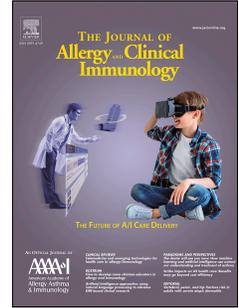# Journal Pre-proof

IL-33 Signaling in Sensory Neurons Promotes Dry Skin Itch

Anna M. Trier, B.A., Madison R. Mack, Ph.D., Avery Fredman, Masato Tamari, M.D. Ph.D., Aaron M. Ver Heul, M.D. Ph.D., Yonghui Zhao, Ph.D., Changxiong J. Guo, B.A., Oshri Avraham, Ph.D., Zachary K. Ford, Ph.D., Landon K. Oetjen, M.D. Ph.D., Jing Feng, Ph.D., Carina Dehner, M.D. Ph.D., Dean Coble, Ph.D., Asima Badic, RMA, Satoru Joshita, M.D. Ph.D., Masato Kubo, Ph.D., Robert W. Gereau, IV, Ph.D., Jennifer Alexander-Brett, M.D. Ph.D., Valeria Cavalli, Ph.D., Steve Davidson, Ph.D., Hongzhen Hu, Ph.D., Qin Liu, Ph.D., Brian S. Kim, M.D. M.T.R.
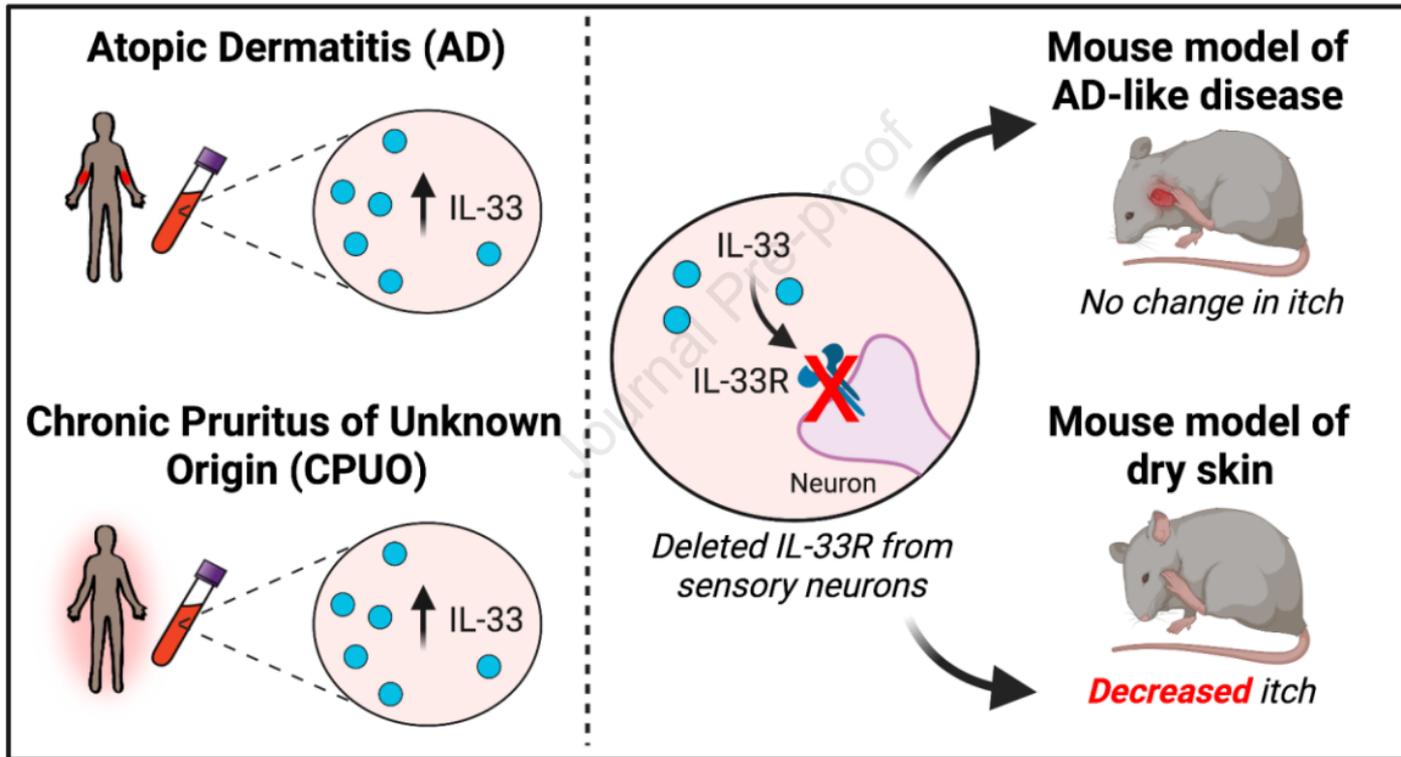
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Neuronal IL-33R is necessary for dry skin itch, but not for itch in atopic dermatitis

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3 Anna M. Trier B.A.[1,2], Madison R. Mack Ph.D.[1,2,14], Avery Fredman[1,2], Masato Tamari M.D.
4 Ph.D.[1,2], Aaron M. Ver Heul M.D. Ph.D.[1,3], Yonghui Zhao Ph.D.[1,4], Changxiong J. Guo B.A.[1,4],
5 Oshri Avraham Ph.D.[5], Zachary K. Ford Ph.D.[6], Landon K. Oetjen M.D. Ph.D.[1,2], Jing Feng
6 Ph.D.[1,4], Carina Dehner M.D. Ph.D.[2], Dean Coble Ph.D.[7], Asima Badic RMA[1,2], Satoru Joshita
7 M.D. Ph.D.[8], Masato Kubo Ph.D.[9,10], Robert W. Gereau IV Ph.D.[4,5,11], Jennifer Alexander-Brett
8 M.D. Ph.D.[12], Valeria Cavalli Ph.D.[5], Steve Davidson Ph.D.[6], Hongzhen Hu Ph.D.[1,4], Qin Liu
9 Ph.D.[1,4] and Brian S. Kim M.D. M.T.R.[1,2,4,13,*]
10

11 [1]Center for the Study of Itch & Sensory Disorders, Washington University School of Medicine,
12 St. Louis, MO 63110, USA.
13 [2]Division of Dermatology, Department of Medicine, Washington University School of Medicine,
14 St. Louis, MO 63110, USA.
15 [3]Division of Allergy and Immunology, Department of Medicine, Washington University School of
16 Medicine, St. Louis, MO 63110, USA.
17 [4]Department of Anesthesiology, Department of Medicine, Washington University School of
18 Medicine, St. Louis, MO 63110, USA.
19 [5]Department of Neuroscience, Washington University School of Medicine, St. Louis, MO 63110,
20 USA.
21 [6]Department of Anesthesiology and Neuroscience Program, University of Cincinnati College of
22 Medicine, Cincinnati, OH 45267, USA.
23 [7]Division of Biostatistics, Washington University School of Medicine, St. Louis, MO 63110, USA.
24 [8]Division of Gastroenterology, Department of Medicine, Shinshu University School of Medicine,
25 3-1-1 Asahi, Matsumoto, Nagano, 390-8621, Japan.
26 [9]Laboratory of Cytokine Regulation, Center for Integrative Medical Science (IMS), RIKEN
27 Yokohama Institute, Yokohama, Kanagawa, Japan.
28 [10]Division of Molecular Pathology, Research Institute for Biomedical Science, Tokyo University
29 of Science, Nodo 278-0022, Chiba Prefecture, Japan.
30 [11]Washington University Pain Center, Washington University School of Medicine, St. Louis, MO
31 63110, USA.
32 [12]Division of Pulmonary and Critical Care, Department of Medicine, Washington University
33 School of Medicine, St. Louis, MO 63110, USA.
34 [13]Department of Pathology and Immunology, Washington University School of Medicine, St.
35 Louis, MO 63110, USA.
36 [14]Present address: Immunology and Inflammation Therapeutic Area, Sanofi, Cambridge, MA
37 02139, USA
38 *Corresponding Author
39
40 Corresponding Author
41 Brian S. Kim
42 Phone: (314) 273-1376
43 Email: briankim@wustl.edu
44 Address: 660 S. Euclid Avenue - Box 8123, St. Louis, MO 63110, USA
45
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80   Locus Biosciences. He also serves on the scientific advisory board for Abrax Japan, Granular

81   Therapeutics, Recens Medical, National Eczema Association, and Cell Reports Medicine. All

82   other authors declare they have no relevant conflicts of interest.

83

84   **Abstract**

85

86   Background: Chronic pruritus, or itch, is common and debilitating, but the neuro-immune

87   mechanisms that drive chronic itch are only starting to be elucidated. Recent studies

88   demonstrate that the IL-33 receptor (IL-33R) is expressed by sensory neurons. However,

89   whether sensory neuron-restricted activity of IL-33 is necessary for chronic itch remains poorly

90   understood.

91

92   Objectives: We sought to determine if IL-33 signaling in sensory neurons is critical for the

93   development of chronic itch in two divergent pruritic disease models.

94

95   Methods: Plasma levels of IL-33 were assessed in patients with atopic dermatitis (AD) and

96   chronic pruritus of unknown origin (CPUO). Mice were generated to conditionally delete IL-33R

97   from sensory neurons. The contribution of neuronal IL-33R signaling to chronic itch

98   development was tested in mouse models that recapitulate key pathologic features of AD and

99   CPUO, respectively.

100

101   Results: IL-33 was elevated in both AD and CPUO as well as their respective mouse models.

102   While neuron-restricted IL-33R signaling was dispensable for itch in AD-like disease, it was

103   required for the development of dry skin itch in a mouse model that mirrors key aspects of

104   CPUO pathology.

105

106   Conclusion: These data highlight how IL-33 may be a predominant mediator of itch in certain

107   contexts, depending on the tissue microenvironment. Further, this study provides insight for

108   future therapeutic strategies targeting the IL-33 pathway for chronic itch.

