

1 **Damage-induced pyroptosis drives endogenous thymic regeneration via**
2 **induction of *Foxn1* by purinergic receptor activation**

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17 **SUMMARY:**

- 18 • Thymocytes rapidly and transiently undergo pyroptosis after acute thymic damage and
19 promote regeneration.
- 20 • Damage-induced redirection of pyruvate acutely enhances mitochondrial OXPHOS in
21 thymocytes.
- 22 • Elevated mitochondrial ROS promotes pyroptosis in thymocytes after acute insult by
23 driving caspase 1 cleavage.
- 24 • Extracellular ATP release promotes *Foxn1* expression in cTECs via activation of P2Y2
- 25 • Therapeutic targeting of the P2Y2 receptor promotes thymic regeneration.

26 **ABSTRACT**

27 Endogenous thymic regeneration is a crucial process that allows for the renewal of immune
28 competence following stress, infection or cytoreductive conditioning. Fully understanding the
29 molecular mechanisms driving regeneration will uncover therapeutic targets to enhance
30 regeneration. We previously demonstrated that high levels of homeostatic apoptosis suppress
31 regeneration and that a reduction in the presence of damage-induced apoptotic thymocytes
32 facilitates regeneration. Here we identified that cell-specific metabolic remodeling after ionizing
33 radiation steers thymocytes towards mitochondrial-driven pyroptotic cell death. We further
34 identified that a key damage-associated molecular pattern (DAMP), ATP, stimulates the cell
35 surface purinergic receptor P2Y2 on cortical thymic epithelial cells (cTECs) acutely after damage,
36 enhancing expression of *Foxn1*, the critical thymic transcription factor. Targeting the P2Y2
37 receptor with the agonist UTPyS promotes rapid regeneration of the thymus *in vivo* following acute
38 damage. Together these data demonstrate that intrinsic metabolic regulation of pyruvate
39 processing is a critical process driving thymus repair and identifies the P2Y2 receptor as a novel
40 molecular therapeutic target to enhance thymus regeneration.

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49 **KEY WORDS:** Thymus regeneration, cell death, pyroptosis, pyruvate, mitochondria, ATP,

50 DAMPs, purinergic receptors, P2Y2

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52 INTRODUCTION

53 Competent T cell development relies on efficient functioning of the thymus, which is extremely
54 sensitive to acute insults, such as that caused by cytoreductive therapies¹. Thymic function
55 progressively declines with age, resulting in reduced export of newly generated naïve T cells and
56 reduced responsiveness to new antigens and vaccines^{2, 3}. The thymus has a remarkable ability
57 to endogenously regenerate^{4, 5, 6}, however, age-related deterioration drastically erodes this
58 regenerative capacity⁷. Harnessing this regenerative capacity has the potential to expedite
59 reconstitution of naïve T cells and improve immune responses. However, much remains unknown
60 about the molecular regulators of this critical process.

61

62 We have previously identified that IL-22 and BMP4 represent two distinct pathways that facilitate
63 endogenous repair in the thymus and, at least in the case of BMP4, is largely mediated by
64 induction in the expression of Foxn1^{5, 8}. FOXN1 is the essential thymic epithelial cell (TEC)
65 transcription factor; not only crucial for the generation and function of TECs, but also for TEC
66 maintenance with declining expression associated with age-related thymic involution⁹. We have
67 previously identified that the constitutively high levels of homeostatic apoptosis in the steady state
68 thymus, which governs negative selection events, is suppressive to the production of BMP4 and
69 IL-23 (the upstream regulator of IL-22), and the depletion of apoptotic thymocytes after injury
70 promotes their production¹⁰. Cell death is a sophisticated and tightly controlled process and much
71 of this is regulated by the mitochondria, and notably necrotic cell death has been tightly linked to
72 regeneration^{11, 12, 13, 14, 15, 16}.

73

74 Given the robust depletion of thymocytes after acute damage concurrent to the activation of these
75 reparative pathways, we hypothesized that a switch to an alternative cell death mechanism may
76 underpin the triggering of tissue regeneration and alleviate the suppressive impact of apoptosis
77 in the thymus. Here we investigated the effects of acute damage on the metabolic landscape of

78 thymocytes and revealed that increased levels of pyruvate are redirected to mitochondrial
79 respiration, reducing glycolysis. This disrupted glycolytic flux drives pyroptosis in the thymus
80 which is rapidly resolved as regeneration begins.

81

82 These findings identify a novel mechanism of metabolic regulation of T cell development and
83 thymic repair and provides a highly targetable therapeutic strategy to enhance immune function.

84

85 **RESULTS**

86 ***DP thymocytes preferentially undergo pyroptosis after damage***

87 Most thymocytes, and in particular CD4+CD8+ double positive (DP) thymocytes, undergo
88 apoptosis as a function of the selection processes fundamental to T cell development^{17, 18}. We
89 previously identified that homeostatic detection of these apoptotic events suppresses the
90 production of multiple regenerative molecules in the thymus by promoting activation of TAM
91 receptors bridging phosphatidylserine (PtdSer) sensing by surrounding stromal cells¹⁰. However,
92 although we had previously found that after acute damage there is a rapid decrease in the
93 detection of PtdSer¹⁰, this declined more rapidly than cell depletion itself (**Fig. 1A**) which led us
94 to hypothesize that alternate forms of cell death may be being induced after acute damage. Given
95 that DP thymocytes are the most numerous, comprising ~80% of a thymus at baseline, and these
96 cells are extremely sensitive to damage and are depleted rapidly after sub-lethal total body
97 irradiation (TBI, 550 cGy) (**Fig. 1B**), we concentrated on this population. Not surprisingly we did
98 find considerable cleavage of caspase-3 (executioner apoptosis caspase) within thymocytes (**Fig.**
99 **1C**), consistent with previous reports demonstrating their sensitivity to damage^{19, 20}. However, we
100 also found significant cleavage of caspase-1 in dying cells suggesting that in addition to
101 immunologically silent apoptosis, there is also considerable pyroptosis occurring amongst
102 thymocytes after acute injury caused by TBI (**Fig. 1D**). In fact, direct comparison revealed similar
103 magnitude of activation of both caspase-3 and caspase-1 after damage in DP thymocyte (**Fig.**

104 **1E**). Consistent with this induction of immunogenic form of cell death, we found increased release
105 of lactate dehydrogenase in the thymus after TBI (**Fig. 1F**) as well as increased levels of
106 gasdermin D (**Fig. 1G**), all suggesting a preferential induction of pyroptosis following acute
107 damage.

