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Enkephalin-mediated modulation of basal somatic sensitivity by regulatory T cells in mice

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Abstract

$CD4^+CD25^+Foxp3^+$ regulatory T cells (Treg) have been implicated in pain modulation in various inflammatory conditions. However, the mechanisms by which Treg hamper pain are still unclear. From a meta-analysis of 11 available transcriptomes, we show that the proenkephalin gene (*Penk)* which encodes the precursor of analgesic opioid peptides, is among the top 10 genes enriched in murine Treg relative to conventional T cells (Tconv). We then show that *Penk* expression in Treg is under the control of TNFR signaling and the transcription factor BATF. Using mice in which *Penk* mRNA expression can be tracked with a fluorescent reporter, we also show that *Penk* expression is restricted to Treg and activated Tconv in non-inflammatory conditions in all examined organs and tissues. Furthermore, inducible ablation of *Penk* in Treg leads to heat hyperalgesia for both male and female mice. Overall, our results indicate that TNFR signaling and BATF regulation of *Penk* in Treg might play a key role at modulating basal somatic sensitivity in mice.

eLife assessment

This study presents a **valuable** finding on a new role of Foxp3+ regulatory T cells in sensory perception, which may have an impact on our understanding of somatosensory perception. The authors identified a previously unappreciated action of enkephalins released by immune cells in the resolution of pain and several upstream signals that can regulate the expression of the proenkephalin gene PENK in Foxp3+ Tregs. However, whereas the generation of transgenic mice with conditional deletion of PENK in Foxp3+ cells and PENK fate-mapping is novel and generates **compelling** data, they show an **incomplete** analysis of Tregs in the control and transgenic mice, proper tamoxifen controls nor the role of PENK+ skin T cells to further support their hypothesis. Nonetheless, the study would be of interest to the biologists working in the field of neuroimmunology and inflammation.

Introduction

Regulatory T-cells (Treg), characterized by the expression of the alpha chain of the interleukin-2 receptor CD25 and the transcription factor Foxp3 [1], are known to be key players in immunoregulation in both humans and mice; too few may lead to autoimmune diseases whereas too many may prevent an efficient immune response to cancer[2,3]. Over the last few years, several new functions of Treg beyond immunoregulation have been identified in tissue regeneration or local regulation of metabolism[4–6] for instance. In addition, there are accumulating evidences of a cross-talk between Treg and the nervous system. These includes promotion of oligodendrocyte differentiation or inhibition of neuroinflammation facilitating CNS repair process after brain injuries and preventing cognitive decline[7–10]. Furthermore, Treg have been involved in the regulation of pain in various model of nerve injury in rats and mice, such as in autoimmune neuritis or chronic constriction of the sciatic nerve[11–13]. Depletion of Treg have been associated with enhanced pain sensitivity whereas increased Treg number or activity limit pain hypersensitivity[14]. Although the current view is that Treg control pain through their immunosuppressive functions [15], whether Treg might regulate pain at steady state is currently unknown. Starting from our previous observation that the expression of the proenkephalin gene (*Penk*), encoding for analgesic enkephalins, is highly enriched in Treg (Aubert et al., 2019 biorXiv, *https://doi.org/10.1101/638072*), we characterize the expression of *Penk* in the immune system and investigate the role of Treg on pain *in vivo* at steady state using advanced genetically-engineered mouse models. Our results uncover a previously unknown mechanism by which Treg modulate basal somatic sensitivity.

Results and Discussion

A meta-signature of murine Treg and *Penk* expression in lymphoid and non-lymphoid organs

Using 11 available transcriptomes retrieved from the GEO website, we generated a Treg molecular signature at steady state in lymphoid tissues, improving the robustness of the analysis by several orders of magnitude relative to individual studies. The 25 most differentially expressed genes are depicted in **Figure 1A** and the complete list is shown in Table S1. As expected from the sorting strategies used to isolate Treg (**Table 1** in the Material and Methods section), we observe that *Il2ra* and *Foxp3* are the most differentially expressed genes in Treg compared to Tconv, followed by the well-known Treg markers *CTLA-4*, *Itgae* (CD103), *Ikzf2* (Helios) and *Klrg1*. However, several unexpected genes are present in that signature, including the proenkephalin gene *Penk*, coding for opioid peptides endowed with analgesic properties. Using the Immuno-Navigator database[16], a batch-corrected collection of RNA quantification across numerous studies, samples and cell types, we confirm that *Penk* is highly correlated to *Foxp3* in mice (R=0.871, **Figure 1B**). Enriched expression of *Penk* in Treg was previously reported in several specific contexts including TCR-transgenic mice[17], UVB-exposure[6] or in the brain of mice recovering from stroke[8]. Using publicly available datasets, we also observe that *Penk* is enriched in Treg in the thymus, the visceral adipose tissue and in muscles (**Figure 1C**). Thus, *Penk* mRNA expression in Treg is consubstantial of their generation and is independent of their tissue localization at steady state.

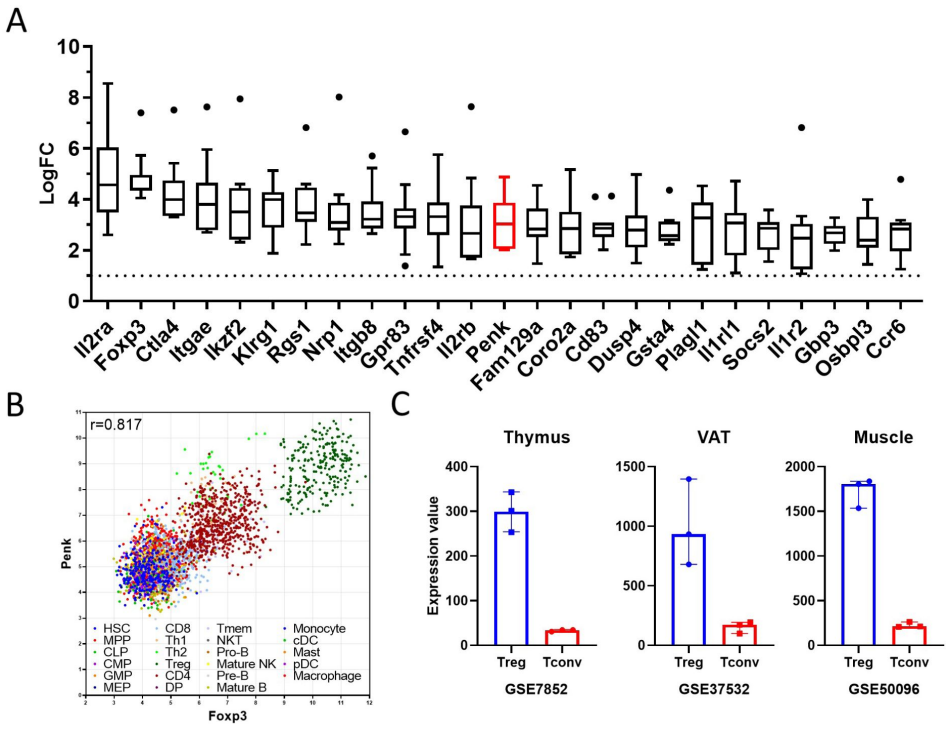

Figure 1

A meta signature of murine Treg and Penk mRNA expression in lymphoid and non lymphoid organs.