109

110   **Key Message**

111   • IL-33 is elevated in two divergent pruritic disease conditions and their respective models

112   • Signaling of the IL-33 receptor in sensory neurons is necessary for dry skin itch, but not

113   itch associated with atopic dermatitis-like disease

114

115   **Capsule Summary**

116   IL-33 signaling in sensory neurons drives chronic itch in dry skin with minimal inflammation and

117   is dispensable in AD-like disease. These findings provide insight on anti-IL-33 mAb therapies

118   currently in phase 2 clinical trials.

119

120   **Key Words**

121   Atopic dermatitis, chronic pruritus of unknown origin, dry skin, IL-33, itch, neuroimmunology,

122   pruriceptor, pruritogen

123

124   **Abbreviations**

125   Ab (antibody), AD (atopic dermatitis), AEW (acetone/ether plus water), bp (base pair), Cap

126   (Capsaicin), CPUO (chronic pruritus of unknown origin), CQ (chloroquine), DRG (dorsal root

127   ganglia), DT (diphtheria toxin), epidermis (Epi), EtOH (ethanol), HC (healthy control), His

128   (histamine), i.d. (intradermal), ILC2s (group 2 innate lymphoid cells), IL-33R (IL-33 receptor), i.p.

4

129 (intraperitoneal), KCl (potassium chloride), LM (littermate), loxP (locus of X-over P1), mAb

130 (monoclonal antibody), MACS (magnetic-activated cell sorting), MasTRECK (mast cell-specific

131 enhancer-mediated toxin receptor-mediated conditional cell knockout), NS (no significance), rh

132 (recombinant human), rm (recombinant mouse), RNA-seq (RNA sequencing), stratum corneum

133 (SC), Veh (Vehicle), WT (wild-type)

134

135 **Introduction**

136

137 Chronic pruritus, or itch, is a debilitating, often intractable condition that causes a reduction in

138 quality of life similar to chronic pain and has a lifetime prevalence of up to 20%.[1,2] Recent

139 studies have identified that various cytokines can function as itch-inducing factors, or

140 pruritogens, at the neuro-immune interface, and there is mounting interest in harnessing the

141 therapeutic potential of blocking these interactions.[3]

142

143 Epithelial cell-derived IL-33 is a potent amplifier of type 2 immune responses and is increasingly

144 implicated in itch, although the mechanisms remain unclear.[4] It has recently been demonstrated

145 that the IL-33 receptor (IL-33R) is expressed in the dorsal root ganglia (DRG) and that IL-33 can

146 directly activate sensory neurons.[5,6] However, whether IL-33R expression in sensory neurons is

147 specifically required for the development of chronic itch, and in what disease setting, remains

148 poorly defined.

149

150 **Results and Discussion**

151

152 IL-33 acts as an 'alarmin' by being rapidly released from damaged epithelial cells to initiate type

153 2 inflammation.[4] In addition to immune cells, IL-33R is also expressed by sensory neurons.[5,6]

154 We confirmed expression of IL-33R (ST2, *Il1rl1*) in mouse DRG (**Fig 1, A**) and, using calcium

155 imaging, found that IL-33 activated 2.1% of DRG neurons (**Fig 1, B and C**). Further, we found

156 that 52% and 63% of IL-33-responsive mouse DRG neurons also responded to histamine and

157 the TRPV1-agonist capsaicin, respectively (**Fig 1, D**). Similarly, we found IL-33R was expressed

158 by human DRG (**Fig 1, E**) and 6.6% of human DRG neurons were responsive to IL-33 (**Fig 1, F**

159 **and G**). Of these neurons, 60% also responded to capsaicin (**Fig 1, H**). Together these findings

160 suggest that IL-33 can directly activate sensory neurons.

161

162 A recent study demonstrated that IL-33R knockdown within the DRG compartment attenuates

163 itch in allergic contact dermatitis.[5] While these findings suggest that neuronal IL-33R signaling

164 may be a critically important itch pathway, the DRG contains a diversity of other cell types. The

165 expression of IL-33R in the DRG, beyond sensory neurons, has yet to be fully assessed. To

166 address this, we analyzed a single cell RNA-seq dataset of naïve mouse DRG (**Fig 1, I**).[7] We

167 found that *Il1rl1* was indeed expressed by another cell type: DRG macrophages (**Fig 1, J**).

168 Similarly, analysis of other neuronally expressed itch-associated cytokine receptors, such as

169 *Il4ra*,[8] revealed expression across numerous cell types (**Fig 1, K**). Taken together, these data

170 underscore that targeted, lineage-specific approaches are likely required to determine the

171 precise contribution of a distinct cell type to itch development. Therefore, the consequence of

172 disrupting IL-33R signaling specifically in sensory neurons remains unknown.

173

174 We generated mice in which loxP sites were inserted into the *Il1rl1* gene locus (IL-33R^flox^ mice)

175 (**Fig 2, A**) and crossed these mice onto the SNS^Cre^ mouse line,[9] generating mice that

176 conditionally lack IL-33R in sensory neurons (IL-33R^Δneuron^ mice). We confirmed the selective

177 loss of *Il1rl1* in sensory neurons, and not immune cells, isolated from IL-33R^Δneuron^ mice (**Fig 2,**

178 **B**). These mice exhibited normal motor function (**Fig 2, C**), thermal pain behavior (**Fig 2, D**), and

179 acute itch response to the classical pruritogens histamine (**Fig 2, E**), chloroquine (**Fig 2, F**), and

6

180 serotonin (**Fig 2, G**), indicating the mice have no gross developmental motor or sensory

181 abnormalities.

182

183 Advances in our understanding of the mechanisms underlying chronic itch have largely drawn

184 from studying inflammatory skin disorders such as atopic dermatitis (AD). AD presents with

185 pruritic skin lesions driven by type 2 inflammation.[10] Given the ability of IL-33 to promote type 2

186 inflammation,[10] there is considerable interest in the therapeutic potential of anti-IL-33

187 monoclonal antibodies (mAbs) in AD.[3,11] Several studies have found elevated levels of IL-33 in

188 both the skin and blood of patients with AD.[10,12,13] In support, we found that patients with

189 moderate-to-severe AD (N = 11, 5.17 ± 1.37) had increased IL-33 in their plasma compared to

190 healthy control (HC) subjects (N = 11, 3.93 ± 1.20) (**Fig 3, A and B, Table E1**). We next utilized

191 a model of AD-like disease, where mice are treated with MC903 (**Fig E1, A**). MC903-treated

192 wild-type (WT) mice developed robust AD-like skin inflammation (**Fig 3, C**).[8] Indeed, analyzing

193 our previously published RNA-seq dataset,[8] we found increased expression of *Il33*, along with

194 transcripts for a number of other pruritogens, in the skin of MC903-treated WT mice compared

195 to controls (**Fig 3, D**). However, while it is well-known that IL-33 is dysregulated in both human

196 and murine AD-associated inflammation, whether IL-33 directly engages the sensory nervous

197 system to elicit itch remains unclear. When we induced AD-like disease in IL-33R$^{\Delta neuron}$ mice,

198 there were no notable differences in clinical or histopathological presentation (**Fig 3, E**), ear

199 thickness (**Fig 3, F**), or scratching bouts (**Fig 3, G**) compared to littermate (LM) controls. Thus,

200 our findings suggest that neuronal IL-33R is dispensable for AD-like disease.

201

202 In AD-like skin, many putative pruritogens are upregulated (**Fig 3, D**) and may override the

203 contribution of IL-33 to itch. Thus, we next sought to test whether neuron-restricted IL-33R may

204 play a more important role in itch that arises in the absence of robust skin inflammation. Chronic

205 pruritus of unknown origin (CPUO) accounts for 10-40% of all chronic itch cases, is poorly

7

206 understood, and lacks effective therapies.[1,14] While patients with AD present with scaly, raised

207 rashes (**Fig 4, A**), chronic itch in CPUO develops in the absence of overt cutaneous

208 inflammation. Additionally, CPUO disproportionately occurs in aged individuals.[14] A key

209 pathogenic factor of CPUO is skin barrier dysfunction, which frequently manifests as dry skin

210 (**Fig 4, B**). The histopathology of CPUO often resembles control skin (**Fig 4, C**), while AD

211 lesional skin exhibits a number of characteristic inflammatory features including irregular

212 epidermal hyperplasia and robust dermal inflammatory infiltrate (**Fig 4, D**). In contrast, CPUO

213 pruritic skin generally exhibits a relatively normal epidermis and mild dermal infiltrate (**Fig 4, E**).