108

109 Since apoptosis is largely suppressive tissue regeneration in the thymus¹⁰, we hypothesized that
110 lytic cell death of DPs may be beneficial to regeneration. Moreover, as thymocyte depletion
111 precedes the period of epithelial regeneration largely driven by enhanced *Foxn1* transcription²¹,
112 we tested if pyroptotic thymocytes could directly influence *Foxn1* expression in TECs. To do this
113 we induced pyroptosis in freshly isolated thymocytes *ex vivo* using Nigericin and LPS and co-
114 cultured the dying cells with cortical thymic epithelial cells (cTECs, using the 1C9 and ANV42.1
115 cell lines) and medullary thymic epithelial cells (mTEC, using the TE-71 cell line) and quantified
116 *Foxn1* expression (**Fig. 1H**). Using this approach, we could demonstrate that the presence of
117 pyroptotic thymocytes directly led to upregulation of *Foxn1* transcription in cTECs but not in
118 mTECs (**Fig. 1H**). This cell-specific regulation of *Foxn1* was notable given that we have previously
119 shown that *Foxn1* upregulation during endogenous regeneration after damage is largely restricted
120 to cTECs⁸.

121

122 ***Mitochondrial dysregulation facilitates pyroptosis in DPs***

123 In addition to the critical role of mitochondria in cellular metabolism, the mitochondria is a
124 gatekeeper of cell death and dysregulated mitochondrial bioenergetics can lead to the induction
125 of intrinsic apoptosis or pyroptosis^{22, 23, 24, 25}. As thymocytes are undergoing such high levels of
126 homeostatic cell death we sought to understand if metabolic adaptations were steering cell death
127 preferences after damage. First, measuring mitochondrial membrane potential using TMRE
128 revealed a marked induction of mitochondrial membrane hyperpolarization (**Fig. 2A**), correlating
129 to increased cleaved caspase 1 (cl-caspase 1) levels (**Fig. 2B**). Importantly, this enhanced

130 mitochondrial activation was resolved by day 7 following damage, in line with what we observed
131 with caspase 1 cleavage and the re-establishment of apoptosis:pyroptosis balance (**Fig. 1E**).
132 Additional evidence of an acute damage-induced dysregulated metabolic phenotype in DPs was
133 revealed with increased mitochondrial mass in DPs after acute damage, which could also be
134 positively correlated with Cas1 activation (**Fig. 2C-D**).

135

136 ***Increased mitochondrial ROS triggers pyroptosis in thymocytes***

137 Bidirectional communication between the mitochondria and the NLRP3 inflammasome has been
138 well characterized and can induce activation of NLRP3 signaling^{26, 27, 28}, while concurrently
139 facilitating a lack of mitophagy driven by cleavage of caspase 1^{29, 30}. This positive feedback loop
140 perpetuates the accumulation of ROS-producing dysfunctional mitochondria due to a lack of
141 mitophagy, which in turns continues to initiate NLRP3-induced pyroptotic cell death^{25, 31}. Next, we
142 hypothesized that transiently enhanced mitochondrial activation in DPs led to increased
143 production of mitochondrial ROS (mitoROS) providing a trigger for pyroptosis. Consistent with
144 mitochondrial hyperpolarization, mitochondrial mass and cl-caspase 1 levels, there was a
145 transient and precipitous elevation in mitoROS levels after damage, again correlating with
146 caspase-1 cleavage (**Fig. 2E-F**). This increase in mitoROS was coupled with a robust and acute
147 induction in glutathione levels (**Fig. S1**), suggesting antioxidant pathways are upregulated. RNA
148 seq analysis of DP thymocytes at baseline and 24 hours after TBI revealed an increase in *Ucp2*
149 and *Mitofusin 2* (**Fig 2G**), which are central facilitators of proton leakage and Nlrp3 activation^{32, 33}.
150 RNA sequencing on DP thymocytes also revealed an enrichment for genes regulating OXPHOS
151 (*Igf2bp2*, *Ybx1*, *Ucp2*) and, importantly, downregulation of pyruvate processing to lactate (*Ldhb*),
152 pointing to a redirection of pyruvate to fuel OXPHOS (**Fig. 2G**). Of note, genes encoding key
153 glycolysis enzymes, such as *Hk1* and *Hk2*, were upregulated after TBI suggesting an increase in
154 glucose uptake, providing increased levels of pyruvate as fuel for mitochondrial metabolism^{34, 35}.

155

156 In order to demonstrate that dysregulated metabolism was driving this shift from apoptosis to
157 pyroptosis after damage, we examined the role of increased mitochondrial metabolism on cell
158 death in thymocytes. Firstly, to assess any damage-induced alterations in glycolytic flux we
159 measured pyruvate and lactate levels in thymocytes at rest and 24 h after damage and the ratio
160 of pyruvate to lactate was significantly increased early after damage (**Fig. 2H**); strengthening our
161 findings of a damage-induced metabolic shift away from glycolysis and towards OXPHOS. Next,
162 to determine if enhancing mitochondrial respiration by increasing pyruvate could induce caspase
163 1 cleavage and cell death, we incubated freshly isolated thymocytes *ex vivo* with high levels of
164 pyruvate (5 mM). This approach demonstrated that pyruvate induced caspase 1 cleavage in
165 thymocytes, but this could not be induced in thymocytes isolated from mice given TBI (**Fig. 2I-J**),
166 suggesting a zenith of pyroptosis, possibly due to the saturation of pyruvate and mitochondrial
167 activity acutely after damage. Consistent with this proposed mechanism, treatment of thymocytes
168 with high levels of pyruvate strongly induced mitoROS (**Fig. 2K**) and targeting mitoROS with the
169 inhibitor TEMPOL reduced cl-caspase 1 levels in thymocytes under pyruvate pressure (**Fig. 2L**).
170 Finally, blocking pyruvate conversion to acetyl co-A with α -ketobutyrate (α -KB) reduced cl-
171 caspase 1 levels, demonstrating a role of the TCA cycle in pyroptosis induction in thymocytes
172 (**Fig. 2M**). These findings were consistent with a demonstration that fueling increased proton
173 leakage and increased mitoROS triggers pyroptosis in thymocytes after damage, confirming DPs
174 preferentially undergo pyroptotic cell death after damage facilitated by increased pyruvate-
175 induced production of mitoROS.