A) Top 25 genes enriched in Treg compared to Tconv from lymphoid tissue from at least 10 of the 11 datasets analyzed ranked by fold change (LogFC) of mean expression relative to Tconv. The Penk gene is highlighted in red. **B)** Correlation of Penk and Foxp3 mRNA expression in all the cell types listed in the legend. The Pearson correlation coefficient is indicated. **C)** Expression of Penk mRNA in Treg (blue) and Tconv (red) isolated from thymus, visceral adipose tissue (VAT) and muscle. The source of the data is indicated below each graph.

Dataset	Genetic background	Age	Sex	Treg sorting	Tissue	Affymetrix Genome array
GSE103216	C57Bl/6	6-8 weeks	Female	Foxp3-RFP	LN	1.0
GSE136582	C57Bl/6	6-8 weeks	NA	Foxp3-eGFP	Spleen	2.0
GSE14308	C57Bl/6	NA	NA	$CD25^{high}$	Spleen and LN	2.0
GSE15907	C57Bl/6	6 weeks	Male	$CD25^{high}$	Spleen	1.0
GSE17580	C57Bl/6	NA	Female	$CD25^{high}$	Mesenteric LN	2.0
GSE24210	C57Bl/6	NA	NA	$CD25^{high}$	Spleen and LN	2.0
GSE37532	C57Bl/6	25 weeks	Male	$CD25^{high}$	LN	1.0
GSE40685	C57Bl/6	NA	NA	Foxp3-GFP	Spleen and LN	2.0
GSE42021	BALB/c	NA	NA	Foxp3-GFP	Spleen and LN	2.0
GSE50096	C57Bl/6	6 weeks	NA	Foxp3-GFP	Spleen	1.0
GSE7460	C57Bl/6	32-36 weeks	NA	$CD25^{high}$	LN	2.0

Table 1

Characteristics of the datasets used for the Treg meta-signature

(NA = not available; LN = Lymph Nodes)

Penk mRNA expression is regulated by TNFR signaling and the BATF transcription factor in Treg

To explore possible mechanisms that could explain the enrichment of Penk mRNA in Treg cells, we examined the Immuno-Navigator dataset to extract the genes that are most correlated with Penk and with each other (**Figure 2A**). Among these genes, we observe several members of the TNFR superfamily (*Tnfrsf1b* (TNFR2), *Tnfrsf4* (OX40), *Tnfrsf9* (4-1BB), *Tnfrsf18* (GITR)) as well as the transcription factor *Batf*. Furthermore, *Batf* expression is highly correlated with *Penk* in Treg (R=0.599, **Figure 2B**). Thus, TNFR and BATF could be involved in the regulation of *Penk* in Treg. To examine this possibility, we reanalyzed our previously published dataset establishing the transcriptome of Treg stimulated with anti-CD3, anti-CD28 antibodies and TNFR agonists *in vitro* [18]. We observe that addition of TNFR2, OX40 or 4-1BB agonists increases *Penk* expression at 36h post-stimulation relative to controls (**Figure 2C**). Interestingly, *Batf* is also increased by TNFR agonists at an earlier time point (18h), suggesting that TNFR signaling may regulate *Penk* expression through the BATF transcription factor. Accordingly, a dramatic decrease in *Penk* expression is observed in Treg lacking BATF [19] (**Figure 2D**). In line with our hypothesis, BATF is a crucial partner of AP-1 transcription factor that has been shown to regulate *Penk* in the hippocampus[20].

Penk is prominently expressed by Treg at steady state

Based on these findings, we further investigated the expression of *Penk* mRNA in various immune cell types across multiple tissues, as defined by a combination of lineage markers. (**Figure S1A**). To do so, we crossed a transgenic mouse model expressing Tamoxifen (TMX)-inducible Cre recombinase under the promoter of *Penk* (Penk$^{\text{Cre-ERT2}}$) with the ROSA26$^{\text{TdTomato}}$ reporter mice. In these mice, any cell that would expressed *Penk* at the time of TMX administration would become permanently tagged with the tdTomato reporter a few days later. In order to improve detection of those cells by flow cytometry, we also used an anti-mCherry that cross-react with TdTomato (**Figure S1B**). In lymphoid tissue, such as in lymph node (LN), *Penk* expression is mainly restricted to Treg and to a small subset of activated CD4^{+} T-cells (**Figure 3A**). Similar results are obtained in non-lymphoid tissues, such as in the colon, although *Penk* expression in activated T cells is further increased relative to LN (**Figure 3B**). As summarized in **Figure 3C**, Treg is the immune subset expressing the highest level of *Penk* in all tissues analyzed. Expression of *Penk* is also detected in Tconv, but at lower frequencies than in Treg, in agreement with the molecular meta-signature established above (**Figure 1**). All other cell types show low or undetectable *Penk* expression. Interestingly, in CD4^{+} T-cells (Tconv and Treg), *Penk* expression is higher in the activated CD62L$^{\text{low}}$ CD44$^{\text{high}}$ fraction compared to the naive CD62L$^{\text{high}}$ CD44$^{\text{low}}$ phenotype (**Figure S2**). Thus, we observed that *Penk* mRNA is predominantly found in Treg and activated CD4^{+} T cells in the immune system at steady state. Among these, the highest expression of *Penk* was detected in Treg cells present in the skin and the colon. However, we cannot exclude the possibility that the distribution of Penk may be broader in an inflammatory context. In line with this, a recent study demonstrated that *Penk* expression in M2 macrophages is dependent on IL-4 and occurs in response to nerve injury[21].