214 We found that patients with CPUO (N = 8, 6.22 ± 2.54) had significantly higher levels of IL-33

215 compared to HCs (N = 11, 3.93 ± 1.20) (**Fig 4, F and G, Table E1**). Thus, we hypothesized that

216 IL-33 may be a key factor in itch physiology associated with CPUO.

217

218 To examine the role of IL-33 in a disease model that recapitulates key pathological features of

219 CPUO, we utilized the acetone/ether plus water (AEW) mouse model (**Fig E1, B**). This model is

220 characterized by the development of dry skin (**Fig 5, A**) and other pathogenic changes that

221 mimic aged skin.[15,16] We have previously utilized the AEW mouse model to identify novel

222 therapeutic approaches that led to proof-of-concept studies in CPUO.[8] In contrast to other

223 mouse models of chronic itch, AEW-elicited itch develops in the absence of notable cutaneous

224 inflammation, similar to CPUO. Indeed, the frequency of cutaneous immune cells (**Fig 5, B**),

225 including mast cells (**Fig 5, C**) and group 2 innate lymphoid cells (ILC2s) (**Fig 5, D**), were

226 comparable between WT mice that were treated with AEW and water-only controls, despite

227 significantly increased itch behavior in AEW-treated mice (**Fig 5, E**). Notably, *Il33* was elevated

228 in the skin of AEW-treated mice compared to controls (**Fig 5, F**).[17] Taken together, our findings

229 demonstrate that AEW-induced itch is associated with IL-33 dysregulation and minimal

230 cutaneous inflammation, similar to CPUO.

231

232 It was recently reported that global deficiency of IL-33 or IL-33R results in decreased AEW-

233 induced itch.[18] However, how IL-33 drives the development of dry skin itch is poorly understood.

234 Indeed, whether IL-33 can promote itch through a mechanism independent of canonical immune

235 circuits remains unknown. Mast cells, and more recently basophils, have been implicated as key

236 mediators of itch.[19–21] To test if these cell types contribute to dry skin itch, we employed

237 MasTRECK mice, which allow for diphtheria toxin (DT)-mediated depletion of mast cells and

238 basophils (**Fig E2**). However, AEW-induced scratching bouts were comparable between DT-

239 treated LM control and MasTRECK mice (**Fig 5, G**). IL-33 also potently activates both ILC2s

240 and T cells to modulate the skin immune responses.[4] However, we found no difference in itch

241 between AEW-treated lymphocyte-deficient $Rag2/Il2rg^{-/-}$ mice and controls (**Fig 5, H**). Finally, we

242 generated mice that conditionally lack IL-33R in immune cells by crossing the IL-33R^{flox} mice

243 with the Vav^{Cre} line (IL-33R$^{\Delta immune}$). Following AEW-treatment, there was no difference in the

244 number of scratching bouts between LM control and IL-33$^{\Delta immune}$ mice (**Fig 5, I**). Collectively,

245 these data suggest that immune cells are largely dispensable for the induction of dry skin itch

246 and instead implicate sensory neurons as the potential primary target of IL-33 for itch

247 development.

248

249 To test the hypothesis that neuronal IL-33R regulates dry skin itch, we utilized the IL-33R$^{\Delta neuron}$

250 mice. Strikingly, AEW-treated IL-33R$^{\Delta neuron}$ mice demonstrated significantly attenuated itch

251 behavior compared to LM controls (**Fig 5, J**). Despite the requirement of neuronal IL-33R for dry

252 skin itch, IL-33 alone was not sufficient to induce robust acute itch responses (**Fig 5, K**), similar

253 to prior reports.[5] This led us to hypothesize that IL-33 may instead sensitize sensory neurons.

254 Indeed, it has been shown that AEW-treated mice exhibit enhanced responsiveness to

255 exogenous pruritogens like chloroquine (CQ).[22,23] However, the mechanisms underlying these

256 observations are not well understood. Using calcium imaging, in a proof-of-concept experiment,

257 we found that IL-33 treatment of DRG neurons increased the number of cells responding to CQ

258 (**Fig 5, L and M**). Thus, although CQ is not a native endogenous pruritogen in dry skin, these

259 studies represent one example by which IL-33 may amplify responses to pruritogens in order to

260 promote chronic itch. Future studies will be required to determine the precise molecular

261 mechanisms by which IL-33 may enhance itch in this manner.

262

263 Our findings suggest that neuron-restricted IL-33R signaling is a critical regulator of itch that

264 arises in the setting of dry skin, independent of immune cells. Furthermore, our findings are

265 consistent with prior studies demonstrating that IL-33 may be dispensable for the development

266 of AD-like disease.[24,25] Together, these findings may help explain why anti-IL-33 mAbs (e.g.

267 etokimab) have failed to meet their primary endpoints or have been discontinued following

268 recent phase 2 clinical trials in AD (NCT03533751, NCT03736967). In contrast, IL-33 may be an

269 important therapeutic target in dry skin itch and CPUO.

270

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341

342 **Figure 1: Mouse and human DRG express IL-33R.** (**A**) Gel of *Il1rl1* RT-PCR product from

343 dorsal root ganglia (DRG) isolated from one wild-type (WT) mouse. Representative of three

344 mice. (**B**) Representative calcium imaging trace of mouse DRG neuron in response to vehicle

345 (Veh), recombinant mouse (rm)IL-33, capsaicin (Cap), and potassium chloride (KCl). (**C**)

346 Percent of rmIL-33-, histamine (His)-, and Cap-responsive DRG neurons out of all KCl-

347 responsive DRG neurons. (**D**) Venn diagrams of overlapping responses between IL-33-

348 responsive (IL-33^{+}) and Cap-responsive (Cap[+]) or His-responsive (His^{+}) neurons. (**B-D**) n > 900

349 neurons from at least 4 WT mice (6 combined experiments). (**E**) Gel of *IL1RL1* RT-PCR product

350 from DRG isolated from one human donor. Representative of three donors. *Ladder has been

351 previously published in Oetjen et al.[8] (**F**) Representative calcium imaging trace of human DRG

352 neuron in response to Veh, recombinant human (rh)IL-33, Cap, and KCl. (**G**) Percent of rhIL-33-

353 and Cap-responsive DRG neurons out of all KCl-responsive DRG neurons. (**H**) Venn diagrams

354 of overlapping responses between IL-33^{+} and Cap^{+} neurons. (**F-H**) N > 200 neurons from 2

355 human subjects (2 combined experiments). (**I**) t-SNE plot of single cell RNA-seq of mouse DRG

356 colored by cell populations. Violin plots of (**J**) *Il1rl1* and (**K**) *Il4ra* gene expression. Full dataset in

357 Avraham et al.[7]

358

359 **Figure 2: Generation of IL-33R conditional deletion mice** (**A**) Map of *Il1rl1* conditional knock-

360 out allele. blue triangles, loxP sites; gray boxes, exons; red box, conditionally deleted region. (**B**)

361 Expression of *Il1rl1* in lymph node-derived immune cells (left) and MACS-sorted sensory

362 neurons (right) from littermate (LM) control and IL-33R[Δneuron] mice by RT-qPCR. n > 3

363 mice/group. (**C-G**) Assessment of (**C**) motor activity (rotarod), (**D**) thermal pain behavior (hot

364 plate), and acute itch behavior following intradermal injection (i.d.) of (**E**) histamine, (**F**)

365 chloroquine, and (**G**) serotonin in LM control and IL-33R[Δneuron] mice. (**C-G**) n > 4 mice/group (**E-**

366 **G**), 2 combined experiments. Not significant (NS), *$p < 0.05$ by unpaired, two-tailed *t* test.

367

Figure 3: IL-33R signaling in sensory neurons is dispensable for chronic itch in AD-like disease. (**A**) Schematic of the measurement of IL-33 in the plasma of 11 healthy control (HC) subjects and 11 patients with atopic dermatitis (AD) by Luminex multiplex ELISA. (**B**) Amount of IL-33 in the plasma of HC subjects and patients with AD. (**C**) Representative clinical images and H&E sections of ear skin from ethanol (EtOH)- or MC903-treated WT mice (day 12). Scale bar is 50 μm. (**D**) Heatmap and hierarchical clustering of significantly differentially expressed genes in the ear skin of EtOH- or MC903-treated WT mice (day 12). The most differentially expressed genes (1,300 genes) are displayed (based on the *t* statistic value). n = 4 mice/group. Full dataset in Oetjen et al.[8] (**E**) Representative clinical images and H&E sections of MC903-treated LM control and IL-33R$^{\Delta neuron}$ mice (day 12). Scale bar is 20 μm. (**F**) Percent change in ear thickness and (**G**) number of scratching bouts from MC903-treated LM control and IL-33R$^{\Delta neuron}$ mice over time (days). n = 13-18 mice/group (2-3 combined experiments). (**B**) *p<0.05 by unpaired, two-tailed *t* test. (**F-G**) NS by multiple *t* test using Holm-Sidak method.

381

Figure 4: IL-33 is elevated in CPUO. Representative clinical images from a patient with (**A**) AD and (**B**) chronic pruritus of unknown origin (CPUO). Black boxes indicate zoomed-in view of skin. Representative H&E skin sections from (**C**) control, (**D**) patient with AD, and (**E**) patient with CPUO. Bracket, stratum corneum (SC); brace, epidermis (Epi); black arrow, spongiosis; gray arrow, vascular dilatation; white arrow, dermal perivascular immune infiltrate. Scale bar represents 100 μm. (**F**) Schematic of the measurement of IL-33 in the plasma of 11 HC subjects (same subjects as in Figure 1) and 8 patients with CPUO by Luminex multiplex ELISA. (**G**) Amount of IL-33 in the plasma of HC subjects and patients with CPUO. *p<0.05 by unpaired, two-tailed *t* test.