176

177 ***Extracellular ATP induces Foxn1 expression in cTECs***

178 Pyroptotic cell death produces a plethora of molecules that act as ligands and messengers to
179 facilitate communication with neighboring cells^{36,37,38}. We have previously shown that extracellular
180 Zn²⁺ can act as a damage-associated molecular pattern (DAMP) after acute damage, inducing
181 expression of the pro-regenerative molecule BMP4 in endothelial cells via the receptor GPR39³⁹.

182 However, activation of GPR39 on TECs failed to induce *Foxn1* expression. We thus sought to
183 identify specific DAMPs that could trigger the induction of *Foxn1* transcription, specifically
184 focusing on cTECs. To this end, we tested the response of the cTEC cell line (1C9) to a panel of
185 DAMPs and identified ATP to be a strong inducer of *Foxn1* transcription (**Fig. 3A**). This finding
186 was confirmed in another cTEC cell line (ANV42.1) (**Fig. 3B**) and, importantly, freshly isolated
187 human TECs (**Fig. 3C**).

188

189 ATP is a ligand for cell surface purinergic receptors and can activate downstream signaling
190 pathways that either induce the influx of extracellular Ca^{2+} or promote the efflux of ER Ca^{2+} via G-
191 coupled signaling^{40, 41, 42}. Previous studies have found that purinergic receptor expression is
192 heterogeneous between thymic epithelial cell subsets, with widespread expression of both P2Y
193 and P2X receptors expressed among all subsets of TECs⁴³. Consistent with this, specific analysis
194 of TEC subsets by RNA sequencing revealed expression of multiple P2X and P2Y receptors
195 across cTECs and mTECs, with expression on cTECs limited to *P2rx1*, *P2rx4*, *P2rx6*, *P2rx7*,
196 *P2ry1*, *P2ry2*, and *P2ry14* (**Fig. 3D**). Baseline expression levels of purinergic receptors were
197 confirmed by qPCR on the 1C9 (cTEC cell line), and on freshly isolated murine cTECs (**Fig. 3E**).
198 Next, as P2 receptor activation induces a downstream increase in intracellular Ca^{2+} levels, we
199 measured Ca^{2+} levels in cTECs and mTECs after damage and demonstrated an increase in Ca^{2+}
200 in cTECs but not mTECs (**Fig. 3F**), with positive correlation between Ca^{2+} levels with *Foxn1*
201 expression (**Fig. 3G**). This data is consistent with the cell-specific effects of pyroptotic thymocytes
202 on *Foxn1* expression in cTECs, suggesting cTECs are central gatekeepers of the ATP-mediated
203 regenerative response. As P2X and P2Y receptor activation regulate Ca^{2+} levels differently⁴⁰, we
204 assessed the effect of ATP on both Ca^{2+} influx and efflux within cTECs. To do this we treated
205 cTECs with tunicamycin, to induce ER release of Ca^{2+} into the cytosol, or thapsigargin, to inhibit
206 ER Ca^{2+} release, and revealed that flooding the cell with Ca^{2+} led to enhanced *Foxn1* expression,
207 while attenuating Ca^{2+} levels restored *Foxn1* expression to baseline (**Fig. 3H**). These results

208 strongly suggested the role of P2Y receptors in mediating the FOXN1 promoting effects of
209 extracellular ATP, however, we further sought to refine our target and identify which P2 receptor
210 was critical to mediate this effect. To confirm this, we treated cTECs with ATP in the presence of
211 antagonists for P2Y2, and P2X4, as P2X4 is highly expressed on cTECs although does not induce
212 Ca²⁺ efflux and demonstrated that inhibition of P2Y2 attenuated ATP-mediated *Foxn1* induction,
213 mirroring the effects ATP elicits as an extracellular DAMP after acute insult (**Fig. 3I**).

214

215 ***Specific activation of P2Y2 receptors can enhance Foxn1 expression and boost thymic***
216 ***function after acute injury***

217 P2 antagonists are of increasing interest therapeutically, with a focus on developing analogous
218 molecules to inhibit or promote these druggable targets in many disease settings, such as
219 epilepsy⁴⁴, rheumatoid arthritis⁴⁵ and ischemic cardiac injury⁴⁶. Moreover, clinical trials have been
220 carried out using antagonists for P2X3⁴⁷, P2X7^{48,49} and P2Y12⁵⁰. To test if P2Y2 could be targeted
221 to enhance FOXN1 we obtained a specific P2Y2 agonist and further demonstrated that stimulation
222 of cTECs with a P2Y2 agonist induced *Foxn1* expression, and inhibition of P2Y2 ablated this
223 response (**Fig. 4A**). We sought to translate our molecular target discovery findings into a
224 therapeutic strategy *in vivo* to test if P2Y2 agonism could enhance thymic regeneration. To do
225 this we treated C57BL/6 mice with SL-TBI and administered the P2Y2 agonist UTPyS
226 intraperitoneally at day 1 following damage and assessed thymic cellularity 13 days after damage
227 and confirmed that UTPyS could enhance thymus regeneration after acute damage (**Fig. 4B**).
228 Additionally, *in vivo* treatment with UTPyS had a global impact on thymocyte populations, with
229 increased regeneration of DPs, CD4+ and CD8+ thymocytes and superior regeneration of the
230 TEC compartment (**Fig. 4C**).

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234 **DISCUSSION**

235 Endogenous thymic regeneration engages complex multicellular signaling networks that
236 intricately communicate within cellular niches to repair and replenish peripheral T cell
237 reconstitution. Here, we demonstrated that an induction of pyroptosis as a preferred cell death
238 mechanism provides the critical ligand ATP, that stimulated P2Y2 receptors on cTECs to promote
239 FOXP1 transcription and enhance regeneration of the thymus. Moreover, we uncovered a cell
240 specific mechanism of metabolically regulated thymus regeneration that is centered on steering
241 pyruvate processing to induce lytic cell death by dysregulating mitochondrial metabolism, acutely
242 increasing mitoROS and triggering NLRP3 activation and pyroptosis. Here we identified the
243 effects of acute damage on the thymocyte metabolic landscape and cell fate.