Heat hyperalgesia in mice deficient for *Penk* in Treg

To determine if enkephalins produced by Treg had an effect on pain at steady state, we generated mice deficient for enkephalins in Treg by crossing TMX-inducible Cre recombinase under the control of the *Foxp3* promoter (Foxp3$^{\text{Cre-ERT2}}$) with mice transgenic for LoxP sequences flanking exon 2 of *Penk* (Penk$^{\text{flox}}$). In these mice (hereafter referred to as Lox), any cell expressing Foxp3 at the time of TMX administration would become deficient for exon 2 of *Penk* a few days later. Since exon 2 is the precursor of Met-Enkephalin, an endogenous opioid that act on thermal pain sensation[22], we evaluated the sensibility of these mice to pain induced by heat (**Figure 4**). As controls, we used mice expressing the Cre recombinase and wild-type (WT) at the locus of LoxP

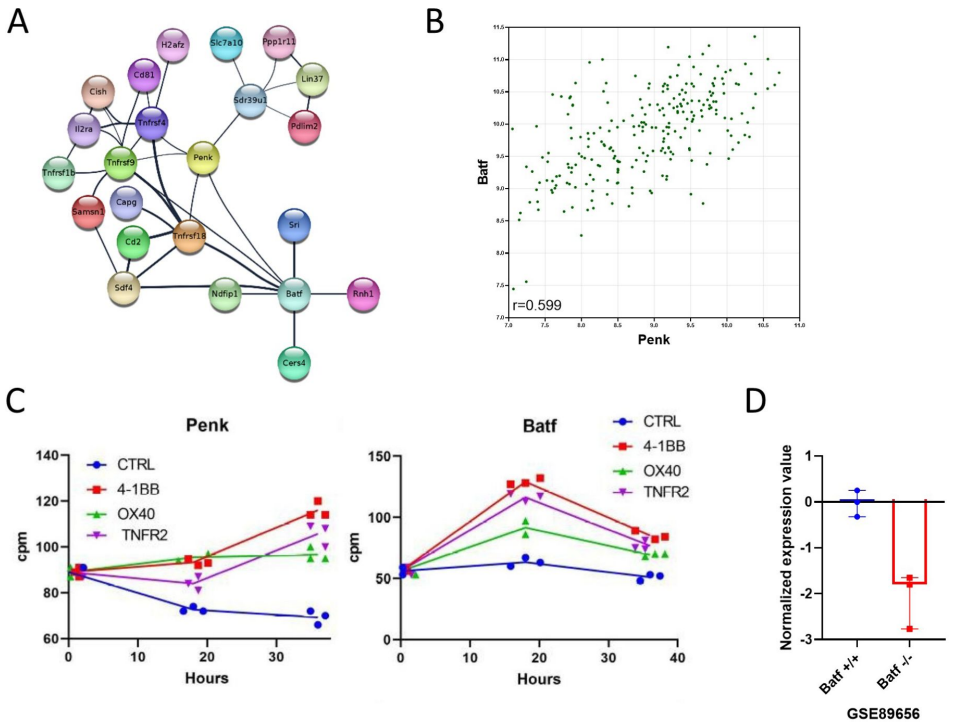

Figure 2

Penk mRNA expression is regulated by TNFR signaling and the BATF transcription factor in Treg.

A) A network of the genes most correlated to Penk in Treg is shown. The Pearson correlation values were extracted from the Immuno-Navigator database (taking only Treg for the analysis) and integrated into Cytoscape v3.730. Each node is a gene linked by edges with width proportional to the Pearson correlation. (edge range: 0.538-0.758). **B)** Illustration of the correlation between expression of Penk and Batf in Treg. Each dot is a sample from the Immuno-Navigator database. The Pearson correlation coefficient is indicated on the figure. **C)** Penk and Batf mRNA expression after in vitro stimulation of purified Treg with the indicated TNFR agonists prior (0), and at 18 and 36hrs after stimulation. Each dot is a biological replicate from a single experiment. **D)** GEO2R analysis of the GSE89656 dataset for Penk mRNA variations between wild type control Treg (WT) and BATF-KO Treg.

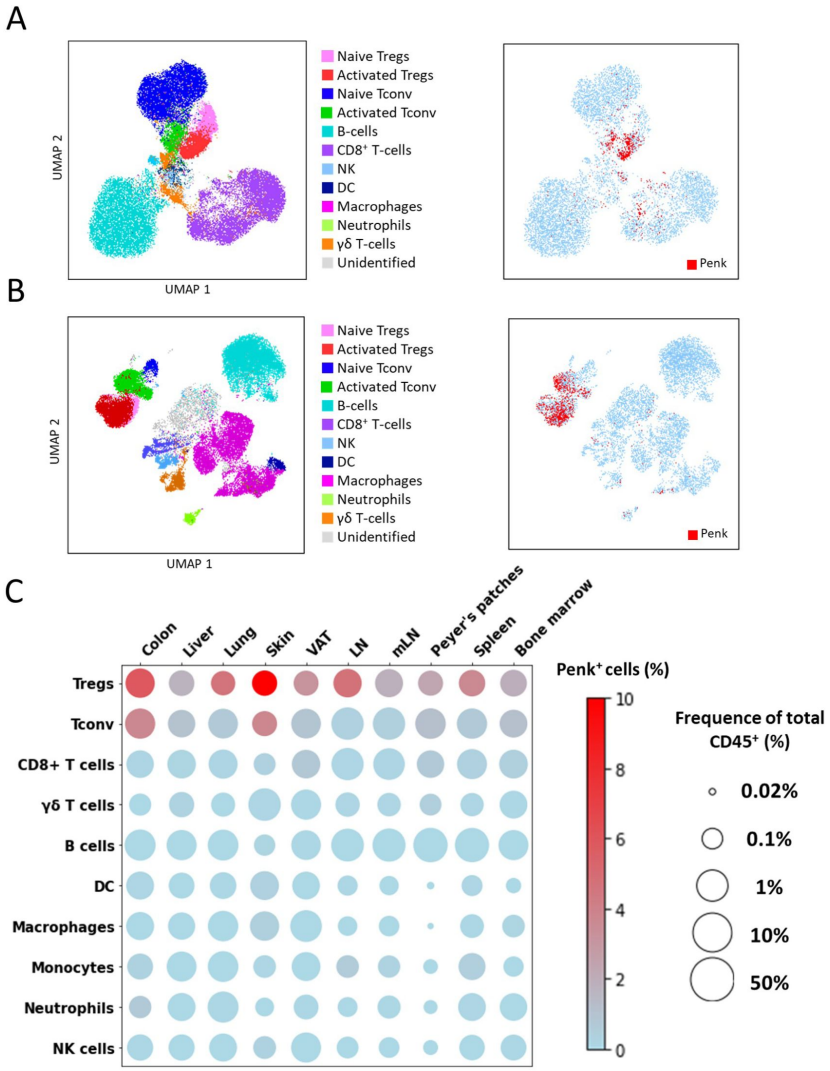

Figure 3

Penk is prominently expressed by Treg at steady state

A-B) (right) UMAP representing all major cell types indicated in the figures determined by flow cytometry from lymph nodes (A) and colon (B) (left). Projection of Penk (blue) on the UMAP of lymph node and colon (right). Subsets were manually gated as depicted in **Figure S1A**. **C)** Scatter plot displaying the average population size and percentage of Penk+ cells for the listed cell populations and organs. Population size was calculated as the percentage out of total CD45+ single cells and represented on a log10 scale. (n = 3 mice for the VAT, spleen and bone marrow, n = 6 mice for the other groups; results cumulative of two independent experiments).

