Regulatory T cell-derived enkephalin gates nociception

Élora Midavaine¹, Beatriz C. Moraes¹, Jorge Benitez¹, Sian R. Rodriguez¹, Joao M. Braz¹, Nathan P. Kochhar¹, Walter L. Eckalbar¹, Ana I. Domingos², John E. Pintar³, Allan I. Basbaum^{1*}, Sakeen W. Kashem^{4,5*}

Affiliations:

¹Department of Anatomy, University of California San Francisco, San Francisco, California, USA.

²Department of Physiology, Anatomy and Genetics, University of Oxford, Oxford, UK. ³Department of Neuroscience and Cell Biology, Rutgers Robert Wood Johnson Medical School, Piscataway, NJ, USA.

4 Department of Dermatology, University of California San Francisco, San Francisco, CA, USA.

5 Dermatology, Veterans Affairs Medical Center, San Francisco, California, USA. *These authors contributed equally

Correspondence:

Allan I. Basbaum, Chair and Professor, Department of Anatomy University of California San Francisco 1550 Rock Hall, Room 345A, San Francisco, California, USA 94158 Email: allan.basbaum@ucsf.edu

Lead correspondence:

Sakeen W. Kashem Assistant Professor, Dermatology University of California San Francisco 1701 Divisadero, 3rd Floor, San Francisco, CA 94115 Email: sakeen.kashem@ucsf.edu

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Summary

T cells have emerged as sex-dependent orchestrators of pain chronification but the sexually dimorphic mechanisms by which T cells control pain sensitivity is not resolved. Here, we demonstrate an influence of regulatory T cells (Tregs) on pain processing that is distinct from their canonical functions of immune regulation and tissue repair. Specifically, meningeal Tregs (mTregs) express the endogenous opioid, enkephalin, and mTreg-derived enkephalin exerts an antinociceptive action through a presynaptic opioid receptor signaling mechanism that is dispensable for immunosuppression. We demonstrate that mTregs are both necessary and sufficient to suppress mechanical pain sensitivity in female, but not male, mice, with this modulation reliant on sex hormones. These results uncover a fundamental sex-specific, and immunologicallyderived endogenous opioid circuit for nociceptive regulation with critical implications for pain biology.

Highlights

1. Gating of allodynia by meningeal Tregs is sex hormone-dependent

3. Treg-derived enkephalin modulates mechanical pain sensitivity, not inflammation

4. Delta opioid receptor on MrgprD⁺ sensory neuron mediates pain processing by mTregs

1 **Introduction**

2 Pain prevale

3 including ner

4 migraine¹. G

5 chronic pain Pain prevalence is significantly higher in women across multiple pain conditions
including nerve injury-induced neuropathic pain, musculoskeletal pain, fibromyalgia and
migraine¹. Gender disparities in pain are further e 3 including nerve injury-induced neuropathic pain, musculoskeletal pain, fibromyalgia and
4 migraine¹. Gender disparities in pain are further evidenced by notable changes in
5 chronic pain severity during hormonal gende migraine 1 migraine¹. Gender disparities in pain are further evidenced by notable changes in
chronic pain severity during hormonal gender affirming care². Here, we identified a
previously unknown immunological mechanism that unde chronic pain severity during hormonal gender affirming care² chronic pain severity during hormonal gender affirming care². Here, we identified a
6 previously unknown immunological mechanism that underlies sex differences in both
6 acute and chronic pain regulation.
8 Regulatory T 6 previously unknown immunological mechanism that underlies sex differences in both
3 acute and chronic pain regulation.
8 Regulatory T cells (Tregs) are a subset of CD4⁺ T cells characterized by their
0 immunosuppressiv

7 acute and chronic pain regulation.

8 Regulatory T cells (Tregs) are

0 immunosuppressive function and

1 transcriptional factor *Foxp3*. In ad 9
0
1
2 Regulatory T cells (Tregs) are a subset of $CD4^+$ 9 Regulatory T cells (Tregs) are a subset of $CD4^+$ T cells characterized by their
10 immunosuppressive function and the expression of the X-linked master regulatory
11 transcriptional factor $Foxp3$. In addition to their 10 immunosuppressive function and the expression of the X-linked master regulatory
11 transcriptional factor $Foxp3$. In addition to their critical function in limiting inflammation,
12 Tregs are also major contributors to 11 transcriptional factor *Foxp3*. In addition to their critical function in limiting inflammation,
12 Tregs are also major contributors to wound healing, they regulate stem cell turnover,
13 maintain metabolic homeostasis 12 Tregs are also major contributors to wound healing, they regulate stem cell turnover,

13 maintain metabolic homeostasis, facilitate placental implantation and promote maternal-

14 fetal tolerance⁸⁻¹⁶. In the contex maintain metabolic homeostasis, facilitate placental implantation and promote maternal-

14 fetal tolerance^{8–16}. In the context of nervous system injury, Tregs mitigate pro-

15 inflammatory cytokine interferon- \Box (IF fetal tolerance^{8–16} 14 fetal tolerance⁸⁻¹⁶. In the context of nervous system injury, Tregs mitigate pro-
15 inflammatory cytokine interferon- \square (IFN- \square)-driven mechanical pain hypersensitivity,
16 suppress microglia-driven nociceptive 15 inflammatory cytokine interferon- \Box (IFN- \Box)-driven mechanical pain hypersensitivity, suppress microglia-driven nociceptive processing and can reduce astrogliosis.
17 Additionally, they contribute to improving remy suppress microglia-driven nociceptive processing and can reduce astrogliosis.
17 Additionally, they contribute to improving remyelination, thereby promoting tissue
18 repair^{17–20}. However, whether and how Tregs can direc 17 Additionally, they contribute to improving remyelination, thereby promoting tissue

18 repair^{17–20}. However, whether and how Tregs can directly alter neuronal activity is still

19 unknown.

20 Here, we demonstrate th repair^{17–20}. However, whether and how Tregs can directly alter neuronal activity is still
19 unknown.
20 Here, we demonstrate that meningeal regulatory T cells (mTregs) are essential
22 contributors to baseline mechanica

unknown.
20
21 Here, we demonstrate that meningeal regulatory T cells (mTregs) are essential
22 contributors to baseline mechanical sensitivity, and to the inhibition of mechanical pain
23 hypersensitivity (allodynia) afte 22
23
24 contributors to baseline mechanical sensitivity, and to the inhibition of mechanical pain

23 hypersensitivity (allodynia) after nerve injury, but only in female mice. Using a well-

25 expanding mTregs can reduce nocicept contributors to baseline mechanical sensitivity, and to the inhibition of mechanical pain

23 hypersensitivity (allodynia) after nerve injury, but only in female mice. Using a well-

25 established spared-nerve injury (SNI 23 hypersensitivity (allodynia) after nerve injury, but only in female mice. Using a well-
24 established spared-nerve injury (SNI) model of neuropathic pain, we further show that
25 expanding mTregs can reduce nociceptive expanding mTregs can reduce nociceptive processing independently of Treg tissue
26 repair programs. We find that mTregs produce the endogenous opioid met-enkephalin,
27 with female mice exhibiting increased numbers of enke 26 repair programs. We find that mTregs produce the endogenous opioid met-enkephalin,
27 with female mice exhibiting increased numbers of enkephalinergic mTregs. mTreg-
28 derived enkephalin is required for the suppression 27 with female mice exhibiting increased numbers of enkephalinergic mTregs. mTreg-
28 derived enkephalin is required for the suppression of mechanical pain hypersensitivity,
29 but not inflammation. This distinction reveal 28 derived enkephalin is required for the suppression of mechanical pain hypersensitivity,
29 but not inflammation. This distinction reveals a novel Treg function that differs from their
30 well-established roles in immuno derived enkephalin is required for the suppression of mechanical pain hypersensitivity,

29 but not inflammation. This distinction reveals a novel Treg function that differs from their

20 well-established roles in immunol 29 but not inflammation. This distinction reveals a novel Treg function that differs from their
20 well-established roles in immunological restraint and tissue repair.
21 The involvement of distinct opioid receptors in the

33 systems (CNS) in the regulation of different pain modalities is well documented. Delta
34 (δOR) and mu (μOR) opioid receptors are differentially expressed among neuronal 32
33
34
35 32 The involvement of distinct opioid receptors in the peripheral (PNS) and central nervous
33 systems (CNS) in the regulation of different pain modalities is well documented. Delta
34 (δ OR) and mu (μ OR) opioid rece 33 systems (CNS) in the regulation of different pain modalities is well documented. Delta
34 (δ OR) and mu (μ OR) opioid receptors are differentially expressed among neuronal
35 subsets and regulate mechanical or ther 34 (δOR) and mu (μOR) opioid receptors are differentially expressed among neuronal
35 subsets and regulate mechanical or thermal pain processing, respectively^{21–23}.
36 Enkephalin can bind to both δOR and μOR, but prefer subsets and regulate mechanical or thermal pain processing, respectively^{21–23}.

S6 Enkephalin can bind to both δ OR and μ OR, but preferentially engages the δ OR. Here

we demonstrate that the anti-allodynic effec 36 Enkephalin can bind to both δOR and μOR, but preferentially engages the δOR. Here
37 we demonstrate that the anti-allodynic effects derived from mTregs are mediated by the
38 δOR that is expressed on the *Mas*-related δOR that is expressed on the *Mas*-related G protein-coupled receptor member D
(MrgprD) subset of unmyelinated primary sensory neurons.
Given the pronounced sex differences observed in Treq-mediated suppression of 38 δOR that is expressed on the *Mas*-related G protein-coupled receptor member D
39 (MrgprD) subset of unmyelinated primary sensory neurons.
40
Given the pronounced sex differences observed in Treg-mediated suppression o

39 (MrgprD) subset of unmyelinated primary sensory neurons.
40
41 Given the pronounced sex differences observed in Treg-me
42 nociceptive processing, we further explored the determinant
43 findings indicate that gonadal ho 41
42
43
44 41 Given the pronounced sex differences observed in Treg-mediated suppression of
42 nociceptive processing, we further explored the determinants of this sex selectivity
43 findings indicate that gonadal hormones, rather th

43 findings indicate that gonadal hormones, rather than sex chromosomes, play a key role
44 in modulating Treg suppression of nociceptive thresholds. Thus, we propose a novel
45 mechanism by which Tregs mediate the suppres 43 findings indicate that gonadal hormones, rather than sex chromosomes, play a key role
44 in modulating Treg suppression of nociceptive thresholds. Thus, we propose a novel
45 mechanism by which Tregs mediate the suppres 44 in modulating Treg suppression of nociceptive thresholds. Thus, we propose a novel
45 mechanism by which Tregs mediate the suppression of nociception, regulated by sex
46 specific hormonal influences. 45 mechanism by which Tregs mediate the suppression of nociception, regulated by sex-
46 specific hormonal influences.

specific hormonal influences.

Results
Results
**Rex-specific suppression of nociceptive thresholds by meningeal Tregs
As the immune system has emerged as a central determinant driving sex differences in
50 pain sensitivity, we sought to investigate Sex-specific suppression of nociceptive thresholds by meningeal Tregs**
49 As the immune system has emerged as a central determinant driving sex diff
50 pain sensitivity, we sought to investigate its contribution to nocice 49 As the immune system has emerged as a central determinant driving sex differences in
50 pain sensitivity, we sought to investigate its contribution to nociceptive processing²⁴.
51 Tregs are a fundamental cell type es pain sensitivity, we sought to investigate its contribution to nociceptive processing²⁴. pain sensitivity, we sought to investigate its contribution to nociceptive processing²⁴.
51 Tregs are a fundamental cell type essential for maintaining and restoring tissue
52 homeostasis. Here we initially focused on id 51 Tregs are a fundamental cell type essential for maintaining and restoring tissue
52 homeostasis. Here we initially focused on identifying Treg localization and function
53 within nervous system tissues. Using confocal m 52 homeostasis. Here we initially focused on identifying Treg localization and function
53 within nervous system tissues. Using confocal microscopy and flow cytometry on whole
54 meningeal sheets, dorsal root ganglia (DRG) 53 within nervous system tissues. Using confocal microscopy and flow cytometry on whole
54 meningeal sheets, dorsal root ganglia (DRG), sciatic nerves, spinal cords (SC) and
55 brain, we localized Tregs to the meninges of meningeal sheets, dorsal root ganglia (DRG), sciatic nerves, spinal cords (SC) and
55 brain, we localized Tregs to the meninges of the CNS, and to the leptomeninges of the
56 DRG (Figure 1A-C). To enhance the sensitivity o brain, we localized Tregs to the meninges of the CNS, and to the leptomeninges of the
56 DRG (Figure 1A-C). To enhance the sensitivity of Treg detection within these organs,
57 we utilized the bright reporter signals from DRG (Figure 1A-C). To enhance the sensitivity of Treg detection within these organs,
57 we utilized the bright reporter signals from the double reporter $F\alpha p3^{eGFP-Cre-}$
58 ^{ERT2}; Rosa26^{LSL-tdTomato} mice. Notably, and co 57
58
59
60
61 ERT2*;Rosa26LSL-tdTomato* 58 $\frac{EN12}{P}$, Rosa26^{LSL-tationato} mice. Notably, and consistent with previous reports we observed
a more pronounced localization of Tregs in the lumbar and caudal segments of the
60 spinal cord meninges (Figure 1A) 59 a more pronounced localization of Tregs in the lumbar and caudal segments of the
60 spinal cord meninges (Figure 1A) 25 . In the DRG, as for most leukocytes other than
61 resident macrophages, Tregs predominated in spinal cord meninges **(Figure 1A)** ²⁵ 61 resident macrophages, Tregs predominated in the leptomeninges, proximal to the
62 dorsal roots entry zone, with sparse presence within the DRG parenchyma (Figure
63 **1B)**²⁶. By intravenous administration of a fluores 62 dorsal roots entry zone, with sparse presence within the DRG parenchyma (Figure 63 **1B)**²⁶. By intravenous administration of a fluorescently-labeled CD45 antibody and using flow cytometry, we distinguished intravascu 62 dorsal roots entry zone, with sparse presence within the DRG parenchyma (Figure $1B)^{26}$. By intravenous administration of a fluorescently-labeled CD45 antibody and using flow cytometry, we distinguished intravascular **1B)** 26 66 nervous and lymphoid systems. We will further refer to the SC meningeal and DRG
67 leptomeningeal Tregs as mTregs (Figure 1C). We observed minimal localization of flow cytometry, we distinguished intravascular Tregs from tissue Tregs **(Figure S1A)**²⁷ flow cytometry, we distinguished intravascular Tregs from tissue Tregs (Figure S1A)²⁷.
65 We quantified the numbers of non-vascular, tissue Tregs in various organs within the
66 nervous and lymphoid systems. We will fur We quantified the numbers of non-vascular, tissue Tregs in various organs within the
66 nervous and lymphoid systems. We will further refer to the SC meningeal and DRG
67 leptomeningeal Tregs as mTregs (Figure 1C). We obse 66 nervous and lymphoid systems. We will further refer to the SC meningeal and DRG leptomeningeal Tregs as mTregs (Figure 1C). We observed minimal localization of Tregs in peripheral nerves and did not detect any Tregs wi 68 Tregs in peripheral nerves and did not detect any Tregs within the parenchyma of the
69 CNS in young, uninjured mice. We observed nearly equivalent numbers of tissue Tregs
70 between male and female mice across tissue (68 Tregs in peripheral nerves and did not detect any Tregs within the parenchyma of the
69 CNS in young, uninjured mice. We observed nearly equivalent numbers of tissue Tregs
70 between male and female mice across tissue (69 CNS in young, uninjured mice. We observed nearly equivalent numbers of tissue Tregs
60 between male and female mice across tissue (Figure 1D)¹⁸.
71 To assess the feasibility of site-specific depletion of mTregs, we pe between male and female mice across tissue **(Figure 1D)**¹⁸

between male and female mice across tissue (Figure 1D)¹⁸.
71 . To assess the feasibility of site-specific depletion of mTregs
73 . (IT) injections of pegylated diphtheria toxin (pegDT) in *Foxp*.
74 . IT iniection of Eva 72
73
74
75 To assess the feasibility of site-specific depletion of mTregs, we performed intrathecal

(IT) injections of pegylated diphtheria toxin (pegDT) in *Foxp3-*DTR mice²⁸. Although an

IT injection of Evan's blue rapidly spre (IT) injections of pegylated diphtheria toxin (pegDT) in *Foxp3-*DTR mice28 Timistion of Evan's blue rapidly spreads through the SC meninges and DRG, into the
75 brain and to the draining cervical and lumbar lymph nodes, pegylated fluorescently
76 labeled molecules (pegDyLight 650) remain restrict 74 IT injection of Evan's blue rapidly spreads through the SC meninges and DRG, into the
75 brain and to the draining cervical and lumbar lymph nodes, pegylated fluorescently
76 labeled molecules (pegDyLight 650) remain re brain and to the draining cervical and lumbar lymph nodes, pegylated fluorescently

17 labeled molecules (pegDyLight 650) remain restricted to the SC meninges and to the

17 DRG (Figure S2A-B). Consistently, a single 20 ng 76 labeled molecules (pegDyLight 650) remain restricted to the SC meninges and to the
77 DRG (Figure S2A-B). Consistently, a single 20 ng dose of pegDT IT selectively
78 depleted >90% of SC and DRG mTregs in both male and 77 DRG (Figure S2A-B). Consistently, a single 20 ng dose of pegDT IT selectively

78 depleted >90% of SC and DRG mTregs in both male and female mice, but spared

79 Tregs located in the brain meninges, draining lymph nodes depleted >90% of SC and DRG mTregs in both male and female mice, but spared
79 Tregs located in the brain meninges, draining lymph nodes, spleen, and peripheral
80 nerves (Figure 1E-F). Importantly, *Foxp3*-DTR mice subjec Tregs located in the brain meninges, draining lymph nodes, spleen, and peripheral

180 nerves (Figure 1E-F). Importantly, *Foxp3*-DTR mice subjected to repeated IT

181 administrations of pegDT do not exhibit the significa 80 nerves (Figure 1E-F). Importantly, *Foxp3*-DTR mice subjected to repeated IT
81 administrations of pegDT do not exhibit the significant weight loss, splenomegaly and
82 mortality that typically develops following system 81 administrations of pegDT do not exhibit the significant weight loss, splenomegaly and
82 mortality that typically develops following systemic autoimmunity in *Foxp3*-DTR mice
63 induced by repeated intraperitoneal (IP) 82 mortality that typically develops following systemic autoimmunity in *Foxp3*-DTR mice

83 induced by repeated intraperitoneal (IP) injections of diphtheria toxin (DT) (**Figure S2C-**

85 **F)**. Clearly, the pegDT IT syste 83 induced by repeated intraperitoneal (IP) injections of diphtheria toxin (DT) **(Figure S2C-**

84 **F)**. Clearly, the pegDT IT system offers a novel method for selective depletion of

85 mTregs while avoiding systemic infl **F)**. Clearly, the pegDT IT system offers a novel method for selective depletion of
85 mTregs while avoiding systemic inflammation.
86 We next evaluated behavioral outcomes in mice following mTreg depletion. A single
88 do

85 mTregs while avoiding systemic inflammation.
86 We next evaluated behavioral outcomes in n
88 dose of pegDT IT induced a profound and prology in naïve female but not male *Foxp3*-DTR mice 87
88
89
90 88 dose of pegDT IT induced a profound and prolonged decrease in mechanical thresholds
89 in naïve female but not male *Foxp3*-DTR mice (**Figure 1G-H)**. Importantly, mechanical
90 thresholds in wildtype (WT) C57BL/6 mice t dose of pegDT IT induced a profound and prolonged decrease in mechanical thresholds
in naïve female but not male *Foxp3*-DTR mice (Figure 1G-H). Importantly, mechanical
90 thresholds in wildtype (WT) C57BL/6 mice treated w 89 in naïve female but not male *Foxp3*-DTR mice **(Figure 1G-H)**. Importantly, mechanical
90 thresholds in wildtype (WT) C57BL/6 mice treated with pegDT IT or *Foxp3*-DTR mice
91 treated with vehicle IT did not differ, rul 90 thresholds in wildtype (WT) C57BL/6 mice treated with pegDT IT or *Foxp3*-DTR mice
91 treated with vehicle IT did not differ, ruling out pegDT or IT injections as the cause of the
92 sex-specific allodynia **(Figure S3A)** 91 treated with vehicle IT did not differ, ruling out pegDT or IT injections as the cause of the
92 sex-specific allodynia (Figure S3A). In addition to evaluating mechanical 92 sex-specific allodynia **(Figure S3A)**. In addition to evaluating mechanical

93 hypersensitivity, which is conveyed by mechanosensitive unmyelinated and myelinated
94 primary afferent nerve fibers, we also assessed mice for noxious heat sensitivity
95 mediated by Trpv1⁺ nociceptors, cold sensit 94 primary afferent nerve fibers, we also assessed mice for noxious heat sensitivity
95 mediated by Trpv1⁺ nociceptors, cold sensitivity that is mediated by Trpm8⁺ nociceptors,
96 pin prick sensitivity mediated by Aδ mediated by Trpv1⁺ nociceptors, cold sensitivity that is mediated by Trpm8⁺ mediated by Trpv1⁺ nociceptors, cold sensitivity that is mediated by Trpm8⁺ nociceptors,
96 pin prick sensitivity mediated by A δ afferents and brush responses mediated by A β
97 fibers. Although depletion of mTre pin prick sensitivity mediated by Aδ afferents and brush responses mediated by Aβ
67 fibers. Although depletion of mTregs selectively induced mechanical allodynia in
68 females, it did not impact any other sensory modalit 97 fibers. Although depletion of mTregs selectively induced mechanical allodynia in females, it did not impact any other sensory modality. Motor function tests using the rotarod also did not differ in either sex (Figure 11 98 females, it did not impact any other sensory modality. Motor function tests using the

99 rotarod also did not differ in either sex (Figure 1I and Figure S3A-H). We conclude that

00 mTregs selectively suppress mechanic 99 rotarod also did not differ in either sex (Figure 1I and Figure S3A-H). We conclude that
00 mTregs selectively suppress mechanical thresholds in a sex-dependent manner,
01 effectively preventing mechanical allodynia in mTregs selectively suppress mechanical thresholds in a sex-dependent manner,
101 effectively preventing mechanical allodynia in a previously uninjured state.
103 **Expansion of mTregs alleviates injury-induced mechanical al**

effectively preventing mechanical allodynia in a previously uninjured state.
102
103 **Expansion of mTregs alleviates injury-induced mechanical allodynia**
104 **independently of tissue repair**
105 **In addition to exploring m**

102
103
104
105
106 **Expansion of mTregs alleviates injury-induced mechanical allodynia**
 independently of tissue repair

105 **In addition to exploring mTreg role in mechanical sensitivity in uninju

106 investigated whether mTreg can suppr** 104 *independently of tissue repair*
105 In addition to exploring mTreg
106 investigated whether mTreg can
107 established spared nerve injury
108 ligated the common peroneal and In addition to exploring mTreg role in mechanical sensitivity in uninjured mice, we

106 investigated whether mTreg can suppress allodynia following nerve injury. Using a well-

107 established spared nerve injury (SNI) mo investigated whether mTreg can suppress allodynia following nerve injury. Using a well-
107 established spared nerve injury (SNI) model of neuropathic pain, we transected and
108 ligated the common peroneal and tibial nerv 107 established spared nerve injury (SNI) model of neuropathic pain, we transected and

108 ligated the common peroneal and tibial nerve branches of the sciatic nerve, sparing the

109 sural nerve. This model induces chro ligated the common peroneal and tibial nerve branches of the sciatic nerve, sparing the

109 sural nerve. This model induces chronic, unremitting, and permanent mechanical

110 hypersensitivity with a non-healing neuroma f 109 sural nerve. This model induces chronic, unremitting, and permanent mechanical

110 hypersensitivity with a non-healing neuroma formation four weeks after the injury

111 **(Figure 2A-B)**^{29,30}. As mice with SNI exhib 110 hypersensitivity with a non-healing neuroma formation four weeks after the injury

111 (Figure 2A-B)^{29,30}. As mice with SNI exhibit mechanical thresholds at the limit of

112 detection with commercially available vo **(Figure 2A-B)**29,30 111 **(Figure 2A-B)**^{29,30}. As mice with SNI exhibit mechanical thresholds at the limit of detection with commercially available von Frey filaments, we conducted single fiber testing using the lowest available 0.008 g von testing using the lowest available 0.008 g von Frey filament. Again, mTreg depletion testing using the lowest available 0.008 g von Frey filament. Again, mTreg depletion
114 increased allodynia following SNI in females, but not in males (Figure 2C-D).
115 We next asked whether expanding mTregs could allevi

increased allodynia following SNI in females, but not in males (Figure 2C-D).
115
116 We next asked whether expanding mTregs could alleviate the mechanica
117 independently of tissue repair. Tregs express the high affinity 116
117
118
119 116 We next asked whether expanding mTregs could alleviate the mechanical allodynia

117 independently of tissue repair. Tregs express the high affinity interleukin-2 receptor, IL-

118 2Ra, and low-doses of IL-2 can effe independently of tissue repair. Tregs express the high affinity interleukin-2 receptor, IL-
118 2Ra, and low-doses of IL-2 can effectively expand Tregs in mice, a therapeutic
119 approach that has been used to treat autoi 2Ra, and low-doses of IL-2 can effectively expand Tregs in mice, a therapeutic
119 approach that has been used to treat autoimmune diseases in humans³¹. IT injections of
120 low-dose IL-2 successfully expanded mTregs in approach that has been used to treat autoimmune diseases in humans 31 120 low-dose IL-2 successfully expanded mTregs in both male and female mice (Figure 121 2F). However, although mTreg expansion promoted significant anti-allodynia in SNI female mice, it did not exhibit a similar effect in 120 low-dose IL-2 successfully expanded mTregs in both male and female mice (Figure 121 2F). However, although mTreg expansion promoted significant anti-allodynia in SNI female mice, it did not exhibit a similar effect in **2F).** However, although mTreg expansion promoted significant anti-allodynia in SNI
122 female mice, it did not exhibit a similar effect in males (Figure 2G-H). It is noteworthy
123 that acute IT injection of IL-2, in unin that acute IT injection of IL-2, in uninjured mice, did not increase nociceptive thresholds,
124 suggesting that an IL-2 or Treg-based therapy could selectively improve neuropathic
125 pain without affecting basal nocicept suggesting that an IL-2 or Treg-based therapy could selectively improve neuropathic
125 pain without affecting basal nociceptive processing (Figure S4A-B). IL-2 injections and
126 mTreg expansion in mice with SNI likewise suggesting that an IL-2 or Treg-based therapy could selectively improve neuropathic
125 pain without affecting basal nociceptive processing (Figure S4A-B). IL-2 injections and
126 mTreg expansion in mice with SNI likewise pain without affecting basal nociceptive processing (Figure S4A-B). IL-2 injections and
126 mTreg expansion in mice with SNI likewise did not alter noxious cold or heat sensitivity,
127 again highlighting the specificity o mTreg expansion in mice with SNI likewise did not alter noxious cold or heat sensitivity,
127 again highlighting the specificity of the sensory modality modulation by mTreg in the
129 **Gonadal hormones, not sex chromosomes** 127 again highlighting the specificity of the sensory modality modulation by mTreg in the
128 context of neuropathic pain (S4C-E).
130 **Gonadal hormones, not sex chromosomes determine sex-selective, anti-**
131 **nociceptive** context of neuropathic pain **(S4C-E)**.