244

245 The thymus is highly hypoxic^{51, 52}, and thymocytes undergo dynamic alterations in respiration
246 during development (specifically between DN and DP stages)⁵³, pointing to their metabolic
247 plasticity. Here we identified the effects of acute damage on the metabolic landscape of
248 thymocytes and revealed that increased levels of pyruvate are redirected toward mitochondrial
249 respiration, reducing glycolysis. Disruption of glycolytic flux has been shown to trigger
250 pyroptosis⁵⁴, and our data demonstrates that an acutely altered metabolic profile in DP
251 thymocytes drives pyroptosis in the thymus and is rapidly resolved as regeneration is initiated.
252 Accompanying damage-induced acute hyperpolarization of the mitochondrial membrane,
253 increased mitochondrial mass and mitochondrial ROS in DP thymocytes, our RNA sequencing
254 data showed an upregulation of genes encoding UCP2 and PARP1, key negative regulators of
255 oxidative stress^{55, 56}, and in Mitofusin 2 which governs mitochondrial integrity⁵⁷. Importantly, our
256 gene signature of damage in DP thymocytes revealed that several genes encoding enzymes
257 critical for glucose processing to pyruvate, such as HK1, HK2, PKM and HIF1 α were upregulated
258 concurrently with a downregulation in genes regulating pyruvate conversion to lactate (*Ldhd*).
259 Functionally resulting in a higher pyruvate to lactate ratio, and redirection towards mitochondrial

260 respiration. ROS reacts with the NLRP3 inflammasome and drives pyroptotic cell death^{25, 58, 59, 60,}
261 ^{61, 62}. Here we confirmed this in the thymus and demonstrated that pyruvate drives this increase
262 in mitochondrial ROS that further triggers caspase-1 cleavage and pyroptosis, strengthening the
263 case for a central role in redirection away from glycolysis as a trigger from thymocyte cell death.

264

265 Both intracellular and extracellular ATP has been previously identified to play a role in tissue
266 repair^{63, 64, 65}, and importantly purinergic receptors are identified to mediate the extracellular ATP
267 response⁶⁶, with interest in pharmacologically targeting these receptors to enhance wound
268 repair^{67, 68}. Activation of purinergic receptors mobilizes intracellular Ca²⁺ in epithelial cells⁶⁹. Here
269 we identified that inhibiting Ca²⁺ efflux from the ER, using thapsigargin, downstream of ATP
270 treatment prevented FOXP1 transcription, which led us to further assess P2Y2 as a specific
271 target. Moreover, P2Y2 has been identified to mediate migration and repair of epithelial cells⁷⁰.
272 Purinergic receptors have been trialed for a range of diseases, for example P2X7 antagonists are
273 being tested in a Phase 2a clinical trial to treat Crohn's disease, while targeting P2X7 to treat
274 rheumatoid arthritis failed to show significance in phase 2 clinical trial⁷¹, and the P2Y2 agonist
275 Diquafosol is currently being tested for the treatment of dry eye⁷². We identified that P2Y2
276 agonism promotes FOXP1 transcription specifically in cTECs and that competition with an
277 antagonist quenches this effect, pointing to receptor specificity. Moreover, our pre-clinical data
278 demonstrates that the P2Y2 agonist UTPγS promotes superior regeneration in acutely injured
279 mice, promoting recovery of thymocyte and both cTEC and mTEC compartments, which is vital
280 for continued maintenance and functioning of the thymus.

281

282 While much remains to be understood regarding mitochondrial regulation of cell death and
283 differentiation in models of chronic damage such as age, these data underline an important
284 mechanism of recovery from acute damage that highlights the significance of metabolic
285 governance of immune function. The question of other fuel sources that drive mitochondrial

286 dysfunction during acute damage, such as lipids or glutamine, is outstanding and may potentially
287 reveal disease specific damage-responses of the thymus, specifically as lipid metabolism has
288 been identified to play a central role in hematopoiesis and T cell differentiation⁷³. However, as
289 these metabolic phenotypes are likely to be variable between cellular compartments, and with our
290 data clearly demonstrating a central role of pyruvate in mitochondrial induced pyroptosis, the
291 convergence of these pathways on mitochondrial ROS is central to pyroptotic driven regeneration.
292 In conclusion, these data describe a complex molecular architecture that govern thymus
293 regeneration and not only provides a platform for therapeutic target discovery and intervention
294 towards enhancing immune function, but also contributes to regenerative medicine by unravelling
295 novel mechanisms of metabolically regulated endogenous tissue regeneration which may be
296 applicable across multiple tissues.

297

298 **MATERIALS AND METHODS**

299 ***Mice***

300 Inbred male and female C57BL/6J mice were obtained from the Jackson Laboratories (Bar
301 Harbor, USA) and all experimental mice were used between 6-8 weeks old. To induce thymic
302 damage, mice were given sub-lethal total body irradiation (SL-TBI) at a dose of 550 cGy from a
303 cesium source mouse irradiator (Mark I series 30JL Shepherd irradiator) with no hematopoietic
304 rescue. Mice were maintained at the Fred Hutchinson Cancer Research Center (Seattle, WA),
305 and acclimatized for at least 2 days before experimentation, which was performed per Institutional
306 Animal Care and Use Committee guidelines.

307

308 ***Reagents***

309 Cells were stained with the following antibodies for analysis CD3-FITC (35-0031, Tonbo
310 Bioscience), CD8-BV711 (100748, BioLegend), CD4-BV650 (100546, BioLegend), CD45-
311 BUV395 (565967, BD Biosciences), CD90-BV785 (105331, BioLegend), MHC-II-Pac Blue

312 (107620, BioLegend), EpCAM-PercPe710 (46-5791-82, eBioscience), Ly51-PE (12-5891-83,
313 eBioscience), UEA1-FITC (FL-1061, Vector Laboratories), TCRbeta-PECy7 (109222,
314 BioLegend), CD62L-APC-Cy7 (104427, BioLegend), CD44-Alexa Fluor RTM700 (56-0441-82,
315 BioLegend), CD25-PercP-Cy5.5 (102030, BioLegend). Flow cytometry analysis was performed
316 on an LSRFortessa X50 (BD Biosciences) and cells were sorted on an Aria II (BD Biosciences)
317 using FACSDiva (BD Biosciences) or FlowJo (Treestar Software).