sequences insertion. Mice were treated with TMX and evaluated for heat sensitivity without further additional treatment at ten different time points (4 before and 6 after administration of TMX, 2 to 3 days apart). Under these conditions, a significant trend towards lower latency periods (signing heat hyperalgesia) is observed in the Lox group compared to WT mice. Interestingly, the effect is not apparent until 7 days after TMX administration (Figure S4). Indeed, before TMX administration, WT and Lox female and male mice do not differ in their response to heat (**Figure 4A-B**). However, after TMX administration, Lox mice develop hyperalgesia compared to control mice, with a 20 percent reduction in their median latency period from D7 onwards (8.1 vs. 6.5 sec, **Figure 4C**). Interestingly, the effect of *Penk* deletion in Treg on heat hyperalgesia is sex independent, since it is observed in both female and male (**Figure 4D**), although the reduction was slightly higher in female (22% reduction in latency period) than in male (16% reduction). This can be explained by the fact that males tend to respond faster (7.9s vs 6.1s) than females (8.4s vs 7.0s), irrespective of the genotype (**Figure 4D**). *Penk* expression by $CD4^+$ T-cells has been associated with analgesia in mice models of visceral pain[23–25], but the specific contribution of Treg was not investigated. In neuroinflammatory settings, such as constriction of the sciatic nerve, pain control by Treg could be dependent on their immuno-suppressive function [12,26–28]. On the contrary, our tests were performed at steady state, ruling out the possibility that hyperalgesia could be due to a defect in the immuno-suppressive function of Treg. They strongly suggest a direct role of enkephalins produced by Treg on nociception, uncovering a new non-immune fundamental function of Tregs on endogenous regulation of basal somatic sensitivity.

Materials & Methods

Extraction of Treg meta-signatures

The datasets used were selected based on a “Treg* AND (Tconv* OR Teff*) AND Mus Musculus” search in the GEO dataset web site (*https://www.ncbi.nlm.nih.gov/gds*). GEO datasets were manually inspected for inclusion of studies comparing fresh Treg with fresh Tconv from lymphoid organs. Characteristics of selected datasets are summarized in **Table 1**. For each dataset, we generated a list of differentially expressed genes with a cutoff based on a false discovery rate (FDR) inferior to 0.05 and a log2 fold change superior to 1 with the GEO2R embedded algorithm.

Mice

All male and female mice were on a C57Bl/6J background. *Foxp3*^{tm9}(EGFP/cre/ERT2)^{Ayr}/J (Foxp3$^{\text{Cre-ERT2}}$) (catalog #016961), *B6.Cg-Gt(ROSA)26Sor*^{tm9}(CAG–tdTomato)^{Hze}*/J* (ROSA $^{\text{tdTomato}}$) (catalog #007909), *B6.Cg-Penk*$^{tm1.1}$(cre/ERT2)^{Hze}*/J,* (Penk$^{\text{Cre-ERT2}}$) (catalog #022862) were purchased from The Jackson Laboratory. *C57BL/6JSmoc-Penk*^{em1}(flox)^{Smoc} (Penk$^{\text{flox}}$) were purchased from Shanghai Model Organisms (catalog NM-CKO-210032). For the *Penk* mapping experiments, Penk$^{\text{Cre-ERT2}}$ were bred with ROSA$^{\text{tdtomato}}$. All mice were confirmed to be homozygous for the inducible Cre and at least heterozygous for tdTomato by touchdown PCR (primers sequences available on request). To specifically knock-out the Penk gene in Tregs, Foxp3$^{\text{Cre-ERT2}}$ were crossed with Penk$^{\text{flox}}$ leading to double heterozygous mice (F1) that were crossed together resulting in double homozygous F2 littermates’ mice. All mice used in this study were of F3 generation. Mice were genotyped by touchdown PCR (primers sequences available on request). Mice were housed under specific pathogen-free conditions and were used for experiments at 8 weeks or older. All protocols were approved by the Ethics Committee for Animal Experimentation Charles Darwin (APAFIS #32284-2021070513305185 v5).

Tamoxifen administration

Tamoxifen (ThermoFisher, Les Ulis, France) was dissolved in peanut oil at the concentration of 8 mg/ml under 37°C agitation and delivered by 200µL oral gavage. All mice were administered tamoxifen only once, 7 days before the start of each experiment.

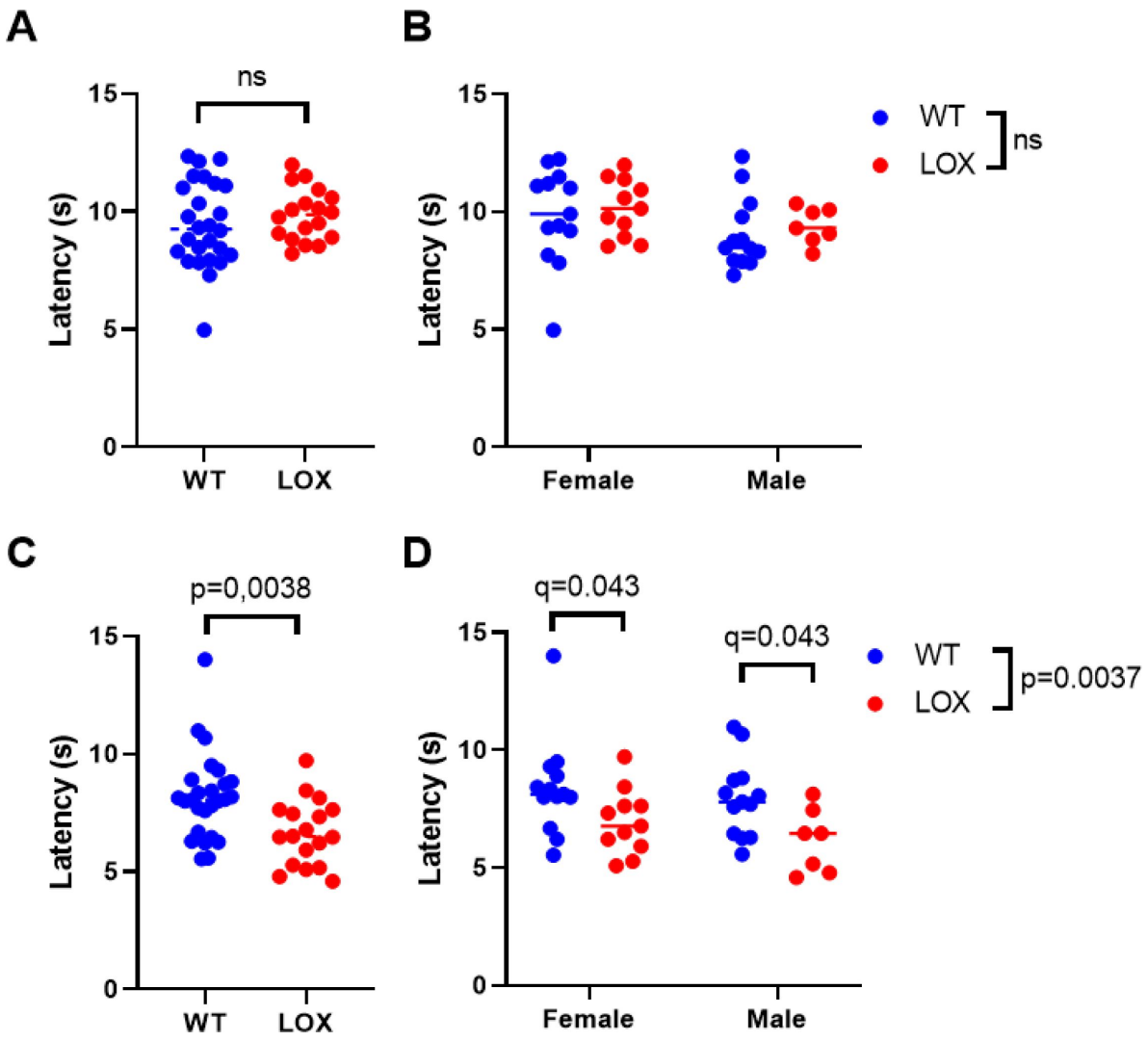

Figure 4

Heat hyperalgesia in mice deficient for *Penk* in Treg.