129
 **130 Gonadal hormones, not sex chromosomes determine sex-selective, anti-

131 nociceptive function of mTregs

132 Foxp3 is an X-linked gene, some of which escape X-inactivation**

130
131
132
133 **Gonadal hormones, not sex chromosomes determine sex-selective, anti-**

131 **nociceptive function of mTregs**

132 *Foxp3* is an X-linked gene, some of which escape X-inactivation. Moreover, r

133 inactivation has been sug 131 *nociceptive function of mTregs*
132 *Foxp3* is an X-linked gene, some
133 inactivation has been suggested
134 females^{32–34}. To test whether sex
135 bhenotype, we used the Four Co Foxp3 is an X-linked gene, some of which escape X-inactivation. Moreover, random X-

133 inactivation has been suggested to be potentially altered during inflammatory state in

134 females^{32–34}. To test whether sex chrom inactivation has been suggested to be potentially altered during inflammatory state in

134 females^{32–34}. To test whether sex chromosomes dosage contributes to our observed

135 phenotype, we used the Four Core Genotype females $^{32-34}$ 134 females^{32–34}. To test whether sex chromosomes dosage contributes to our observed

135 phenotype, we used the Four Core Genotypes (FCG) mouse model in which gonadal

136 sex in mice is independent of sex chromosomes 135 phenotype, we used the Four Core Genotypes (FCG) mouse model in which gonadal
136 sex in mice is independent of sex chromosomes^{35,36}. FCG mice harbor a deficiency in
137 the sex determining region Y protein (*Sry*) sex in mice is independent of sex chromosomes 35,36 136 sex in mice is independent of sex chromosomes^{35,36}. FCG mice harbor a deficiency in 137 the sex determining region Y protein (Sry) on the Y chromosome and instead feature an 138 autosomal transgenic insertion of Sry 137 the sex determining region Y protein (*Sry*) on the Y chromosome and instead feature an autosomal transgenic insertion of *Sry*. This genetic configuration enables the autosomal transgenic insertion of *Sry*. 138 autosomal transgenic insertion of *Sry.* This genetic configuration enables the discrimination of sex chromosome dose influence from the contribution of gonadal
140 hormones (Figure 2I). Both XX and XY chromosome gonadal female mice displayed
141 mTreg-mediated alleviation of mechanical allodynia afte 140 hormones (Figure 2I). Both XX and XY chromosome gonadal female mice displayed
141 mTreg-mediated alleviation of mechanical allodynia after SNI; XX- and XY- gonadal
142 male mice did not (Figure 2J-L). Similarly, after mTreg-mediated alleviation of mechanical allodynia after SNI; XX- and XY- gonadal

142 male mice did not (Figure 2J-L). Similarly, after mTreg depletion in *Foxp3*-DTR mice

143 crossed to the FCG system, we found that fem male mice did not (Figure 2J-L). Similarly, after mTreg depletion in *Foxp3*-DTR mice
143 crossed to the FCG system, we found that female specific gonadal hormones, but not
144 sex chromosome, mediate the mTreg suppression 144 sex chromosome, mediate the mTreg suppression of nociceptive thresholds in the
145 absence of injury (Figure 2M-N). Based on our findings in both uninjured and chronic
146 injury states, we conclude that there is a pro 145 absence of injury (Figure 2M-N). Based on our findings in both uninjured and chronic
146 injury states, we conclude that there is a profound and consistent sex hormone-
147 dependent contribution of mTregs to the modul injury states, we conclude that there is a profound and consistent sex hormone-
147 dependent contribution of mTregs to the modulation mechanical pain sensitivity.
148 **Regulatory T cells express the endogenous opioid pept** injury states, we conclude that there is a profound and consistent sex hormone-
147 dependent contribution of mTregs to the modulation mechanical pain sensitivity.
148 **Regulatory T cells express the endogenous opioid pept**

dependent contribution of mTregs to the modulation mechanical pain sensitivity.

148
 Regulatory T cells express the endogenous opioid peptide enkephalin

150 To investigate the molecular mechanisms though which Tregs su 150
151
152 **Regulatory T cells express the endogenous opioid peptide enkephalin**
150 To investigate the molecular mechanisms though which Tregs suppress
151 thresholds, we first interrogated public genomic resources. We hypoth
152 me 150 To investigate the molecular mechanisms though which Tregs suppress nociceptive
151 thresholds, we first interrogated public genomic resources. We hypothesized that
152 meningeal tissue Tregs could exhibit an activated thresholds, we first interrogated public genomic resources. We hypothesized that
152 meningeal tissue Tregs could exhibit an activated lymphoid Treg phenotype rather than
153 a resting Treg phenotype. Tregs have increased meningeal tissue Tregs could exhibit an activated lymphoid Treg phenotype rather than
153 a resting Treg phenotype. Tregs have increased expression of the *Penk* gene, which
154 encodes for *Proenkephalin*, a peptide precu 153 a resting Treg phenotype. Tregs have increased expression of the *Penk* gene, which
154 encodes for *Proenkephalin*, a peptide precursor of both Met- and Leu-enkephalin, in
155 various tissues including the nervous sys encodes for *Proenkephalin*, a peptide precursor of both Met- and Leu-enkephalin, in

155 various tissues including the nervous system, in both mice and humans^{19,37–39}. Here we

156 re-analyzed raw public RNA-seq data of various tissues including the nervous system, in both mice and humans^{19,37–39}. Here we various tissues including the nervous system, in both mice and humans^{19,37–39}. Here we
156 re-analyzed raw public RNA-seq data of activated Tregs, resting Tregs, as well as
157 activated and resting CD4⁺ Foxp3 convent 156 re-analyzed raw public RNA-seq data of activated Tregs, resting Tregs, as well as

157 activated and resting CD4⁺ Foxp3 conventional T cells (Tconv)⁴⁰. Strikingly, in activated

158 versus resting Tregs, we observ activated and resting CD4⁺ Foxp3⁻ conventional T cells (Tconv)⁴⁰. Strikingly, in activated

158 versus resting Tregs, we observed a significant upregulation of *Penk* expression

159 **(Figure 3A)**. We also investigat 158 versus resting Tregs, we observed a significant upregulation of *Penk* expression
159 **(Figure 3A)**. We also investigated other opioid ligand and receptor genes but only
160 recorded a very sparse expression of other 159 **(Figure 3A)**. We also investigated other opioid ligand and receptor genes but only recorded a very sparse expression of other opioid-related genes among the CD4⁺ T cell subsets **(Figure 3B)**. Based on our prior exp recorded a very sparse expression of other opioid-related genes among the CD4⁺ T cell 160 recorded a very sparse expression of other opioid-related genes among the CD4⁺ T cell
161 subsets (Figure 3B). Based on our prior experience defining mechanical sensitivity
162 through enkephalin- δ OR signaling²¹ 161 subsets (**Figure 3B**). Based on our prior experience defining mechanical sensitivity
162 through enkephalin-δOR signaling²¹, we therefore focused on Treg expression of *Penk*.
163 By ATAC-seq analysis, we observed through enkephalin-δOR signaling²¹
Py ATAC seg apolysis, we absen through enkephalin- δ OR signaling²¹, we therefore focused on Treg expression of *Penk.*
163 By ATAC-seq analysis, we observed open chromatin regions of the *Penk* locus in
164 activated Tregs, but not in other CD4⁺ 163 By ATAC-seq analysis, we observed open chromatin regions of the *Penk* locus in activated Tregs, but not in other CD4⁺ T cell subsets. This open chromatin was similar to the open chromatin, promoter and enhancer regi activated Tregs, but not in other CD4+ 164 activated Tregs, but not in other CD4⁺ T cell subsets. This open chromatin was similar
165 to the open chromatin, promoter and enhancer regions of the developing forebrain, an
166 established enkephalin-producing ar to the open chromatin, promoter and enhancer regions of the developing forebrain, an
166 established enkephalin-producing area of the murine CNS (Figure 3C)⁴¹. By analyzing
167 the raw dataset from the Immunological Gen established enkephalin-producing area of the murine CNS **(Figure 3C)**⁴¹ established enkephalin-producing area of the murine CNS (Figure $3C)^{41}$. By analyzing
167 the raw dataset from the Immunological Genome Project⁴², we also explored *Penk*
168 expression within cell populations of the the raw dataset from the Immunological Genome Project⁴² the raw dataset from the Immunological Genome Project⁴², we also explored *Penk*
expression within cell populations of the immune system. We observed a strikingly
greater *Penk* expression in Tregs, compared to other imm expression within cell populations of the immune system. We observed a strikingly

169 greater *Penk* expression in Tregs, compared to other immune cells (**Figure 3D)**.

170 Furthermore, *Penk* expression in Tregs increase quater *Penk* expression in Tregs, compared to other immune cells (**Figure 3D)**.
170 Furthermore, *Penk* expression in Tregs increase significantly following stimulation with
171 IL-2, compared to other common gamma chain

172
173 Folestablish whether Tregs indeed produce the endogenous opioid peptide enkephalin,
174 Fwe screened commercially available anti-Met-enkephalin antibodies. Met-enkephalin 174 we screened commercially available anti-Met-enkephalin antibodies. Met-enkephalin
175 was chosen over leu-enkephalin as the latter can be cleaved from both proenkephalin 173
174
175
176 173 To establish whether Tregs indeed produce the endogenous opioid peptide enkephalin,
174 we screened commercially available anti-Met-enkephalin antibodies. Met-enkephalin
175 was chosen over leu-enkephalin as the latte we screened commercially available anti-Met-enkephalin antibodies. Met-enkephalin

175 was chosen over leu-enkephalin as the latter can be cleaved from both proenkephalin

176 and prodynorphin peptides⁴³. These antibodi was chosen over leu-enkephalin as the latter can be cleaved from both proenkephalin
176 and prodynorphin peptides⁴³. These antibodies were validated using *Penk^{-/-}* mice as
177 negative controls. Figure 3F shows that and prodynorphin peptides⁴³. These antibodies were validated using *Penk^{-/-}* 276 and prodynorphin peptides⁴³. These antibodies were validated using *Penk*^{-/-} mice as
177 negative controls. Figure 3F shows that mTregs produce met-enkephalin, but meningeal
178 CD4⁺ T cells and lymphoid Tregs p negative controls. Figure 3F shows that mTregs produce met-enkephalin, but meningeal
178 CD4⁺ T cells and lymphoid Tregs produce very low levels even after cytokine
179 stimulation (**Figure 3F)**. We validated this findin CD4⁺ T cells and lymphoid Tregs produce very low levels even after cytokine 178 CD4⁺ T cells and lymphoid Tregs produce very low levels even after cytokine
179 stimulation (**Figure 3F)**. We validated this finding by generating *Penk^{Cre};Rosa26*^{tdTomato}
181 observed very similar number of enke stimulation (**Figure 3F)**. We validated this finding by generating *Penk*Cre*;Rosa26*tdTomato 179
180
181
182
183 mice, which fate-labeled enkephalinergic cells. Consistent with our antibody finding, we
181 observed very similar number of enkephalinergic lineage (tdTomato positive) mTregs in
182 naïve mice. Very few lymphoid or intrav 181 observed very similar number of enkephalinergic lineage (tdTomato positive) mTregs in
182 naïve mice. Very few lymphoid or intravascular Tregs were tdTomato labeled (Figure
183 3G). Most interestingly, female mice exhi 182 naïve mice. Very few lymphoid or intravascular Tregs were tdTomato labeled (Figure 183 3G). Most interestingly, female mice exhibited significantly greater numbers of enkephalin-positive Tregs in the meninges, but not 183 **3G)**. Most interestingly, female mice exhibited significantly greater numbers of enkephalin-positive Tregs in the meninges, but not in the lymphoid organs. This 184 enkephalin-positive Tregs in the meninges, but not in the lymphoid organs. This distinction suggests that Treg fate and function variation across the sexes may be organ
186 system specific (Figure 3H).
187 *mTreg-derived enkephalin is required for suppressing nociceptive processing***
189 Using** *Penk^{C*}

186 system specific **(Figure 3H)**.
187 **mTreg-derived enkephalin**
189 Using Penk^{Cre};Rosa26^{tdTomato}
190 cells in the meninges and the 187
188
189
190
191 188 **mTreg-derived enkephalin is required for suppressing nociceptive processing**
189 Using Penk^{Cre};Rosa26^{tdTomato} mice, we next investigated enkephalin lineage posi
191 the representation of mTregs in the tdTomato-pos Using *Penk*^{Cre};*Rosa26*^{tdTomato} mice, we next investigated enkephalin lineage positive cells in the meninges and the DRG. Interestingly, we observed a significant increase in the representation of mTregs in the tdToma 190 cells in the meninges and the DRG. Interestingly, we observed a significant increase in

191 the representation of mTregs in the tdTomato-positive enkephalin subpopulation

192 compared to tdTomato-negative cells (**Fi** 191 the representation of mTregs in the tdTomato-positive enkephalin subpopulation

192 compared to tdTomato-negative cells (**Figure 4A-B)**. In order to manipulate the

193 enkephalin-producing immune cells, we generated 192 compared to tdTomato-negative cells (Figure 4A-B). In order to manipulate the

193 enkephalin-producing immune cells, we generated bone marrow chimeric mice by

194 transplanting *Penk^{Cre};Rosa26*^{DTR} bone marrow int enkephalin-producing immune cells, we generated bone marrow chimeric mice by
194 transplanting $Penk^{Cre}$; Rosa26^{DTR} bone marrow into irradiated CD45.2 congenically
195 marked WT mice (Figure 4C). This strategy enables a s transplanting *Penk*Cre*;Rosa26*DTR transplanting $Penk^{Cre}; Rosa26^{DIR} bone marrow into irradiated CD45.2 congenically marked WT mice (**Figure 4C**). This strategy enables a selective DT-induced depletion of hematopoietic enkephalinergic cells, that spares depletion of non-hematopoietic enkephalinergic cells of the nervous system and the stroma. Importantly, CD4⁺ T cells of the meninges are predominantly bone marrow-derived and exhibit a tissue circularory$ 196 of hematopoietic enkephalinergic cells, that spares depletion of non-hematopoietic
197 enkephalinergic cells of the nervous system and the stroma. Importantly, CD4⁺ T cells
198 of the meninges are predominantly bone 196 of hematopoietic enkephalinergic cells, that spares depletion of non-hematopoietic

197 enkephalinergic cells of the nervous system and the stroma. Importantly, CD4⁺ T cells

198 of the meninges are predominantly bon enkephalinergic cells of the nervous system and the stroma. Importantly, $CD4^+$ T cells enkephalinergic cells of the nervous system and the stroma. Importantly, CD4⁺ T cells
198 of the meninges are predominantly bone marrow-derived and exhibit a tissue circulatory
199 characteristic rather than acquiring ti 198 of the meninges are predominantly bone marrow-derived and exhibit a tissue circulatory

199 characteristic rather than acquiring tissue residency⁴⁴. Consistently, we found that

100 mTregs are indeed bone marrow-der characteristic rather than acquiring tissue residency⁴⁴ characteristic rather than acquiring tissue residency⁴⁴. Consistently, we found that

200 mTregs are indeed bone marrow-derived, similar to lymphoid Tregs, and differ from

201 spinal microglia and skin Langerhans cells mTregs are indeed bone marrow-derived, similar to lymphoid Tregs, and differ from
201 spinal microglia and skin Langerhans cells, which are host-derived (Figure S5A-C).
202 pegDT IT administrations in *Penk*DTR^{Aheme} chim Let 201 spinal microglia and skin Langerhans cells, which are host-derived (Figure S5A-C).

202 pegDT IT administrations in *Penk*DTR^{Δheme} chimeric mice decreased the number of

203 mTregs and led to profound mechanical pegDT IT administrations in PenkDTR^{Δheme} pegDT IT administrations in *Penk*DTR^{Aneme} chimeric mice decreased the number of mTregs and led to profound mechanical hypersensitivity in female, but not male mice, in both uninjured and nerve injured states (Figure 4D-204 both uninjured and nerve injured states (Figure 4D-G). We conclude that blood-derived
205 meningeal enkephalinergic cells gate mechanical hypersensitivity in females.
207 Having established a female-specific contributi 204 both uninjured and nerve injured states (Figure 4D-G). We conclude that blood-derived

205 meningeal enkephalinergic cells gate mechanical hypersensitivity in females.

207 Having established a female-specific contribu

meningeal enkephalinergic cells gate mechanical hypersensitivity in females.
206
207 Having established a female-specific contribution of the bone marre
208 enkephalin system, we next used female mice to dissect the mechan 208
209
210 enkephalin system, we next used female mice to dissect the mechanism of pain
209 regulation by mTregs. To establish whether bone marrow-derived enkephalin is required
210 for the regulation of nociceptive thresholds, we ge enkephalin system, we next used female mice to dissect the mechanism of pain regulation by mTregs. To establish whether bone marrow-derived enkephalin is required for the regulation of nociceptive thresholds, we generated regulation by mTregs. To establish whether bone marrow-derived enkephalin is required
210 for the regulation of nociceptive thresholds, we generated bone marrow chimeras in
211 which *Penk* deficient bone marrow is transp 210 for the regulation of nociceptive thresholds, we generated bone marrow chimeras in

211 which *Penk* deficient bone marrow is transplanted into irradiated hosts, thus generating

212 *Penk*^{Aheme} mice. As predicted, which *Penk* deficient bone marrow is transplanted into irradiated hosts, thus generating

212 Penk^{Aheme} mice. As predicted, these Penk^{Aheme} mice display decreased nociceptive

213 thresholds during uninjured state, co 212 Penk^{Aneme} mice. As predicted, these Penk^{Aneme} mice display decreased nociceptive
213 thresholds during uninjured state, compared to vehicle-injected Penk^{Aheme} mice, which
214 supports our conclusion that hematopo thresholds during uninjured state, compared to vehicle-injected Penk^{Aheme} thresholds during uninjured state, compared to vehicle-injected *Penk*^{Aneme} mice, which
214 supports our conclusion that hematopoietic cell-derived enkephalin controls basal
215 mechanical sensitivity but only in females 214 supports our conclusion that hematopoietic cell-derived enkephalin controls basal
215 mechanical sensitivity but only in females (Figure 4H).
216 We recognize that recombination-based selective ablation of enkephalin o

mechanical sensitivity but only in females (Figure 4H).
216
217 We recognize that recombination-based selective ablat
218 using $Foxp3^{Cre}$ or $Foxp3^{Cre-ERT2}$ has multiple limitations
219 systemic targeting of Tregs. including 216
217
218
219
220 217 We recognize that recombination-based selective ablation of enkephalin on Tregs,

218 using $F\alpha p3^{Cre}$ or $F\alpha p3^{Cre-ERT2}$ has multiple limitations and caveats. These include

219 systemic targeting of Tregs, including e using *Foxp3Cre* or *Foxp3Cre-ERT2* using *Foxp3^{Cre}* or *Foxp3^{Cre-ER12*} has multiple limitations and caveats. These include
219 systemic targeting of Tregs, including enkephalinergic Tregs in the skin, potential
220 compensatory *Penk* regulation upon co systemic targeting of Tregs, including enkephalinergic Tregs in the skin, potential

220 compensatory *Penk* regulation upon constitutive ablation, potential side effects of

221 cre/Cre-ERT2 mice, and the impact of random compensatory *Penk* regulation upon constitutive ablation, potential side effects of

221 tamoxifen, potential stochastic deletion of *Penk* outside of Tregs in homozygote

222 Cre/Cre-ERT2 mice, and the impact of random X 221 tamoxifen, potential stochastic deletion of *Penk* outside of Tregs in homozygote
222 Cre/Cre-ERT2 mice, and the impact of random X-inactivation on heterozygous n
223 In light of these concerns, we also generated mixed Cre/Cre-ERT2 mice, and the impact of random X-inactivation on heterozygous mice 37 Cre/Cre-ERT2 mice, and the impact of random X-inactivation on heterozygous mice³⁷.