318

319 ***Thymus digestion and cell isolation***

320 Single cell suspensions of freshly dissected thymuses were obtained and either mechanically
321 suspended or enzymatically digested as previously described^{5, 74} and counted using the Z2
322 Coulter Particle and Size Analyzer (Beckman Coulter, USA). For studies sorting rare populations
323 of cells in the thymus, multiple identically treated thymuses were pooled so that sufficient number
324 of cells could be isolated; however, in this instance separate pools of cells were established to
325 maintain individual samples as biological replicates.

326

327 ***Cell death assays***

328 Thymuses from untreated and SL-TBI treated mice were harvest, enzymatically digested and
329 stained with cell surface markers for thymus populations. Cells were further stained for caspase
330 1 cleavage with Caspase-1 (active) Staining Kit (Abcam, ab219935), or fixed for caspase 3
331 analysis using Cleaved Caspase-3 (Asp175) Antibody (Alexa Fluor® 488 Conjugate) (Cell
332 signaling, 9669S). Apoptosis and pyroptosis was assessed by adding Annexin V-FITC
333 (Biolegend, 640906), Annexin V binding buffer (BioLegend, 422201) and Propidium Iodide
334 (Invitrogen, BMS500PI). Gasdermin D was measured in freshly isolated thymocytes using
335 Gasdermin D (mouse) ELISA Kit (Adipogen Life Sciences, AG-45B-0011-KI01). Lactate
336 dehydrogenase was assessed from the supernatant of harvested thymocytes using Lactate
337 Dehydrogenase assay (Abcam, ab102526).

338

339 ***In vitro assays***

340 *Co-culture assays:* thymocytes were isolated from untreated C57BL/6 mice and incubated with
341 Nigericin (10 μ M, Tocris, 4312) and LPS (1 ng/ml, Invivogen, tlr1-eb1ps) for 3 h and co-cultured
342 with 1C9s, ANV42.1 or TE-71 cell lines for 20 h before lysis for qPCR. *DAMP stimulation:* 1C9s
343 were stimulated with ATP (100 μ M, Tocris 3312), HMGB1 (1 μ g/ml, Abcam, ab78940), IL1a (50
344 ng/ml, Tocris, 400-ML-005/CF), IL-33 (50 ng/ml, Tocris, 3626-ML-010/CF), or uric acid (50 μ g/ml,
345 Sigma, U2625) for 20 h and lysed for qPCR analysis. *UTP γ S assays:* 1C9s or ANV42, cells were
346 stimulated with UTP γ S (100 μ M, R&D Systems, 3279), or UTP γ S plus AR-C 118925XX (20 μ M,
347 Tocris, 4890) for 20 h before lysis for qPCR. BzATP triethylammonium salt (100-300 μ M, Tocris,
348 3312), was used for ATP stimulation.

349

350 ***qPCR and RNA sequencing***

351 RNA was extracted from exECs or DCs using a RNeasy Mini kit (74104, Qiagen), and from sorted
352 cells using a RNeasy Plus Micro kit (74034, Qiagen). cDNA was synthesized using the iScript
353 gDNA Clear cDNA Synthesis kit (1725035, Bio-Rad, USA) and a Bio-Rad C1000 Touch
354 ThermoCycler (Bio-Rad). RNA expression was assessed in the Bio-Rad CFX96 Real Time
355 System (Bio-Rad), using iTaq Universal SYBR Green Supermix (1725122, Bio-Rad), and the
356 following primers: β -Actin (F 5'-CACTGTCGAGTCGCGTCC-3'; R 5'-
357 TCATCCATGGCGAACTGGTG-3'); PrimePCR™ SYBR® Green Assay: Foxn1, Mouse (Biorad,
358 10025637, qMmuCED0044924).

359

360 RNAseq was performed on freshly isolated and FACS purified CD4+CD8+ thymocytes. To obtain
361 sufficient RNA for every timepoint, thymi of 2 mice were pooled for untreated mice and 6 for
362 irradiated mice. All samples underwent a quality control on a bioanalyzer to exclude degradation
363 of RNA.

364

365 ***Ex vivo metabolic assays***

366 Thymuses from untreated and SL-TBI treated mice were harvested and enzymatically digested
367 and stained from flow cytometry analysis of thymocyte populations as above. Further analysis of
368 mitochondrial bioenergetics were assessed using TMRE (Abcam, ab113852), MitoTracker™
369 Green FM (Invitrogen, M7514), MitoSOX™ Red Mitochondrial Superoxide Indicator
370 (ThermoFisher, M36008), and Intracellular glutathione (GSH) Detection Assay Kit (Abcam,
371 ab112132). Thymocytes were isolated from untreated and TBI-treated mice and intracellular
372 pyruvate and lactate levels were measured by absorbance using Pyruvate Assay kit (Abcam,
373 ab65342) or Lactate-Glo™ Assay (Promega, J5021). Thymocytes were isolated from untreated
374 and TBI-treated mice and incubated in RPMI with 5 mM sodium pyruvate (Gibco, 11360070) for
375 3 h at 37 °C and stained for flow cytometry analysis and cl-caspase 1 levels. Cells were further
376 incubated with 5 mM sodium pyruvate plus 200 µM α-ketobutyrate (Sigma-Aldrich) or 100 µM
377 TEMPOL (Tocris, 3082) for 3 h and cells were prepared for flow cytometry analysis.

378

379 ***Intracellular Ca²⁺ assay***

380 The thymuses from untreated and SL-TBI-treated mice were harvested and processed for flow
381 cytometry as above. The intracellular Ca²⁺ dye BAPTA-AM/Indo-AM was added (Sigma-Aldrich).
382 Unbound intracellular Ca²⁺ was assessed in cTECs and mTECs by measuring BAPTA-AM levels
383 on the BUV-496 filter.

384

385 ***In vivo UTPyS administration***

386 For *in vivo* studies of UTPyS administration, mice were given SL-TBI (550cGy) and subsequently
387 received intraperitoneal injections of 1 mg/kg UTPyS (R&D systems, 3279), or 1x PBS as control,
388 on day 1 following TBI. Thymuses were harvested 13 days after SL-TBI and cellularity was
389 assessed and populations were analyzed by flow cytometry.

390

391 **Statistics**

392 All analysis between two groups was performed with a non-parametric Mann-Whitney test.

393 Statistical comparison between 3 or more groups in Figs. 1A, 1C, 1D, 1E, 1F, 1G, 2A, 2B, 2C,

394 2E, 3A, 3H, 3I, and 4A were performed using a one-way ANOVA with Tukey's multiple comparison

395 post-hoc test. All statistics were calculated using Graphpad Prism and display graphs were

396 generated in Graphpad Prism or R.