A) Withdrawal latency of WT and Lox mice before the administration of TMX (baseline). The mean of all 4 measures before administration of TMX for each mouse is shown. Statistical modeling was performed using a non parametric unpaired Mann-Whitney t-test and was deemed as non-significant (ns). **B)** Withdrawal latency of WT and Lox mice before administration of TMX (baseline) shown separately for female and male mice, as in A. Statistical modeling was performed using two-way ANOVA and the effect of the genotype on the results was deemed non-significant (ns). **C)** Withdrawal latency of WT and Lox mice post-TMX treatment. The mean of all 4 measures taken from D7 onward is represented for each mouse. Statistical modeling was performed using a non parametric unpaired Mann-Whitney t-test and was deemed significant (p=0,0038). **D)** Withdrawal latency of WT and Lox mice post-TMX treatment shown separately for female and male mice, as in C. Statistical modeling was performed using two-way ANOVA and the effect of the genotype on the results was deemed significant (p=0,0037). A multiple comparisons test using the Benjamini-Hochberg False Discovery Rate model was used. The adjusted p values were deemed significant for both female and male mice (q=0,043). Results shown in this figure are cumulative of two independent experiments with n = 44 mice (26 WT and 18 Lox).

Preparation of cell suspensions

Organs were harvested on ice in PBS 3% fetal calf serum (FCS). Inguinal, brachial and mesenteric LNs and spleens were directly mashed through a 70µm filter and resuspended in

PBS 3% FCS. Lungs, colons, livers, VAT and skin were dissected, minced then incubated in appropriate digestion buffers (Miltenyi Biotech, Paris, France) at 37°C for various duration according to manufacturer protocols. Cell suspensions were then passed through a 70µm cell strainer and suspended in PBS 3% FCS. To eliminate dead cells and debris, liver cell suspensions was isolated on a 70:30 Percoll gradient. Rings were collected, washed, and cell pellets were resuspended in PBS 3% FCS. ACK Lysing Buffer was used to lyse red blood cells in the lungs, livers and spleens (lysis performed for 1 min at RT, followed by two washes with PBS), prior to staining for flow cytometry.

Antibodies and flow cytometry analysis

The monoclonal antibodies (mAb) and fluorescent reagents used in this study are listed below in the Table. Up to 4.10^6 cells were incubated for 30 min at 4°C with fixable live/dead dye and with the anti-CD16/CD32 (clone 2.4G2) to block FcgRII and FcgRII receptors. Cells were then stained with a combination of antibodies. Cell-surface staining was performed in PBS 3% FCS for 20 min at 4°C. Permeabilization and intracellular staining was performed using the Foxp3/Transcription Factor Staining Buffer Set kit and protocol (ThermoFisher). Stained cells were washed with PBS 1X before acquisition on a Cytek Aurora flow cytometer (Cytek Bioscience, Fremont, CA, USA). Data were analyzed and UMAPs were generated using FlowJo software, version 10.8.1 (TreeStar, Ashland, OR, USA).

mAb or reagent (dye)	Provider	Clone	Dilution
Anti-mouse F4/80 (AF647)	BioLegend	BM8	1/400
Anti-mouse CD62L (AF700)	BD Biosciences	MEL-14	1/400
Anti-mouse CD25 (APC Fire 750)	Biolegend	PC61	1/100

Anti-mouse CD4 (BUV 496)	Miltenyi Biotec	GK1.5	1/400
Anti-mouse CD3 (BUV563)	BD Biosciences	145-2C11	1/400
Anti-mouse CD69 (BUV661)	BD Biosciences	H1.2F3	1/200
Anti-mouse Nkp46 (BUV737)	BD Biosciences	29A1.4	1/200
Anti-mouse CD8a (BUV805)	BD Biosciences	53-6.7	1/800
Anti-mouse Ly6G (BV570)	BioLegend	1A8	1/200
Anti-mouse CD11b (BV650)	BD Biosciences	M1/70	1/1000
Anti-mouse CD45 (NovaFluor Blue 660-120S)	eBioscience	30-F11	1/200
Anti-mouse CD11c (PE CF594)	BD Biosciences	HL3	1/150
Anti-mouse Ly6C (PE Cy7)	BD Biosciences	AL-21	1/1600
Anti-mouse CD44 (PerCP)	BioLegend	IM7	1/200
Anti-mouse CD19 (PerCP Cy5.5)	BD Biosciences	1D3	1/400
Anti-mouse IA/IE (V500)	BD Biosciences	M5/114.15.2	1/300
Anti-mouse TCRγδ (BV421)	eBioscience	GL3	1/200
Anti-mouse Ki67 (AF532)	Invitrogen/eBiosience	SolA15	1/1600
Anti-mouse mCherry (Pacific Blue)	Invitrogen/eBiosience	16D7	1/50
Anti-mouse GATA3 (PE Cy5)	Invitrogen/eBioscience	TWAJ	1/400
Anti-mouse Foxp3 (FITC)	Invitrogen/eBioscience	FJK-16s	1/800
Fixable viability dye (Live Dead Blue)	Invitrogen/eBioscience		1/2000
Anti-mouse CD16/CD32 (BD Fc Block)	BD Biosciences	2.4G2	1/80

Hot plate test

The method used was described by Baker et al. (2002)[29]. Briefly, mice were placed on a metal hot plate maintained at 55°C. The plate was enclosed with Plexiglas walls so that mice could not escape. The response latency, which is the time taken to observe a nocifensive behavior such as jumping, licking or flicking of the hind paws, was recorded. The mice were then immediately removed from the plate upon the recording of a reaction, or within 25s if no response was observed to prevent tissue damage. The test was repeated every two days for a total of four measures before the administration of tamoxifen and four measures starting from D3 post-tamoxifen. Male and female mice were tested separately. Mice remained in their home cage except when being tested on the hot plate. The experimenter was blind to the genotype of the mice until the end of the experiment

Statistical analysis

All statistical tests are reported in the figure legends and were performed with Prism v8 (Graph Pad Inc, La Jolla, CA, USA). The statistical power of the analyses (alpha) was set at 0.05.