1223 In light of these concerns, we also generated mixed bone marrow chimeras using 1:1

1224 ratio of *Foxp3*-DTR and *Penk^{-/-}* bone 223 In light of these concerns, we also generated mixed bone marrow chimeras using 1:1
224 ratio of $Foxp3$ -DTR and $Penk^{\prime}$ bone marrow and implanted these chimeras in irradiate
225 WT mice. Intrathecal injection of pegDT ratio of *Foxp3*-DTR and *Penk^{-/-}* bone marrow and implanted these chimeras in irradiated The matio of Foxp3-DTR and Penk⁷⁻ bone marrow and implanted these chimeras in irradiated

225 WT mice. Intrathecal injection of pegDT into these mice results in ablation of FoxP3-

226 DTR Tregs; the remaining Tregs are WT mice. Intrathecal injection of pegDT into these mice results in ablation of *FoxP3*-
226 DTR Tregs; the remaining Tregs are left deficient for *Penk* (*Penk*^{AmTreg} mice) while
227 preserving other immune cell types. I DTR Tregs; the remaining Tregs are left deficient for *Penk* (*Penk*ΔmTreg 226 DTR Tregs; the remaining Tregs are left deficient for *Penk* (*Penk*^{Am reg} mice) while
227 preserving other immune cell types. Importantly, this approach circumnavigated
228 potential depletion of any previously unr preserving other immune cell types. Importantly, this approach circumnavigated

potential depletion of any previously unrecognized non-hematopoietic cells that e
 $Foxp3$. At baseline, uninjected mixed chimeric mice had si 228 potential depletion of any previously unrecognized non-hematopoietic cells that express
229 *Foxp3*. At baseline, uninjected mixed chimeric mice had similar mechanical thresholds
230 as WT^{Aheme} control chimeras. As p *Foxp3*. At baseline, uninjected mixed chimeric mice had similar mechanical thresholds
230 as $WT^{\Delta \text{heme}}$ control chimeras. As predicted, pegDT IT injection led to mechanical as $WT^{\Delta \text{heme}}$ control chimeras. As predicted, pegDT IT injection led to mechanical
8

hypersensitivity in uninjured Penk^{ΔmTreg} but not WT^{Δ heme control mice and exacerbated

231 by persensitivity in uninjured *Penk*^{Am reg} but not WT^{aneme} control mice and exacerbated

232 nerve injury-induced hypersensitivity (Figure 4I-J). Having shown that IL-2-induced

233 mTreg expansion and expressio

nerve injury-induced hypersensitivity (**Figure 4I-J)**. Having shown that IL-2-induced

233 mTreg expansion and expression of enkephalin alleviates neuropathic pain, we next

234 investigated whether this could be mediated 233 mTreg expansion and expression of enkephalin alleviates neuropathic pain, we next

234 investigated whether this could be mediated by the δOR, the preferred receptor for

235 enkephalin. In these studies, we co-admin

234 investigated whether this could be mediated by the δOR, the preferred receptor for
235 enkephalin. In these studies, we co-administered IL-2 and naltrindole, a selective
236 antagonist of the δOR, and observed that I

- enkephalin. In these studies, we co-administered IL-2 and naltrindole, a selective

236 antagonist of the δ OR, and observed that IL-2-induced anti-allodynia was abolishe

237 (Figure 4K-L). We conclude that mTreg-deriv 236 antagonist of the δOR, and observed that IL-2-induced anti-allodynia was abolished
237 **(Figure 4K-L)**. We conclude that mTreg-derived enkephalin is required for suppress
238 mechanical pain hypersensitivity and that **(Figure 4K-L)**. We conclude that mTreg-derived enkephalin is required for suppressing

238 mechanical pain hypersensitivity and that this suppression is mediated by the δOR.

240 *Treg-derived enkephalin is dispensable*
-

238 mechanical pain hypersensitivity and that this suppression is mediated by the δOR.
239
240 **Treg-derived enkephalin is dispensable for immune suppression**
241 Previously, Tregs have been shown to suppress hyperalgesi 239
240
241
242
243 **Treg-derived enkephalin is dispensable for immune suppression**
241 Previously, Tregs have been shown to suppress hyperalgesia followii
242 suppressing IFN- \square -induced primary afferent sensitization¹⁷. Thus, we
243 a po Previously, Tregs have been shown to suppress hyperalgesia following nerve injury by
242 suppressing IFN- \square -induced primary afferent sensitization¹⁷. Thus, we hypothesized that
243 a potential mechanism by which Tregsuppressing IFN-⁻induced primary afferent sensitization¹⁷
a patential meabonism by which Trea derived ankenholing suppressing IFN- \square -induced primary afferent sensitization¹⁷. Thus, we hypothesized that
243 a potential mechanism by which Treg-derived enkephalin mediates the suppression of
244 nociceptive thresholds involves modula 243 a potential mechanism by which Treg-derived enkephalin mediates the suppression of

244 nociceptive thresholds involves modulation of immunological responses. To address

245 this possibility, we tested the nociceptiv 244 nociceptive thresholds involves modulation of immunological responses. To address
245 this possibility, we tested the nociceptive thresholds of immunodeficient $Rag2^{-/-}$ mice
246 which are missing both T and B cells an this possibility, we tested the nociceptive thresholds of immunodeficient *Rag2-/* this possibility, we tested the nociceptive thresholds of immunodeficient $Rag2^{-/-}$ mice

246 which are missing both T and B cells and compared them to immunocompetent

247 littermates. In these studies, we mated $Rag2^{+/-}$ which are missing both T and B cells and compared them to immunocompetent

247 littermates. In these studies, we mated $Rag2^{+\prime}$ mice and were surprised to observe

248 decreased nociceptive thresholds in the $Rag2^{-\prime}$ of littermates. In these studies, we mated *Rag2^{+/-}* mice and were surprised to observe littermates. In these studies, we mated $Rag2^{+\prime}$ mice and were surprised to observe

248 decreased nociceptive thresholds in the $Rag2^{-\prime}$ offspring compared to their $Rag2^{+\prime +}$ and

249 $Rag2^{+\prime -}$ littermates. This find decreased nociceptive thresholds in the *Rag2-/-* offspring compared to their *Rag2+/+* decreased nociceptive thresholds in the $Rag2^{-/-}$ offspring compared to their $Rag2^{+/+}$ and $Rag2^{+/+}$ littermates. This finding suggests that there may be a mechanism of Treg-
250 mediated control of nociceptive thresholds *Rag2+/-* Rag2^{+/-} littermates. This finding suggests that there may be a mechanism of Treg-
250 mediated control of nociceptive thresholds, which is independent of exaggerated
251 lymphocyte-driven inflammation (Figure 5A). Furthe 250 mediated control of nociceptive thresholds, which is independent of exaggerated lymphocyte-driven inflammation (Figure 5A). Furthermore, consistent with this conclusion, depleting macrophages through liposomal clodron 252 conclusion, depleting macrophages through liposomal clodronate administration did not
253 reverse the mechanical allodynia observed in female mice deficient in mTreg (Figure
254 5B)⁴⁵. 252 conclusion, depleting macrophages through liposomal clodronate administration did not
253 reverse the mechanical allodynia observed in female mice deficient in mTreg (Figure
254 5B)⁴⁵.
256 We also used the *Penk*DTR 5B $)^{45}$.

z53 reverse the mechanical allodynia observed in female mice deficient in mTreg (Figure
254 5B)⁴⁵.
255 We also used the *Penk*DTR^{Aheme} bone marrow chimeric mice to deplete all bone
257 marrow-derived enkephalin lineage 254 **5B)**⁴⁵.
255 We al
257 marrov
258 regulat 256
257
258
259 We also used the *Penk*DTRΔheme 256 We also used the *Penk*DTR^{Aneme} bone marrow chimeric mice to deplete all bone
257 marrow-derived enkephalin lineage cells and assessed their contribution to the
258 regulation of immune responses. Unlike *Foxp3*-DTR marrow-derived enkephalin lineage cells and assessed their contribution to the

258 regulation of immune responses. Unlike *Foxp3*-DTR mice, we observed no changes in

269 mouse weight or spleen size in *Penk*DTR^{Aheme} bo regulation of immune responses. Unlike *Foxp3*-DTR mice, we observed no changes in

259 mouse weight or spleen size in *Penk*DTR^{Δheme} bone marrow chimeric mice chronically

260 injected with systemic DT (**Figure S5D-E)**. mouse weight or spleen size in PenkDTR^{Δheme} mouse weight or spleen size in *Penk*DTR^{Aneme} bone marrow chimeric mice chronically

260 injected with systemic DT (Figure S5D-E). Furthermore, we also did not observe any

261 specific alterations in CD4⁺ T cell cytok 260 injected with systemic DT (Figure S5D-E). Furthermore, we also did not observe any specific alterations in CD4⁺ T cell cytokine production after nerve injury in the meninges or lymphoid organs. We conclude, therefore specific alterations in CD4⁺ specific alterations in CD4⁺ T cell cytokine production after nerve injury in the meninges
262 or lymphoid organs. We conclude, therefore that peripheral enkephalin does not
263 contribute to T cell-driven inflammatory r

266 conventional T cell proliferation. We assessed T cell suppression capacity by cocontribute to T cell-driven inflammatory responses (Figure S5F-G).
264
265 We next investigated the contribution of Treg-derived enkephalin
266 conventional T cell proliferation. We assessed T cell suppressi
267 culturing 265
266
267
268 265 We next investigated the contribution of Treg-derived enkephalin in the regulation of

266 conventional T cell proliferation. We assessed T cell suppression capacity by co-

267 culturing naïve conventional CD4⁺ T c 266 conventional T cell proliferation. We assessed T cell suppression capacity by co-

267 culturing naïve conventional CD4⁺ T cells with either WT *Penk^{+/+}* or *Penk^{-/-}* Tregs. We

268 observed no difference in the culturing naïve conventional CD4⁺ T cells with either WT *Penk+/+* or *Penk*-/ culturing naïve conventional CD4⁺ T cells with either WT *Penk*^{+/+} or *Penk*^{-/-} Tregs. We
268 observed no difference in the suppressive capacity of *Penk*^{-/-} Treg compared to control
269 Tregs (Figure 5C-D). Next, observed no difference in the suppressive capacity of *Penk-/-* 268 observed no difference in the suppressive capacity of *Penk*^{-/-} Treg compared to control
269 Tregs (Figure 5C-D). Next, we transplanted equal amounts of CD45.1 *Penk*^{+/+} or
270 CD45.2 *Penk^{-/-}* CD4⁺ T cells in Tregs **(Figure 5C-D)**. Next, we transplanted equal amounts of CD45.1 *Penk+/+* Tregs (Figure 5C-D). Next, we transplanted equal amounts of CD45.1 *Penk*^{+/+} or
270 CD45.2 *Penk^{-/-}* CD4⁺ T cells into *Rag2^{-/-}* mice and performed SNI to measure chimerism
271 of congenic markers among CD4⁺ T ce CD45.2 *Penk-/-* CD4+ T cells into *Rag2-/-* 270 CD45.2 Penk^{-/-}CD4⁺ T cells into Rag2^{-/-} mice and performed SNI to measure chimerism

271 of congenic markers among CD4⁺ T cells. We did not observe a competitive advantage

272 or disadvantage amongst Penk⁻ of congenic markers among CD4⁺ T cells. We did not observe a competitive advantage 271 of congenic markers among $CD4^+$ T cells. We did not observe a competitive advantage

272 or disadvantage amongst *Penk*^{-/-} CD4⁺ T cells across various tissues (Figure 5E-G).

273 Restimulating harvested T cells f or disadvantage amongst *Penk-/-* CD4+ or disadvantage amongst *Penk*⁻⁻ CD4⁺ T cells across various tissues **(Figure 5E-G)**.

273 Restimulating harvested T cells from distinct tissues with PMA/lonomycin revealed no

274 differences in T cell differentiation 273 Restimulating harvested T cells from distinct tissues with PMA/Ionomycin revealed no
274 differences in T cell differentiation across Tregs, T helper 1 (Th1) and Th17 subsets
275 between the *Penk*-sufficient or defici 274 differences in T cell differentiation across Tregs, T helper 1 (Th1) and Th17 subsets
275 between the *Penk*-sufficient or deficient T cells (Figure 5H and Figure S5H-I). In
276 addition, we noticed no difference in we 275 between the *Penk-*sufficient or deficient T cells (Figure 5H and Figure S5H-I). In addition, we noticed no difference in weight, health, or spleen size between *Penk*^{Aheme} addition, we noticed no difference in weight, health, or spleen size between Penk^{Δheme}

and WT^{Aheme} bone marrow chimeric mice further revealing that peripheral enkephalin and WT^{Aneme} bone marrow chimeric mice further revealing that peripheral enkephalin

278 has a very limited, if any, role in the suppressing of systemic inflammatory responses

280 **(Figure S5J-K)**.

281 Finally, using an

178 has a very limited, if any, role in the suppressing of systemic inflammatory responses
279 **(Figure S5J-K)**.
280 Finally, using an adoptive transfer-based graft versus host disease (GVHD) model, we
282 assessed whether 279 **(Figure S5J-K)**.
280
281 Finally, using an
282 assessed wheth
283 responses. As ex 282
283
284 282 assessed whether Treg-derived enkephalin is required for suppressing immune
283 responses. As expected, we observed a profound Th1 response in mice transferred with
284 activated Tconv alone. However, mice that receive 282 assessed whether Treg-derived enkephalin is required for suppressing immune

283 responses. As expected, we observed a profound Th1 response in mice transferred with

284 activated Tconv alone. However, mice that recei responses. As expected, we observed a profound Th1 response in mice transferred with

284 activated Tconv alone. However, mice that received additional transfers of either

285 Penk^{+/+} or Penk^{-/-} Tregs equally suppress 286 and mitigated weight dysregulation (Figure 5 I-J and S5L-N). In summary, we conclude
287 that Treg-derived enkephalin does not contribute to any inflammatory response restraint
288 mechanism. Rather, we conclude that T *Penk^{+/+}* or *Penk^{-/-}* Tregs equally suppressed Th1 responses, reduced GVHD severity Penk^{+/+} or Penk^{-/-} Tregs equally suppressed Th1 responses, reduced GVHD severity

286 and mitigated weight dysregulation (Figure 5 I-J and S5L-N). In summary, we conclude

287 that Treg-derived enkephalin does not cont 286 and mitigated weight dysregulation (Figure 5 I-J and S5L-N). In summary, we conclude that Treg-derived enkephalin does not contribute to any inflammatory response restraint mechanism. Rather, we conclude that Tregs can 287 that Treg-derived enkephalin does not contribute to any inflammatory response restraint
288 mechanism. Rather, we conclude that Tregs can suppress pain sensitivity through a
290
291 **Delta opioid receptor signaling on** 288 mechanism. Rather, we conclude that Tregs can suppress pain sensitivity through a
289 mechanism that is independent of their function in immunosuppression.
291 **Delta opioid receptor signaling on MrgprD⁺ primary affe**

mechanism that is independent of their function in immunosuppression.
290
291 **Delta opioid receptor signaling on MrgprD⁺ primary afferent DRG n
292 required for the anti-allodynic function of mTregs**
293 Enkephalin is 290
291
292
293
294 *Delta opioid receptor signaling on MrgprD+*

**Delta opioid receptor signaling on MrgprD⁺ primary afferent DRG neurons is

292 required for the anti-allodynic function of mTregs**

293 Enkephalin is a potent agonist at the δOR, and, to a lesser extent the μOR. I

2 *required for the anti-allodynic function of mTregs*
293 Enkephalin is a potent agonist at the δOR, and, to
294 previous studies, we demonstrated the divergence o
295 and μOR in mediating distinct pain modalities. Sp
296 293 Enkephalin is a potent agonist at the δOR, and, to a lesser extent the μOR. In our
294 previous studies, we demonstrated the divergence of expression and function of δOR
295 and μOR in mediating distinct pain modalit 294 previous studies, we demonstrated the divergence of expression and function of δOR
295 and μOR in mediating distinct pain modalities. Specifically, δOR is expressed on
296 nonpeptidergic IB4⁺ unmyelinated as well a 295 and μOR in mediating distinct pain modalities. Specifically, δOR is expressed on

296 nonpeptidergic IB4⁺ unmyelinated as well as myelinated primary afferents and

297 selectively regulates mechanical thresholds an nonpeptidergic IB4⁺ 296 nonpeptidergic IB4⁺ unmyelinated as well as myelinated primary afferents and selectively regulates mechanical thresholds and nerve injury-induced mechanical hypersensitivity²¹. Conversely, the μ OR is expressed 297 selectively regulates mechanical thresholds and nerve injury-induced mechanical

298 hypersensitivity²¹. Conversely, the μ OR is expressed on Trpv1⁺ nociceptors and

299 selectively regulates thermal hyperalgesi hypersensitivity²¹. Conversely, the μOR is expressed on Trpv1⁺
cologitively requistes thermal byperclassic, in addition a spinal δ 299 selectively regulates thermal hyperalgesia. In addition, a spinal δOR can dampen
300 mechanical hypersensitivity by inhibiting the excitability of somatostatin-positive dorsal
301 horn interneurons²³. 299 selectively regulates thermal hyperalgesia. In addition, a spinal δOR can dampen
300 mechanical hypersensitivity by inhibiting the excitability of somatostatin-positive dorsal
302
303 To assess the requirement of PNS horn interneurons 23 .

300 mechanical hypersensitivity by inhibiting the excitability of somatostatin-positive dorsal
301 horn interneurons²³.
303 To assess the requirement of PNS or CNS δOR circuits in coordinating the anti-
304 allodynic ef 301 horn interneurons²³.
302 To assess the requ
304 allodynic effect of m
305 with AAV.PHP.S-C. 303
304
305
306 303 To assess the requirement of PNS or CNS δ OR circuits in coordinating the anti-
304 allodynic effect of mTregs, we intravenously injected *Oprd1*^{+/+} control or *Oprd1^{†/†}* mice
305 with AAV.PHP.S-CAG-Cre or AAV.P allodynic effect of mTregs, we intravenously injected Oprd1^{+/+} control or Oprd1^{f/fl} allodynic effect of mTregs, we intravenously injected *Oprd1*^{+/+} control or *Oprd1*[™] mice
305 with AAV.PHP.S-CAG-Cre or AAV.PHP.eB-CAG-Cre. This approach selectively
306 introduces Cre recombinase and targets deletion with AAV.PHP.S-CAG-Cre or AAV.PHP.eB-CAG-Cre. This approach selectively
306 introduces Cre recombinase and targets deletion of δOR into to the PNS (DRG) or CNS
307 (spinal cord and brain), respectively (**Figure 6A).** Thre 306 introduces Cre recombinase and targets deletion of δOR into to the PNS (DRG) or CNS
307 (spinal cord and brain), respectively (**Figure 6A).** Three weeks after the AAV injection,
308 in the PNS lost the capacity to res 307 (spinal cord and brain), respectively (Figure 6A). Three weeks after the AAV injection, we performed SNI and four weeks later administered IL-2. Mice selectively lacking δOR in the PNS lost the capacity to respond to 308 we performed SNI and four weeks later administered IL-2. Mice selectively lacking δOR
309 in the PNS lost the capacity to respond to the anti-allodynic effect of IL-2, but the effects
310 of IL-2 were preserved in mic 309 in the PNS lost the capacity to respond to the anti-allodynic effect of IL-2, but the effects
310 of IL-2 were preserved in mice lacking δOR in the CNS (Figure 6B-C). We conclude
311 that a sensory neuron-expressed, p 310 of IL-2 were preserved in mice lacking δOR in the CNS (Figure 6B-C). We conclude
311 that a sensory neuron-expressed, presynaptic δOR coordinates mTreg suppression of
312 mechanical pain hypersensitivity.
313 Next. we 311 that a sensory neuron-expressed, presynaptic δOR coordinates mTreg suppression of
312 mechanical pain hypersensitivity.
313 Next, we identified the specific sensory neuron subset that coordinates the anti-
315 nocicep

312 mechanical pain hypersensitivity.
313 Next, we identified the specific
315 nociception mediated by mTre
316 expression on DRG sensory ne 313
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315
316
317 314 Next, we identified the specific sensory neuron subset that coordinates the anti-
315 nociception mediated by mTreg-derived enkephalin. Previous studies of δ OR
316 expression on DRG sensory neurons using $Oprd1^{eGFP}$ 315 nociception mediated by mTreg-derived enkephalin. Previous studies of δOR expression on DRG sensory neurons using $Oprd1^{eGFP}$ reporter mice revealed that approximately half of the reporter-positive cells in the DRG a expression on DRG sensory neurons using *Oprd1^{eGFP}* reporter mice revealed that
317 approximately half of the reporter-positive cells in the DRG are myelinated neurons,
318 while approximately 36% are IB4⁺ non-peptider 317 approximately half of the reporter-positive cells in the DRG are myelinated neurons,
318 while approximately 36% are IB4⁺ non-peptidergic neurons expressing the MrgprD
320 receptor. Using established single cell RNA while approximately 36% are IB4⁺ while approximately 36% are IB4⁺ non-peptidergic neurons expressing the MrgprD
319 receptor. Using established single cell RNA sequencing resources, we found that the
320 MrgprD⁺ subset of DRG sensory neurons not only receptor. Using established single cell RNA sequencing resources, we found that the
320 MrgprD⁺ subset of DRG sensory neurons not only expresses *Oprd1*, but also other
321 receptors for Treg ligands, including *II10ra a* MrgprD⁺ subset of DRG sensory neurons not only expresses *Oprd1*, but also other 320 MrgprD⁺ subset of DRG sensory neurons not only expresses *Oprd1*, but also other receptors for Treg ligands, including *II10ra and Icosl*, which have been implicated in suppression of pain thresholds^{46–48} (Figure 6 321 receptors for Treg ligands, including *II10ra and Icosl*, which have been implicated in
322 suppression of pain thresholds^{46–48} (Figure 6D). The total proportion of sensory neurons suppression of pain thresholds^{46–48} (Figure 6D). The total proportion of sensory neurons
10

expressing the *Oprd1* transcript matches previous data using *Oprd1eGFP* expressing the *Oprd1* transcript matches previous data using *Oprd1*^{eGPP} reporter mice
324 **(Figure 6E)**. Our subsequent flow cytometry-based profiling of *Oprd1*^{eGPP} reporter
325 expression on DRG cells confirmed GFP **(Figure 6E)**. Our subsequent flow cytometry-based profiling of *Oprd1eGFP* **(Figure 6E)**. Our subsequent flow cytometry-based profiling of $Oprd1^{eGFP}$ reporter

325 expression on DRG cells confirmed GFP expression specifically on IB4⁺ CD45⁻ Thy1⁺

326 sensory neurons, which corresponds to expression on DRG cells confirmed GFP expression specifically on IB4⁺ CD45 Thy1⁺ 325
326
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328 sensory neurons, which corresponds to the Mrgpr D^+ nonpeptidergic nociceptive neuron sensory neurons, which corresponds to the MrgprD⁺ nonpeptidergic nociceptive neuron
327 population. Importantly, the flow analysis confirmed the absence of GFP expression on
328 broadly defined CD45⁺ CD90.2⁺ IB4⁻ l population. Importantly, the flow analysis confirmed the absence of GFP expression on

328 broadly defined CD45⁺ CD90.2⁺ IB4⁻ lymphoid cells and CD45⁺ CD11b⁺ CD90.2⁻ IB4⁻

329 myeloid cells (Figure 6F). We al broadly defined CD45⁺ CD90.2⁺ IB4⁻ lymphoid cells and CD45⁺ CD11b⁺ CD90.2⁻ IB4⁻ 329
330
331
332 329 myeloid cells (**Figure 6F)**. We also confirmed the absence of GFP expression on microglia as well as on immune cells profiled from the draining lymph nodes (data not shown)²³. Based on this selective Oprd1 expressio 330 microglia as well as on immune cells profiled from the draining lymph nodes (data not
331 shown)²³. Based on this selective Oprd1 expression profile, we generated mice in which
332 MrgprD⁺ neurons lack the δ OR 331 shown)²³. Based on this selective Oprd1 expression profile, we generated mice in which
332 MrgprD⁺ neurons lack the δ OR (*MrgprD*^{Cre-ERT2}; *Oprd1*^{fl/fl}). Female *MrgprD*^{Cre-}
333 ^{ERT2}; *Oprd1*^{fl/fl} mic MrgprD⁺ neurons lack the δOR (*MrgprD*^{Cre-ERT2}; Oprd1^{fl/fl}). Female *MrgprD*^{Cre-}
ERT2: Oprd1^{fl/fl} mise but not their male counterparts aybibited exergencted mechanical 333
334
335
336 frace, but not their male counterparts, exhibited exaggerated mechanical hypersensitivity after SNI compared to tamoxifen-injected sex-matched littermate
335 controls (Figure 6G-H). Female mice lacking δOR on MrgprD⁺ sensory neurons and
336 treated with IL-2 IT four weeks following SNI displ 334 hypersensitivity after SNI compared to tamoxifen-injected sex-matched littermate
335 controls (Figure 6G-H). Female mice lacking δOR on MrgprD⁺ sensory neurons and
336 treated with IL-2 IT four weeks following SNI d controls (Figure 6G-H). Female mice lacking δOR on MrgprD⁺ sensory neurons and controls (Figure 6G-H). Female mice lacking δOR on MrgprD⁺ sensory neurons and
336 treated with IL-2 IT four weeks following SNI displayed a complete deficiency in IL-2
337 anti-allodynic efficacy (Figure 6I-J). We conc 336 treated with IL-2 IT four weeks following SNI displayed a complete deficiency in IL-2
337 anti-allodynic efficacy (Figure 6I-J). We conclude that, the enkephalin receptor δOR,
838 expressed specifically by MrgprD⁺ s 337 anti-allodynic efficacy (Figure 6I-J). We conclude that, the enkephalin receptor δOR,
338 expressed specifically by MrgprD⁺ sensory neurons, mediates the anti-nociceptive
539 function of mTregs.
340 expressed specifically by MrgprD⁺ sensory neurons, mediates the anti-nociceptive
339 function of mTregs.
340
341 **Discussion**