391

13

Figure 5: Dry skin itch is dependent on IL-33 signaling in sensory neurons. (**A**) Representative clinical images of skin from water- or AEW-treated WT mice (day 5). Frequency of (**B**) immune cells (**C**) mast cells and (**D**) group 2 innate lymphoid cells (ILC2s) in the skin of water- or AEW-treated WT mice (day 5). n = 6-8 mice/group (2 combined experiments). (**E**) Number of scratching bouts from water- or AEW-treated WT mice (day 5). n = 5 mice/group (representative of 3 experiments). (**F**) Expression of *Il33* by RT-qPCR in water- or AEW-treated skin of WT mice (day 4). n = 5-7 mice/group (2 combined experiments). Number of scratching bouts from AEW-treated (**G**) LM control and MasTRECK mice, (**H**) control (Cont) and *Rag2/Il2rg*$^{-/-}$ mice, (**I**) LM control and IL-33R$^{\Delta immune}$ mice, and (**J**) LM control and IL-33R$^{\Delta neuron}$ mice (day 5). (G-J) n = 9-18 mice/group (2 combined experiments). (**K**) Number of scratching bouts following i.d. injection of Veh or rmIL-33 in WT mice. n = 6-8 mice/group (2 combined experiments). (**L**) Representative calcium traces of mouse DRG neurons responding to chloroquine (CQ) after exposure to Veh or rmIL-33. Each trace represents one neuron. (**M**) Percent of CQ-responsive neurons out of all KCl-responsive neurons following exposure to Veh or rmIL-33. n = >400 neurons from 3 mice (2 combined experiments). (**B-J, M**) NS, *p<0.05, **p<0.01, ****p<0.0001 by unpaired, two-tailed t test. (**K**) NS by one-way ANOVA with multiple comparisons.

1 **Online Repository**

2

3 **Methods**

4 **Human subjects**

5 Studies were conducted in accordance with The Code of Ethics of the World Medical

6 Association. Written consent was provided by all donors prior to sample collection, and

7 human studies were approved by the Washington University in St. Louis Institutional

8 Review Board. For cytokine profile assessment, blood was collected from patients with

9 moderate-to-severe atopic dermatitis (AD) and chronic pruritus of unknown origin

10 (CPUO) seen by the Division of Dermatology at WUSM/BJH between March 2015 to

11 November 2018 as well as from recruited healthy controls (HCs). Board-certified

12 dermatologists determined the diagnoses of AD based on criteria outlined in Eichenfield

13 et al,[E1] while the diagnoses of CPUO was based on Kim et al.[E2] Healthy control and

14 patient demographics are included in **Table E1**. Dorsal root ganglia (DRG) samples

15 were obtained from de-identified US transplant donors under an IRB-exempt protocol at

16 the University of Cincinnati. Control skin sections for H&E were obtained from the skin

17 of patients that presented to the hospital either for an amputation due to chronic ulcers

18 or for cancer resection. There had to be a clear margin for sectioning (at least 10 cm for

19 samples from amputations and 1 cm from tumor resections), which was re-reviewed by

20 a board-certified pathologist.

21

22 **Research animals**

23 All animal experiments were conducted using protocols approved by the Washington

24 University Institutional Animal Care and Use Committee. Mice were maintained in

25 standard husbandry conditions (social housing, 12 hr light-dark cycle, 23°C, food and

26 water *ad libitum*). Wild-type (WT) C56BL/6J were purchased from the Jackson

27 Laboratory. *Rag2/Il2rg$^{-/-}$* double knockout mice were purchased from Taconic

28 Biosciences. MasTRECK mice were donated by Dr. Seiji Nishino (Stanford University).

29 SNS^{Cre} mice were donated by Dr. Rohini Kuner (Heidelberg University). IL-33R^{flox} were

30 generated by Cyagen Biosciences Inc., California, USA on a C57Bl6/J background. IL-

31 33R$^{\Delta neuron}$ mice were generated by crossing SNS^{Cre} with IL-33R^{flox} mice. Experiments

32 were conducted with independent cohorts of male and female mice that were 8 -12

33 weeks old except for calcium imaging experiments, where 4-7 week old mice were

34 used. No phenotypic differences based on sex were observed.

35

36 **Chronic itch mouse models**

37 For induction of AD-like disease, the bilateral ear skin of mice (ventral side only) was

38 treated with MC903 (1 nmol in 10 µL of ethanol, Tocris Bioscience) or ethanol (EtOH)

39 control once daily for 12 days as previously described.[E3] Ear thickness measurements

40 were performed with dial calipers as previously described. [E4, E5] Percent change was

41 calculated from baseline (day 0).

42

43 To induce dry skin itch, we used the acetone/ether plus water (AEW) model as

44 previously described.[E6] At least two days prior to the first treatment, we shaved the

45 nape or cheek skin. On treatment days, a 1:1 ratio of acetone (Sigma-Aldrich) + diethyl

46   ether (Sigma-Aldrich) was applied using a cotton pad for 15 seconds to the shaved skin

47   (cheek or nape) followed immediately by application of a cotton pad soaked in distilled

48   water for 30 seconds. Cotton pads used for water treatment were never re-used. Mice

49   received treatments twice daily (~8 hrs apart) for five days.

50

51   **Itch behavior assessment (chronic and acute)**

52   For assessment of itch behavior, mice were acclimated to the test chambers two days

53   prior to the initiation of the experiment. Mice were additionally acclimated for at least 5

54   minutes before each recording. For chronic itch models, we recorded the mice in the

55   morning (before they received any treatment). To assess acute itch responses,

56   acclimated mice where given an intradermal injection of 20 µL of either histamine (1

57   mg/mL, Sigma-Aldrich), chloroquine (100 µg, Thermo Fisher Scientific), serotonin (1

58   mM, Sigma-Aldrich), recombinant mouse (rm)IL-33 (50, 300 or 1000 ng; R&D Systems),

59   or saline control into their right cheek (shaved two days prior) and then itch behavior

60   was immediate recorded. Video recordings were manually scored for the number of

61   scratching bouts in a 30-minute period. A scratching bout was defined as a continuous

62   scratching motion by the hindpaw that ended when the mouse placed its paw on the

63   floor or in its mouth. Data for acute itch model only contains cheek-directed scratching

64   bouts (site of pruritogen administration).

65

66   **Pain behavior assessment (thermal sensitivity)**

67   Thermal sensitivity was assessed using the hotplate assay. Hotplate temperature was

68   set to 50°C. Latency was measured as time from mouse being placed on the hotplate

69   and removal upon either flicking/licking of its hind paw or jumping was observed. Data

70   was averaged across two trials taken over two days.

71

72   **Rotarod testing**

73   To test for potential defects in coordinated motor activity, mice were tested using a

74   rotarod system (Ugo Basile) where mice were placed on a rotating treadmill that was

75   accelerated from 5 rotations per minute (rpm) to 40 rpm over 5 minutes (built-in program

76   of the apparatus). Time was recorded from acceleration initiation until mice fell from the

77   rod or completed one passive rotation (time to failure). Training occurred over 3 days

78   prior to testing day with 3 trials conducted per day with a 10-minute break between each

79   trial. On testing day, 3 trials were completed with a 10-minute break between each trial

80   and data was averaged across all trials.

81

82   **Basophil and mast cell depletion**

83   Basophil and mast cell depletion was performed as previously described.[E7] Briefly,

84   MasTRECK and littermate (LM) mice were treated daily with intraperitoneal (i.p.)

85   injections of diphtheria toxin (DT; 250 ng in 100 µL of PBS; Sigma-Aldrich) for five

86   consecutive days immediately prior to initiation of AEW treatments. Depletion was

87   verified by flow cytometry (**Fig E2**).

88

89   **Histological analysis**

90   To assess murine AD-like histopathology, ears were harvested from EtOH- or MC903-

91   treated mice and were fixed in 4% paraformaldehyde (PFA, Thermo Fisher Scientific),

92   embedded in paraffin, sectioned, and stained with hematoxylin and eosin (H&E).

93   Histology for Fig 3, C was performed by WUSM Digestive Disease Research Core

94   Center and slides were imaged using the NanoZoomer 2.0-HT System (Hamatsu).

95   Histology for Fig 3, E was performed by HistoWiz Inc (histowiz.com) using a standard

96   operating procedure and fully automated workflow. For human histopathology, 4 mm

97   punch biopsies of human skin were collected and fixed in 10% PFA. Samples were

98   embedded in paraffin and sections were stained with H&E. Slides were imaged using

99   the NanoZoomer 2.0-HT System (Hamatsu).

100

101 **Plasma cytokine measurement**

102 IL-33 levels in human samples were assessed using a custom Luminex ELISA kit (R&D

103 Systems) as previously described.[E8] Plasma was isolated following Ficoll gradient

104 separation of peripheral blood drawn from patients with AD, patients with CPUO, or

105 healthy control subjects (**Table E1**). Before the detection assay, plasma was diluted (1:1

106 in assay diluent), loaded onto Protein L-coated plates (Thermo Fisher Scientific), and

107 incubated on an orbital shaker at room temperature for 90 minutes. FLEXMAP three-

108 dimensional system (Thermo Fisher Scientific) was used to collect the data.

109

110 **RNA-seq data analysis**

111 We reanalyzed RNA-seq data of mouse ear skin treated with MC903 or EtOH (in Fig 3,

112 D). Dataset was obtained from NCBI GEO[E9] under the accession number GSE90883.

113 Full methods on sample processing are available in Oetjen et al. [E3] For our re-analysis,

114 duplicate genes were removed, and genes were filtered for protein coding designation.

115 Using row mean (counts), the top 12,000 genes were selected for additional

116 downstream analysis. Differential gene expression was calculated using the *limma* R

117 package with the online Phantasus software (https://artyomovlab.wustl.edu/phantasus/)

118 along with hierarchical clustering (one minus pearson correlation) and heatmap

119 generation. Only top 1,300 most differentially expressed genes are displayed in figure

120 (based on *t* value).