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Supplementary figures and table

Table S1: List of the differentially expressed genes between Tconv and Treg. Only genes upregulated in 10 out of 11 datasets are presented with the associated log2 fold change as determined by GEO2R. (NA = not available)

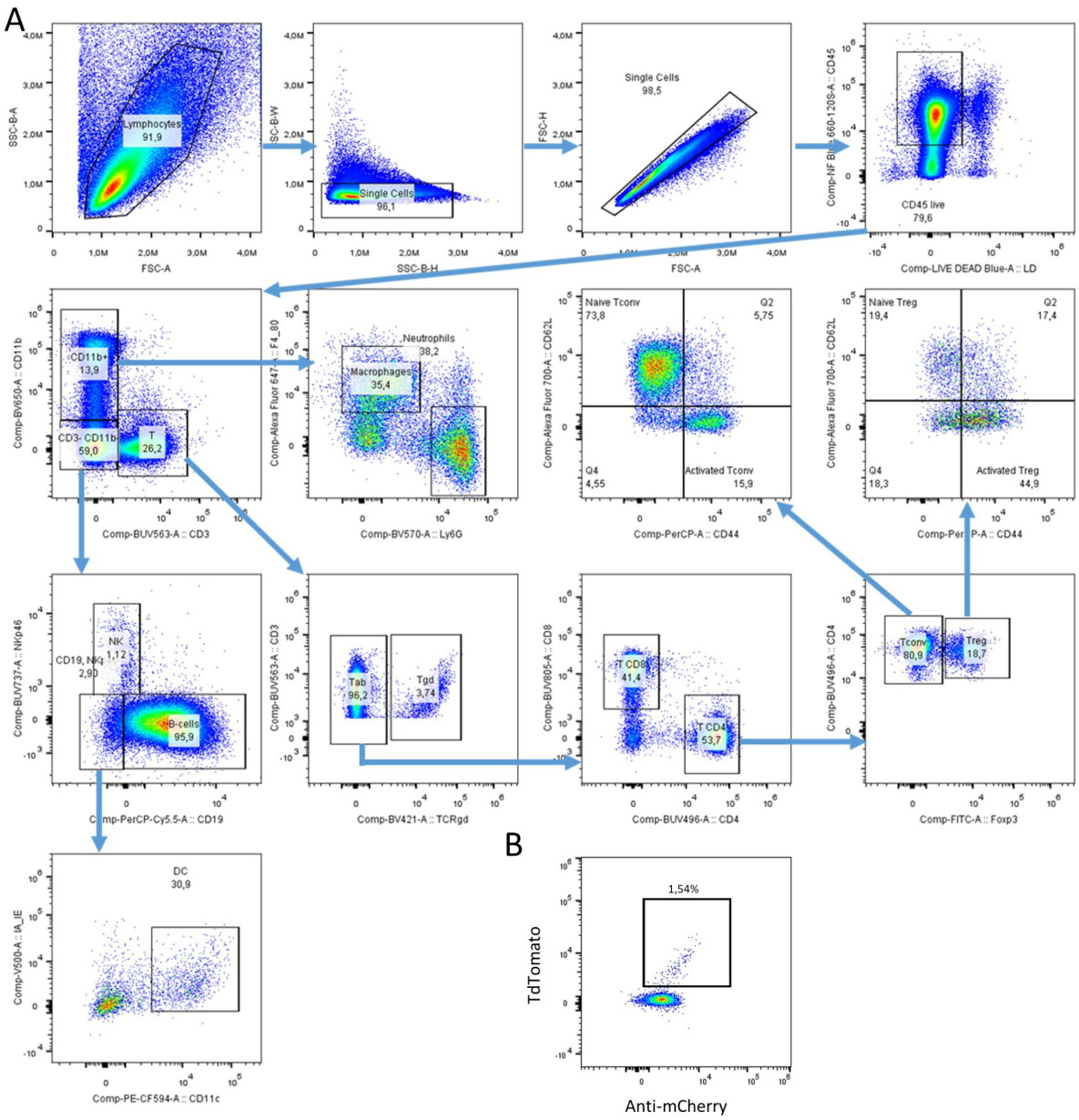

Figure S1.

Representative flow cytometry profiles in Penk$^{Cre\text{-}ERT2}$x ROSA^{tdtomato}

A) Gating strategy used to define the population for the mapping of *Penk* expression by immune cells. **B)** Representative staining of TdTomato and mCherry

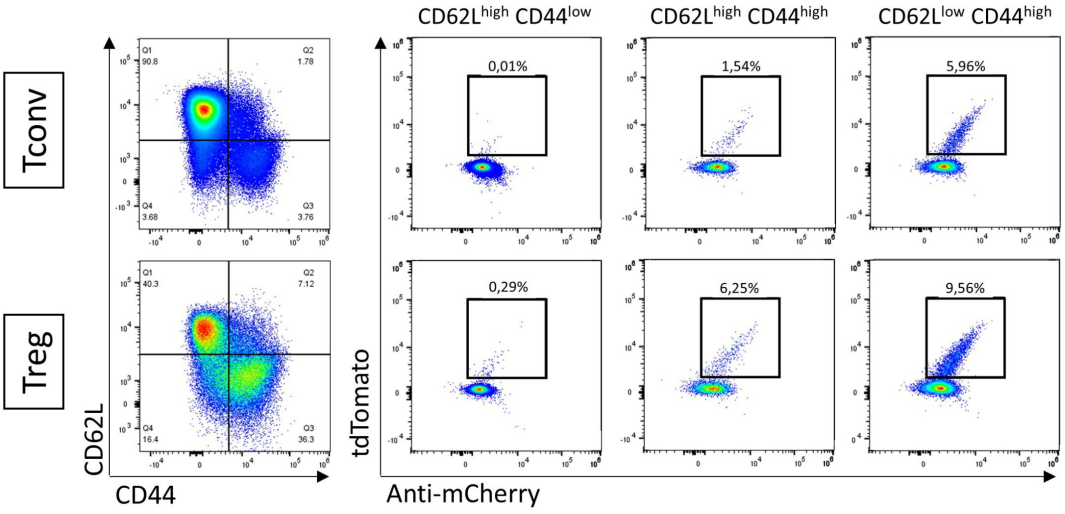

Figure S2.

Penk expression in Tconv and Treg according to activation status in lymph node

Representative staining of TdTomato and mCherry in naive (CD62L^{high} CD44^{low}), central memory (CD62L^{high} CD44^{high}) and effector memory (CD62L^{low} CD44^{high}) Tconv (top) and Treg (bottom).

