pain models,[18] to assess pain outcomes. Although the partitioned heritability analysis did not provide the direction of the observed contribution of the adaptive immune system, we started from the hypothesis that B and T cells were needed for pain resolution in CPSP, testing *Rag1*$^{-/-}$ mice which lack mature B and T lymphocytes.[19] Withdrawal thresholds to evoked mechanical stimulation at the plantar hind paw were measured before and at multiple time points up to 84 days after hind-paw incision in C57BL/6J (wild-type) and *Rag1* null mutant (*Rag1*$^{-/-}$) mice (Fig. 3a). Repeated measures ANOVA revealed significant interactions between strain and sex ($F_{13,221}$=2.1, P=0.015), but with both sexes clearly displaying robust allodynia and a large genotype effect (Supplementary Fig. S2); collapsing the data across sex, the strain interaction was also highly significant ($F_{13,247}$=2.3, P=0.007). Although the mechanical allodynia produced by the incision was fully resolved in wild-type mice by 5 days and certainly 7 days after surgery, exacerbated and prolonged allodynia was observed in *Rag1*$^{-/-}$ mice, with a statistically significant strain difference, corrected by multiple comparisons, up to 42 days after surgery (Fig. 3a). There was no main effect of repeated measure ($F_{13,247}$=1.1, P=0.29) or repeated measure by strain interaction on the contralateral hind paw ($F_{13,247}$=1.7, P=0.07; data not shown).

A similar pattern of responses was observed in the laparotomy assay, with no main effects of, or interactions with, sex observed; therefore, the data were collapsed across sex. A two-between (genotype, surgery), one-within repeated measures ANOVA revealed significant main effects and interactions, including a genotype × surgery × repeated measures interaction ($F_{6,49}$=6.4, P<0.001). Pain hypersensitivity in this assay was observed only in mice after laparotomy, which was increased and prolonged in duration in *Rag1*$^{-/-}$ mutant mice (Fig. 3b). This experiment improves both external validity (generalisability across another surgery type) and internal validity (replication in an independent laboratory) of the conclusion that B cells, T cells, or both are relevant to post-surgical pain in mice.

Next, purified B cells, T cells, or both were injected in *Rag1*$^{-/-}$ mice to determine whether a specific lymphocyte lineage was responsible for the differences in pain outcomes



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Peak	Tissue / cell type
I	Umbilical cord blood CD34+ CD38-HSC
II	Bone marrow CD34+ CD38+ HSC
III	Umbilical cord blood CD34+ B cells
IV	Prel B cells
V	Umbilical cord blood CD34+ T cells
VI	Peripheral naive CD4+ T cells
VII	Thymic CD34+ CD38+ CD1A- T cells
VIII	Central memory T cells
IX	Th2 T cells
X	Th1 T cells
XI	Thymic CD34+ CD38+ CD1A+ T cells
XII	Effector memory T cells
XIII	Tonsils CD4+ T cells

Fig 2. Partitioned heritability of fixed and random effects meta-analyses. Partitioned heritability in a fixed effects (a) and random effects (b) models meta-analysis. Enrichment tracked in 106 tissues or cell lines from Benita and colleagues.[15] Tissues are grouped by central nervous system (C, purple, n=21), peripheral nervous system (P, blue, n=4), muscle (M, red, n=3), stem cells (S, green, n=11), myeloid cells (Y, orange, n=16), B cells (B, brown, n=8), T cells (T, pink, n=22), and other tissues or cell lines (O, grey, n=21). Shown are false discovery rate (FDR)-corrected P-values for enrichment. (c) Tissues and cell lines significantly enriched at the FDR 20% level, numbered from left to right using Roman numerals as in panels a and b.