339 function of mTregs.
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341 **Discussion**
343 In this report. we de 341
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344 341 342 **Discussion**
343 In this repor
345 expand Treg
346 the mening In this report, we describe a novel, sexually dimorphic mechanism for pain regulation by
344 the immune system. Using a range of site-selective targeting strategies to deplete or
345 expand Tregs within the recently recogn expand Tregs within the recently recognized borders of the nervous system, specifically
346 the meninges, we find that meningeal Tregs (mTregs) can profoundly modulate
347 mechanical hypersensitivity. Strikingly, this pain the meninges, we find that meningeal Tregs (mTregs) can profoundly modulate
347 mechanical hypersensitivity. Strikingly, this pain regulatory function of mTregs is sex-
348 specific and controlled by gonadal hormones. Alth the meninges, we find that meningeal Tregs (mTregs) can profoundly modulate
347 mechanical hypersensitivity. Strikingly, this pain regulatory function of mTregs is sex-
348 specific and controlled by gonadal hormones. Alth mechanical hypersensitivity. Strikingly, this pain regulatory function of mTregs is sex-
348 specific and controlled by gonadal hormones. Although proenkephalin expression by
350 had not been explored. Here, we demonstrat Tregs has been observed in sequencing studies, its functional relevance to nociception
350 had not been explored. Here, we demonstrate that enkephalin secreted by mTregs acts
351 on δ-opioid receptors (δOR) on primary sen had not been explored. Here, we demonstrate that enkephalin secreted by mTregs acts
351 on δ-opioid receptors (δOR) on primary sensory neurons to selectively modulate
352 mechanical sensitivity. Our findings provide the f 351 on δ-opioid receptors (δOR) on primary sensory neurons to selectively modulate
352 mechanical sensitivity. Our findings provide the first mechanism of Treg-mediated
353 suppression of nociception and establish regulato 352 mechanical sensitivity. Our findings provide the first mechanism of Treg-mediated
353 suppression of nociception and establish regulatory T cells as key sentinels of pain
354 homeostasis. suppression of nociception and establish regulatory T cells as key sentinels of pain

356 To assess for sex differences in transcriptional identify after nerve injury, primary
357 afferent neurons, including those expressing MrgprD, have recently been sorted for 354 homeostasis.
355 To assess fc
357 afferent neuro
358 deep RNA se 356
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358
359 356 To assess for sex differences in transcriptional identify after nerve injury, primary
357 afferent neurons, including those expressing MrgprD, have recently been sorted for
358 deep RNA sequencing⁵². Surprisingly, ve afferent neurons, including those expressing MrgprD, have recently been sorted for
358 deep RNA sequencing⁵². Surprisingly, very few differences were found between the
359 sexes, suggesting a lack of a strong intersectio deep RNA sequencing⁵² sexes, suggesting a lack of a strong intersection of sex and injury in the transcriptional
360 identity of peripheral neurons. The lack of transcriptional differences may also suggest
361 that non-neuronal cells may be a p sexes, suggesting a lack of a strong intersection of sex and injury in the transcriptional
360 identity of peripheral neurons. The lack of transcriptional differences may also suggest
361 that non-neuronal cells may be a p identity of peripheral neurons. The lack of transcriptional differences may also suggest

361 that non-neuronal cells may be a primary determinant of sex-selective pain modulation.

362 Indeed, preclinical research has dem that non-neuronal cells may be a primary determinant of sex-selective pain modulation.
362 Indeed, preclinical research has demonstrated the involvement of T cells in driving pain
363 phenotypes in female mice⁵³. Additio 362 Indeed, preclinical research has demonstrated the involvement of T cells in driving pain
363 phenotypes in female mice⁵³. Additionally, human leukocyte antigen (HLA) risk alleles
364 have been identified for human ch phenotypes in female mice 53 phenotypes in female mice⁵³. Additionally, human leukocyte antigen (HLA) risk alleles
364 have been identified for human chronic pain conditions, further suggesting a potential
365 role for T cells in pain modulation^{24,} have been identified for human chronic pain conditions, further suggesting a potential
365 nole for T cells in pain modulation^{24,54}.
366 Regulatory T cells display broad tissue supportive roles that extend beyond their
3 role for T cells in pain modulation^{24,54}.

365 role for T cells in pain modulation^{24,54}.
366 Regulatory T cells display broad tis
368 originally described function in suppre 366 367 Regulatory T cells display broad tissue supportive roles that extend beyond their
368 originally described function in suppressing inflammation^{8,11,12,20}. A major advantage of originally described function in suppressing inflammation^{8,11,12,20}. A major advantage of
11 our analysis is that we utilized a site-selective mTreg ablation strategy that preserved
370 peripheral Tregs and avoided systemic inflammation. Using this approach, we identify a
371 novel, sex-specific mechanism by which peripheral Tregs and avoided systemic inflammation. Using this approach, we identify a

371 novel, sex-specific mechanism by which Tregs modulate nociceptor activity to regulate

372 pain sensitivity in the context of heal novel, sex-specific mechanism by which Tregs modulate nociceptor activity to regulate

372 pain sensitivity in the context of health and nerve injury. Although, proenkephalin-

373 expressing Tregs have been identified in pain sensitivity in the context of health and nerve injury. Although, proenkephalin-
373 expressing Tregs have been identified in various tissues, their functional assessment
374 has been limited^{19,37,38}. Somewhat parado expressing Tregs have been identified in various tissues, their functional assessment
374 has been limited^{19,37,38}. Somewhat paradoxically, a recent pre-print study demonstrated
375 a small but statistically significant has been limited^{19,37,38} has been limited^{19,37,38}. Somewhat paradoxically, a recent pre-print study demonstrated
375 a small but statistically significant decrease in basal heat sensitivity in both female and
376 male mice conditionally depleted a small but statistically significant decrease in basal heat sensitivity in both female and
376 male mice conditionally depleted for *Penk* expression in systemic Tregs. Mechanical
373 thresholds and other sensory modaliti male mice conditionally depleted for *Penk* expression in systemic Tregs. Mechanical
377 thresholds and other sensory modalities were however not examined⁵⁵. In distinct
378 contrast, we uncover a sensory modality selec thresholds and other sensory modalities were however not examined⁵⁵ 377 thresholds and other sensory modalities were however not examined⁵⁵. In distinct
378 contrast, we uncover a sensory modality selective function of mTreg that is consistent
379 with prior findings of δ OR agonism, 378 contrast, we uncover a sensory modality selective function of mTreg that is consistent
379 with prior findings of δ OR agonism, namely providing relief of mechanical but not heat
380 pain^{21,56}. Whether mTregs toni 379 with prior findings of δOR agonism, namely providing relief of mechanical but not heat
380 pain^{21,56}. Whether mTregs tonically restrain nociception is difficult to conclude. Our
381 finding of increased basal sensit pain^{21,56}. Whether mTregs tonically restrain nociception is difficult to conclude. Our 380 pain^{21,56}. Whether mTregs tonically restrain nociception is difficult to conclude. Our finding of increased basal sensitivity in $Rag2^{-/-}$ or $Penk^{\text{Aheme}}$ mice, is supportive of this hypothesis, however, alternativ finding of increased basal sensitivity in *Rag2-/-* or *Penk*Δheme finding of increased basal sensitivity in Rag2^{-/-} or Penk^{Aneme} mice, is supportive of this
382 hypothesis, however, alternative possibilities exist. It is conceivable that mTreg
383 deficiency could lead to inflammatio 383 deficiency could lead to inflammation, which may alter nociceptor sensitivity.
384 Additionally, the endogenous opioid signaling pathway may have a role in opioid
385 induced analgesia. It is significant that naloxone, deficiency could lead to inflammation, which may alter nociceptor sensitivity.
384 Additionally, the endogenous opioid signaling pathway may have a role in opioid
385 induced analgesia. It is significant that naloxone, a n 384 Additionally, the endogenous opioid signaling pathway may have a role in opioid
385 induced analgesia. It is significant that naloxone, a non-selective opioid receptor
386 antagonist IV injection, does not induce pain 385 induced analgesia. It is significant that naloxone, a non-selective opioid receptor
386 antagonist IV injection, does not induce pain in healthy individuals. Whether
387 experiments in uninjured female mice have been p 386 antagonist IV injection, does not induce pain in healthy individuals. Whether
387 experiments in uninjured female mice have been performed is unclear⁵⁷.
389 Our observations suggest that sex hormones, rather than sex experiments in uninjured female mice have been performed is unclear 57 .

- experiments in uninjured female mice have been performed is unclear⁵⁷.
1888 . Our observations suggest that sex hormones, rather than sex chromoson
190 . main drivers of mTreg-induced anti-nociception. While the specific 388
389
390
391
392 390 main drivers of mTreg-induced anti-nociception. While the specific sex hormones
391 involved in regulating mTreg function in pain remain unclear, previous studies have
392 implicated estrogen and progesterone in modula 391 involved in regulating mTreg function in pain remain unclear, previous studies have
392 implicated estrogen and progesterone in modulating neuropathic pain associated wi
393 SNI⁴⁹. Estrogen administration increases 391 involved in regulating mTreg function in pain remain unclear, previous studies have
392 implicated estrogen and progesterone in modulating neuropathic pain associated wi
393 SNI⁴⁹. Estrogen administration increases implicated estrogen and progesterone in modulating neuropathic pain associated with
393 SNI⁴⁹. Estrogen administration increases *Penk* expression in the whole spinal cord⁵⁸.
394 Additionally, in CD4⁺ T cells, the es SNI⁴⁹. Estrogen administration increases *Penk* expression in the whole spinal cord⁵⁸. SNI⁴⁹. Estrogen administration increases *Penk* expression in the whole spinal cord⁵⁸.
394 Additionally, in CD4⁺ T cells, the estrogen receptor engages conserved non-coding
395 Sequences (CNS) in *Foxp3* enhancer Additionally, in CD4⁺ Additionally, in CD4⁺ T cells, the estrogen receptor engages conserved non-coding
395 sequences (CNS) in *Foxp3* enhancer regions⁵⁹. Importantly, we reveal an anti-
396 nociceptive role for regulatory T cells that is d sequences (CNS) in *Foxp3* enhancer regions⁵⁹ sequences (CNS) in $Foxp3$ enhancer regions⁵⁹. Importantly, we reveal an anti-
nociceptive role for regulatory T cells that is distinct from their well-established functions
in immune suppression and tissue repair. Furth nociceptive role for regulatory T cells that is distinct from their well-established functions
397 in immune suppression and tissue repair. Furthermore, we demonstrate that this
398 mechanism operates within nervous system 397 in immune suppression and tissue repair. Furthermore, we demonstrate that this
398 mechanism operates within nervous system tissues, at a site distant from the per
199 nerve injury, highlighting the immune system's rem 398 mechanism operates within nervous system tissues, at a site distant from the peripheral
399 nerve injury, highlighting the immune system's remarkable ability to modulate
400 nociception.
401 399 nerve injury, highlighting the immune system's remarkable ability to modulate
400 nociception.
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- 400 nociception.
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- **Methods**
 Mice

All mouse experiments were approved by UCSF Institutional Animal Care and Use

422 Committee and conducted in accordance with the guidelines established by the
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- 420 **Mice**
421 All mo
422 Comr
423 Institu
424 All mi All mouse experiments were approved by UCSF Institutional Animal Care and Use

422 Committee and conducted in accordance with the guidelines established by the

423 Institutional Animal Care and Use Committee and Laborator Committee and conducted in accordance with the guidelines established by the
423 Institutional Animal Care and Use Committee and Laboratory Animal Resource (
424 All mice experiments were performed on age-matched adult mal
- Institutional Animal Care and Use Committee and Laboratory Animal Resource Center.

424 All mice experiments were performed on age-matched adult male and female mice at a

425 starting age between 8 and 14 weeks old. Litte
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- All mice experiments were performed on age-matched adult male and female mice at a
425 Starting age between 8 and 14 weeks old. Littermate controls were used for all
426 Sexperiments when feasible. Mice were bred in-house starting age between 8 and 14 weeks old. Littermate controls were used for all

426 experiments when feasible. Mice were bred in-house and backcrossed over 10

427 generations to C56BL/6 breeders obtained from Jackson labs experiments when feasible. Mice were bred in-house and backcrossed over 10
427 generations to C56BL/6 breeders obtained from Jackson labs. Experimental mic
428 co-housed to maintain the same microbiome. They were maintaine generations to C56BL/6 breeders obtained from Jackson labs. Experimental mice were

428 co-housed to maintain the same microbiome. They were maintained in a temperature

429 (21°C) - and light (12h light/dark cycle)-contro
- co-housed to maintain the same microbiome. They were maintained in a temperature

429 (21°C) and light (12h light/dark cycle)-controlled environment and were provided with

430 food and water *ad libidum*. The following
- 429 (21°C) and light (12h light/dark cycle)-controlled environment and were provided with
430 food and water *ad libidum*. The following mouse strains are used: C57BL/6J (JAX
431 #000664), *Foxp3*-DTR (B6.129(Cg)-Foxp3tm
-
-
- *Foxp3*eGFP-Cre-ERT2
- 430 food and water *ad libidum.* The following mouse strains are used: C57BL/6J (JAX
431 #000664), *Foxp3*-DTR (B6.129(Cg)-Foxp3tm3 (Hbegf/GFP)Ayr/J, JAX# 016958),
432 *Foxp3^{eGFP-Cre-ERT2 (Foxp3tm9 (EGFP/cre/ERT2)Ayr/J; J*} 431 #000664), *Foxp3*-DTR (B6.129(Cg)-Foxp3tm3 (Hbegf/GFP)Ayr/J, JAX# 016958),
432 *Foxp3*^{eGFP-Cre-ERT2} (Foxp3tm9 (EGFP/cre/ERT2)Ayr/J; JAX#016961), Ai9 (B6.Cg-
433 Gt(ROSA)26Sor^{tm14(CAG-tdTomato)Hze}/J; JAX#007914), Fo 432 Foxp3^{eGFP-Cre-ER12} (Foxp3tm9 (EGFP/cre/ERT2)Ayr/J; JAX#016961), Ai9 (B6.Cg-
433 Gt(ROSA)26Sor^{tm14(CAG-tdTomato)Hze}/J; JAX#007914), Four Core Genotypes (B6.Cg-
434 Tg(Sry)2Ei Srydl1Rlb T(XTmsb4x-Hccs;Y)1Dto/ArnoJ; J *Gt(ROSA)26Sortm14(CAG-tdTomato)Hze*
-
-
- 433 *Gt(ROSA)26Sor^{tm14(CAG-ta1omato)Hze*/J; JAX#007914), Four Core Genotypes (B6.Cg-
434 Tg(Sry)2Ei Srydl1Rlb T(XTmsb4x-Hccs;Y)1Dto/ArnoJ; JAX#010905), Penk-IRES2-
435 Cre(B6;129S-Penktm2(cre)Hze/J; JAX#025112), Rosa26-IS} 436 Gt(ROSA)26Sortm1(HBEGF)Awai/J;JAX#008040),CD45.1 (B6.SJL-Ptprca Pepcb/BoyJ;
437 JAX# 002014), B6C3F1/J (JAX#100010), *Rag2^{-/-}* (B6.Cg-Rag2tm1.1Cgn/J;
438 JAX#008449), Mrgprd-CreERT2 (Mrgprdtm1.1(cre/ERT2)Wgl/J; JAX# 436 Gt(ROSA)26Sortm1(HBEGF)Awai/J;JAX#008040),CD45.1 (B6.SJL-Ptprca Pepcb/BoyJ;
437 JAX# 002014), B6C3F1/J (JAX#100010), *Rag2^{-/-} (*B6.Cg-Rag2tm1.1Cgn/J;
438 JAX#008449), Mrgprd-CreERT2 (Mrgprdtm1.1(cre/ERT2)Wql/J; J
- JAX# 002014), B6C3F1/J (JAX#100010), *Rag2^{-/-}* (B6.Cg-Rag2tm1.1Cgn/J;
- 438 JAX#008449), Mrgprd-CreERT2 (Mrgprdtm1.1(cre/ERT2)Wql/J; JAX# 031286),
439 Oprd1fl/fl (B6;129-Oprd1tm1.1Cgrf/KffJ; JAX# 030075), DOR-eGFP (B6;129S2-
- 437 JAX# 002014), B6C3F1/J (JAX#100010), *Rag2^{-/-} (*B6.Cg-Rag2tm1.1Cgn/J;
438 JAX#008449), Mrgprd-CreERT2 (Mrgprdtm1.1(cre/ERT2)Wql/J; JAX# 0312
439 Oprd1fl/fl (B6;129-Oprd1tm1.1Cgrf/KffJ; JAX# 030075), DOR-eGFP (B6;12
4 438 JAX#008449), Mrgprd-CreERT2 (Mrgprdtm1.1(cre/ERT2)Wql/J; JAX# 031286),
439 Oprd1fl/fl (B6;129-Oprd1tm1.1Cgrf/KffJ; JAX# 030075), DOR-eGFP (B6;129S2-
440 Oprd1tm2Kff/J; JAX#029012). *Penk^{-/-}* were kindly provided by D
- Oprd1tm2Kff/J; JAX#029012). *Penk-/-*
- 439 Oprd1fl/fl (B6;129-Oprd1tm1.1Cgrf/KffJ; JAX# 030075), DOR-eGFP (B6;129S2-
440 Oprd1tm2Kff/J; JAX#029012). *Penk^{-/-}* were kindly provided by Dr. John Pintar on
441 C57BL/6 background (MGI 3628668)⁶⁰. *Foxp3*-DTR (X-440 Oprd1tm2Kff/J; JAX#029012). *Penk^{-/-}* were kindly provided by Dr. John Pintar on a
441 C57BL/6 background (MGI 3628668)⁶⁰. *Foxp3*-DTR (X-linked) was mated with mal
442 Four Core Genotypes XY^{Sry-}Sry^{Tg} and the X 441 C57BL/6 background (MGI 3628668)⁶⁰. *Foxp3*-DTR (X-linked) was mated with male
442 Four Core Genotypes XY^{Sry-}Sry^{Tg} and the X^{Foxp3-DTRYSry-Sry^{Tg} male mice were mated to
443 **homozygous** *Foxp3***-DTR female mice**.}
- Four Core Genotypes XY^{Sry-}Sry^{Tg} and the X^{Foxp3-DTRYSry-Sry^{Tg}} Four Core Genotypes XY^{Sry-}Sry^{1g} and the X^{Foxp3-DTRYSry-Sry^{1g} male mice were mated to
443 homozygous *Foxp3*-DTR female mice.
445 **Bone marrow transplantation**
446 CD45 mismatched host recipient mice were irradiated}
-
-

- 143 homozygous *Foxp3*-DTR female mice.
1445 **Bone marrow transplantation**
146 CD45 mismatched host recipient mice v
147 and iniected retro-orbitally with 5x10⁶ ce 444
445
446
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448 445 **Bone marrow transplantation**
446 **CD45** mismatched host recipier
447 and injected retro-orbitally with
448 *Penk^{Cre};Rosa26*^{DTR} *Penk^{-/-}* or a
449 week old mice. Mice were kept 446 CD45 mismatched host recipient mice were irradiated at 550 cGy twice, 5 hours apart
447 and injected retro-orbitally with $5x10^6$ cells from the bone marrow of CD45.1 WT,
448 Penk^{Cre}; Rosa26^{DTR} Penk^Z or a 1:1 m and injected retro-orbitally with 5x10 6 and injected retro-orbitally with 5x10° cells from the bone marrow of CD45.1 WT,
448 Penk^{Cre}; Rosa26^{DTR} Penk^{-/-} or a 1:1 mix of Foxp3-DTR and Penk^{-/-} sex-matched 7-
449 week old mice. Mice were kept on doxycycline *Penk*Cre;*Rosa26*DTR *Penk-/-* or a 1:1 mix of *Foxp3*-DTR and *Penk-/-* Penk^{Cre}; Rosa26^{OTR} Penk²⁺ or a 1:1 mix of Foxp3-DTR and Penk²⁺ sex-matched 7-10
449 week old mice. Mice were kept on doxycycline chow for the first week (Bioserv #S38
450 and chimerism was assessed at 8 weeks postweek old mice. Mice were kept on doxycycline chow for the first week (Bioserv #S3888)
450 and chimerism was assessed at 8 weeks post-transplant.
451 **Pharmacological interventions**
453 Animals were randomly assigned to veh
-

- at and chimerism was assessed at 8 weeks post-transplant.
451
Pharmacological interventions
453 Animals were randomly assigned to vehicle control or treatment group. Pegylated
454 diphtheria toxin (peaDT) was a generous 452
453
454
455 452 **Pharmacological interventions**
453 Animals were randomly assigned
454 diphtheria toxin (pegDT) was a ge
455 previously described⁶¹. pegDT (20
456 (PBS) control were iniected intratl
- Animals were randomly assigned to vehicle control or treatment group. Pegylated
454 diphtheria toxin (pegDT) was a generous gift from Ana I. Domingos and generated
455 previously described⁶¹. pegDT (20 ng) or correspondi
- previously described⁶¹
- diphtheria toxin (pegDT) was a generous gift from Ana I. Domingos and generated as
455 previously described⁶¹. pegDT (20 ng) or corresponding phosphate-buffered saline
456 (PBS) control were injected intrathecally (IT) i (PBS) control were injected intrathecally (IT) in a volume of 5µL in naive mice below
457 lumbar level L4. All intrathecal injections were performed in non-anesthetized, lightly
458 restrained mice and injections were vali
-
- lumbar level L4. All intrathecal injections were performed in non-anesthetized, lightly
458 restrained mice and injections were validated by a sudden flick of the tail. Of note, the
459 10 µL injection distributes predomin 458 restrained mice and injections were validated by a sudden flick of the tail. Of note, the 5-
459 10 µL injection distributes predominantly to the lumbo-sacral cord given lidocaine IT
injection at that segment paralyzes
- lumbar level L4. All intrathecal injections were performed in non-anesthetized, lightly

458 restrained mice and injections were validated by a sudden flick of the tail. Of note, the

459 10 µL injection distributes predom 10 µL injection distributes predominantly to the lumbo-sacral cord given lidocaine IT
160 injection at that segment paralyzes the hindpaws but not forepaws. Non-pegylated
160 injection at that segment paralyzes the hindpaw 460 injection at that segment paralyzes the hindpaws but not forepaws. Non-pegylated

-
- diphtheria toxin (30 ng/g, Sigma Cat#322326) or corresponding saline control were

462 administered in a volume of 200 μl every three days intraperitoneally (IP). IL-2 (0.1 μ

7 Peprotech, Cat# 212-12) or PBS vehicle cont
-
-
- administered in a volume of 200 μl every three days intraperitoneally (IP). IL-2 (0.1 μg,

Peprotech, Cat# 212-12) or PBS vehicle control were administered daily for three

consecutive days IT. Selective δOR agonist, [D-A Peprotech, Cat# 212-12) or PBS vehicle control were administered daily for three
464 consecutive days IT. Selective δOR agonist, [D-Ala2]-Deltorphin 2 (15 μg, Abcam
465 #ab120708, CAS 122752-16-3) and selective δOR antago 464 consecutive days IT. Selective δOR agonist, [D-Ala2]-Deltorphin 2 (15 μg, Abcam Cat
465 #ab120708, CAS 122752-16-3) and selective δOR antagonist, naltrindole (5 μg, Sigma
466 Cat# N115) administrations were performed 465 #ab120708, CAS 122752-16-3) and selective δOR antagonist, naltrindole (5 μg, Sigma
466 Cat# N115) administrations were performed 30 min before behavior experiments.
468 **Tamoxifen injections**
Mrgpro^{CreERT2}:Oprd1^{fi}
-
-