121

122 **Single cell RNA-seq data analysis**

123 We reanalyzed single cell RNA-sequencing dataset of mouse dorsal root ganglia (in Fig.

124 1, I-K). Dataset was obtained from NCBI GEO[E9] under the accession number

125 GSE158892. Full methods on sample processing are available in Avraham et al. [E10]

126 Data analysis and processing was performed using commercial code from Partek Flow

127 package at https://www.partek.com/partek-flow/. Processed data are publicly available

128 at https://mouse-drg-injury.cells.ucsc.edu/.

129

130 **Mouse RNA isolation and qRT-PCR**

131 For RNA isolation from the whole dorsal root ganglia (DRG), DRG were harvested from

132 naïve wild-type (WT) mice and homogenized in 1 mL Trizol Reagent (Life Technologies)

133 with a bead homogenizer (BioSpec). RNA was extracted using the RNeasy Mini Kit

134 (QIAGEN) and DNA was digested using Turbo DNA-free Kit (Invitrogen) following the

135 manufacturer's instructions.

136

137 For RNA isolation from purified DRG neurons, DRG were harvested from LM control

138 and IL-33R[Δneuron] mice then digested as previously described.[E3,E11] Briefly, DRG was

139 incubated in Ca^{2+}/Mg^{2+}-free HBSS containing collagenase type I (342 U/mL; GIBCO)

140 and dispase II (3.8 U/mL; GIBCO) at 37°C on a rotator for 30-40 minutes. Sample was

141 triturated then transferred into MACS buffer (0.5% BSA in DPBS). Sensory neurons

142 were negatively selected from the DRG using the MACS Neuron Isolation kit (130-115-

143 389; Miltenyi Biotec) as previously described by Thakur et al.[E12] RNA was extracted

144 using the Nucleospin RNA XS kit (Takara Bio) according to manufacturer's instructions.

145

146 For RNA isolation from immune cells, lymph nodes were harvested (inguinal,

147 mesenteric, and superficial cervical) from naïve LM control and IL-33R$^{\Delta neuron}$ mice

148 before being manually homogenized through a 70 µm cell strainer and washed (5%

149 FBS in DMEM). RNA was extracted using the Nucleospin RNA kit (Takara Bio)

150 according to manufacturer's instructions.

151

152 For RNA isolation from skin, water control- or AEW-treated cheek skin from WT mice

153 were harvested 4 hours following the last treatment of AEW on day 4 of AEW mouse

154 model. Skin samples were stored in RNAlater (Invitrogen) at 4°C before transfer to -

155 80°C. Following tissue homogenization with a bead homogenizer (BioSpec) in 350 µL of

156 RNA lysis buffer (Nucleospin RNA, Takara Bio), RNA was isolated using the Nucleospin

157 RNA kit (Takara Bio) per the manufacturer's instructions.

158

159 Following all upstream protocols of RNA isolation, cDNA was synthesized using the

160 High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems). For relative

161 quantification of *Il33* mRNA, qRT-PCR was performed with 10 ng of cDNA, pre-

162   validated commercial primers (Mm.PT.58.31135215; Integrated DNA Technologies),

163   and the Fast SYBER Green Master Mix (Applied Biosystems) on a StepOnePlus

164   machine (Applied Biosystems). For relative quantification of *Il1rl1* mRNA, qRT-PCR was

165   performed with 10 ng of cNDA, pre-validated commercial primer-probe assay

166   (Mm.PT.58.11610831.g; Integrated DNA Technologies), and the TaqMan Gene

167   Expression Master Mix (Applied Biosystems) on a StepOnePlus machine (Applied

168   Biosystems). Gene expression was normalized to *Gapdh* (Mm.PT.39a.1; Integrated

169   DNA Technologies). Fold change was calculated using the $2^{-\Delta\Delta CT}$ method.[E13] Products

170   from *Il1rl1* qRT-PCR reaction were run on a 2% agarose gel with 1 mg/mL of ethidium

171   bromide at 140 V.

172

173   **Human RNA isolation and RT-PCR**

174   Human dorsal root ganglia (hDRG) were dissected from donors and the fat, dura, and

175   connective tissues were removed as previously described.[E14] Samples were kept in

176   RNAlater (Sigma-Aldrich) at -80°C. One half of a single DRG was homogenized in 1 mL

177   of Trizol Reagent according to manufacturer's instructions for total RNA extraction. For

178   genomic DNA elimination and cDNA synthesis, the Maxima H Minus First Strand cDNA

179   Synthesis Kit with dsDNase (Thermo Scientific) was used with the *IL1RL1* primer set

180   (Forward: CAG GGA GCG GCA GGA ATG T, Reverse: CTT GCA TTT ATC AGC CTC

181   CAG AGA A; Millipore Sigma) in accordance with manufacturer's instructions. For gel

182   electrophoresis, RT-PCR product was loaded onto a 2% agarose gel with 1 mg/mL of

183   ethidium bromide at 100 V.

184

185 **Calcium imaging of mouse DRG neurons**

186 We isolated mouse DRG neurons and performed calcium imaging as previously

187 described.[E3,11] Following euthanasia via CO_2 inhalation, the DRG were harvested.

188 Nerve fibers were trimmed, and connective tissue was removed from the DRGs. For

189 enzymatic dissociation, DRGs were incubated in 1 mL of Ca^{2+}/Mg^{2+}-free HBSS

190 containing collagenase type I (342 U/mL; Worthington Biochemical Corporation) and

191 dispase II (4 U/mL; GIBCO) at 37°C on a rotator for 30 - 40 minutes. DRGs were then

192 triturated to generate a single cell suspension. Dissociated DRG neurons were then

193 seeded on 8 mm glass pre-coated with poly-D-lysine (20 mg/mL, Fisher) and laminin

194 (20 mg/mL, Sigma). Cells were cultured overnight at 37°C with 5% CO_2 in Neurobasal-A

195 culture medium (GIBCO) supplemented with nerve growth factor (100 ng/mL; Sigma-

196 Aldrich), glial cell-derived neurotrophic factor (20 ng/mL; Sigma-Aldrich), B-27 (2%;

197 GIBCO), penicillin (100 U/mL; Sigma-Aldrich), streptomycin (100 mg/mL; Sigma-

198 Aldrich), and 10% FBS (Sigma-Aldrich).

199

200 The following day, the cultured DRG neurons were loaded with the calcium indicator

201 dye Fura-2 AM (4 µM; Invitrogen) for 20-45 min then washed with calcium imaging

202 buffer (130 mM NaCl, 3 mM KCl, 2.5 mM $CaCl_2$, 0.6 mM $MgCl_2$, 10 mM HEPES, 10 mM

203 glucose, 1.2 mM NaHCO3). Slides were imaged with alternating 340 and 380 nm

204 excitation wavelengths using an inverted Nikon Ti-S microscope, CoolSNAP CCD

205 camera (Photometrics), and NIS-elements software (Nikon Instruments). Capsaicin (300

206 nM; Sigma-Aldrich), histamine (50 µM, Sigma-Aldrich), and KCl (100 mM; Sigma-

207 Aldrich) were applied to DRG neurons via perfusion. rmIL-33 (1 µg/mL; R&D Systems)

208   and vehicle control (0.1% BSA (Sigma-Aldrich) in PBS) were manually loaded by gently

209   pipetting the solution into the recording chamber. Cells were washed with calcium

210   imaging buffer for at least 3 minutes between stimuli. For sensitization experiments, we

211   evaluated the response of DRG neurons to chloroquine (1 mM; MP Biomedicals)

212   immediately after stimulation with vehicle control (0.1% BSA) or rmIL-33 (4 μg/mL) with

213   no wash between exposure to vehicle or rmIL-33 and chloroquine. Fluorescence ratios

214   (340/380) were normalized to baseline. A change in the fluorescence ratio (340/380) of

215   greater than 10% was considered to be a cellular response.

216

217   **Calcium imaging of human DRG neurons**

218   We isolated human DRG neurons and performed calcium imaging as previously

219   described[E3,14]. Following DRG dissociation, cells were plated on glass coverslips and

220   incubated for 3 days in Neurobasal-A media supplemented with B-27, penicillin (100

221   U/mL) plus streptomycin, Glutamax (2 mM; GIBCO) and FBS (5%) at 37°C with 5%

222   CO_2. Cells were loaded with Fura-2 AM (3 μg in 3 μL DMSO in 1 mL of media) before

223   imaging on an Olympus BX51 microscope with Rolera Bolt camera (Q-Imaging) and a

224   CoolLED pE-4000 (365/385) illumination system controlled via MetaFluor software

225   (Molecular Devices). Recombinant human IL-33 (1 μg/mL; R&D Systems), capsaicin

226   (250 nM) and KCl (60 mM) were administered to the bath. A change in the fluorescence

227   ratio (340/380) of greater than 10% was considered to be a cellular response.

228

229   **Flow cytometry**

230 Samples were collected on day 5 of the AEW mouse model. For skin digestion, tissue

231 was minced then incubated in 500 µL of Liberase TL (0.25 mg/mL; Roche) in DMEM

232 (Sigma-Aldrich) at 37°C and 5% CO_2 for 90 min. To obtain a single-cell suspension,

233 skin and spleen samples were then manually homogenized through a 100 µm cell

234 strainer. To lyse erythrocytes in spleen samples, samples were resuspended in 2 mL of

235 Red Blood Cell Lysis Buffer Hybri-Max (Sigma-Aldrich) and incubated for 5 min. To test

236 for cellular viability, all cells were stained with Zombie UV dye (1:500; Biolegend) for 20

237 minutes at room temperature. Blocking solution (anti-mouse CD16/CD32 - 2.4G2 clone;

238 2 µg/mL; Bio X Cell) was applied to cells for 10 min (4°C) before cells were stained with

239 primary antibodies diluted in BD Horizon Brilliant Stain Buffer for 30 min (4°C). Cells

240 were then stained with secondary streptavidin-conjugated antibodies for 15 min (4°C).