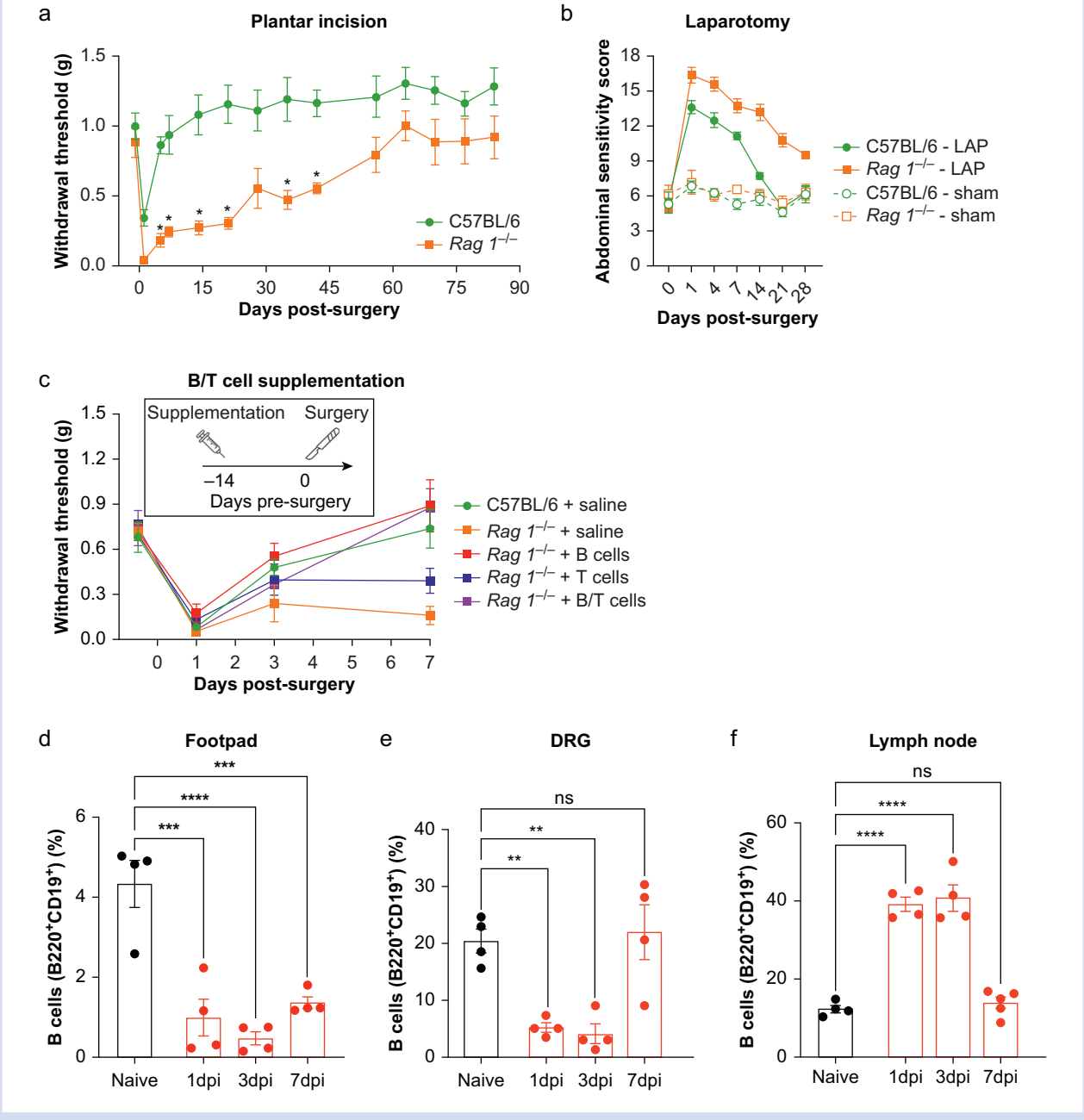

Fig 3. Correlation of pain behaviour with B-cell recruitment in mice in the plantar and abdominal incision pain assays. (a–c) Rag1$^{-/-}$ mice lacking T and B cells display increased and prolonged postsurgical pain. (a) Mechanical pain thresholds before and after plantar incision in the ipsilateral hind paw in C57BL/6 (wild-type; n=11) and *Rag1*$^{-/-}$ mice (n=10). (b) Abdominal sensitivity scores (see Methods) before and after laparotomy (LAP) or sham surgery in C57BL/6 and *Rag1*$^{-/-}$ mice (n=8 per surgery per genotype), performed in a different laboratory. (c) Supplementation of B cells resolves pain hypersensitivity in *Rag1*$^{-/-}$ mice. Mice received saline, or tail vein injection of purified total T cells, B cells, or both (n=5 per group), 14 days pre-surgery (inset; icons from iStock). Symbols in all graphs represent mean (SEM). *P<0.05 compared with wild-type (a, b) or as indicated (c). (d–f) B-cell fractions vary with time in a tissue-dependent fashion after surgery. CD45+B220+CD19+ B cells were isolated from mice at 1, 3, and 7 days post-injury (dpi), with naive tissue used as a control and are represented as a percentage of all CD45+ immune cells in each tissue. Cell quantification by flow cytometry. Significance levels: *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001, non-significant (ns). Tissues are: (d) footpad, (e) dorsal root ganglia (DRG), and (f) lymph node.