- 466 Cat# N115) administrations were performed 30 min before behavior experiments.
467 **Tamoxifen injections**
469 *Mrgprd^{CreERT2};Oprd1^{fI/fI} and Foxp3^{eGFP-Cre-ERT2};Rosa26^{tdTomato} mice were injected IP
470 tamoxifen (* 468
469
470
471 468 **Tamoxifen injections**
469 *Mrgprd^{CreERT2};Oprd1^{ft/ft}
470 tamoxifen (Sigma Cat #
471 consecutive days to inc
472 Mrgprd^{CreERT2};Oprd1^{fl/fl} and <i>Foxp3^{eGFP-Cre-ERT2};Rosa26^{tdTomato} mice were injected IP with*
470 tamoxifen (Sigma Cat #5648) 100 mg/kg in corn oil (Sigma Cat #8267) for five
471 consecutive days to induce Cre-media
- tamoxifen (Sigma Cat #5648) 100 mg/kg in corn oil (Sigma Cat #8267) for five
471 consecutive days to induce Cre-mediated recombination.
472 **Intrathecal dye tracing**
-
-

- 471 consecutive days to induce Cre-mediated recombination.
472 **Intrathecal dye tracing**
474 Naive mice were intrathecally injected with 5µL of Evans I
475 #E2129) or pegylated DyLight 650-4xPEG NHS Ester (Th 473 **Intrathecal dye tracing**
474 Naive mice were intrathe
475 #E2129) or pegylated Dy
476 24 hours post-injection, r
477 decapitation. Spinal cord
- 474
475
476
-
- #E2129) or pegylated DyLight 650-4xPEG NHS Ester (Thermo Fischer, Cat #62274).
476 24 hours post-injection, mice were anesthetized with avertin and euthanized by
477 decapitation. Spinal cord and brain meninges, spinal cor #E2129) or pegylated DyLight 650-4xPEG NHS Ester (Thermo Fischer, Cat #62274).
476 24 hours post-injection, mice were anesthetized with avertin and euthanized by
decapitation. Spinal cord and brain meninges, spinal cord, b
- 24 hours post-injection, mice were anesthetized with avertin and euthanized by
477 decapitation. Spinal cord and brain meninges, spinal cord, brain, dorsal root gar
478 trigeminal ganglia, sciatic nerve and lymph nodes wer 477 decapitation. Spinal cord and brain meninges, spinal cord, brain, dorsal root ganglia and
478 trigeminal ganglia, sciatic nerve and lymph nodes were assessed for dye uptake.
480 **Animal behavior**
481 For all behavioral
-

- 178 trigeminal ganglia, sciatic nerve and lymph nodes were assessed for dye uptake.
179
180 **Animal behavior**
181 For all behavioral tests, the experimenter was blind to genotype and treatment an
182 performed during the l
- 479
480
481
482
483
- 480 **Animal behavior**
481 For all behavioral 1
482 performed during 1
483 experimenters but
484 investigator⁶². 481 For all behavioral tests, the experimenter was blind to genotype and treatment and
482 performed during the light cycle. The project utilized both male and female
483 experimenters but a predominant number of experimen 482 performed during the light cycle. The project utilized both male and female
483 experimenters but a predominant number of experiments were performed k
485 **von Frev measurement of mechanical hvpersensitivitv** 483 experimenters but a predominant number of experiments were performed by a female
484 investigator⁶².
485 **von Frey measurement of mechanical hypersensitivity**
487 Mice were acclimatized once to the von Frey apparatus investigator 62 .
-

- 484 investigator⁶².
485 **von Frey measurement of mechanical hypersensitivity**
487 Mice were acclimatized once to the von Frey apparatus for
488 plantar surface of the ipsilateral and contralateral hind paws 486
487
488
489
-
- Mice were acclimatized once to the von Frey apparatus for two hours. The lateral

488 plantar surface of the ipsilateral and contralateral hind paws (sural innervation) was

489 stimulated with von Frey hairs of logarithmi
- plantar surface of the ipsilateral and contralateral hind paws (sural innervation) was

489 stimulated with von Frey hairs of logarithmically increasing stiffness (Stoelting Cat #

490 58011). Animals were habituated on a
- plantar surface of the ipsilateral and contralateral hind paws (sural innervation) was

489 stimulated with von Frey hairs of logarithmically increasing stiffness (Stoelting Cat #

490 58011). Animals were habituated on a 489 stimulated with von Frey hairs of logarithmically increasing stiffness (Stoelting Cat # 58011). Animals were habituated on a wire mesh for 1 hour, after which they were tested with von Frey filaments (0.008, 0.02, 0.0
- g) using the Dixon up–down method^{63,64}
- 490 58011). Animals were habituated on a wire mesh for 1 hour, after which they were
491 tested with von Frey filaments (0.008, 0.02, 0.04, 0.07, 0.16, 0.4, 0.6, 1, 1.4, 2, 4 a
492 g) using the Dixon up–down method^{63,64} tested with von Frey filaments (0.008, 0.02, 0.04, 0.07, 0.16, 0.4, 0.6, 1, 1.4, 2, 4 and 8
492 g) using the Dixon up–down method^{63,64}. The von Frey hairs were held for 3 sec with
493 intervals of several minutes between
-
- q q) using the Dixon up-down method^{63,64}. The von Frey hairs were held for 3 sec with

493 intervals of several minutes between each stimulation. For the Dixon up-down method

494 we recorded 2 days of baseline mechanic intervals of several minutes between each stimulation. For the Dixon up-down method
494 we recorded 2 days of baseline mechanical sensitivity which were averaged. After SNI
195 nearly all mice reached a 50% paw withdrawal we recorded 2 days of baseline mechanical sensitivity which were averaged. After SNI,

195 nearly all mice reached a 50% paw withdrawal threshold of the lowest filament (0.008

196 g), thus we utilized a single fiber metho
-
- nearly all mice reached a 50% paw withdrawal threshold of the lowest filament (0.008 g), thus we utilized a single fiber method of testing to achieve resolution of allodynia severity. Mice were stimulated 10 times with the 496 g), thus we utilized a single fiber method of testing to achieve resolution of allodynia
497 severity. Mice were stimulated 10 times with the 0.008 g filament. The filament was
498 applied for 3 seconds and the number 497 severity. Mice were stimulated 10 times with the 0.008 g filament. The filament was
498 applied for 3 seconds and the number of positive responses across the 10 stimulation
499 were registered as percent nociceptive re
-
-

- applied for 3 seconds and the number of positive responses across the 10 stimulations
499 were registered as percent nociceptive responses.
500 **Hargreaves measurement of heat hypersensitivity**
502 Mice were acclimatized f were registered as percent nociceptive responses.
500
501 **Hargreaves measurement of heat hypersensitivity**
502 Mice were acclimatized for 30 min in plexiglass cylinde
503 the glass of a Hargreaves apparatus and the latenc 501
502
503
504
-
- 502 Mice were acclimatized for 30 min in plexiglass cylinders. The mice were then placed on
503 the glass of a Hargreaves apparatus and the latency to withdraw the paw from the heat
504 source was recorded. Each paw was te 503 the glass of a Hargreaves apparatus and the latency to withdraw the paw from the heat
504 Source was recorded. Each paw was tested three times and latencies were averaged
505 Over the trials. 503 the glass of a Hargreaves apparatus and the latency to withdraw the paw from the heat
504 source was recorded. Each paw was tested three times and latencies were averaged
505 over the trials.
506 504 source was recorded. Each paw was tested three times and latencies were averaged
505 over the trials.
506
- 505 over the trials.
-

507 **Acetone induced cold sensitivity**
508 Mice were habituated for 60 min on
509 to spray 50 µl of acetone (Thermo S
510 of the paw and the behaviors were \
511 a Sony HDR-CX440 camera. The le Mice were habituated for 60 min on a mesh in plexiglass cylinders. A syringe was used
509 to spray 50 µl of acetone (Thermo Scientific Cat # 423240010) onto the plantar surface
510 of the paw and the behaviors were video r

- to spray 50 µl of acetone (Thermo Scientific Cat # 423240010) onto the plantar surface
510 of the paw and the behaviors were video recorded for 30 seconds after each trial using
511 a Sony HDR-CX440 camera. The left hind p 510 of the paw and the behaviors were video recorded for 30 seconds after each trial using
511 a Sony HDR-CX440 camera. The left hind paw was tested five times and positive
512 responses included withdrawals, shakes, licks 511 a Sony HDR-CX440 camera. The left hind paw was tested five times and positive
512 responses included withdrawals, shakes, licks and jumps. Results are displayed a
513 total number of behaviors across the five trials.
5 responses included withdrawals, shakes, licks and jumps. Results are displayed as the
513 total number of behaviors across the five trials.
515 **Tail flick measurement of heat hypersensitivity**
516 Mice were placed in a re
-
-

513 total number of behaviors across the five trials.
514 **Tail flick measurement of heat hypersensitiv**
516 Mice were placed in a restrainer and 2 cm of the
517 water bath. The latency (seconds) to withdraw t 514
515
516
517
518 **Tail flick measurement of heat hypersensitivity**
516 Mice were placed in a restrainer and 2 cm of the tip
517 water bath. The latency (seconds) to withdraw the t
518 cut-off of 15 s was set to prevent tissue damage an
519 517 water bath. The latency (seconds) to withdraw the tail from the water was recorded. A
518 cut-off of 15 s was set to prevent tissue damage and testing was performed with
intervals of several minutes between each stimul water bath. The latency (seconds) to withdraw the tail from the water was recorded. A

s18 cut-off of 15 s was set to prevent tissue damage and testing was performed with

intervals of several minutes between each stimulat 518 cut-off of 15 s was set to prevent tissue damage and testing was performed with
519 intervals of several minutes between each stimulation. Mice were tested three tin
520 withdrawal latencies were averaged.
521 **Hot pla**

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521
522 intervals of the seasurement of heat hypersensitivity
523 In Mice were acclimated to the testing environment as described above. The hot plate 522 Hot plate measurement of heat hypersensitivity
523 Mice were acclimated to the testing environment as
524 temperature was set to 52°C. The mouse was place 522
523
524
525 **Hot plate measurement of heat hypersensitivity**
523 Mice were acclimated to the testing environment as
524 temperature was set to 52°C. The mouse was place
525 shake, lick or bite a hindpaw was scored. A cut-off d
526 dam

523 Mice were acclimated to the testing environment as described above. The hot plate
524 temperature was set to 52°C. The mouse was placed on the plate and the latency to
525 shake, lick or bite a hindpaw was scored. A cu 524 temperature was set to 52°C. The mouse was placed on the plate and the latency to
525 shake, lick or bite a hindpaw was scored. A cut-off of 20 s was set to prevent tissue
527 **Pin prick withdrawal test** 525 shake, lick or bite a hindpaw was scored. A cut-off of 20 s was set to prevent tissue
526 damage.
523 **Pin prick withdrawal test**
529 Mice were habituated for 60 min on a mesh in plexiglass cvlinders. A 27G needle wa

damage.
527
528 **Pin prick withdrawal test**
529 Mice were habituated for 60 min on a mesh in plexiglass cylinders. A 27G needle was
530 gentle applied onto the hindpaws, with minutes between each stimulation for a total of 528
529
530
531 528 **Pin prick withdrawal test**
529 Mice were habituated for 60
530 gentle applied onto the hino
531 stimulations per paw. Mice
532 paw for 1 sec. 3: mice liftino

Mice were habituated for 60 min on a mesh in plexiglass cylinders. A 27G needle was
530 gentle applied onto the hindpaws, with minutes between each stimulation for a total of
531 stimulations per paw. Mice were scored as f 531 stimulations per paw. Mice were scored as follow: 1: brief withdrawal, 2: mice lifting their
532 paw for 1 sec, 3: mice lifting their paw for 2 sec or shake, 4: lick. Results are displayed
533 as the total number of be 532 paw for 1 sec, 3: mice lifting their paw for 2 sec or shake, 4: lick. Results are displayed
533 as the total number of behaviors across the five trials.
534 **Brush withdrawal test** 532 paw for 1 sec, 3: mice lifting their paw for 2 sec or shake, 4: lick. Results are displayed
533 as the total number of behaviors across the five trials.
535 **Brush withdrawal test**
536 Mice were habituated for 60 min o

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533 as the total number of behaviors across the five trials.
534 **Brush withdrawal test**
535 **Brush withdrawal test**
536 Mice were habituated for 60 min on a mesh in plexigla
537 aently applied onto the hindpaws, with minu 536
537
538 535 **Brush withdrawal test**
536 Mice were habituated fo
537 gently applied onto the h
538 stimulations per paw. Mi
539 paw for 1 sec. 3: mice lif

537 gently applied onto the hindpaws, with minutes between each stimulation for a total of 5
538 stimulations per paw. Mice were scored as follow: 1: brief withdrawal, 2: mice lifting their
539 paw for 1 sec, 3: mice lifti

- 538 stimulations per paw. Mice were scored as follow: 1: brief withdrawal, 2: mice lifting their
539 paw for 1 sec, 3: mice lifting their paw for 2 sec or shake, 4: lick. Results are displayed
540 as the total number of be 538 stimulations per paw. Mice were scored as follow: 1: brief withdrawal, 2: mice lifting their
539 paw for 1 sec, 3: mice lifting their paw for 2 sec or shake, 4: lick. Results are displayed
540 as the total number of be
-
-

- 539 paw for 1 sec, 3: mice lifting their paw for 2 sec or shake, 4: lick. Results are displayed
540 as the total number of behaviors across the five trials.
542 **Rotarod Mice were acclimatized to the testing room and train** 542 **Rotarod**
543 Mice were acclimatized to the testing room and trained by placing them on an
544 accelerating rotarod for a maximum of 60 seconds at low speed, three times with
- 542
543
544
545
- 542 **Rotarod**
543 Mice wer
545 training ta
545 cutoff of 3 Mice were acclimatized to the testing room and trained by placing them on an
544 accelerating rotarod for a maximum of 60 seconds at low speed, three times w
545 training taking place on two consecutive days. Latency to fa 544 accelerating rotarod for a maximum of 60 seconds at low speed, three times with
545 training taking place on two consecutive days. Latency to fall was measured with
546 cutoff of 300 seconds. The procedure was repeated
- 545 training taking place on two consecutive days. Latency to fall was measured with a
546 cutoff of 300 seconds. The procedure was repeated three times and latencies avera
543 **Spared Nerve injury**
-
-

- 546 cutoff of 300 seconds. The procedure was repeated three times and latencies averag
547 across trials.
548 **Spared Nerve injury**
550 We employed an established, robust and reliable spared nerve injury (SNI) model to 547 across trials.
548 **Spared Nerv**
550 We employed
551 induce a chro
- 549
550
551
552 549 **Spared Nerve injury**
550 We employed an esta
551 induce a chronic neuro
552 intervention on two bra
- 550 We employed an established, robust and reliable spared nerve injury (SNI) model to
551 induce a chronic neuropathic injury⁶⁵. This model utilizes non-healing surgical
intervention on two branches of the sciatic nerve induce a chronic neuropathic injury⁶⁵. This model utilizes non-healing surgical
552 intervention on two branches of the sciatic nerve (the common peroneal and ti intervention on two branches of the sciatic nerve (the common peroneal and tibial
15

553 branches), while sparing the third branch (the sural branch) for sensory testing on the
554 lateral portion of the hindpaw. Briefly, mice were anesthetized with isoflurane (3% for
555 induction and 1.5% for maintenance 557 alternatively. A 1 cm incision was performed on the upper thigh skin, near the division
558 point of the sciatic nerve. A 2% lidocaine solution was applied and the biceps femoris induction and 1.5% for maintenance, mixed with oxygen). The fur on the left hind leg
556 was shaved and disinfected with 3 passages of alcohol and iodine solution,
557 alternatively. A 1 cm incision was performed on the up was shaved and disinfected with 3 passages of alcohol and iodine solution,
557 alternatively. A 1 cm incision was performed on the upper thigh skin, near th
558 point of the sciatic nerve. A 2% lidocaine solution was appli alternatively. A 1 cm incision was performed on the upper thigh skin, near the division
558 point of the sciatic nerve. A 2% lidocaine solution was applied and the biceps femoris
559 muscle was gently separated through a b point of the sciatic nerve. A 2% lidocaine solution was applied and the biceps femoris

559 muscle was gently separated through a blunt opening to reveal the sciatic nerve's

560 common peroneal, tibial, and sural branches muscle was gently separated through a blunt opening to reveal the sciatic nerve's
560 common peroneal, tibial, and sural branches. The common peroneal and tibial ner
561 were ligated with non-dissolvable 8-0 silk sutures (common peroneal, tibial, and sural branches. The common peroneal and tibial nerves
561 were ligated with non-dissolvable 8-0 silk sutures (Fine Science Tools Cat # 12052-08).
562 Subsequently, a 2 mm segment from both the

were ligated with non-dissolvable 8-0 silk sutures (Fine Science Tools Cat # 12052-08).
562 Subsequently, a 2 mm segment from both the common peroneal and tibial nerves was
563 transected, ensuring the sural nerve remained 562 Subsequently, a 2 mm segment from both the common peroneal and tibial nerves was
563 transected, ensuring the sural nerve remained undisturbed. The muscle and the skin
564 were stitched using 6–0 sutures (Henry Schein

transected, ensuring the sural nerve remained undisturbed. The muscle and the skin

564 were stitched using 6–0 sutures (Henry Schein Surgical suture Cat #101-2636), and t

565 skin was further sealed with a tissue adhesiv

were stitched using 6–0 sutures (Henry Schein Surgical suture Cat #101-2636), and the
565 skin was further sealed with a tissue adhesive (3M Vetbond Cat # 1469SB), after an
566 ethanol solution application. Mice were kept 565 skin was further sealed with a tissue adhesive (3M Vetbond Cat # 1469SB), after an
566 ethanol solution application. Mice were kept on heating pad until they regained
567 consciousness and demonstrated stable, balanced

consciousness and demonstrated stable, balanced locomotion. Mice were transferred
into their home cage and observed meticulously for the next two days.
Immunohistochemistry

571 Avertin-anesthetized mice were transcardially perfused with 10 ml of 1x PBS followed into their home cage and observed meticulously for the next two days.
569
570 **Immunohistochemistry**
571 Avertin-anesthetized mice were transcardially perfused with 10 ml of 1:
572 by 30 ml of 4% paraformaldehyde (PFA. The 570
571
572
573 570 **Immunohistochemistry**
571 Avertin-anesthetized mic
572 by 30 ml of 4% paraform
573 PBS. After perfusion, spii
574 DRG were collected. pos

571 Avertin-anesthetized mice were transcardially perfused with 10 ml of 1x PBS followed
572 by 30 ml of 4% paraformaldehyde (PFA, Thermo Scientific Cat # 119690010) diluted ir
573 PBS. After perfusion, spinal cord, sciat

572 by 30 ml of 4% paraformaldehyde (PFA, Thermo Scientific Cat # 119690010) diluted in
573 PBS. After perfusion, spinal cord, sciatic nerves, lymph nodes, spleens, brains and
574 DRG were collected, postfixed in 4% PFA s

573 PBS. After perfusion, spinal cord, sciatic nerves, lymph nodes, spleens, brains and
574 DRG were collected, postfixed in 4% PFA solution at 4°C for 5⊡h and then
575 cryoprotected in 30% sucrose in PBS at 4°C.
577 Spin

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-

DRG were collected, postfixed in 4% PFA solution at 4°C for 5□h and then
575 cryoprotected in 30% sucrose in PBS at 4°C.
576 Spinal meninges were harvested from fixed spinal cords. Spinal cords were transferred
578 in PBS 578 in PBS and meninges were gently peeled into a single sheet onto a microscope slide
579 after a longitudinal hemisection of the spinal cord. Brain meninges were similarly 577
578
579
580 S77 Spinal meninges were harvested from fixed spinal cords. Spinal cords were transferred
578 in PBS and meninges were gently peeled into a single sheet onto a microscope slide
579 after a longitudinal hemisection of the 578 in PBS and meninges were gently peeled into a single sheet onto a microscope slide
579 after a longitudinal hemisection of the spinal cord. Brain meninges were similarly
580 harvested from the skull. Frozen tissues we after a longitudinal hemisection of the spinal cord. Brain meninges were similarly

harvested from the skull. Frozen tissues were embedded at −35°C in O.C.T. com

and 30□μm transverse spinal cord sections were generated 580 harvested from the skull. Frozen tissues were embedded at –35°C in O.C.T. compound
581 and 30⊡µm transverse spinal cord sections were generated using a Leica SM220R
582 sliding microtome and 20 µm DRG sections were ge and 30□µm transverse spinal cord sections were generated using a Leica SM220R
582 sliding microtome and 20 µm DRG sections were generated using a cryostat (Therm
583 Fisher Scientific) on SuperFrost Plus slides. Spinal c sackspoke and 20 pm DRG sections were generated using a cryostat (Thermo

583 Fisher Scientific) on SuperFrost Plus slides. Spinal cord sections were processed as

584 free-floating. Sections were blocked (10% NGS, 1% BSA, Fisher Scientific) on SuperFrost Plus slides. Spinal cord sections were processed as
584 free-floating. Sections were blocked (10% NGS, 1% BSA, 0.05% Tween-20, 0.1% Tri
585 X-100 in PBS) and incubated in 0.3 M glycine cont 584 free-floating. Sections were blocked (10% NGS, 1% BSA, 0.05% Tween-20, 0.1% T
585 X-100 in PBS) and incubated in 0.3 M glycine containing 0.2% Tween 20. Sections
586 were labeled in blocking buffer for 24 hours at 4°C. 586 were labeled in blocking buffer for 24 hours at 4°C. Slides were coverslipped with
587 Fluoromount-G (Thermo Fisher Scientific). Fluorescence images were acquired us
588 an Olympus FV3000 confocal microscope and quanti Fluoromount-G (Thermo Fisher Scientific). Fluorescence images were acquired using
an Olympus FV3000 confocal microscope and quantified using ImageJ (Fiji).
Tissue clearing 587 Fluoromount-G (Thermo Fisher Scientific). Fluorescence images were acquired using
588 an Olympus FV3000 confocal microscope and quantified using ImageJ (Fiji).
590 **Tissue clearing**
591 Whole DRG or spinal cords 588 an Olympus FV3000 confocal microscope and quantified using ImageJ (Fiji).
589 **Tissue clearing**
591 Whole DRG or spinal cords from *Foxp3^{eGFP-Cre-ERT2*;Ai9 mice were cleared aft
592 fixation using SHIELD tissue}

Whole DRG or spinal cords from *Foxp3*eGFP-Cre-ERT2

590
591
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593 590 **Tissue clearing**
591 Whole DRG or sp
592 fixation using SH
593 PBS then proces
594 epoxy solution (S Whole DRG or spinal cords from *Foxp3*^{eGFP-Cre-ER12};Ai9 mice were cleared after PFA
592 fixation using SHIELD tissue clearing (LifeCanvas PCK-500)⁶⁶. Tissues were washed
593 PBS then processed as previously described⁶ fixation using SHIELD tissue clearing (LifeCanvas PCK-500) 66

PBS then processed as previously described⁶⁶

fixation using SHIELD tissue clearing (LifeCanvas PCK-500)⁶⁶. Tissues were washed in
593 . PBS then processed as previously described⁶⁶. Briefly, the tissues were incubated in
594 . epoxy solution (SHIELD OFF) for 10 h overnight at 37°C in SHIELD ON-Epoxy solution for epoxy crosslinking. DRG were then

- space oppoxy solution (SHIELD OFF) for 10 hours at 4°C with gentle shaking then incubated
595 overnight at 37°C in SHIELD ON-Epoxy solution for epoxy crosslinking. DRG were then
596 further incubated in SHIELD ON solution 595 overnight at 37°C in SHIELD ON-Epoxy solution for epoxy crosslinking. DRG were then
596 further incubated in SHIELD ON solution for 10h and delipidated for two days (DRG) to
597 five days (spinal cord) at 45°C with sha 596 further incubated in SHIELD ON solution for 10h and delipidated for two days (DRG) to
597 five days (spinal cord) at 45°C with shaking then washed with PBS. Whole mount DRG
1997 five days (spinal cord) at 45°C with sha
- 597 five days (spinal cord) at 45°C with shaking then washed with PBS. Whole mount DRG
- 598 and spinal cords were acquired in FocusClear reflexive index matching solution
599 (CelExplorer, FC-102).
600 **Tissue digestion**
602 Mice were iniected intravenously with 50 ul of anti-ARTC2 nanobody (Biolegend
-
-