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436 **AUTHOR CONTRIBUTIONS**

437 S.K. and J.A.D. conceived of the idea of this manuscript. J.A.D., and S.K. designed, analyzed and
438 interpreted experiments, and drafted the manuscript; C.A.E., K.C., L.I., P.d.R, A.C., K.S.H.,
439 C.W.S., and D.G. performed experiments; L.S., E.V., and J.A.D. supervised experiments. All
440 authors contributed to the article and approved the submitted version.

441

442 **CONFLICT OF INTEREST**

443 J.A.D., S.K., and L.I., have submitted a patent application pending around these findings to
444 promote thymus regeneration.

445

446

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698

699 **FIGURE LEGENDS**

700 **Figure 1: A switch from apoptotic to pyroptotic DP thymocytes triggers thymus**
701 **regeneration. A-E**, Thymus was analyzed from 6-8 week old C57/BL/6 mice at days 0, 1, 2, 3 and
702 7 following sublethal total body irradiation (TBI, 550cGy). **A**, Total thymic cellularity and proportion
703 of cellularity as a function of baseline cellularity (n=15-19/timepoint from 5 independent
704 experiments). **B**, Concatenated flow cytometry plot of CD4 vs CD8 (gated on viable CD45+ cells)
705 (n=9-13 from 3-4 independent experiments). **C**, Concatenated flow cytometry plot from one
706 experiment showing cleaved caspase-3 on DP thymocytes (Gated on CD45+CD4+CD8+ cells)
707 and bar graph showing proportion of cleaved-cas3+ DP thymocytes (n=15-16/timepoint from 5
708 independent experiments). **D**, Concatenated flow cytometry plot from one experiment showing
709 cleaved-caspase1 and PI (gated on CD45+CD4+CD8+ cells) and bar graph with proportions
710 (n=13/timepoint from 4 independent experiments). **E**, Magnitude of change in expression of
711 cleaved-caspase-1 and cleaved-caspase-3 in DP thymocytes after TBI. (n=10-
712 15/timepoint/condition from 4-5 independent experiments). **F**, Lactate dehydrogenase levels were
713 measured in the thymus supernatant of mice (n=4 mice/group). **G**, Gasdermin D levels were
714 measured in CD45+ cells from the thymus at days 0, 1, 2, 3, 7, and 14 post TBI (n=3-4 mice/group
715 from 2-3 independent experiments). **H**; Cells were co-cultured with freshly isolated thymocytes
716 treated to induce or inhibit pyroptosis and Foxn1 expression was measured by qPCR, 20 h after
717 co-culture, in 1C9s (n =8-11 thymuses from 3 separate experiments), ANV42.1 (n=8 thymuses

718 from 3 separate experiments), and TE-71 (n=11 from 3 separate experiments). Data represents
719 mean \pm SEM.

720

721 **Figure 2: Dysregulated metabolism redirects pyruvate to fuel OXPHOS in thymocytes after**

722 **damage. A-F,** Mitochondrial function was analyzed in the thymus isolated from 6-8 week old

723 C57/BL/6 mice at days 0, 1, 2, 3 and 7 following sublethal total body irradiation (TBI, 550cGy). **A,**

724 Mitochondrial membrane potential assessed by staining of TMRE. Concatenated histogram of

725 TMRE on DP thymocytes (left), quantification of TMRE+ proportions (right) (n=8-12 mice from 3

726 separate experiments). **B,** Correlation of TMRE expression with Caspase-1 MFI (n=3-8 from 3

727 independent experiments). **C,** Mitochondrial mass assessed by Mitotracker Green. Concatenated

728 histogram of TMRE on DP thymocytes (left), quantification of TMRE+ proportions (right) (n=9-10

729 mice from 3 independent experiments). **D,** Correlation of TMRE expression with Caspase-1 MFI

730 (n=6/timepoint from 3 independent experiments). **E,** Mitochondrial ROS was assessed by staining

731 for MitoSOX. Concatenated histogram of TMRE on DP thymocytes (left), quantification of TMRE+

732 proportions (right) (n=5-7 mice from 2 separate experiments). **F,** Correlation of TMRE expression

733 with Caspase-1 MFI (n=5-7/timepoint from 2 independent experiments). **G,** RNA seq was carried

734 out on FACS purified CD4+CD8+ thymocytes from untreated and TBI-treated (1 day post TBI)

735 mice (n=3/group). Displayed are heatmaps for expression of key genes involved with OXPHOS

736 and glycolysis. **H,** Intracellular lactate and pyruvate levels were measured in freshly isolated

737 thymocytes from untreated and TBI-treated mice (n=5 mice/group from 2 separate experiments).

738 **I-K,** Thymocytes isolated from mice at days 0 or 1 post TBI were incubated with RPMI

739 supplemented with pyruvate (5 mM) for 4 h. **I,** Concatenated histogram showing expression of

740 cleaved-caspase-1 on DP thymocytes in cells incubated with media alone, high pyruvate (on

741 day 0 thymocytes) or normal media with thymocytes isolated at d1 following TBI. **J,** Proportion of

742 Cleaved-caspase-1+GhostDye+ cells (n=6 mice from 2 separate experiments). **K,** Proportion of

743 mitoSOX+ DP thymocytes (n=6 mice from 2 separate experiments). **L,** Freshly isolated

744 thymocytes from untreated mice were incubated with RPMI supplemented with pyruvate (5 mM)
745 for 4 h plus TEMPOL (100 μ M). cl-caspase 1 levels were measured using flow cytometry (n=6
746 mice from 2 separate experiments). **M**, Freshly isolated thymocytes from untreated mice were
747 incubated with RPMI supplemented with pyruvate (5 mM) for 4 h plus α -ketobutyrate (200 μ M).
748 cl-caspase 1 levels were measured using flow cytometry (n=13-14 mice from 4 separate
749 experiments). Data represents mean \pm SEM.

