H2-O deficiency promotes regulatory T cell differentiation and CD4 hyperactivity

Robin A. Welsh[1], Nianbin Song[1], Chan-su Park[1,2], J. David Peske[1], Scheherazade Sadegh-Nasseri[1,4*]

[1]Department of Pathology, Johns Hopkins School of Medicine, Baltimore, MD, USA
[2]Current Address: Department of Pharmaceutics, College of Pharmacy, Chungbuk National University, 1 Chungdae-ro, Seowon-gu, Cheongju 28644, Korea
[4] Senior Author
*Corresponding Author: ssadegh@jhmi.edu

Abstract

Regulatory T cells (Treg) are crucial immune modulators, yet the exact mechanism of thymic Treg development remains controversial. Here, we present the first direct evidence for H-2O, a peptide editing molecular chaperon for MHC class II antigen processing, on selection of thymic Tregs. We demonstrate that lack of H2-O in the thymic medulla promotes thymic Treg development and leads to an increased peripheral Treg frequency. Single-cell RNA-sequencing (scRNA-seq) analysis of splenic CD4 T cells revealed an enrichment of both effector-like Tregs and activated CD4 T cells in the absence of H2-O. H2-O has been shown to increase the presentation of high-affinity class II epitopes, thus leading to proper negative selection in the medullary thymus. In spite of this notion, our data support two concepts; a) lack of expression of H2-O in thymic medulla creates an environment permissive to Treg development and, b) that loss of H2-O drives increased basal auto-stimulation of CD4 T cells. These findings can help in better understanding of autoimmunity and design of therapeutics for treatment of autoimmune diseases.

Introduction

T cells are key players in humoral immune responses. Upon infection with a pathogen, CD4 T cells utilize their T cell receptor (TCR) to survey peptides presented by professional antigen presenting cells (APCs) on class II molecules (pMHCII) for recognition. Identification of a cognate pMHCII complex by the TCR leads to CD4 T cell activation and ultimately clearance of the foreign pathogen. Faulty activation, however, can lead to deleterious inflammation causing possible autoimmune diseases and cancer development. Therefore, multiple regulatory processes exist to ensure proper T cell activation.

Regulation of T cell activation begins during thymic development where immature thymocytes are screened for self-reactivity. Broadly divided into positive and negative selection, this process ensures that CD4 T cells expressing high avidity self-reactive TCRs are either eliminated (1), or become CD4 regulatory T cells (Tregs) (2). Critical to the screening process for identifying auto-reactive T cells is the Class II antigen processing machinery expressed by medullary thymic epithelium cells (mTECs) and thymic antigen presenting cells (APCs). Two chaperone proteins, H2-M (murine; human, HLA-DM), and H2-O (murine; human, HLA-DO) are major components of the MHC II processing pathway. While H2-M is expressed in all APCs, H2-O is expressed mainly in thymic medulla and B cells (3). H2-M plays a critical role in MHC class II antigen processing by dissociating the Class II Invariant Chain peptide (CLIP) from the newly synthesized MHC II. Dissociation of CLIP promotes a peptide-receptive MHC II conformation that can readily scan for the best MHC II groove fitting epitopes of denatured antigens. However, a peptide-receptive MHC II conformation is highly transient (4, 5, 6, 7). We have proposed that H2-O binds to H2-M (8) and works cooperatively with H2-M by stabilizing the peptide-receptive MHC II conformation in order to optimize epitope selection (9). Together, these two molecules ensure that the best MHC II groove fitting epitopes are presented to cognate CD4 T cells.

While the exact mechanism of Treg selection remains to be fully understood (10), two critical requirements have been identified as necessary for successful thymic Treg development. First, thymic Treg development requires relatively strong TCR signaling in the thymic medulla (11), and second, Treg development relies on signaling by common γ chain (γC) cytokines, mainly IL-2, for driving Foxp3 expression (12). Considering that strong TCR signaling during negative selection normally leads to CD4 T cell deletion (1), when investigating Treg selection, both the nature and density of medulla presented self-peptides must be considered. If epitopes are in high abundance and more ubiquitously expressed, then cognate CD4 T cells will undergo clonal deletion. However, if epitopes are in lower abundance and have a sparser expression, leading to discontinuous TCR stimulation, then cognate CD4 T cells might undergo Treg selection (10, 13, 14). This model of Treg selection relies on the level of TCR signaling that medulla localized CD4 single-positive (SP) T cells receive. Indeed, loss of H2-O function has been shown to alter both the repertoire and density of peptides presented by naïve peripheral B cells (15, 16). Thus, loss of H2-O could affect whether CD4 T cells are signaled for deletion, or survival during thymic deletion. Should a lower density of self pMHCII be presented by thymic epithelium, an increased number of self-reactive CD4 T cells might escape deletion leading to

an increased frequency of auto-reactive T cell clones in the periphery. Or, alternatively, presentation of a lower density of self pMHCII could promote selection of regulatory T cells.

Here, we demonstrate that loss of H2-O generates a more stimulatory *in vivo* environment impacting both the thymic development and peripheral activation of regulatory T cells.

Results

Loss of H2-O increases the activation state of auditing medulla CD4 T cells

Previously, we demonstrated that loss of H2-O expression correlated with both an increased B cell presentation of low-affinity MHC II peptides, and an increased frequency of a MOG_{35-55} specific, self-reactive CD4 T cell (15). Because of H2-O expression in the medullary thymus we speculated that H2-O deficiency might lead to presentation of lower densities of high-affinity self-peptides in the medulla, thereby causing an altered clonal deletion process. Medulla CD4 T cells can be divided into two groups based upon expression of the pro-apoptotic protein Caspase-3 (17); "Auditing" CD4 T cells (CCR7+ CD4+ Caspase-3_{neg}) that are still undergoing self-auditing, and "Clonally Deleted" CD4 T cells (CCR7+ CD4+ Caspase-3_{pos}) that have been selected for thymic deletion.