599 (CelExplorer, FC-102).
600
601 **Tissue digestion**
602 Mice were injected intra
603 149802) in 200 uL of Pl 600
601
602
603
604 601 **Tissue digestion**
602 Mice were injected
603 149802) in 200 µL
604 from purinergic-me
605 intravenously with Mice were injected intravenously with 50 µl of anti-ARTC2 nanobody (Biolegend Cat # 149802) in 200 µL of PBS 30 minutes before euthanasia to protect Treg during harvest from purinergic-mediated cell death⁶⁷. 5 minutes b 149802) in 200 μ L of PBS 30 minutes before euthanasia to protect Treg during harvest
604 from purinergic-mediated cell death⁶⁷. 5 minutes before harvest mice were injected
605 intravenously with 6 μ g of FITC-conju from purinergic-mediated cell death⁶⁷ from purinergic-mediated cell death^{o'}. 5 minutes before harvest mice were injected

foos intravenously with 6 µg of FITC-conjugated anti-CD45 antibody to label blood immu

cells in 200 µL of PBS 27 . Avertin-anesthet for intravenously with 6 μg of FITC-conjugated anti-CD45 antibody to label blood immune
606 cells in 200 μL of PBS²⁷. Avertin-anesthetized mice were decapitated, and spinal cord
607 meninges, brain meninges, L4-6 DRG, cells in 200 μ L of PBS²⁷ cells in 200 μ L of PBS²⁷. Avertin-anesthetized mice were decapitated, and spinal cord

formeninges, brain meninges, L4-6 DRG, sciatic nerves, lymph nodes, brains and spleens

were harvested. Spinal cord meninges, bra 608 were harvested. Spinal cord meninges, brain meninges, DRG, sciatic nerves and brains
609 were crushed with the back of a 3ml syringe in a serrated 24 well plate and triturated in
610 digestion media (Liberase TM (0.20 were harvested. Spinal cord meninges, brain meninges, DRG, sciatic nerves and brains
609 were crushed with the back of a 3ml syringe in a serrated 24 well plate and triturated in
610 digestion media (Liberase TM (0.208 WU/ were crushed with the back of a 3ml syringe in a serrated 24 well plate and triturated in
610 digestion media (Liberase TM (0.208 WU/ml) (Roche Cat # 054010200001) and DNase
611 (40 ug/ml) (Sigma Cat # DN25) in 1.0 ml cRPM 610 digestion media (Liberase TM (0.208 WU/ml) (Roche Cat # 054010200001) and DNase 611 I (40 ug/ml) (Sigma Cat # DN25) in 1.0 ml cRPMI (RPMI supplemented with 10% (vol/vol) fetal bovine serum (FBS), 1% (vol/vol) HEPES, 1% (vol/vol) Sodium Pyru 1% (vol/vol) penicillin-streptomycin). They were digested fo 612 (vol/vol) fetal bovine serum (FBS), 1% (vol/vol) HEPES, 1% (vol/vol) Sodium Pyruvate,
613 1% (vol/vol) penicillin-streptomycin). They were digested for 30 min at 37°C, 220 RPM
614 and triturated every 15 minutes. Dige

-
- 1% (vol/vol) penicillin-streptomycin). They were digested for 30 min at 37°C, 220 RPM
614 and triturated every 15 minutes. Digested samples were again triturated and passed
615 over a 40 µm cell strainer and any remaining 614 and triturated every 15 minutes. Digested samples were again triturated and passed
615 over a 40 µm cell strainer and any remaining tissue pieces mashed through the cell
616 strainer. Cell strainers were flushed with
-
- strainer. Cell strainers were flushed with staining media (PBS w/o Mq^{2+} and 616 strainer. Cell strainers were flushed with staining media (PBS w/o Mg²⁺ and 617 Ca²⁺ supplemented with 3% FBS, 2 mM EDTA and 0.05% NaN₃). Single-cel suspensions were centrifuged at 500 g at 4°C, washed and resus
- Ca^{2+} Ca²⁺ supplemented with 3% FBS, 2 mM EDTA and 0.05% NaN₃). Single-cell
618 suspensions were centrifuged at 500 g at 4°C, washed and resuspended in s
619 media. Spleens and lymph node immune cells were obtained by mashi
- 615 over a 40 µm cell strainer and any remaining tissue pieces mashed through the cell
616 strainer. Cell strainers were flushed with staining media (PBS w/o Mg²⁺ and
617 Ca²⁺ supplemented with 3% FBS, 2 mM EDTA and 0
- 619 media. Spleens and lymph node immune cells were obtained by mashing the tissues
620 over a 40 µm cell strainer and washed with staining media.
622 **Cell stimulation** media. Spleens and lymph node immune cells were obtained by mashing the tissues
620 over a 40 µm cell strainer and washed with staining media.
622 **Cell stimulation**
623 Isolated single cell suspensions were incubated for
-

- over a 40 µm cell strainer and washed with staining media.
621 **Cell stimulation**
623 **Cell stimulation**
623 Isolated single cell suspensions were incubated for 4 hr at 3
624 (supplemented with 10% FBS. 2 mM L-alutamine. 1
- 622
623
624
625 622 **Cell stimulation**
623 Isolated single ce
625 1% penicillin-stre
626 acetate) PMA. lo
-
- 624 (supplemented with 10% FBS, 2 mM L-glutamine, 10 mM HEPES, 1% sodium pyruvate,
625 1% penicillin-streptomycin, 50 µM 2-mercaptoethanol) with (phorbol 12-myristate 13-
626 acetate) PMA, lonomycin in the presence of Bref 1% penicillin-streptomycin, 50 µM 2-mercaptoethanol) with (phorbol 12-myristate 13-
626 acetate) PMA, lonomycin in the presence of Brefeldin A and Monensin (Tonbo, TNB-
627 4975). 1% penicillin-streptomycin, 50 μM 2-mercaptoethanol) with (phorbol 12-myristate 13-
626 acetate) PMA, lonomycin in the presence of Brefeldin A and Monensin (Tonbo, TNB-
628 **Flow cytometry** 626 acetate) PMA, Ionomycin in the presence of Brefeldin A and Monensin (Tonbo, TNB-
627 4975).
628 **Flow cytometry**
630 Single-cell suspensions were stained in a 96 well plate. Briefly, they were washed wit
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627 4975).

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- 629
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631
632 629 **Flow cytometry**
630 Single-cell suspe
631 250 µL of staining
632 antibodies (1:100
633 washed twice in s
-
- 630 Single-cell suspensions were stained in a 96 well plate. Briefly, they were washed with
631 250 µL of staining media and stained with viability dye (1:500) and cell surface
632 antibodies (1:100) in 100 µL of staining 631 250 µL of staining media and stained with viability dye (1:500) and cell surface
632 antibodies (1:100) in 100 µL of staining media with Fc shield (1:100). Samples
633 washed twice in staining media and stained for in
-
- 632 antibodies (1:100) in 100 μ L of staining media with Fc shield (1:100). Samples were
633 washed twice in staining media and stained for intracellular cytokines or for Foxp3
634 according to manufacturer recommendati washed twice in staining media and stained for intracellular cytokines or for Foxp3
634 according to manufacturer recommendations with BD Cytofix/Cytoperm
635 Fixation/Permeabilization Kit (BD 554714, AB_2869008). Samples according to manufacturer recommendations with BD Cytofix/Cytoperm
635 Fixation/Permeabilization Kit (BD 554714, AB_2869008). Samples were further stained
636 with conjugated intracellular antibodies (1:100) overnight at 4
-
- Fixation/Permeabilization Kit (BD 554714, AB_2869008). Samples were further stained
636 with conjugated intracellular antibodies (1:100) overnight at 4°C in BD
637 permeabilization/wash buffer. Samples were washed with pe 636 with conjugated intracellular antibodies (1:100) overnight at 4°C in BD
637 permeabilization/wash buffer. Samples were washed with permeabiliza
638 twice and resuspended in 200 μ L of staining media. For visualizati
-
- 639 reporter signal and intranuclear Foxp3 signal, cells were fixed in 200 µL of freshly
640 prepared 2% formaldehyde (EM grade) in PBS for 60 minutes exactly and washed with
641 eBioscience Permeabilization buffer (eBiosc twice and resuspended in 200 μ L of staining media. For visualization of both tdTomato
639 reporter signal and intranuclear Foxp3 signal, cells were fixed in 200 μ L of freshly
640 prepared 2% formaldehyde (EM grade)
-
-
- For exporter signal and intranuclear Foxp3 signal, cells were fixed in 200 µL of freshly

fead prepared 2% formaldehyde (EM grade) in PBS for 60 minutes exactly and washed

eBioscience Permeabilization buffer (eBioscience 640 prepared 2% formaldehyde (EM grade) in PBS for 60 minutes exactly and washed with
641 eBioscience Permeabilization buffer (eBioscience Foxp3 perm-kit) and stained
642 overnight in 1x eBioscience Permeabilization buffe overnight in 1x eBioscience Permeabilization buffer at $4^{\circ}C^{68}$. Cells were washed twice

in the buffer and resuspended in staining media. For Met-enkephalin staining, we
 $\frac{1}{2}$
- 641 eBioscience Permeabilization buffer (eBioscience Foxp3 perm-kit) and stained
642 overnight in 1x eBioscience Permeabilization buffer at 4°C⁶⁸. Cells were washe
643 in the buffer and resuspended in staining media. Fo 643 in the buffer and resuspended in staining media. For Met-enkephalin staining, we
-
- screened multiple commercially available antibodies and selected an antibody that
645 showed positive staining in wildtype mTreg but not $Penk^{-/}$ mTreg. The antibody was
646 conjugated to fluorescent phycoerythrin with Lig showed positive staining in wildtype mTreg but not *Penk-/* showed positive staining in wildtype mTreg but not $Penk^{-/-}$ mTreg. The antibody was

conjugated to fluorescent phycoerythrin with Lightning-link conjugation kit (ab102918)

and utilized after cell stimulation and intra
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-
-
- conjugated to fluorescent phycoerythrin with Lightning-link conjugation kit (ab102918)
647 and utilized after cell stimulation and intracellular cytokine staining. Cells were counted
648 with 50 µL of counting beads (Therm and utilized after cell stimulation and intracellular cytokine staining. Cells were counted
648 with 50 μ L of counting beads (Thermo Fisher Scientific Cat # C36950) and samples
650 were analyzed using a BD FACSCanto2 o with 50 μ L of counting beads (Thermo Fisher Scientific Cat # C36950) and samples
649 were analyzed using a BD FACSCanto2 or BD FACS Aria Fusion flow cytometer (BI
650 Biosciences). Positive and negative selection gates were analyzed using a BD FACSCanto2 or BD FACS Aria Fusion flow cytometer (BD
650 Biosciences). Positive and negative selection gates were set using fluorescence minus
651 unstained cells. For negative control of enkephali
- 650 Biosciences). Positive and negative selection gates were set using fluorescence minus
651 unstained cells. For negative control of enkephalin staining, *Penk*^{-/-} samples were used
652 for gating. Fluorescence intensi unstained cells. For negative control of enkephalin staining, *Penk-/-*
- unstained cells. For negative control of enkephalin staining, *Penk^{-/-}* samples were used
652 for gating. Fluorescence intensity distribution was analyzed using FlowJo 10 software
653 (BD Biosciences). Antibodies for flo
-
- 652 for gating. Fluorescence intensity distribution was analyzed using FlowJo 10 software (BD Biosciences). Antibodies for flow cytometry are listed in the resource table. Lineaged as exclusion markers include viability dy 653 (BD Biosciences). Antibodies for flow cytometry are listed in the resource table. Lineage
654 exclusion markers include viability dye, CD11b (to exclude myeloid cells), B220 (to
655 exclude B cells), Ter119 (to exclude
-
-

- 654 exclusion markers include viability dye, CD11b (to exclude myeloid cells), B220 (to
655 exclude B cells), Ter119 (to exclude red blood cells).
657 **Cell sorting**
658 Spleens were mashed on a cell strainer and cells wer exclude B cells), Ter119 (to exclude red blood cells).
656
657 **Cell sorting**
658 Spleens were mashed on a cell strainer and cells wer
659 4°C. Cells were stained for viability and lineage exclu 656
657
658
659
660
-
- 657 **Cell sorting**
658 Spleens wer
659 4°C. Cells w
660 CD4, CD45F
661 washed and 4°C. Cells were stained for viability and lineage exclusion markers, in addition to CD45,
660 CD4, CD45RB to stain naïve Tconv and CD25 to stain Tregs. Cells were pelleted,
661 washed and incubated with CD4 negative select
-
- 661 washed and incubated with CD4 negative selection beads and purified on a LS
662 magnetic column (Miltenyi Biotec). Cells were double sorted in BD FACS Aria F
663 Singlet, Live, CD45⁺ CD4⁺ CD45RB⁺ CD25⁻ for Tcon
- 660 CD4, CD45RB to stain naïve Tconv and CD25 to stain Tregs. Cells were pelleted,
661 washed and incubated with CD4 negative selection beads and purified on a LS
662 magnetic column (Miltenyi Biotec). Cells were double so washed and incubated with CD4 negative selection beads and purified on a LS
662 magnetic column (Miltenyi Biotec). Cells were double sorted in BD FACS Aria F
663 Singlet, Live, CD45⁺ CD4⁺ CD45RB⁺ CD25⁻ for Tconv or 662 magnetic column (Miltenyi Biotec). Cells were double sorted in BD FACS Aria Fusion for
663 Singlet, Live, CD45⁺ CD4⁺ CD45RB⁺ CD25⁻ for Tconv or Singlet, Live, CD45⁺ CD4⁺
665 **T cell suppression assav** Singlet, Live, CD45⁺ CD4⁺ CD45RB⁺ CD25⁻ for Tconv or Singlet, Live, CD45⁺ CD4⁺
- CD25+ CD45RB-
- 664
665
666

- 664 CD25⁺ CD45RB⁻ for Treg into complete IMDM.
665 **T cell suppression assay**
667 Sorted Tconv from lymphoid organs of CD45.1
668 violet (Thermo-Fisher, Scientific #C34571) acco 667
668
669 666 **T cell suppression assay**
667 Sorted Tconv from lymphoio
668 violet (Thermo-Fisher, Scie
669 0.25x10⁵ Tconv per well we
670 *Penk* deficient lymphoid ord Sorted Tconv from lymphoid organs of CD45.1 female mice were labeled with cell trace
668 violet (Thermo-Fisher, Scientific #C34571) according to manufacturer instructions.
669 0.25x10⁵ Tconv per well were cultured with d violet (Thermo-Fisher, Scientific #C34571) according to manufacturer instructions.
669 0.25x10⁵ Tconv per well were cultured with distinct dilution of Treg from CD45.2 W
670 Penk deficient lymphoid organs. Cells were the $0.25x10^5$ 670 Penk deficient lymphoid organs. Cells were then washed and resuspended with mouse
671 anti-CD3/CD28 Dynabeads at a 1:1 ratio of beads to Tconv. Cells were incubated for 96
672 hours in a humidified incubator at 37° For *Penk* deficient lymphoid organs. Cells were then washed and resuspended with mouse

anti-CD3/CD28 Dynabeads at a 1:1 ratio of beads to Tconv. Cells were incubated for 96

hours in a humidified incubator at 37°C. Cells 671 anti-CD3/CD28 Dynabeads at a 1:1 ratio of beads to Tconv. Cells were incubated for 96
672 hours in a humidified incubator at 37°C. Cells were washed and resuspended in staining
673 media and suppression ratio was calc 672 hours in a humidified incubator at 37°C. Cells were washed and resuspended in staining
673 media and suppression ratio was calculated by dividing the percent proliferated cells
674 from incubated Tconv⁺ Treg samples media and suppression ratio was calculated by dividing the percent proliferated cells
674 from incubated Tconv⁺ Treg samples by percent proliferated cells from Tconv only
676 **T cell adoptive transfer** from incubated Tconv⁺ Treg samples by percent proliferated cells from Tconv only
575 samples⁶⁹.
576 **T cell adoptive transfer**
578 *Competition assav* samples⁶⁹.
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676
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679
680

- 675 samples^{s»}.
676 **T cell adoptive transfer**
678 *Competition assay*
679 1x10⁶ negatively selected bulk CD4⁺
- 677 **T cell adoptive transfer**
678 *Competition assay*
679 1x10⁶ negatively selected
680 lymphoid organs were tra
681 organs were collected 28 678 *Competition assay*
679 1x10⁶ negatively se
680 lymphoid organs w
681 organs were collect
682 1x10° negatively selected bulk CD4⁺ T cells from WT female and *Penk* deficient
680 lymphoid organs were transplanted into female *Rag2^{-/-}* mice. SNI was performed
681 organs were collected 28 days later for cell stimu lymphoid organs were transplanted into female *Rag2^{-/-}* mice. SNI was performed and
681 organs were collected 28 days later for cell stimulation and flow cytometry.
683 Graft versus host disease (GVHD)
684 GVHD was estab
-
-
- organs were collected 28 days later for cell stimulation and flow cytometry.
682
683 *Graft versus host disease (GVHD)*
684 GVHD was established as previously described. Briefly, WT or *Penk* deficient female
685 mice bone 683 *Graft versus host disease (GVHD)*
684 *GVHD was established as previous*
685 mice bone marrow was transplante
686 activate T cells. 0.25x10⁶ sorted W
687 induce chronic GVHD either withou
- 684 GVHD was established as previously described. Briefly, WT or *Penk* deficient female
685 mice bone marrow was transplanted into MHC mismatched B6C3F1/J female mice to
686 activate T cells. 0.25x10⁶ sorted WT Tconv we
- 685
686 activate T cells. 0.25x10⁶ sorted WT Tconv were transplanted into male *Rag2^{-/-}*
- 685 mice bone marrow was transplanted into MHC mismatched B6C3F1/J female mice to
686 activate T cells. 0.25x10⁶ sorted WT Tconv were transplanted into male $Rag2^{-/-}$ mice to
687 induce chronic GVHD either without Tregs 686 activate T cells. $0.25x10^6$ sorted WT Tconv were transplanted into male $Rag2^{-r}$ mice to induce chronic GVHD either without Tregs or in the presence of 0.125×10^6 WT or *Penk* deficient Tregs. Mice were measured induce chronic GVHD either without Tregs or in the presence of 0.125 $x10^6$ WT or Penk
- formation of SVHD either without Tregs or in the presence of 0.125×10^6 WT or *Penk* deficient Tregs. Mice were measured for weight changes and GVHD score. Mice were euthanized then harvested for cytokine secretion as
- 688 deficient Tregs. Mice were measured for weight changes and GVHD score. Mice were
689 euthanized then harvested for cytokine secretion assay by flow cytometry^{70,71}. GVHD
 $\frac{1}{2}$ euthanized then harvested for cytokine secretion assay by flow cytometry^{70,71}. GVHD
 18
-
- 690 scoring is as follows: $0 = no$ signs of GVHD, 1 = visible signs of GVHD (hunching, lethargy, ruffled fur), 2 = no weight gain, 3 = 0-5% weight loss, 4 = >5% weight loss
692 One Tconv mouse did not survive for harvesting
- 691 lethargy, ruffled fur), 2 = no weight gain, 3 = 0-5% weight loss, 4 = >5% weight loss.
692 One Tconv mouse did not survive for harvesting for cytokine stimulation.
693 **Analysis of sequencing data**
695 *Bulk RNA-seq* 692 One Tconv mouse did not survive for harvesting for cytokine stimulation.
693 **Analysis of sequencing data**
695 *Bulk RNA-seq*
696 Raw files GSM4677053-064 from GEO dataset GSE154680. and all files
-

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- 694 **Analysis of sequencing data**
695 *Bulk RNA-seq*
696 Raw files GSM4677053-064 fr
697 GSE180020 were gathered an
698 (outFilterMultimapNmax 1–outl
- 695 *Bulk RNA-seq*
696 Raw files GSM
697 GSE180020 w
698 (outFilterMultin
699 0000). Data wa Raw files GSM4677053-064 from GEO dataset GSE154680, and all files from GSE
697 GSE180020 were gathered and aligned using STAR for uniquely mapped reads
698 (outFilterMultimapNmax 1–outFilterMatchNmin 30–alignIntronMin 20– 697 GSE180020 were gathered and aligned using STAR for uniquely mapped reads
698 (outFilterMultimapNmax 1–outFilterMatchNmin 30–alignIntronMin 20–alignIntron
699 0000). Data was annotated with GENCODE GRCm38/mm10 genome as
- 698 (outFilterMultimapNmax 1–outFilterMatchNmin 30–alignIntronMin 20–alignIntronMax 1-
699 0000). Data was annotated with GENCODE GRCm38/mm10 genome assembly. Raw
700 count tables were normalized by median of ratios method 699 0000). Data was annotated with GENCODE GRCm38/mm10 genome assembly. Raw
700 count tables were normalized by median of ratios method with DESeq2 package from
701 Bioconductor to analyze for differential expression.
703
- 700 count tables were normalized by median of ratios method with DESeq2 package from
701 Bioconductor to analyze for differential expression.
703 ATAC-seq
704 Fastq files were gathered from SRR12264679-94 from GSE154680. R
- Bioconductor to analyze for differential expression.
-
-
-
- 705
706
-
- 705 mapped to the mouse mm10 genome assembly using STAR alignment (--
706 outFilterMismatchNoverLmax 0.04 --outFilterMismatchNmax 999 --
707 alignSJDBoverhangMin 1 --outFilterMultimapNmax 1 --alignIntronMin 20 --704 Fastq files were gathered from SRR12264679-94 from GSE154680. Raw reads were
705 mapped to the mouse mm10 genome assembly using STAR alignment (--
706 outFilterMismatchNoverLmax 0.04 --outFilterMismatchNmax 999 --
707
- 705 mapped to the mouse mm10 genome assembly using STAR alignment (-- -706 outFilterMismatchNoverLmax 0.04 --outFilterMismatchNmax 999
1707 alignSJDBoverhangMin 1 --outFilterMultimapNmax 1 --alignIntronM
1708 alignIntronMax 1000000 --alignMatesGapMax 1000000). Bam files
1709 STAR. PCR duplic alignSJDBoverhangMin 1 --outFilterMultimapNmax 1 --alignIntronMin 20 --
708 alignIntronMax 1000000 --alignMatesGapMax 1000000). Bam files were go
709 STAR. PCR duplicates were removed by Picard, and peak calling performe
7
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-
- alignIntronMax 1000000 --alignMatesGapMax 1000000). Bam files were generated by
709 STAR. PCR duplicates were removed by Picard, and peak calling performed using
710 MACS2 (--keep-dup 1 --bw 500 -n output --nomodel --extsi 709 STAR. PCR duplicates were removed by Picard, and peak calling performed using
710 MACS2 (--keep-dup 1 --bw 500 -n output --nomodel --extsize 400 --slocal 5000 --llc
711 100000 -q 0.01) PMID: 22936215). To generate bigw 710 MACS2 (--keep-dup 1 --bw 500 -n output --nomodel --extsize 400 --slocal 5000 --llocal
711 100000 -q 0.01) PMID: 22936215). To generate bigwig files for ATAC-seq datasets, all
712 aligned bam files were merged by condit
-
-
- 100000 -q 0.01) PMID: 22936215). To generate bigwig files for ATAC-seq datasets, all

112 aligned bam files were merged by condition using samtools merge. Bedtools

113 genomecov was run to covert the merged bam files into z aligned bam files were merged by condition using samtools merge. Bedtools

713 genomecov was run to covert the merged bam files into a bedgraph files. Finally,

714 bedGraphToBigWig (ucsc-tools/363) was used to generate 713 genomecov was run to covert the merged bam files into a bedgraph files. Finally,
714 bedGraphToBigWig (ucsc-tools/363) was used to generate the bigwig files display
715 browser tracks using the IGV browser and compared
- 714 bedGraphToBigWig (ucsc-tools/363) was used to generate the bigwig files displayed on
715 browser tracks using the IGV browser and compared to existing encode ATAC and
717 sc*RNA-seq*
-
-
-
- 717
718 *scRNA-seq*
719 Fastq files were gathered from GEO from datasets GSE139088 GSE201653 and initial 716 Chip-Seq peaks.
717 sc*RNA-seq*
719 Fastq files were g
720 counts were obta
- Fastq files were gathered from GEO from datasets GSE139088 GSE201653 and initial

720 counts were obtained using the Cell Ranger pipeline^{48,72}. Using Seurat v4, individual

721 cells were removed from the data set if the counts were obtained using the Cell Ranger pipeline^{48,72}
- 720
721 718 *scRNA-seq*
-
- genes/features, fewer than 1000 UMI or greater than 10% reads mapping to
723 mitochondrial genes. 2000 variable genes were found for each normalized library
724 anchors were selected for integration with dimensionality of genes/features, fewer than 1000 UMI or greater than 10% reads mapping to
723 mitochondrial genes. 2000 variable genes were found for each normalized lik
724 anchors were selected for integration with dimensionality of each
- cells were removed from the data set if they had fewer than 1000 discovered
722 genes/features, fewer than 1000 UMI or greater than 10% reads mapping to
723 mitochondrial genes. 2000 variable genes were found for each norm mitochondrial genes. 2000 variable genes were found for each normalized library, and

724 anchors were selected for integration with dimensionality of each dataset set at 30. Glia

725 cells noted for the markers of *Sparc*
- 224 anchors were selected for integration with dimensionality of each dataset set at 30. Glial

725 cells noted for the markers of *Sparc* and *Mpz* and non-neuronal cells lacking the

226 expression of Avil were excluded.
-
- cells noted for the markers of *Sparc* and *Mpz* and non-neuronal cells lacking the

reverses a expression of Avil were excluded. Variable genes were identified from the merge

dataset, and PCA and UMAP were ran to generat
-
- expression of Avil were excluded. Variable genes were identified from the merged

727 dataset, and PCA and UMAP were ran to generate new UMAP coordinates with a

728 dimensionality of 30 and clustering was performed with a dataset, and PCA and UMAP were ran to generate new UMAP coordinates with a

728 dimensionality of 30 and clustering was performed with a resolution of 0.5.