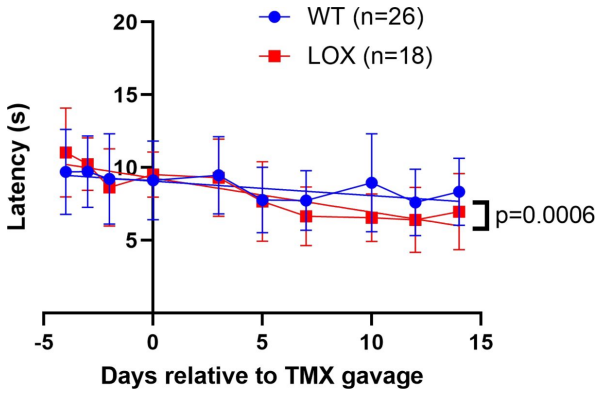

Figure S3.

A kinetics of latency periods in response to heat in WT and Lox mice.

Each dot is the mean value (± SD) of latency period in response to heat stimulation for all mice of the WT or Lox genotype. The results are represented as a function of days relative to TMX gavage (D0). Statistical modeling of the results was performed with linear regression curve fitting with a comparison of intercept and slopes. The low p value allow to reject the hypothesis that one curve fits all the datasets.

References

1. Hatzioannou A., et al. (2021) **Regulatory T Cells in Autoimmunity and Cancer: A Duplicitous Lifestyle** *Front. Immunol* **12**

2. Kim J. M., Rasmussen J. P., Rudensky A. Y. (2007) **Regulatory T cells prevent catastrophic autoimmunity throughout the lifespan of mice** *Nat. Immunol* **8**:191–197

3. Nishikawa H., Sakaguchi S. (2010) **Regulatory T cells in tumor immunity** *Int. J. Cancer* **127**:759–767

4. Xiao T., et al. (2022) **Tregs in visceral adipose tissue up-regulate circadian-clock expression to promote fitness and enforce a diurnal rhythm of lipolysis** *Sci. Immunol* **7**

5. Meng F., Hao P., Du H. (2023) **Regulatory T cells differentiation in visceral adipose tissues contributes to insulin resistance by regulating JAZF-1/PPAR-γ pathway** *J. Cell. Mol. Med* **27**:553–562

6. Shime H., et al. (2020) **Proenkephalin + regulatory T cells expanded by ultraviolet B exposure maintain skin homeostasis with a healing function** *Proc. Natl. Acad. Sci* **117**:20696–20705

7. Dombrowski Y., et al. (2017) **Regulatory T cells promote myelin regeneration in the central nervous system** *Nat. Neurosci* **20**:674–680

8. Ito M., et al. (2019) **Brain regulatory T cells suppress astrogliosis and potentiate neurological recovery** *Nature* **565**:246–250

9. Huang Y., Liu Z., Cao B.-B., Qiu Y.-H., Peng Y.-P. (2020) **Treg Cells Attenuate Neuroinflammation and Protect Neurons in a Mouse Model of Parkinson's Disease** *J. Neuroimmune Pharmacol. Off. J. Soc. NeuroImmune Pharmacol* **15**:224–237

10. Lemaitre P., et al. (2023) **Molecular and cognitive signatures of ageing partially restored through synthetic delivery of IL2 to the brain** *EMBO Mol. Med* **15**

11. Austin P. J., Kim C. F., Perera C. J., Moalem-Taylor G. (2012) **Regulatory T cells attenuate neuropathic pain following peripheral nerve injury and experimental autoimmune neuritis** *Pain* **153**:1916–1931

12. Duffy S. S., et al. (2019) **Regulatory T Cells and Their Derived Cytokine, Interleukin-35, Reduce Pain in Experimental Autoimmune Encephalomyelitis** *. J. Neurosci. Off. J. Soc. Neurosci* **39**:2326–2346

13. Kuhn J. A., et al. (2021) **Regulatory T-cells inhibit microglia-induced pain hypersensitivity in female mice** *eLife* **10**

14. Lees J. G., Duffy S. S., Perera C. J., Moalem-Taylor G. (2015) **Depletion of Foxp3+ regulatory T cells increases severity of mechanical allodynia and significantly alters systemic cytokine levels following peripheral nerve injury** *Cytokine* **71**:207–214

15. Bethea J. R., Fischer R. (2021) **Role of Peripheral Immune Cells for Development and Recovery of Chronic Pain** *Front. Immunol* **12**

16. Vandenbon A., et al. (2016) **Immuno-Navigator, a batch-corrected coexpression database, reveals cell type-specific gene networks in the immune system** *Proc. Natl. Acad. Sci* **113**:E2393–E2402

17. Zelenika D., et al. (2002) **Regulatory T Cells Overexpress a Subset of Th2 Gene Transcripts** *J. Immunol* **168**:1069–1079

18. Lubrano di Ricco M., et al. (2020) **Tumor necrosis factor receptor family costimulation increases regulatory T-cell activation and function via NF-κB** *Eur. J. Immunol* **50**:972–985

19. Hayatsu N., et al. (2017) **Analyses of a Mutant Foxp3 Allele Reveal BATF as a Critical Transcription Factor in the Differentiation and Accumulation of Tissue Regulatory T Cells** *Immunity* **47**:268–283

20. Sonnenberg J. L., Rauscher F. J., Morgan J. I., Curran T. (1989) **Regulation of proenkephalin by Fos and Jun** *Science* **246**:1622–1625

21. Celik M. Ö., Labuz D., Keye J., Glauben R., Machelska H. (2020) **IL-4 induces M2 macrophages to produce sustained analgesia via opioids** *JCI Insight* **5**

22. Aman Y., Pitcher T., Simeoli R., Ballard C., Malcangio M. (2016) **Reduced thermal sensitivity and increased opioidergic tone in the TASTPM mouse model of Alzheimer's disease** *PAIN* **157**

23. Boué J., et al. (2014) **Endogenous regulation of visceral pain via production of opioids by colitogenic CD4(+) T cells in mice** *Gastroenterology* **146**:166–175

24. Basso L., et al. (2016) **Endogenous analgesia mediated by CD4(+) T lymphocytes is dependent on enkephalins in mice** *J. Neuroinflammation* **13**

25. Basso L., et al. (2018) **T-lymphocyte-derived enkephalins reduce Th1/Th17 colitis and associated pain in mice** *J. Gastroenterol* **53**:215–226

26. Davoli-Ferreira M., et al. (2020) **Regulatory T cells counteract neuropathic pain through inhibition of the Th1 response at the site of peripheral nerve injury** *Pain* **161**:1730–1743

27. Ledeboer A., et al. (2007) **Intrathecal Interleukin-10 Gene Therapy Attenuates Paclitaxel-Induced Mechanical Allodynia and Proinflammatory Cytokine Expression in Dorsal Root Ganglia in Rats** *Brain. Behav. Immun* **21**:686–698

28. Shen K.-F., et al. (2013) **Interleukin-10 down-regulates voltage gated sodium channels in rat dorsal root ganglion neurons** *Exp. Neurol* **247**:466–475

29. Baker A. K., Hoffmann V. L. H., Meert T. F. (2002) **Dextromethorphan and ketamine potentiate the antinociceptive effects of mu-but not delta- or kappa-opioid agonists in a mouse model of acute pain** *Pharmacol. Biochem. Behav* **74**:73–86

30. Shannon P., et al. (2003) **Cytoscape: A Software Environment for Integrated Models of Biomolecular Interaction Networks** *Genome Res* **13**:2498–2504

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Reviewer #1 (Public Review):

Summary:
The authors explore mechanisms through which T-regs attenuate acute pain using a heat sensitivity paradigm. Analysis of available transcriptomic data revealed expression on the proenkephalin (Penk) gene in T-regs. The authors explore the contribution of T-reg Penk in the resolution of heat sensitivity.