observed. As before, we observed a prolonged duration of mechanical allodynia in (saline-treated) *Rag1*$^{-/-}$ mice compared with wild-type mice. As expected, combined administration of T and B cells to *Rag1*$^{-/-}$ mice prior to plantar incision resolved allodynia in a kinetics similar to lymphocyte-replete animals (Fig. 3c). However, reconstitution of mice with only T cells did not significantly affect the time course of mechanical allodynia. Remarkably, transfer of purified B cells alone led to a significant acceleration of pain resolution at day 7 after surgery (day 7 one-way ANOVA: $F_{4,20}$=6.9, P=0.001; Tukey *post hoc* analysis revealed two homogenous subsets at P<0.05; see Fig. 3c).

Tissue-specific recruitment of B cells to the dorsal root ganglion in mechanical allodynia

Using the plantar incision assay, we determined the recruitment patterns of B and T cells in the footpad, L4–6 DRG, and inguinal lymph nodes over the first 7 days post-injury, by which time post-incisional allodynia is no longer observed (Fig. 3a and c). Flow cytometry analysis of cells collected from these tissues showed a marked decline in the proportion of CD45+CD19+B220+ B cells in the footpad and DRG as early as 24 h post-incision (Fig. 3d and e; Supplementary Fig. S3). These cells did not return in the footpad for the duration of the study but were seen again in the DRG at 7 days post-injury. There was an early and significant increase in the proportion of B cells in the inguinal lymph nodes at 1 and 3 days post-incision, returning to baseline levels by 7 days (Fig. 3f; Supplementary Fig. S3). There were no significant changes in T-cell infiltration of either the footpad or the DRG (Supplementary Figs 4a, b, and 5). A more in-depth characterisation of specific T-cell subsets in the lymph node showed a small significant increase in CD8+ T cells at 24 h and a decrease in CD4+ T cells at 7 days post-injury (Supplementary Figs 4c and 5).

Discussion

The aim of this study was to identify genetic variants associated with CPSP severity in the general surgical population. We used a GWAS approach in six independent cohorts grouped into five surgical types to interrogate the common variants associated with CPSP severity. Although this was a GWAS of several relatively small-scale studies, we were able to identify the first genome-wide significant hits associated with CPSP severity in the majority of the individual surgeries. Our study thus serves as a starting point for more large-scale efforts to identify mechanisms underlying CPSP across various types of surgeries.

Our data produced the first estimation of SNP-based heritability of CPSP incidence across an array of surgeries. The estimate of 35% heritability is in line with a recent study on overall chronic pain heritability in the UK Biobank, which found an estimate of 38.4%.[20] This further solidifies chronic pain in general, and CPSP specifically, as a heritable trait. Our GWAS analysis identified a total of 77 genome-wide significant SNPs across 24 loci associated with the severity of CPSP. Furthermore, hundreds of genes with individual or aggregated SNP effects and biological pathways were found significant at the FDR 20% level.