730 Findallmarkers function utilizing a Wilcoxon rank-sum test wa dimensionality of 30 and clustering was performed with a resolution of 0.5.

729 Findallmarkers function utilizing a Wilcoxon rank-sum test was used to find

730 specific markers and annotation was performed as recently es
- 729 Findallmarkers function utilizing a Wilcoxon rank-sum test was used to find cluster
730 specific markers and annotation was performed as recently established⁴⁸. Number
731 Oprd1 expressing cells were defined by a thr specific markers and annotation was performed as recently established 48
-
- 732
- specific markers and annotation was performed as recently established⁴⁸. No
131 0 *Oprd1* expressing cells were defined by a threshold of non-zero expression.
132 **Statistical analysis**
134 Statistical analysis was perfo 733 **Statistical analysis**
- 734 Statistical analysis was performed using GraphPad Prism 9 software. Data are
735 presented as mean ± SEM. Differences pre- and post-injection within a single c 734 Statistical analysis was performed using GraphPad Prism 9 software. Data are
735 presented as mean ± SEM. Differences pre- and post-injection within a single g
735 presented as mean ± SEM. Differences pre-%735 presented as mean \pm SEM. Differences pre- and post-injection within a single group
19

were assessed using a Wilcoxon matched-pairs signed rank test. Differences between

T37 two groups were assessed using a Mann-Whitney test. Statistical analysis for multiple

comparisons were performed using Kruskal-Walli 739 comparison test or a Two-Way ANOVA followed by Sidak's multiple comparison test.
740 *p*□<□0.05 (*), *p*□<□0.01 (**), *p*□<□0.001 (***).
741 742 Acknowledgement: We thank Dr. Dena Dubal and her laboratory for the FCG mouse, 739 comparison test or a Two-Way ANOVA followed by Sidak's multiple comparison test.
740 p□<□0.05 (*), p□<□0.01 (**), p□<□0.001 (***).
741 **Acknowledgement:** We thank Dr. Dena Dubal and her laboratory for the FCG mouse
74 740 *p*□<□0.05 (*), *p*□<□0.01 (**), *p*□<□0.001 (***).
741 **Acknowledgement:** We thank Dr. Dena Dubal
743 Dr. Kevin Yackle for *Penk^{Cre}* mice, Dr. Ari Molofs
744 mice. Dr. Amvnah Pradhan for *Oprd1^{eGFP}* mice a 743
744 742 **Acknowledgement:** We thank Dr. Dena Dubal and her laboratory for the FCG mouse,
743 Dr. Kevin Yackle for *Penk^{Cre}* mice, Dr. Ari Molofsky for *Foxp3^{Cre-ERT2} Rosa26*^{TdTomato}
745 with Penk^{-/-} tissue isolation. W Dr. Kevin Yackle for *Penk*Cre mice, Dr. Ari Molofsky for *Foxp3*Cre-ERT2*Rosa26*TdTomato 745
746
747 mice, Dr. Amynah Pradhan for *Oprd1^{eGFP}* mice and Dr. Mike Ansonoff for assistance

with Penk^{-/-} tissue isolation. We thank additional members of the Basbaum laboratory

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746 and UCSF ImmunoX for critical feedback. Funding for this work was supported by

747 grants Canadian Institute of Health Research (746 and UCSF ImmunoX for critical feedback. Funding for this work was supported by
747 grants Canadian Institute of Health Research (CIHR) (to É.M.), the Fonds de Rech
748 en Santé-Québec (to É.M.), the Dermatology Foundat en Santé-Québec (to É.M.), the Dermatology Foundation (Career Development Award

1749 to S.W.K.), the Sandler Foundation PBBR (to S.W.K), Grunfeld Scholar Award from

1750 SFVAMC (to S.W.K), T32AR007175-44 (to S.W.K), NIH 748 en Santé-Québec (to É.M.), the Dermatology Foundation (Career Development Award
749 to S.W.K.), the Sandler Foundation PBBR (to S.W.K), Grunfeld Scholar Award from
750 SFVAMC (to S.W.K), T32AR007175-44 (to S.W.K), NIH 749 to S.W.K.), the Sandler Foundation PBBR (to S.W.K), Grunfeld Scholar Award from
750 SFVAMC (to S.W.K), T32AR007175-44 (to S.W.K), NIH NSR35NS097306 (to A.I.B.
751 and Open Philanthropy (to A.I.B.). Figures were generat 750 SFVAMC (to S.W.K), T32AR007175-44 (to S.W.K), NIH NSR35NS097306 (to A.I.B.)
751 and Open Philanthropy (to A.I.B.). Figures were generated with BioRender.com.
753 **Disclosures:** Authors have no conflicts of interests to 751 and Open Philanthropy (to A.I.B.). Figures were generated with BioRender.com.
752 **Disclosures:** Authors have no conflicts of interests to declare.
754 **Author contributions:** É.M. and S.W.K. designed experiments. É.M. 752
753
754
755
756 **Disclosures:** Authors have no conflicts of interests to declare.
754 **Author contributions:** É.M. and S.W.K. designed experiment
756 S.R. N.P.K. W.L.E. and S.W.K. performed experiments, data a
757 J.E.P. and A.I.D. provid 757
758 756 S.R. N.P.K. W.L.E. and S.W.K. performed experiments, data analysis or visualization.
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762 Sakeen.kashem@ucsf.edu or allan.basbaum@ucsf.edu.

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765 values. At le 762 sakeen.kashem@ucsf.edu or allan.basbaum@ucsf.edu.
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767 764 **Diversity, equity, and inclusion statement:** Authors support diversity and inclusion
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769 766 least one author identifies as an under-represented minority and/or as an immigrant.
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Figure Legends

783
 Figure 1. mTreg suppress mechanical pain hypersensitivity in female mice.

785 Representative whole mount maximum projection confocal microscopy image of the (A)

786 Iumbar spinal cord meninges and 784
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786
787 **Figure 1. mTreg suppress mechanical pain hypersensitivity in female mice.**
785 Representative whole mount maximum projection confocal microscopy image of t
786 Iumbar spinal cord meninges and (B) DRG showing Tregs (green-Representative whole mount maximum projection confocal microscopy image of the (A)

786 Iumbar spinal cord meninges and (B) DRG showing Tregs (green-red: yellow) and

787 nerves (autofluorescence, red) in *Foxp3*^{eGFP-CreE} Treas (green-red: yellow) and

Treas (autofluorescence, red) in $Foxp3^{\text{GFP-CreeRT2}}$; Rosa26^{dTomato} reporter mice. In

Treas showing DRG magnification. Scale bar represents 100 µm in A) and 150 µm in B).

Treas Arrows indic nerves (autofluorescence, red) in *Foxp3*eGFP-CreERT2*;Rosa26*tdTomato showing DRG magnification. Scale bar represents 100 µm in A) and 150 µm in B).
789 Arrows indicate Tregs. (C) Total number of weight-adjusted tissue Tregs across organs,
790 in both sexes combined. (D) Relative number of t 5788 showing DRG magnification. Scale bar represents 100 µm in A) and 150 µm in B).

789 Arrows indicate Tregs. (C) Total number of weight-adjusted tissue Tregs across org

790 in both sexes combined. (D) Relative number o 789 Arrows indicate Tregs. (C) Total number of weight-adjusted tissue Tregs across organs,
790 in both sexes combined. (D) Relative number of tissue Tregs from male (white) and
791 female (black) mice per organ. 100% repre in both sexes combined. (D) Relative number of tissue Tregs from male (white) and

791 female (black) mice per organ. 100% represents mean number of female Tregs per

792 organ. Comparison is made between each individual o 792 organ. Comparison is made between each individual organ. (E) Representative

793 concatenated flow cytometry plots of tissue Treg after a single intrathecal (IT) injecti

794 of 20 ng of pegylated diphtheria toxin (peg concatenated flow cytometry plots of tissue Treg after a single intrathecal (IT) injection
794 of 20 ng of pegylated diphtheria toxin (pegDT). (F) Relative quantifications of tissue
795 Treg depletion 2 days after a single concatenated flow cytometry plots of tissue Treg after a single intrathecal (IT) injection

of 20 ng of pegylated diphtheria toxin (pegDT). (F) Relative quantifications of tissue

Treg depletion 2 days after a single IT pe of 20 ng of pegylated diphtheria toxin (pegDT). (F) Relative quantifications of tissue

795 Treg depletion 2 days after a single IT pegDT injection across organs. 100% represe

796 mean number of tissue Tregs in IT vehicle Treg depletion 2 days after a single IT pegDT injection across organs. 100% represents

The mean number of tissue Tregs in IT vehicle-injected mice per organ. (G and H) 50% paw

withdrawal thresholds measured using von Fre mean number of tissue Tregs in IT vehicle-injected mice per organ. (G and H) 50% paw

797 withdrawal thresholds measured using von Frey filaments before (day 0) and after a

798 single dose of 20 ng of IT pegDT or vehicle withdrawal thresholds measured using von Frey filaments before (day 0) and after a

798 single dose of 20 ng of IT pegDT or vehicle in female (G) or male (H) *Foxp3*-DTR mion

799 (I) Summary of significant behavioral diff 5798 single dose of 20 ng of IT pegDT or vehicle in female (G) or male (H) *Foxp3*-DTR mice.

799 (I) Summary of significant behavioral differences comparing IT pegDT- and control-

800 injected female and male mice. Total (I) Summary of significant behavioral differences comparing IT pegDT- and control-

800 injected female and male mice. Total number of mice for G-I is presented in Figure

801 ScMg= Spinal cord meninges, BrMg= Brain mening 800 injected female and male mice. Total number of mice for G-I is presented in Figure S3.
801 ScMg= Spinal cord meninges, BrMg= Brain meninges, LN= Lymph nodes, ns = not
802 significant, **p*<0.05, ***p*<0.01, ****p*<0.00

801 ScMg= Spinal cord meninges, BrMg= Brain meninges, LN= Lymph nodes, ns = not
802 significant, *p<0.05, **p<0.01, ***p<0.001. Related to Figure S1-S3.
803 Figure 2. Expanding mTreg alleviates nociception dependent on sex significant, *p<0.05, **p<0.01, ***p<0.001. Related to Figure S1-S3.
803
804 Figure 2. Expanding mTreg alleviates nociception dependent on sex hormones
805 and independent of tissue repair.
806 (A) Schematic representation

805
806
807 **Figure 2. Expanding mTreg alleviates nociception dependent on sex hormones**
 and independent of tissue repair.

(A) Schematic representation of the spared nerve injury (SNI) surgery. (B) Long-term

assessment of mechani 805 **and independent of tissue repair.**
806 (A) Schematic representation of the
807 assessment of mechanical thresholc
808 combined, no difference between the
809 response to 0.008 g von Frev filame (A) Schematic representation of the spared nerve injury (SNI) surgery. (B) Long-term
807 assessment of mechanical thresholds in mice following SNI surgery (both sexes
808 combined, no difference between the sexes). n = 7-8 807 assessment of mechanical thresholds in mice following SNI surgery (both sexes combined, no difference between the sexes). $n = 7-8$ mice per group. (C and D) is response to 0.008 g von Frey filament in mice with SNI (d combined, no difference between the sexes). n = 7-8 mice per group. (C and D) Percent
809 response to 0.008 g von Frey filament in mice with SNI (day 0) and treated with IT
810 pegDT or vehicle every 4 days. n = 8 per grou response to 0.008 g von Frey filament in mice with SNI (day 0) and treated with IT
810 pegDT or vehicle every 4 days. $n = 8$ per group for females and 9-10 per group for
811 males. (E) Schematic representation of mTreg ex 810 pegDT or vehicle every 4 days. $n = 8$ per group for females and 9-10 per group for males. (E) Schematic representation of mTreg expansion in mice 4 weeks after SN 317 injections of low-dose IL-2 (0.1 µg). (F) Total mT 811 males. (E) Schematic representation of mTreg expansion in mice 4 weeks after SNI by
812 3 IT injections of low-dose IL-2 (0.1 µg). (F) Total mTreg number in meninges after low-
813 dose IL-2 or vehicle IT injections (b 3 IT injections of low-dose IL-2 (0.1 µg). (F) Total mTreg number in meninges after low-
813 dose IL-2 or vehicle IT injections (both sexes combined, no differences between the
814 sexes). (G and H) Nociceptive thresholds dose IL-2 or vehicle IT injections (both sexes combined, no differences between the
814 sexes). (G and H) Nociceptive thresholds of females (G) and male (H) mice given lov
815 dose IL-2 or vehicle IT 4 weeks after SNI. (I) dose IL-2 or vehicle IT 4 weeks after SNI. (I) Schematic representation of the mating
816 strategy of Four Core Genotypes (FCG) *FoxP3*-DTR mice demonstrating resulting XX
817 and XY females and XX and XY male mice. (J and 816 strategy of Four Core Genotypes (FCG) *FoxP3*-DTR mice demonstrating resulting XX
817 and XY females and XX and XY male mice. (J and K) Nociceptive thresholds of FCG
818 female (J) and male (K) mice following low-dose strategy of Four Core Genotypes (FCG) *FoxP3*-DTR mice demonstrating resulting XX
817 and XY females and XX and XY male mice. (J and K) Nociceptive thresholds of FCG
818 female (J) and male (K) mice following low-dose IL-2 817 and XY females and XX and XY male mice. (J and K) Nociceptive thresholds of FCG
818 female (J) and male (K) mice following low-dose IL-2 or vehicle IT injections 4 weeks
819 after SNI. (L) Anti-nociceptive efficacy det female (J) and male (K) mice following low-dose IL-2 or vehicle IT injections 4 weeks

819 after SNI. (L) Anti-nociceptive efficacy determined as post IL-2/vehicle injection

820 threshold divided by baseline mechanical th after SNI. (L) Anti-nociceptive efficacy determined as post IL-2/vehicle injection
820 threshold divided by baseline mechanical threshold in male and female mice wit
821 (white) or XY (pink) chromosomes. (M) Nociceptive th threshold divided by baseline mechanical threshold in male and female mice with XX
821 (white) or XY (pink) chromosomes. (M) Nociceptive thresholds of FCG *FoxP3*-DTR
822 female and male mice following a single IT pegDT or 821 (white) or XY (pink) chromosomes. (M) Nociceptive thresholds of FCG *FoxP3*-DTR
822 female and male mice following a single IT pegDT or vehicle injection. (N) Percent
823 baseline nociceptive thresholds determined as p 822 female and male mice following a single IT pegDT or vehicle injection. (N) Percent
823 baseline nociceptive thresholds determined as post pegDT/vehicle injection threshold
824 divided by baseline mechanical threshold baseline nociceptive thresholds determined as post pegDT/vehicle injection threshold
824 divided by baseline mechanical threshold in male and female mice with XX (white) or
825 XY (pink) chromosomes. ns = not significant, 824 divided by baseline mechanical threshold in male and female mice with XX (white) or
825 XY (pink) chromosomes. ns = not significant, **p*<0.05, ***p*<0.01,****p*<0.001. **Related t**
826 **Figure S4.**
827 825 XY (pink) chromosomes. ns = not significant, **p*<0.05, ***p*<0.01,****p*<0.001. **Related to** 826 **Figure S4.**

Figure 3. mTregs express and produce enkephalin.

829 (A) Volcano plot of transcription fold change of activated Treg (aTreg) vs resting Treg

830 (rTreg) and (B) heatmap of relative log2 expression value of aTreg, rTreg (Treg) and (B) heatmap of relative log2 expression value of aTreg, rTreg and activated

831 and resting CD4⁺ CD25 conventional T cells (aTconv and rTconv) from public dataset

832 GSE154680 (n=3). (C) Averaged ATAC seque 830 (rTreg) and (B) heatmap of relative log2 expression value of aTreg, rTreg and activated
831 and resting CD4⁺ CD25 conventional T cells (aTconv and rTconv) from public dataset
832 GSE154680 (n=3). (C) Averaged ATAC se and resting CD4⁺ CD25 832 GSE154680 (n=3). (C) Averaged ATAC sequencing (ATACseq) of open chromatin
833 accessibility peaks on the *Penk* locus in different T cell subsets (n=4 per group,
834 GSE154680), compared to ATACseq, and histone modific 832 GSE154680 (n=3). (C) Averaged ATAC sequencing (ATACseq) of open chromatin
833 accessibility peaks on the *Penk* locus in different T cell subsets (n=4 per group,
834 GSE154680), compared to ATACseq, and histone modific accessibility peaks on the *Penk* locus in different T cell subsets (n=4 per group,
834 GSE154680), compared to ATACseq, and histone modification Chip-Seq from p
835 ENCODE dataset of the p0 developing forebrain, a known e 834 GSE154680), compared to ATACseq, and histone modification Chip-Seq from public
835 ENCODE dataset of the p0 developing forebrain, a known enkephalinergic region. (D
836 and E) Log2 values of *Penk* expression by differ and E) Log2 values of *Penk* expression by different unstimulated immune cell types
837 from Immgen dataset GSE180020. (E) Treg *Penk* expression fold change after cytokine
838 stimulation compared to vehicle control. (F) and E) Log2 values of *Penk* expression by different unstimulated immune cell types
837 from Immgen dataset GSE180020. (E) Treg *Penk* expression fold change after cytol
838 stimulation compared to vehicle control. (F) Rep 837 from Immgen dataset GSE180020. (E) Treg *Penk* expression fold change after cytokine
838 stimulation compared to vehicle control. (F) Representative PMA:Ionomycin stimulated
839 mTregs, meningeal CD4⁺ T cells (mCD4) 838 stimulation compared to vehicle control. (F) Representative PMA:Ionomycin stimulated
839 mTregs, meningeal CD4⁺ T cells (mCD4) from WT mice or *Penk^{-/-}* mTreg (control). (G)
840 Representative flow cytometry plots mTregs, meningeal CD4+ T cells (mCD4) from WT mice or *Penk-/* mTregs, meningeal CD4⁺ T cells (mCD4) from WT mice or *Penk^{-/-}* mTreg (control). (G)
840 Representative flow cytometry plots of Tregs from meninges or secondary lymphoid
841 organs (SLO) from *Penk*^{Cre}*Rosa26*^{tdtoma} Representative flow cytometry plots of Tregs from meninges or secondary lymphoid

841 organs (SLO) from *Penk*^{Cre} Rosa26^{tdtomato} mice. Pink represents non-vascular, tissue

842 Tregs from transgenic *Penk* lineage repo organs (SLO) from *Penk*Cre*Rosa*26tdtomato organs (SLO) from *Penk*^{Cre} Rosa26^{tatomato} mice. Pink represents non-vascular, tissue

842 Tregs from transgenic *Penk* lineage reporter mice. Gray represents vascular Treg in

843 reporter mice while Blue corresponds Tregs from transgenic *Penk* lineage reporter mice. Gray represents vascular Treg in

843 reporter mice while Blue corresponds to tissue Treg from non-transgenic control mice

844 (H-I) Number of enkephalin lineage fate r reporter mice while Blue corresponds to tissue Treg from non-transgenic control mice.