Strengths:
Investigating the potential role of T-reg Penk in the resolution of acute pain is a strength.

Weaknesses:
The overall experimental design is superficial and lacks sufficient rigor to draw any meaningful conclusions.

For instance:

1. The were no TAM controls. What is the evidence that TAM does not alter heat-sensitive receptors.
2. There are no controls demonstrating that recombination actually occurred. How do the authors know a single dose of TAM is sufficient?
3. Why was only heat sensitivity assessed? The behavioral tests are inadequate to derive any meaningful conclusions. Further, why wasn't the behavioral data plotted longitudinally

https://doi.org/10.7554/eLife.91359.1.sa2

Reviewer #2 (Public Review):

Summary:
The present study addresses the role of enkephalins, which are specifically expressed by regulatory T cells (Treg), in sensory perception in mice. The authors used a combination of transcriptomic databases available online to characterize the molecular signature of Treg. The proenkephalin gene Penk is among the most enriched transcripts, suggesting that Treg plays an analgesic role through the release of endogenous opioids. In addition, in silico analysis suggests that Penk is regulated by the TNFR superfamily; this being experimentally confirmed. Using flow cytometry analysis, the authors then show that Penk is mostly expressed in Treg of the skin and colon, compared to other immune cells. Finally, genetic conditional excision of Penk, selectively in Treg, results in heat hypersensitivity, as assessed by behavior analysis.

Strengths:
The manuscript is clear and reveals a previously unappreciated role of enkephalins, as released by immune cells, in sensory perception. The rationale in this manuscript is easy to follow, and conclusions are well supported by data.

Weaknesses:
The sensory deficit of Penk cKO appears to be quite limited compared to control littermates.

https://doi.org/10.7554/eLife.91359.1.sa1

Reviewer #3 (Public Review):

Summary:
Aubert et al investigated the role of PENK in regulatory T cells. Through the mining of publicly available transcriptome data, the authors confirmed that PENK expression is selectively enriched in regulatory but not conventional T cells. Further data mining suggested that OX40, 4-1BB as well as BATF, can regulate PENK expression in Tregs. The authors generated fate-mapping mice to confirm selective PENK expression in Tregs and activated effector T cells in the colon and spleen. Interestingly, transgenic mice with conditional deletion of PENK in Tregs resulted in hypersensitivity to heat, which the authors attributed to heat hyperalgesia.

Strengths:
The generation of transgenic mice with conditional deletion of PENK in foxp3 and PENK fate-mapping is novel and can potentially yield significant findings. The identification of upstream signals that regulate PENK is interesting but unlikely to be the main reason why PENK is predominantly expressed in Tregs as both BATF and TNFR are expressed in effector T cells.

Weaknesses:
There is a lack of direct evidence and detailed analysis of Tregs in the control and transgenic mice to support the authors' hypothesis. PENK was previously reported to be expressed in skin Tregs and play a significant role in regulating skin homeostasis: this should be considered as an alternative mechanism that may explain the changed sensitivity to heat observed in the paper.

https://doi.org/10.7554/eLife.91359.1.sa0

Author Response

> *eLife assessment*
>
> *This study presents a valuable finding on a new role of Foxp3+ regulatory T cells in sensory perception, which may have an impact on our understanding of somatosensory perception. The authors identified a previously unappreciated action of enkephalins released by immune cells in the resolution of pain and several upstream signals that can regulate the expression of the proenkephalin gene PENK in Foxp3+ Tregs. However, whereas the generation of transgenic mice with conditional deletion of PENK in Foxp3+ cells and PENK fate-mapping is novel and generates compelling data, they show an incomplete analysis of Tregs in the control and transgenic mice, proper tamoxifen controls nor the role of PENK+ skin T cells to further support their hypothesis. Nonetheless, the study would be of interest to the biologists working in the field of neuroimmunology and inflammation.*
>
> ***Public Reviews:***
>
> ***Reviewer #1 (Public Review):***
>
> *Summary:*

> *The authors explore mechanisms through which T-regs attenuate acute pain using a heat sensitivity paradigm. Analysis of available transcriptomic data revealed expression on the proenkephalin (Penk) gene in T-regs. The authors explore the contribution of T-reg Penk in the resolution of heat sensitivity.*
>
> *Strengths:*
>
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>
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>
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>
> *For instance:*
>
> 1. *The were no TAM controls. What is the evidence that TAM does not alter heat-sensitive receptors.*

Author response : By comparing panel A and C, it appears that heat-sensitivity in controls (blue dots) is slightly different before and after TMX administration, suggesting that heat-sensitive receptors are moderately altered by TMX per se. However, heat sensitivity is increased by two fold in KO animals. Thus, a possible effect of TAM on heat receptors is not responsible for the heat hyperalgesia seen in KO, as shown in figure 4 and S3.

> 1. *There are no controls demonstrating that recombination actually occurred. How do the authors know a single dose of TAM is sufficient?*

Author response : these experiments are in progress. Specificity of the deletion will be presented in an updated version of the manuscript in the near future.

> 1. *Why was only heat sensitivity assessed? The behavioral tests are inadequate to derive any meaningful conclusions. Further, why wasn't the behavioral data plotted longitudinally*

Author response : We respectfuly point the reviewer to figure S3 where the longitudinal data are presented. New behavorial tests are being performed. The results will be presented in a revised version.

> ***Reviewer #2 (Public Review):***
>
> *Summary:*
>
> *The present study addresses the role of enkephalins, which are specifically expressed by regulatory T cells (Treg), in sensory perception in mice. The authors used a combination of transcriptomic databases available online to characterize the molecular signature of Treg. The proenkephalin gene Penk is among the most enriched transcripts, suggesting that Treg plays an analgesic role through the release of endogenous opioids. In addition, in silico analysis suggests that Penk is regulated by the TNFR superfamily; this being experimentally confirmed. Using flow cytometry analysis, the authors then show that Penk is mostly expressed in Treg of the skin and colon, compared to other immune cells. Finally, genetic conditional excision of Penk, selectively in Treg, results in heat hypersensitivity, as assessed by behavior analysis.*
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Author response : Supplementary figures are being prepared and new results are being collected to show that the KO do not perturb immune and/or skin homeostasis at the time of the experiments. These will be presented in a revised version.