After performing GWAS, we carried out functional analyses of our genetic results to gain insights into biological processes contributing to CPSP. We first aimed to uncover the basic mechanism underlying CPSP by identifying disease-relevant tissues and cell types, independent of the surgery site and sex. For our functional analysis we used the results of the GWAS meta-analysis across different surgeries, using sex as a co-variable. Our results were thus related to the overall strongest identified genetic effect but not to any particular SNP. Our work led to unexpected findings when we tested for tissue-specific partitioned heritability. Previous GWASs of chronic pain indicated enriched heritability in brain regions, with the *DCC* gene and the axonal guidance pathway showing the strongest genetic associations.[21] These studies pointed to a *neuronal* origin hypothesis of chronic pain. Brain connectivity has been found to be a predictor for the transition to chronic pain,[22] and effective treatment of chronic pain has been shown to reverse abnormal brain anatomy and function.[23] In contrast, we observed an enrichment of heritability in adaptive immune cells, specifically in B and T lymphocytes. The enrichment pattern further suggests differentiation of B and T cells, judged by enrichment for haematopoietic progenitor stem cells. Importantly, we cannot exclude the importance of other cells or tissues, such as central or peripheral neuronal tissues, just because their signals did not reach significance in our analyses of this relatively small cohort. However, an immune origin of CPSP is clearly highlighted by our results as the adaptive immune contribution appeared as the strongest signal.

A possible explanation for this stronger immune response elicited by surgery-related injury might be related to associated inflammatory responses.[24] Another possible explanation could be the difference in time spans for the assessment of the reported pain. Typically, chronic pain patients have pain lasting for years, whereas in our study, cases were evaluated at 3- and 6-month time points. Therefore, our results point to a more substantial role for the immune system in earlier stages of persistent pain (i.e. 3–6 months after surgery). Although the majority of these cases resolve over time, contribution of the nervous system could be more critical in the late phase of CPSP (i.e. years after surgery). These findings are supported by multiple longitudinal studies of chronic pain neuroinflammation that show a strong immune response early after injury,[25] and are further supported here by our GWAS.

The physiological response to surgical trauma features a complex yet distinctive profile of coordinated, overlapping, and time-dependent events categorised into three phases: (1) an apoptosis-inducing inflammatory reaction, (2) proliferative events that lead to restoration of affected tissue, and (3) remodelling of the affected tissue.[26] In all phases, a significant role for T lymphocytes is found. CD4+ T helper cells are attracted by the interferon-γ secreted by macrophages and contribute to the initial proinflammatory environment, γδ T cells promote proliferation of keratinocytes thereby affecting tissue remodelling, and regulatory T cells reduce the inflammation by secretion of anti-inflammatory mediators.[27] However, our previous studies show that although γδ T cells respond early after injury, they do not contribute to pain signalling.[28] Furthermore, αβ T cells do not infiltrate the skin until well after the incisional wound has healed and pain has subsided.[29]

Although the exact role of B lymphocytes within the wound healing process has not yet been solidified, CD19 (a key regulator of B cells) controls wound healing through a hyaluronan-induced TLR4 signalling process. TLR4-dependent pro-inflammatory signalling induces B-cell proliferation and cytokine excretion, indicating a role for B lymphocytes.[30] Although there is evidence that various T-cell populations

contribute to wound healing, these often use the punch biopsy model (~1 cm diameter wound), whereas the model we used is a very discrete sterile incision followed by close apposition and suturing of the skin. Thus, wound healing is likely unaffected in our injury model. In part, the detrimental effect of prolonged inflammation after injury results from the deregulation and desynchronisation of wound-healing events, impairing the transition towards the proliferation phase in which both B and T cells play critical roles.[27] As corticosteroids are often administered as a standard of care after surgery, their impact on adaptive immunity must also be taken into account.[31]

When we validated our results obtained by genetic approaches using *in vivo* mouse assays, we found evidence for a B cell, but not T cell, contribution to resolving CPSP. After hind paw or abdominal surgical incision, mice with a genetic deficiency in B and T cells displayed higher peak levels of pain hypersensitivity, and thus B or T cells might play a role in post-surgical pain initiation. The mutant mice remained in an allodynic state significantly longer than their wild-type counterparts, indicating a clear role for B or T cells in the maintenance post-surgical pain. To identify which immune cell type was directly responsible for this prolonged allodynia, we attempted to rescue the allodynic effect in the $Rag1^{-/-}$ mice using purified B or T cells. The rescue was observed convincingly in the presence of supplemented B cells, thus establishing their critical role. Furthermore, the recruitment pattern of T and B cells suggests a minor change in T cells across key sites (footpad, DRG, inguinal lymph node) in the acute stage after an incisional wound. Conversely, B-cell numbers showed a robust and significant decrease in the footpad and DRG early after incision (days 1–3) with a concomitant increase in inguinal lymph nodes. By day 7, however, B cells returned to baseline levels in the lymph nodes and DRG but remained largely absent in the footpad, which suggests that redistribution of B cells during the course of wound healing is fundamental to pain resolution in this animal model. The specific mechanisms by which B lymphocytes contribute to the control of CPSP remain unknown. However, our conclusions build on our previous results demonstrating the importance of the active contribution of the immune system to prevention of pain development.[32] In contrast to our previous findings, obtained via blood transcriptomics from which immune system changes would be expected, the current results were obtained from GWAS analysis and are thus completely unbiased.