241 Finally, cells were fixed overnight at 4°C with BD Cytoperm/Cytofix reagent before

242 sample acquisition on a LSR Fortessa X-20 (BD Biosciences). Data were analyzed with

243 FlowJo 10 (Tree Star). Immune cells were defined as live CD45$^+$ cells.

244

245 Group 2 innate lymphoid cells (ILC2s) were defined as live IL7R$^+$ KLRG1$^+$ ST2$^+$ cells

246 that were negative for the lineage (Lin) markers: CD11b, CD11c, NK1.1, CD19, FcεR1α,

247 CD3ε, and CD4. Thus, cells were stained with the following antibodies to determine

248 ILC2 frequencies: CD11b PerCP/Cy5.5 (M1/70; eBioscience), CD11c PerCP/Cy5.5

249 (N418; eBioscience), NK1.1 PerCP/Cy5.5 (PK136; eBioscience), CD19 PerCP/Cy5.5

250 (1D3; eBioscience), FcεR1α PerCP/Cy5.5 (MAR-1; Biolegend), CD3ε Pe/Cy7 (145-

251 2C11; Biolegend), CD4 BV421 (GK1.5; Biolegend), IL7Rα (CD127) BV650 (A7R34;

252   Biolegend), KLRG1 PE/Daz (MAFA; Biolegend), ST2 Biotin (RMST2-2; eBioscience),

253   and Strepavidin FITC (Biolegend).

254

255   Basophils were defined as live FcεR1α/IgE⁺ CD49b⁺ cells that were negative for c-KIT,

256   Siglec-F and the Lin markers: CD5, CD11c, CD19, and NK1.1. Mast cells were defined

257   as live c-KIT⁺ FcεR1α/IgE⁺ cells that were negative for SiglecF and the Lin markers:

258   CD5 or CD3ε, CD11c, CD19, and NK1.1. Thus, cells were stained with the following

259   antibodies to evaluate basophil and mast cell frequencies: CD5 PerCP/Cy5.5 (53-7.3;

260   eBioscience) or CD3e PerCP/Cy5.5 (145-2C11; eBioscience), CD11c PerCP/Cy5.5

261   (N418; eBioscience), CD19 PerCP/Cy5.5 (1D3; eBioscience), NK1.1 PerCP/Cy5.5

262   (PK136; eBioscience), Siglec-F BV421 (E50-2440; BD Bioscience), c-KIT (CD117)

263   Pe/Cy7 (2B8, eBioscience) or c-KIT BV605 (ACK2; Biolegend), FcεR1α FITC (MAR-1;

264   eBioscience), IgE FITC (23G3; eBioscience), and CD49b APC (DX5; eBioscience).

265

266   **Statistical analysis**

267   All data are presented as mean ± standard deviation. Data from independent

268   experiments were pooled where indicated. Statistical significance for two groups were

269   determined using a two-tailed, unpaired Student's *t* test or multiple *t* test using Holm-

270   Sidak method. Differences were considered significant if $p < 0.05$. Venn diagrams were

271   generated using BioVenn.[E15] Graphical results and statistical testing for RNA-seq were

272   conducted using Phantasus (https://artyomovlab.wustl.edu/phantasus/). scRNAseq

273   analysis was generated using Partek Flow package (https://www.partek.com/partek-

274   flow/). Outliers were identified based on the ROUT method (Q = 1%; GraphPad Prism7

275   Software). The remainder of graphs were generated and statistical analysis were

276   preformed using GraphPad Prism7 Software. Significance is labeled as: N.S., not

277   significant, $*p < 0.05$, $**p < 0.01$, $****p < 0.0001$.

278

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323

324

Characteristic	Control	AD	CPUO
Subjects (N)	11	11	8
Mean age (yr)	37.1 ± 12.6	43.7 ± 15.0	77.8 ± 5.3
Percent female	54.5%	45.5%	12.5%

325

326 **Table E1: Demographics for subjects analyzed in Fig. 2 & 3, plasma cytokines.**

327 Data are represented as mean ± SD.

328

329 **Figure E1: Mouse models of chronic itch.** (**A**) Schematic of treatment course for

330 inducing AD-like disease. Mice are treated once daily for 12 days with ethanol (EtOH) or

331 MC903. (**B**) Schematic of treatment course for inducing dry skin itch, a model that

332 mirrors CPUO. Mice are treated twice daily for 5 days with water-only control or AEW

333 (acetone/ether plus water).

334

335 **Figure E2: Depletion of mast cells and basophils following DT treatment in**

336 **MasTRECK mice.** (**A**) Schematic of selective depletion of mast cells and basophils.

337 Littermate (LM) control and MasTRECK mice received daily intraperitoneal (i.p.)

338 injections of diphtheria toxin (DT) for five days prior to the initiation of AEW treatment.

339 (**B**) Representative flow cytometry plots and frequency of mast cells in the skin of AEW-

340 treated LM control and MasTRECK mice (day 5). n = 5-9 mice/group. Representative

341 flow cytometry plots and frequency of basophils (**C**) in the spleen and (**D**) the skin of

342 AEW-treated LM control and MasTRECK mice (day 5). n = 5-9 mice/group. Data are

343   representative of two independent experiments. Unpaired, two-tailed *t* test was used for

344   all statistical analyses. NS, ****$p < 0.0001$ by unpaired, two-tailed *t* test.
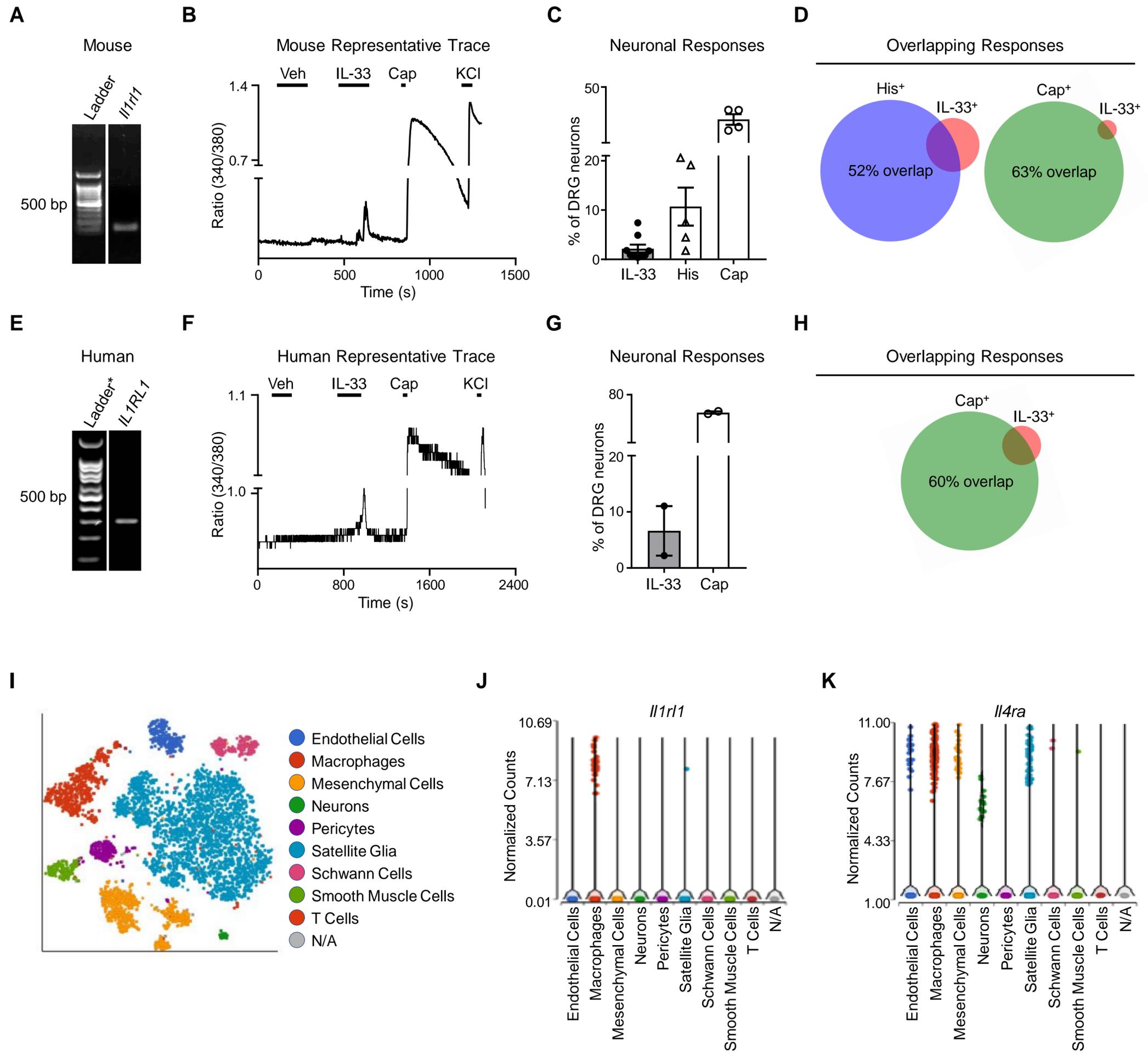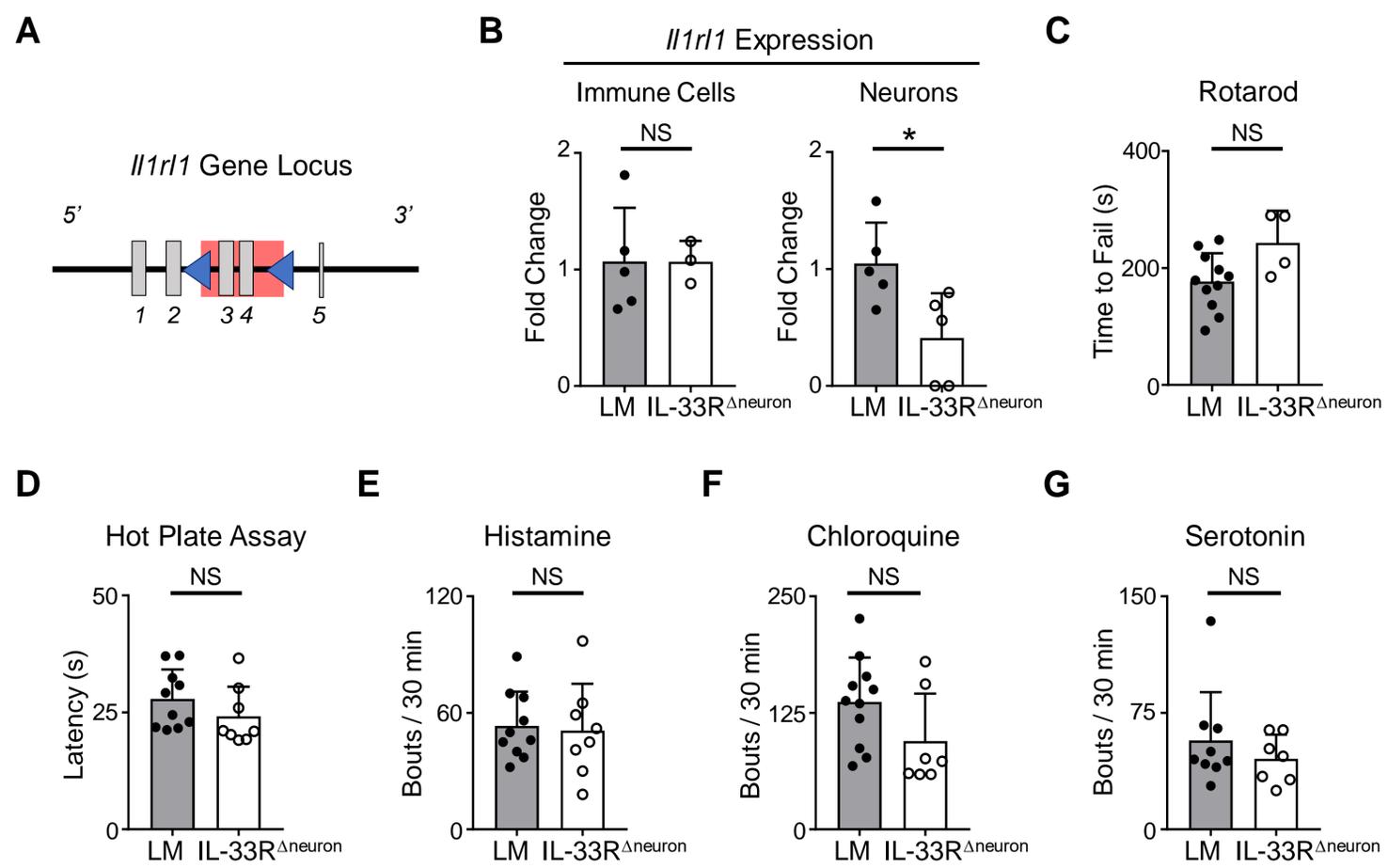
Figure 1: Mouse and human DRG express IL-33R