750

751 **Figure 3: Activation of the P2Y2 receptor with extracellular ATP induces FOXN1 in cTECs.**

752 **A**, The cTEC cells line 1C9 was treated with a panel of DAMPs and *Foxn1* transcription was
753 measured in by qPCR 20 h following incubation (n=3-4 separate experiments). **B**, A second
754 cTEC cell line (ANV42.1) was treated with ATP (100 μ M) and *Foxn1* expression was measured
755 after 20 h (n=3 separate experiments). **C**, Freshly isolated human cTECs were treated with ATP
756 (100 μ M) and *Foxn1* expression was measured after 20 h (n= 2). **D**, cTECs, MHCII^{hi} mTEC, and
757 MHCII^{lo} mTEC were isolated from untreated 6wo C57BL/6 mice and RNA sequencing performed.
758 Displayed is expression of purinergic receptor family members (n=3/cell population). **E**, Purinergic
759 receptor expression in FACS purified cTECs from untreated mice measured by qPCR (n=2-3
760 pooled mouse thymuses). **F**, Intracellular free Ca²⁺ levels were measured by flow cytometry in
761 untreated and TBI-treated (day 4 post TBI) cTECs (n=10 mice from 2 separate experiments) and
762 mTECs (n=8-10 mice from 2 separate experiments). **G**, Correlation of free Ca²⁺ and *Foxn1*
763 expression at days 0 and 4 after SL-TBI (n=3/timepoint). **H**, 1C9 (cTEC) were treated with
764 tunicamycin (1 μ M) or thapsigargin (100 nM) for 20 h and *Foxn1* expression was measured by
765 qPCR (n=4 independent experiments). **I**, 1C9 (cTECs) were treated with ATP and either
766 antagonists for P2Y2 or P2X4 and *Foxn1* expression was measured by qPCR 20 h after
767 incubation (n=5 separate experiments). Data represents mean \pm SEM.

768

769 **Figure 4: Activation of the P2Y2 enhances thymus regeneration after damage. A,** ANV42.1
770 (cTEC) cells were treated with the P2Y2 agonist UTP γ S and the P2Y2 antagonist ARC-118925XX
771 for 20 h and *Foxn1* expression was measured by qPCR after 20 h (n=3 separate experiments);
772 **B-D,** 6wo C57BL/6 mice were treated with UTP γ S (1 mg/kg) IP at day 1 following SL-TBI and the
773 thymuses were harvested at day 13. **B,** Total thymus cellularity. **C,** Number of CD4-CD8- double
774 negative (DN), DP, of CD4 or CD8+ single positive (SP4 or SP8, respectively) thymocytes (n=23-
775 24 mice from 3 independent experiments). Data represents mean \pm SEM.

776 **Supplementary Figure 1**

777 Thymuses from 6-8 week old C57BL/6 mice were harvested at days 0, 1, 2, 3 and 7 following TBI
778 and glutathione levels were measured by flow cytometry in DP thymocytes (n=3 mice).