B-cell involvement has been well characterised in chronic pain conditions, such as rheumatoid arthritis and complex regional pain syndrome, however, in the direction opposite to that proposed by our results. Indeed, the monoclonal antibody rituximab, targeting the B lymphocyte antigen CD20, has been shown to alleviate chronic pain.[33–36] Furthermore, immunoglobulin G (IgG) from fibromyalgia patients, when transferred to mice, induces pain-like behaviour.[37] These findings suggest that B lymphocytes and autoantibodies could contribute to the pathology of chronic pain. However, our results suggest that they are protective against chronic pain development at the acute-to-chronic pain transition state, most likely through their critical contributions to wound healing through hyaluronan-induced TLR4 signalling.[30,38] Thus, our current findings are reminiscent of the apparently bidirectional role of neutrophils, which variously have been shown to contribute to pain development[39] or pain resolution.[32]

There were a number of limitations to this study: (1) small sample sizes for GWAS analysis, though larger datasets are unavailable; (2) several types of surgeries, with potentially different pain intensities and chronification rates; (3) the majority of clinical cases were evaluated at the 3-month time point post-surgery, when patients might have resolved their pain by 6 months; (4) unbalanced male and female representation as the majority of participants (75%) were women, although this skew in data is apparent in many clinical pain studies; (5) an absence of *bona fide* replication in independent cohorts, which will be necessary in follow-up studies; (6) a high probability for hospital- and country-specific post-surgical care affecting our results, such as drug treatments, post-surgical care, length of stay, and diet; and 7) an overlap in genes expressed in both B and T cells present in partitioned heritability methodology, obscuring the ability to distinguish between B and T cells unequivocally. However, we were able to validate our GWAS results in the mouse experiments addressing many of these concerns and suggesting that B cells are the main effectors of pain after surgery, displaying a net protective effect.

In conclusion, we identified 77 SNPs spread over 24 genomic loci associated with CPSP severity, a debilitating condition affecting up to 85% of patients undergoing surgery and varying with surgery type. Functional analysis strongly suggested a critical contribution of the adaptive immune system in the genetic factors associated with CPSP. Furthermore, B cells rather than T cells were able to rescue the prolonged allodynia experienced by immune-compromised mice after paw incisional surgery. These insights into the aetiology of CPSP severity warrant clinical and preclinical studies to further specify the underlying immune mechanisms for CPSP and suggest a new direction for disease-modifying treatment.

Authors' contributions

Study conceptualisation: BAR, CDB, EAJJ, EKA, HK, ILK, JEC, JSM, LD, MA, MID, NG, WFFAB
Methodology – clinical: BAR, EAJJ, EKA, HK, JEC, MA, MDG, NJvdH, SK, WFFAB
Methodology – bioinformatics: MP, RRIvR, SK
Methodology – mouse assays: CDB, EK, GT, GP, ILK, JRS, JSM, MID, MK, NG
Investigation: MP, RRIvR, SK
Results interpretation: CDB, ILK, JSM, LD, MID, MP, NG, RRIvR, SK
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Editing of the manuscript: JSM, LD, MP
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Declarations of interest

EAJJ is a consultant for Boston Scientific Inc; LD was a consultant for ONO PHARMA USA Inc.; WFFAB was member of the advisory board and a lead investigator of the Prodigy Study at Medtronic, a coordinating investigator of the PHOENICS study supported by Fresenius Kabi and B. Braun Melsungen, and is a consultant for Fresenius Kabi. The remaining authors have no conflicts of interest to declare.

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Data availability statement

All data are available upon request.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bja.2024.04.053.

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