A Mouse

B Mouse Representative Trace

C Neuronal Responses

D Overlapping Responses

E Human

F Human Representative Trace

G Neuronal Responses

H Overlapping Responses

I

Endothelial Cells
Macrophages
Mesenchymal Cells
Neurons
Pericytes
Satellite Glia
Schwann Cells
Smooth Muscle Cells
T Cells
N/A

J *Il1rl1*

K *Il4ra*

Figure 2: Generation of novel conditional IL-33R knock-out mice

A *Il1rl1* Gene Locus
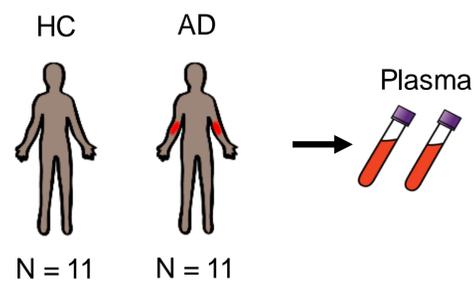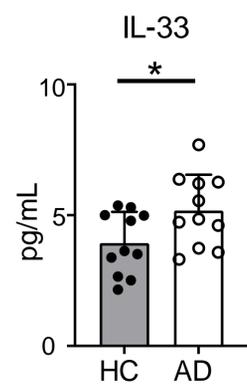
B *Il1rl1* Expression — Immune Cells / Neurons