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Figure 1

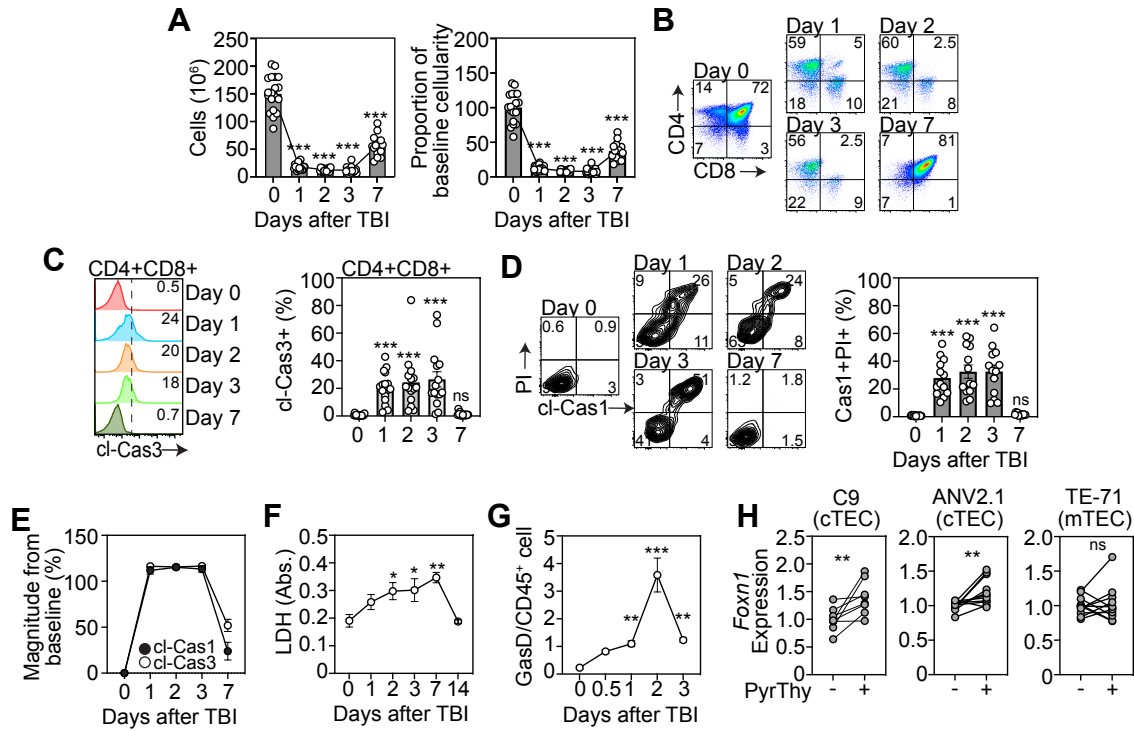


Figure 2

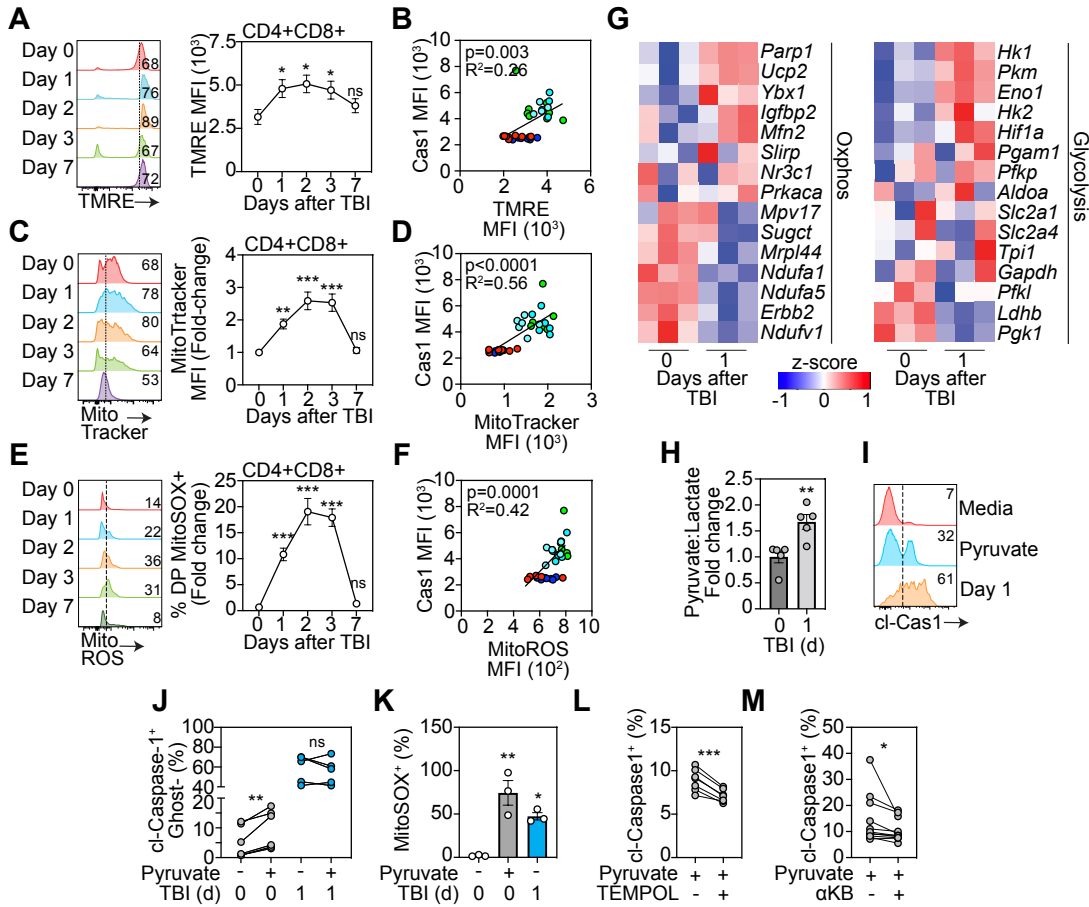


Figure 3

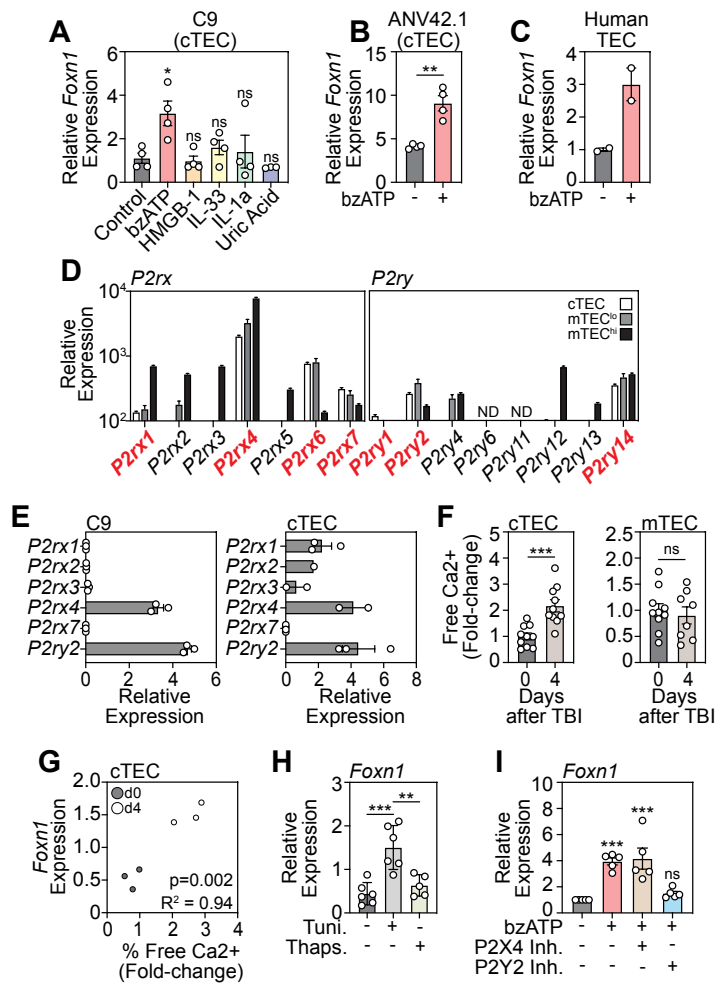


Figure 4

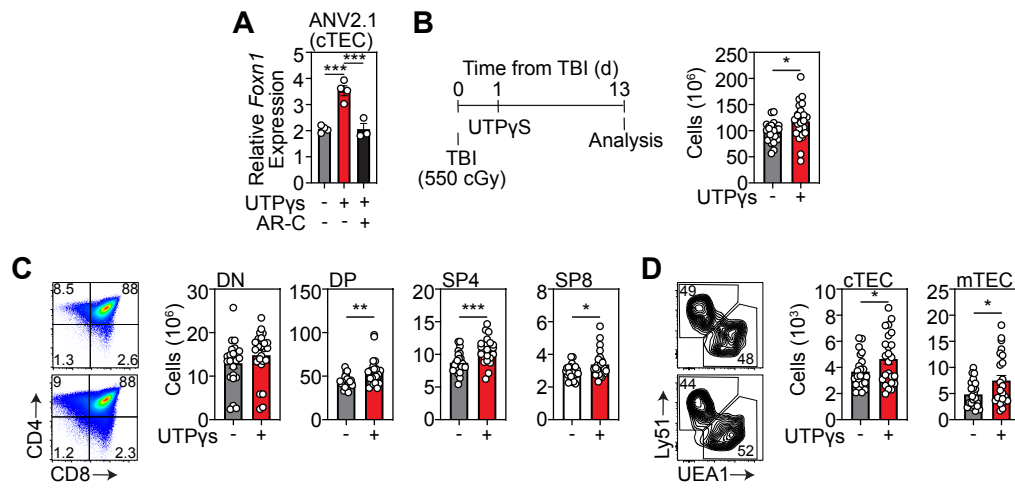


Figure S1