844 (H-I) Number of enkephalin lineage fate reporter positive tissue Tregs in (H) meninges

845 and (I) secondary lymphoid organs (SLO 844 (H-I) Number of enkephalin lineage fate reporter positive tissue Tregs in (H) meninges
845 and (I) secondary lymphoid organs (SLO) in male and female mice. NK: Natural Killer
846 cells, Tgd: γδ T cells, Mo: Monocytes, and (I) secondary lymphoid organs (SLO) in male and female mice. NK: Natural Killer

cells, Tgd: γδ T cells, Mo: Monocytes, MF.rp: Red pulp macrophages, CD4T: CD4⁺ T

cells, CD8T: CD8⁺ T cells, B.fo: splenic follicula cells, Tgd: γδ T cells, Mo: Monocytes, MF.rp: Red pulp macrophages, CD4T: CD4⁺ T cells, Tgd: γδ T cells, Mo: Monocytes, MF.rp: Red pulp macrophages, CD4T: CD4⁺ T
847 cells, CD8T: CD8⁺ T cells, B.fo: splenic follicular B cells, DC8: CD8⁺ dendritic cells,
848 pDC: splenic plasmacytoid, MF. cells, CD8T: CD8⁺ T cells, B.fo: splenic follicular B cells, DC8: CD8⁺ cells, CD8T: CD8⁺ T cells, B.fo: splenic follicular B cells, DC8: CD8⁺ dendritic cells,
848 pDC: splenic plasmacytoid, MF.pc: peritoneal macrophages, MC: myeloid cells, B.mz:
849 splenic marginal zone B cells, ns = not 849 splenic marginal zone B cells, ns = not significant, *p<0.05, **p<0.01,***p<0.001.
850 **Figure 4. mTreg-derived enkephalin controls nociceptive thresholds.**
852 (A) Representative flow cytometry plots of Cre positive (852 (A) Representative flow cytometry plots of Cre positive (pink) or Cre negative (blue)
853 CD45⁺ non-vascular cells from the meninges and the DRG, combined, from 851
852
853
854 **Figure 4. mTreg-derived enkephalin controls nociceptive thresholds.**

852 (A) Representative flow cytometry plots of Cre positive (pink) or Cre negati

853 CD45⁺ non-vascular cells from the meninges and the DRG, combine 852 (A) Representative flow cytometry plots of Cre positive (pink) or Cre negative (blue)
853 CD45⁺ non-vascular cells from the meninges and the DRG, combined, from
854 Penk^{Cre}Rosa26^{tdTomato} mice. (B) Flow plot shows CD45⁺ non-vascular cells from the meninges and the DRG, combined, from 853 CD45⁺ non-vascular cells from the meninges and the DRG, combined, from
854 Penk^{Cre}Rosa26^{tdTomato} mice. (B) Flow plot shows representative tdTomato ne
855 right plot shows tdTomato positive leukocytes, demonstrati *Penk*^{Cre}*Rosa26*^{tdTomato} mice. (B) Flow plot shows representative tdTomato negative and

855 ight plot shows tdTomato positive leukocytes, demonstrating the more pronounced

856 mTreg representation in the enkephaline right plot shows tdTomato positive leukocytes, demonstrating the more pronounced

856 mTreg representation in the enkephalinergic fate cell population. (C) Schematic

857 representation of bone marrow transplants to genera 856 mTreg representation in the enkephalinergic fate cell population. (C) Schematic
857 representation of bone marrow transplants to generate a global depletion of
858 enkephalinergic cells, a depletion of hematopoietic-d example are representation of bone marrow transplants to generate a global depletion of

858 enkephalinergic cells, a depletion of hematopoietic-derived enkephalin or a d

869 Fenk^{Cre}Rosa26^{DTR} \rightarrow irradiated WT recip enkephalinergic cells, a depletion of hematopoietic-derived enkephalin or a depletion of

859 Freg-derived enkephalin, respectively. (D-G) Bone marrow chimera of

860 Penk^{Cre}Rosa26^{DTR} \rightarrow irradiated WT recipients. Noc 859 Treg-derived enkephalin, respectively. (D-G) Bone marrow chimera of
860 Penk^{Cre}Rosa26^{DTR} \rightarrow irradiated WT recipients. Nociceptive thresholds
861 pegDT (pink) or vehicle (white) IT injection in female (D) and mal *Penk*^{Cre}*Rosa26*^{DTR} \rightarrow irradiated WT recipients. Nociceptive thresholds after a single pegDT (pink) or vehicle (white) IT injection in female (D) and male (E) mice. n= 5 pe group. Nociceptive thresholds after SNI an 861 pegDT (pink) or vehicle (white) IT injection in female (D) and male (E) mice. n= 5 per group. Nociceptive thresholds after SNI and pegDT (pink) or vehicle (white) IT injectic in female (F) and male (G) mice. (H) Nocic group. Nociceptive thresholds after SNI and pegDT (pink) or vehicle (white) IT injection

863 in female (F) and male (G) mice. (H) Nociceptive thresholds at baseline of female WT \rightarrow

864 WT (black) or *Penk^{-/-}* \rightarrow W 863 in female (F) and male (G) mice. (H) Nociceptive thresholds at baseline of female WT \rightarrow 864 WT (black) or *Penk^{-/-}* \rightarrow WT (white) bone marrow chimeras. (I) Female *Foxp3*-DTR + *Penk^{-/-}* (1:1) \rightarrow WT mice and WT (black) or *Penk*^{\div} \rightarrow WT (white) bone marrow chimeras. (I) Female *Foxp3*-DTR + WT (black) or *Penk^{-/-}* \rightarrow WT (white) bone marrow chimeras. (I) Female *Foxp3*-DTR +
865 *Penk^{-/-}* (1:1) \rightarrow WT mice and tested for nociceptive thresholds after pegDT (pink) or
866 vehicle (white) IT, n=10 per group *Penk^{* $-$ *}* (1:1) \rightarrow WT mice and tested for nociceptive thresholds after pegDT (pink) or 865 $Penk^{\prime\prime}$ (1:1) \rightarrow WT mice and tested for nociceptive thresholds after pegDT (pink) or
866 vehicle (white) IT, n=10 per group. (J) Nociceptive thresholds in SNI mice after pegD
67 (pink) or vehicle (white) IT injec vehicle (white) IT, n=10 per group. (J) Nociceptive thresholds in SNI mice after pegDT
867 (pink) or vehicle (white) IT injections. (K) WT SNI female mice given low-dose IL-2 and
868 naltrindole. (L) Nociceptive efficacy c 867 (pink) or vehicle (white) IT injections. (K) WT SNI female mice given low-dose IL-2 and
868 naltrindole. (L) Nociceptive efficacy calculated as percent compared to baseline
869 threshold. ns = not significant, *p<0.05, 868 naltrindole. (L) Nociceptive efficacy calculated as percent compared to baseline
869 threshold. ns = not significant, *p<0.05, **p<0.01,***p<0.001.
871
871 threshold. ns = not significant, $p < 0.05$, $\frac{p}{q} < 0.01$, $\frac{p}{q} < 0.001$.

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- 872

874 **Figure 5. Treg-derived enkephalin is dispensable for suppressing inflammation.** (A) Baseline nociceptive thresholds of uninjured *Rag2+/+, +/- or -/-* 875 (A) Baseline nociceptive thresholds of uninjured $Rag2^{4+}$, $4-$ or $4-$ female mice. (B)
876 Nociceptive thresholds of female $Foxp3$ -DTR mice injected with pegDT IT + IV
877 clodronate (pink) or control (white) lipos

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- Nociceptive thresholds of female *Foxp3*-DTR mice injected with pegDT IT + IV
877 clodronate (pink) or control (white) liposomes showing peripheral macrophages
878 mediate the nociception induced by mTreg depletion, n=5 pe clodronate (pink) or control (white) liposomes showing peripheral macrophages do not

878 mediate the nociception induced by mTreg depletion, n=5 per group. (C) Representative

889 flow cytometry histograms of proliferate mediate the nociception induced by mTreg depletion, n=5 per group. (C) Representative
879 flow cytometry histograms of proliferated conventional T cells (Tconv) alone (pink) or
880 4:1 with WT Tregs (gray), *Penk^{-/-}* Tre 879 flow cytometry histograms of proliferated conventional T cells (Tconv) alone (pink) or 4:1 with WT Tregs (gray), *Penk^{-/-}* Tregs (yellow), or unstimulated, un-proliferated cell
-
-
- 4:1 with WT Tregs (gray), *Penk^{-/-}* Tregs (yellow), or unstimulated, un-proliferated cell
881 trace violet-stained control (blue). Histogram shows cells that have not proliferated. (I
882 Suppression of Tconv cell prolif or *Penk^{-/-}* Tregs (yellow). (E) Schematic representation of competition experiment
- 881 trace violet-stained control (blue). Histogram shows cells that have not proliferated. (D)
882 Suppression of Tconv cell proliferation by different concentrations of WT Tregs (white)
883 or *Penk^{-/-}* Tregs (yellow). 882 Suppression of Tconv cell proliferation by different concentrations of WT Tregs (white)
883 or *Penk^{-/-}* Tregs (yellow). (E) Schematic representation of competition experiment
884 showing 1:1 transfer of WT or *Penk[*] or Penk^{-/-} Tregs (yellow). (E) Schematic representation of competition experiment
884 Showing 1:1 transfer of WT or Penk^{-/-} T cells into Rag2^{-/-} mice. SNI surgery was
885 performed and organs were harvested 4 weeks showing 1:1 transfer of WT or *Penk-/-* T cells into *Rag2-/-*
- *Penk* sufficient CD45.1 and *Penk* deficient CD45.2 CD4⁺ T cells in the meninges
- S84 showing 1:1 transfer of WT or *Penk^{-/-}* T cells into *Rag2^{-/-}* mice. SNI surgery was

ses performed and organs were harvested 4 weeks later for F-H. (F) Equal competition of
 Penk sufficient CD45.1 and *Penk* de performed and organs were harvested 4 weeks later for F-H. (F) Equal competition of

886 Penk sufficient CD45.1 and Penk deficient CD45.2 CD4⁺ T cells in the meninges

represented as a concatenated flow cytometry plot, n
-
- 886 Penk sufficient CD45.1 and Penk deficient CD45.2 CD4⁺ T cells in the meninges
887 represented as a concatenated flow cytometry plot, n=4 per group. Representative
888 cytometric plots. (G) Pooled proportion of *Penk* CD45.2 CD4⁺ T cells in different organs, n=4 per genotype. (H) Percent of FoxP3⁺
- represented as a concatenated flow cytometry plot, n=4 per group. Representative flow

888 cytometric plots. (G) Pooled proportion of *Penk* sufficient CD45.1 and *Penk* deficient

890 \Box ⁺ and IL-17A⁺ CD4⁺ T cells cytometric plots. (G) Pooled proportion of *Penk* sufficient CD45.1 and *Penk* deficient

889 CD45.2 CD4⁺ T cells in different organs, n=4 per genotype. (H) Percent of FoxP3⁺, IF

890 \Box ⁺ and IL-17A⁺ CD4⁺ T cel 889 CD45.2 CD4⁺ T cells in different organs, n=4 per genotype. (H) Percent of FoxP3⁺, IFN-
890 \Box ⁺ and IL-17A⁺ CD4⁺ T cells from G. (I) Representative flow cytometric plots of cytokine
891 secretion by CD4⁺ \Box ⁺ and IL-17A⁺ CD4⁺
cooration by CD4⁺ T.6 890 □ ⁺ and IL-17A⁺ CD4⁺ T cells from G. (I) Representative flow cytometric plots of cytokine
891 secretion by CD4⁺ T cells after GVHD induced by transfer of pre-activated Tconv alone
892 or combined with *Penk^{+/*} secretion by CD4⁺ T cells after GVHD induced by transfer of pre-activated Tconv alone
- 891 secretion by CD4⁺ T cells after GVHD induced by transfer of pre-activated Tconv alone
892 or combined with *Penk^{+/+}* or *Penk^{-/-}* Tregs. (J) Weight curves of GVHD mice, n=3-4 per
893 group. ns = not significant, or combined with *Penk+/+* or *Penk-/-*
-
-

892 or combined with *Penk^{+/+}* or *Penk^{-/-}* Tregs. (J) Weight curves of GVHD mice, n=3-4 per
893 group. ns = not significant, **p*<0.05, ***p*<0.01,****p*<0.001. **Related to Figure S5.**
895 **Figure 6. δOR on MrgprD⁺** 893 group. ns = not significant, **p*<0.05, ***p*<0.01,****p*<0.001. **Related to Figure S5.** 894
895
896
897
898 **Figure 6.** δ**OR on MrgprD+**

- **Figure 6. 5OR on MrgprD⁺ sensory neurons is required for mTreg mediated anti-

nociception.**

(A) Schematic representation of AAV-induced ablation of *Oprd1* in the PNS (B-C) or

CNS. (B) Nociceptive thresholds of fema 896 **nociception.**
897 (A) Schematic
898 CNS. (B) Noci
899 expansion cor
900 No difference (A) Schematic representation of AAV-induced ablation of *Oprd1* in the PNS (B-C) or
898 CNS. (B) Nociceptive thresholds of female mice lacking *Oprd1* in the PNS after mTre
899 expansion compared to controls. (C) Anti-noci 898 CNS. (B) Nociceptive thresholds of female mice lacking *Oprd1* in the PNS after mTreg
899 expansion compared to controls. (C) Anti-nociceptive efficacy of mTreg expansion. (D)
900 No difference in nociceptive threshold expansion compared to controls. (C) Anti-nociceptive efficacy of mTreg expansion. (D)

900 No difference in nociceptive thresholds in female mice lacking *Oprd1* in the CNS after

901 mTreg expansion compared to controls. No difference in nociceptive thresholds in female mice lacking *Oprd1* in the CNS after

mTreg expansion compared to controls. (E) Heatmap of row normalized expression

from DRG sensory neurons clusters from combined GSE1 mTreg expansion compared to controls. (E) Heatmap of row normalized expression

902 from DRG sensory neurons clusters from combined GSE139088 and GSE201653. (

903 Proportions of sensory neuron clusters expressing *Oprd1* 902 from DRG sensory neurons clusters from combined GSE139088 and GSE201653. (F
903 Proportions of sensory neuron clusters expressing *Oprd1* from E. (G) Representative
904 flow cytometry plot of δ OR-GFP (green) expres Proportions of sensory neuron clusters expressing *Oprd1* from E. (G) Representative

904 flow cytometry plot of δOR-GFP (green) expression on IB4⁺ MrgprD⁺ DRG sensory

905 neurons compared to cells from non-reporter flow cytometry plot of δOR-GFP (green) expression on IB4⁺ MrgprD⁺
pourane compared to selle from non-reporter mise (purple). Quarleid flow cytometry plot of δOR-GFP (green) expression on IB4⁺ MrgprD⁺ DRG sensory
905 neurons compared to cells from non-reporter mice (purple). Overlaid are lymphoid
906 CD45⁺ CD90.2⁺ cells and myeloid CD45⁺ CD11b neurons compared to cells from non-reporter mice (purple). Overlaid are lymphoid

906 CD45⁺ CD90.2⁺ cells and myeloid CD45⁺ CD11b⁺ cells from the DRG. (H-I) Percen

907 response to 0.008 g von Frey fiber stimulati $CD45^+$ CD90.2⁺ cells and myeloid CD45⁺ CD11b⁺ CD45⁺ CD90.2⁺ cells and myeloid CD45⁺ CD11b⁺ cells from the DRG. (H-I) Percent
907 response to 0.008 g von Frey fiber stimulation after SNI in female (H) or male (I) mic
2008 conditionally lacking δOR on MrgprD⁺ response to 0.008 g von Frey fiber stimulation after SNI in female (H) or male (I) mice

conditionally lacking δOR on MrgprD⁺ neurons (pink) or controls (white). (J) Nociceptiv

thresholds after mTreg expansion in femal conditionally lacking δOR on MrgprD⁺ neurons (pink) or controls (white). (J) Nociceptive conditionally lacking δOR on MrgprD⁺ neurons (pink) or controls (white). (J) Nociceptive
909 thresholds after mTreg expansion in female mice lacking *Oprd1* in MrgprD⁺ neurons
910 compared to controls. (K) Anti-nocice thresholds after mTreg expansion in female mice lacking *Oprd1* in MrgprD+ thresholds after mTreg expansion in female mice lacking Oprd1 in MrgprD⁺ neurons

910 compared to controls. (K) Anti-nociceptive efficacy of mTreg expansion. SA-LTMR=

911 Slowly adapting low-threshold mechanoreceptor, R 910 compared to controls. (K) Anti-nociceptive efficacy of mTreg expansion. SA-LTMR=
911 Slowly adapting low-threshold mechanoreceptor, RA-LTMR= rapidly-adapting low-
912 threshold mechanoreceptor, MrgprD= Mas-related G pr 911 Slowly adapting low-threshold mechanoreceptor, RA-LTMR= rapidly-adapting low-
912 threshold mechanoreceptor, MrgprD= Mas-related G protein-coupled receptor D, F
913 proprioceptor, SST: somatostatin, Trpm8= Transient re 912 threshold mechanoreceptor, MrgprD= Mas-related G protein-coupled receptor D, Prop
913 proprioceptor, SST: somatostatin, Trpm8= Transient receptor potential cation channel
914 subfamily M member 8, TAM= Tamoxifen, cKO= 913 proprioceptor, SST: somatostatin, Trpm8= Transient receptor potential cation channel
914 subfamily M member 8, TAM= Tamoxifen, cKO= Conditional KO, ns = not significant,
915 *p<0.05, **p<0.01,***p<0.001.
917 914 subfamily M member 8, TAM= Tamoxifen, cKO= Conditional KO, ns = not significant,
915 *p<0.05, **p<0.01,***p<0.001.
916
917 915 **p*<0.05, ***p*<0.01,****p*<0.001.
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921 **Supplemental Figure Legends**
922 **Figure S1. Flow cytometric ga**t
924 (A) Gating strategy to quantify tis
925 Mice were iniected IT with ARTC

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- 925
926 **Figure S1. Flow cytometric gating strategy of meningeal Tregs.**

924 (A) Gating strategy to quantify tissue Treg numbers. Numbers indica

925 Mice were injected IT with ARTC2 nanobody to minimize Treg apop

926 with anti-925 Mice were injected IT with ARTC2 nanobody to minimize Treg apoptosis and injected IV
926 with anti-CD45 FITC (pink) antibody or vehicle injected (blue) to label vascular immune
928 with anti-CD45 FITC (pink) antibody or vehicle injected (blue) to label vascular immune
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929 Figure S2. pegDT IT injection avoids systemic inflammation and weight loss in
930 *Foxp3-*DTR mice. 927 cells.
928 **Figur**
930 **Foxp**.
931 (A)Ev 929
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931
932 **Figure S2. pegDT IT injection avoids systemic inflammation and weight loss in**
930 **Foxp3-DTR mice.**
931 (A) Evan's blue staining after IT injection showing diffusion into the cerebellum, the
932 olfactory bulb, the cervi

- 930 **Foxp3-DTR mice.**
931 (A) Evan's blue sta
932 olfactory bulb, the c
933 lumbar DRG. (B) po
934 Evan's blue. (C) W (A) Evan's blue staining after IT injection showing diffusion into the cerebellum, the

932 olfactory bulb, the cervical and lumbar lymph nodes, the spinal cord meninges and t

933 lumbar DRG. (B) pegDyLight650 IT injectio
- olfactory bulb, the cervical and lumbar lymph nodes, the spinal cord meninges and the
933 Lumbar DRG. (B) pegDyLight650 IT injection exhibits a more limited diffusion than
934 Evan's blue. (C) Weight curves of *Foxp3*-DTR
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- 933 lumbar DRG. (B) pegDyLight650 IT injection exhibits a more limited diffusion than
934 Evan's blue. (C) Weight curves of $Foxp3$ -DTR mice injected with IT pegDT, IP DT or IT
935 vehicle every 3 days demonstrating a lack 934 Evan's blue. (C) Weight curves of *Foxp3*-DTR mice injected with IT pegDT, IP DT or IT
935 vehicle every 3 days demonstrating a lack of weight loss after site-selective Treg
ablation. Arrows represent DT injections. (D vehicle every 3 days demonstrating a lack of weight loss after site-selective Treg
936 ablation. Arrows represent DT injections. (D and E) Representative images of sple
937 sizes and spleen weights from mice in (C). (F) Su 936 ablation. Arrows represent DT injections. (D and E) Representative images of spleen
937 sizes and spleen weights from mice in (C). (F) Survival curves of mice in (C). ns = not
939 significant, *p<0.05, **p<0.01,***p<0.
-
-

937 sizes and spleen weights from mice in (C). (F) Survival curves of mice in (C). ns = not
938 significant, *p<0.05, **p<0.01,***p<0.001.
939 **Figure S3. mTreg depletion selectively induces mechanical hypersensitivity in
** 938 significant, **p*<0.05, ***p*<0.01,****p*<0.001.
939 **Figure S3. mTreg depletion selectively**
941 **female mice.**
942 (A) von Frev. (B) Hargreaves. (C) hotplate 939
940
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943 **Figure S3. mTreg depletion selectively induces mechanical hypersensitivity in**

941 **female mice.**

(A) von Frey, (B) Hargreaves, (C) hotplate, (D) tail flick, (E) acetone, (F) pinprick, (G)

943 brush and (G) rotarod beh

- 941 **female mice.**
942 (A) von Frey, (
943 brush and (G)
944 of IT pegDT. n
945 942 (A) von Frey, (B) Hargreaves, (C) hotplate, (D) tail flick, (E) acetone, (F) pinprick, (G)
943 brush and (G) rotarod behavioral testing in *FoxP3*-DTR mice injected with a single do
945 **Figure S4. mTreg expansion sele**
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943 brush and (G) rotarod behavioral testing in *FoxP3*-DTR mice injected with a single dose
944 of IT pegDT. n = 4-20 mice per group. ns = not significant,*****p*<0.0001.
945 **Figure S4. mTreg expansion selectively improv** 944 of IT pegDT. n = 4-20 mice per group. ns = not significant,*****p*<0.0001.
945 **Figure S4. mTreg expansion selectively improves mechanical hyper
947 injured female mice.**
948 (A) mTreg expansion using IL-2 IT injectio 945
946
947
948
949 946 **Figure S4. mTreg expansion selectively improves mechanical hypersensitivity in**
947 **injured female mice.**
948 (A) mTreg expansion using IL-2 IT injections induces no changes in nociceptive
950 mice induces no changes

-
- injured female mice.

948 (A) mTreg expansion using IL-2 IT injections induces no changes in nociceptive

950 thresholds in uninjured naive male and female mice. mTreg expansion in nerve-

950 mice induces no changes in (B 948 (A) mTreg expansion using IL-2 IT injections induces no changes in nociceptive
949 thresholds in uninjured naive male and female mice. mTreg expansion in nerve-i
950 mice induces no changes in (B) acetone, (C) Hargreav 950 mice induces no changes in (B) acetone, (C) Hargreaves and (D) hotplate behavioral
951 testing. ns = not significant.
952 **Figure S5. Functional characterization of enkephalin from CD4⁺ T cells.** 950 mice induces no changes in (B) acetone, (C) Hargreaves and (D) hotplate behavioral
951 testing. ns = not significant.
952 **Figure S5. Functional characterization of enkephalin from CD4⁺ T cells.
954 (A) Chimerism of**
-

Figure S5. Functional characterization of enkephalin from CD4+

- 951 testing. ns = not significant.
952 **Figure S5. Functional chai**
954 (A) Chimerism of meningeal
955 (B) pooled chimerism compa 954
955
956 Figure S5. Functional characterization of enkephalin from CD4⁺ T cells.

954 (A) Chimerism of meningeal Tregs and spinal cord microglia in PenkDTR^{Δhem}

955 (B) pooled chimerism comparing Tregs in the meninges and in th (A) Chimerism of meningeal Tregs and spinal cord microglia in PenkDTR $^{\Delta$ heme (A) Chimerism of meningeal Tregs and spinal cord microglia in PenkDTR^{Aneme} mice and
955 (B) pooled chimerism comparing Tregs in the meninges and in the lymphoid organs
956 compared to tissue macrophages of the spinal cor 955 (B) pooled chimerism comparing Tregs in the meninges and in the lymphoid organs
956 compared to tissue macrophages of the spinal cord (microglia) and the epidermis
957 (Langerhans cells, LC). (C) mTreg number after IP external compared to tissue macrophages of the spinal cord (microglia) and the epidermis

957 (Langerhans cells, LC). (C) mTreg number after IP DT injection in PenkDTR^{∆heme} r

958 (D) Weight curves of PenkDTR^{∆heme} mice (Langerhans cells, LC). (C) mTreg number after IP DT injection in PenkDTR^{Aheme} 957 (Langerhans cells, LC). (C) mTreg number after IP DT injection in PenkDTR^{Aneme} mice.
958 (D) Weight curves of PenkDTR^{Aheme} mice injected with IP DT every three days showing
959 peripheral penk ablation doesn't indu (D) Weight curves of PenkDTR^{Δheme} 958 (D) Weight curves of PenkDTR^{Aneme} mice injected with IP DT every three days showing
959 peripheral penk ablation doesn't induce weight loss. (E) Unaltered spleen weight and
960 (F) meningeal and (G) spleen CD4⁺ T c peripheral penk ablation doesn't induce weight loss. (E) Unaltered spleen weight and
960 (F) meningeal and (G) spleen CD4⁺ T cell populations after IP DT. Unaltered CD4 T c
961 populations in the (H) spleen and (I) in th (F) meningeal and (G) spleen $CD4^+$ 960 (F) meningeal and (G) spleen CD4⁺ T cell populations after IP DT. Unaltered CD4 T cell
961 populations in the (H) spleen and (I) in the nerve and unaltered (J) mouse weight and
962 (K) spleen weight. (L) Graft Versu 961 populations in the (H) spleen and (I) in the nerve and unaltered (J) mouse weight and
962 (K) spleen weight. (L) Graft Versus Host Disease (GVHD) score in Rag2^{-/-} mice injecte
963 with pre-activated Tconv alone or wi (K) spleen weight. (L) Graft Versus Host Disease (GVHD) score in *Rag2-/-* 962 (K) spleen weight. (L) Graft Versus Host Disease (GVHD) score in *Rag2*^{-/-} mice injected
963 with pre-activated Tconv alone or with *Penk^{+/+}* or *Penk^{-/-}* Tregs, n=3-4 mice per group.
964 (M) IFN-□⁺ and (N) ILwith pre-activated Tconv alone or with *Penk+/+* or *Penk-/-*
- 963 with pre-activated Tconv alone or with *Penk^{+/+}* or *Penk*^{-⁄-} Tregs, n=3-4 mice per group.
964 (M) IFN-□⁺ and (N) IL-17⁺ CD4⁺ T cells after GVHD induction. ns = not significant,
965 *p<0.05, ***p<0.001. 964 (M) IFN- \Box ⁺ and (N) IL-17⁺ CD4⁺ T cells after GVHD induction. ns = not significant,
965 *p<0.05, ***p<0.001.

- 965 **p*<0.05, ****p*<0.001.

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Figure 4 Midavaine et al., 2024