C Rotarod

D Hot Plate Assay

E Histamine

F Chloroquine

G Serotonin

Figure 3: IL-33R signaling in sensory neurons is dispensable for chronic itch in AD-like disease
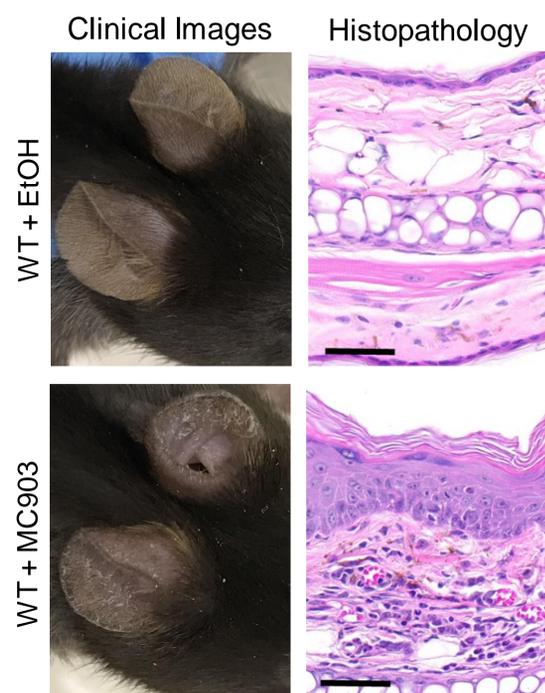
Figure 4: IL-33 is elevated in CPUO
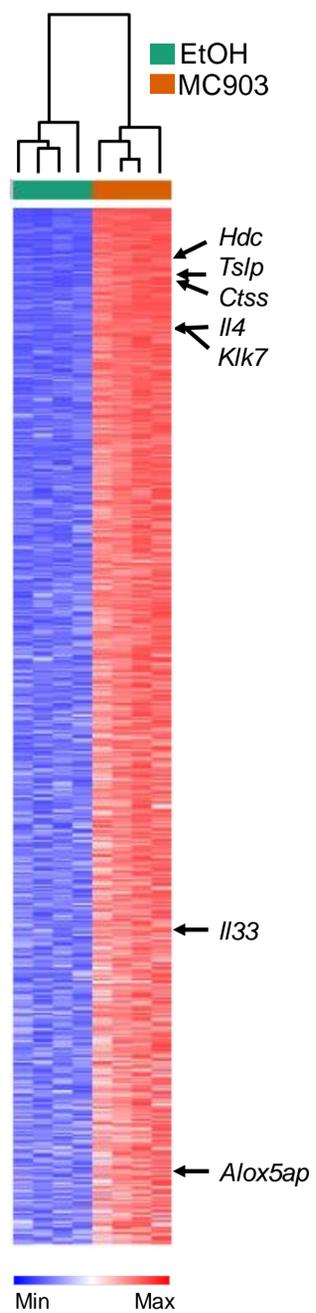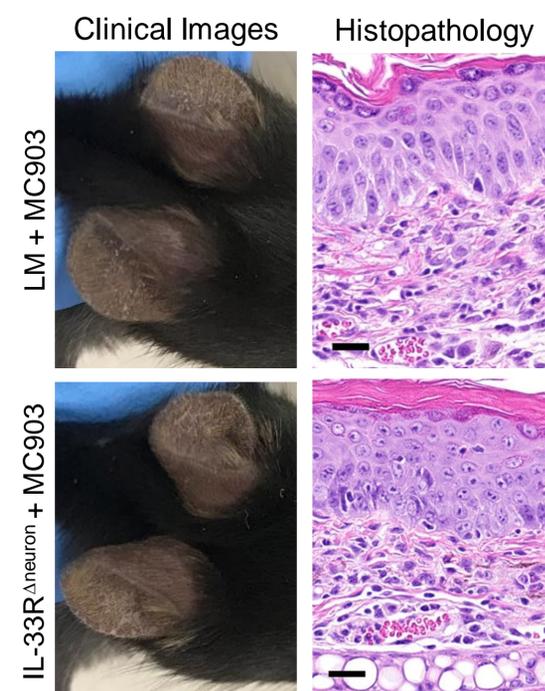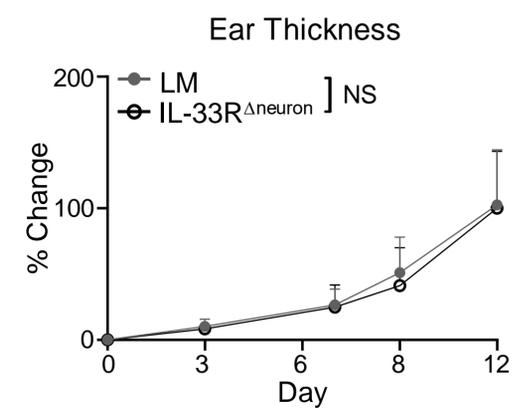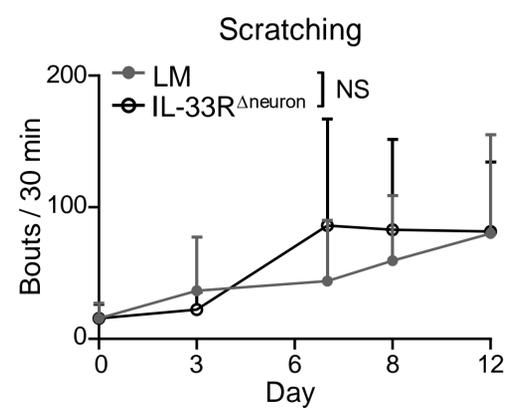
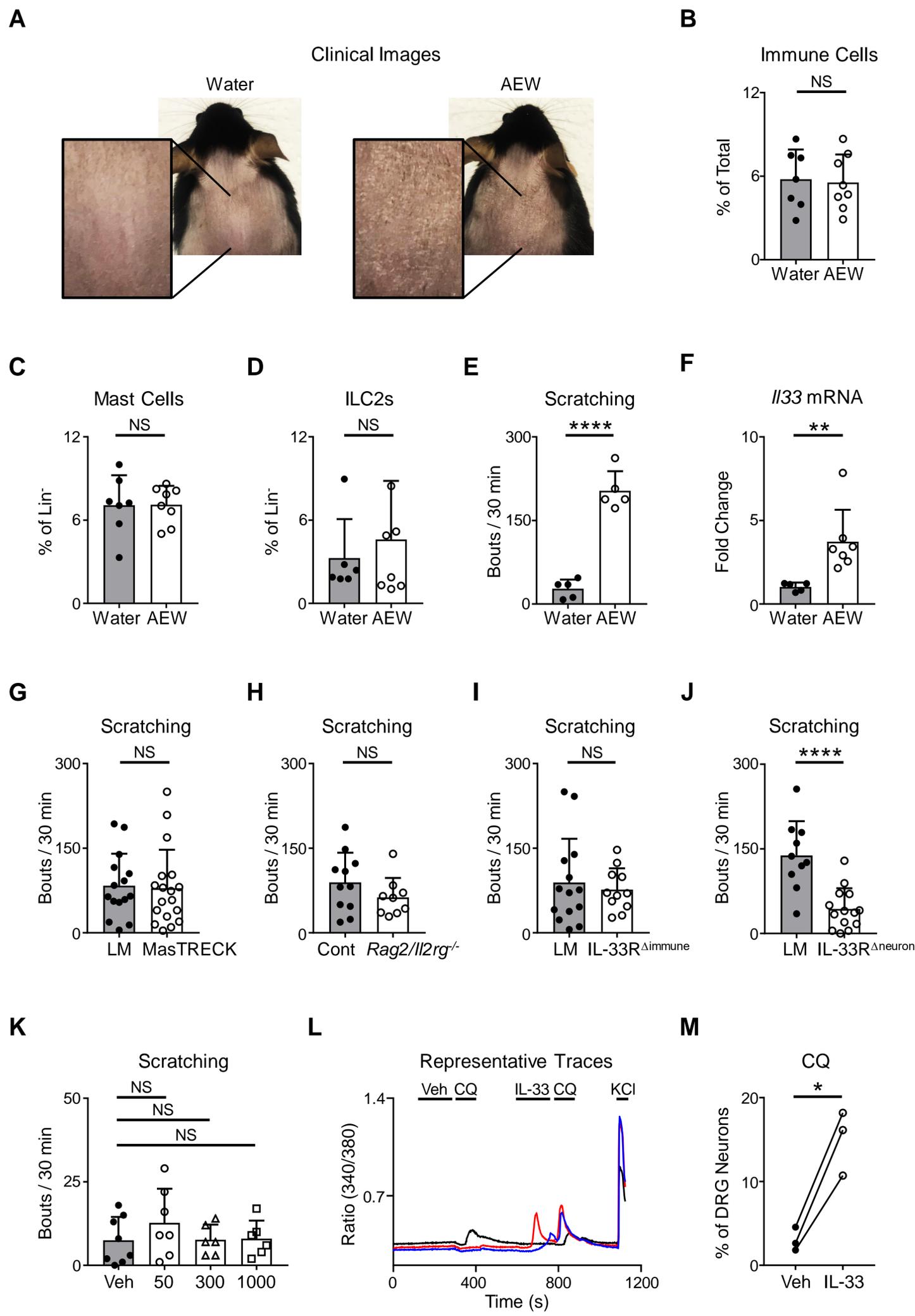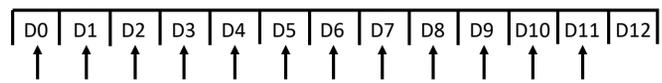**Figure 5: Dry skin itch is dependent on IL-33 signaling in sensory neurons**

A — Clinical Images (Water, AEW)

B — Immune Cells; NS; % of Total; Water AEW

C — Mast Cells; NS; % of Lin⁻; Water AEW

D — ILC2s; NS; % of Lin⁻; Water AEW

E — Scratching; ****; Bouts / 30 min; Water AEW

F — *Il33* mRNA; **; Fold Change; Water AEW

G — Scratching; NS; Bouts / 30 min; LM MasTRECK

H — Scratching; NS; Bouts / 30 min; Cont *Rag2/Il2rg⁻/⁻*

I — Scratching; NS; Bouts / 30 min; LM IL-33R^Δimmune

J — Scratching; ****; Bouts / 30 min; LM IL-33R^Δneuron

K — Scratching; NS; Bouts / 30 min; Veh 50 300 1000

L — Representative Traces; Veh CQ IL-33 CQ KCl; Ratio (340/380); Time (s)

M — CQ; *; % of DRG Neurons; Veh IL-33

Figure E1: Mouse models of chronic itch

A

MC903 Model

| D0 | D1 | D2 | D3 | D4 | D5 | D6 | D7 | D8 | D9 | D10 | D11 | D12 |

EtOH   MC903

B

Acetone / Ether + Water Model

| D0 | D1 | D2 | D3 | D4 | D5 |
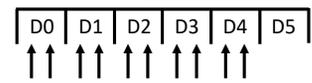
Water   AEW
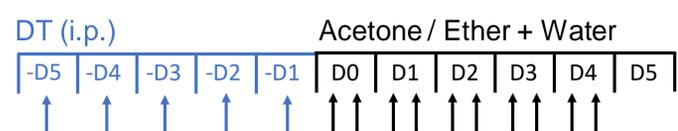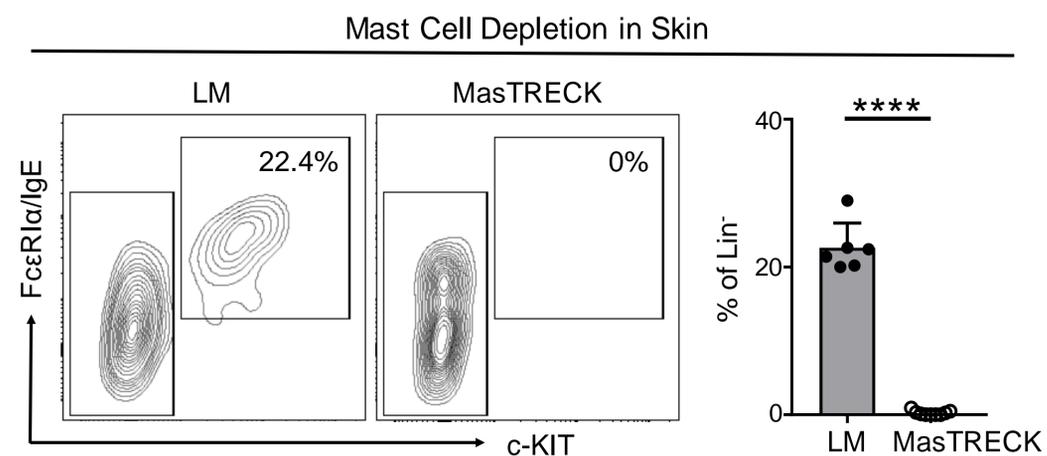
Figure E2: Depletion of mast cells and basophils following DT treatment in MasTRECK mice

A

DT (i.p.)   Acetone / Ether + Water

| -D5 | -D4 | -D3 | -D2 | -D1 | D0 | D1 | D2 | D3 | D4 | D5 |

B

Mast Cell Depletion in Skin

LM — 22.4%

MasTRECK — 0%

FcεRIα/IgE (y-axis), c-KIT (x-axis)

Bar graph: % of Lin⁻, ****, LM ~22, MasTRECK ~0

C

Basophil Depletion in Spleen

LM — 0.82%

MasTRECK — 0.07%

FcεRIα/IgE (y-axis), c-KIT (x-axis)

Bar graph: % of Lin⁻, ****, LM ~1, MasTRECK ~0.1

D

Basophil Depletion in Skin

LM — 0%

MasTRECK — 0%

FcεRIα/IgE (y-axis), c-KIT (x-axis)

Bar graph: % of Lin⁻, NS, LM ~0.15, MasTRECK ~0