We found that loss of H2-O significantly increased expression of the activation marker CD69 on auditing, but not CD4 T cells selected for clonal deletion (**Fig 1a**). CD69 in combination with MHC-I levels defines 3 medullary maturation stages: semi-mature (CD69+ MHC-I -), mature 1 (CD69+ MHC-I +), and mature 2 (CD69- MHC-I -) (17). Subdivision of auditing H2-O-KO CD4 T cells identified a significant increase in the Mature 1 stage (**Fig 1b, middle**). Conversely, both the semi-mature and mature 2 stages were decreased in H2-O KO mice (**Fig 1b left/right**). H2-O deficiency, however, did not appear to alter the rate of CD4 T cells undergoing clonal deletion (**Fig 1c**). Importantly, no differences were observed in thymocytes undergoing positive selection (**sFig1**). These data suggest that loss of H2-O drives a more stimulatory thymic medulla environment, but with similar levels of clonal deletion. It is therefore likely that the increased peripheral frequencies of MOG-specific CD4 T cells previously identified is due to increased peripheral expansion of the MOG-reactive clone, not a general altered clonal deletion.

H2-O KO thymi have increased regulatory T cell development

The increase in the Mature 1 (M1) state was noteworthy as Treg development has been shown to begin in the M1 stage (17). With increased levels of peripheral Treg previously identified in H2-O KO mice (15), we wondered if H2-O deficiency was affecting Treg selection. In fact, one model of thymic Treg selection centers around the concept of antigen density (13, 18). Within this "mosaic" model, sporadic MHC-TCR interactions with sparsely presented self-epitopes leads to Treg development. Having previously shown that peripheral loss of H2-O leads to increased presentation of lower affinity peptides (15), we postulated that altering the level of self-epitopes present in the medulla could alter Treg selection. In support of this idea, we found an increased frequency of CD25+Foxp3+ T cells in the H2-O KO CD4 SP population (**Fig 2a**). Furthermore, H2-O KO thymic Tregs (tTregs) expressed higher levels of CD25, CD5 and the orphan nuclear receptor Nur77 (**Fig 2b**). As Nur77 has been associated with the level of TCR engagement (19), increased Nur77 signal was particularly interesting since it indicates that absence of H2-O leads to increased TCR engagement suggesting an increased self-reactivity. We

also found that H2-O KO tTregs were enriched in the M1 maturation stage (**Fig 2c**). This could mean two things; (1) loss of H2-O leads to increased presentation of specific Treg epitopes thereby increasing the frequency of Treg selection, or (2) lower antigen density allows an increased proportion of self-reactive CD4 T cells to engage their cognate ligands at suboptimal levels for clonal deletion but optimal ranges for Treg selection. Cumulatively, evaluation of H2-O KO thymi showed that only CD4 T cells undergoing the auditing process were affected by H2-O loss.

Loss of H2-O correlates with increased peripheral CD4 T cell activation

In light of the observation that lack of H2-O KO did not alter clonal deletion frequencies, we next evaluated whether peripheral loss of H2-O increased CD4 T cells activation. We found that H2-O KO spleens had a dramatic increase in the proportion of CD3+ CD4 T cells (**Fig 3a**). Critically, unimmunized H2-O KO spleens had increased levels of CD4 T cells co-expressing the key activation markers CD44+ and CD69+ (**Fig 3b**). We further assessed the levels of "non-activated" (CCR7+ CD62L+) versus "activated" (CCR7- CD62L-) CD4 T cells (20), and found an overall decreased percentage of CCR7 expressing CD4 T cells in H2-O KO spleens (**Fig 3c**) and lower frequencies of non-activated CD4 T cells in H2-O KO mice (**Fig 3d**). Importantly, this correlated with an increase in percentage of activated CD4 T cells (**Fig 3d**). Collectively, these phenotypic analyses support the idea that loss of H2-O leads to increased basal levels of activated CD4 T cells in unimmunized H2-O KO mice.

As discussed above, H2-O KO thymi promoted Tregs selection. Consistent with these observations and our previously published data (15, 21), H2-O KO spleens had an increased frequency of CD25+ Foxp3+ Tregs (**Fig 4a**). Furthermore, H2-O KO Tregs showed decreased levels of CD62L expression indicating these cells are likely more activated and circulating through the periphery (**Fig 4b**). Evaluation of the intracellular levels of Nur77 expression again revealed an increase, suggesting a higher TCR stimulation (**Figure 4c)** further validating the observations that loss of H2-O is driving a more activated state in both conventional CD4 T cells and Treg cells.

Single cell RNA-Sequencing of H2-O KO splenic CD4 T cells confirms increased activation

Based upon the strong FACS data above exhibiting increased numbers of Tregs, and more activated CD4 T cells, we attempted single-cell RNA-sequencing (scRNA-seq) to gain a more holistic unbiased characterization. CD3+ CD4+ NK1.1- CD19- cells were sorted from spleens of 3 unimmunized H2-O WT and 3 H2-O KO mice and subjected to 10x Genomics scRNA-seq analyses. Principle Component Analysis (PCA) of the normalized scRNA-seq data identified 11 distinct CD4 T cell clusters (**Fig 5a & sFig 2**). Separation of the clusters based upon H2-O WT or H2-O KO genotypes identified a dramatic increase in cluster 0, cluster 2 and cluster 3 in the H2-O KO samples. Conversely, clusters 1 and 4 were significantly increased in H2-O WT samples (**Fig 5b**).

Comparing the genes from Clusters 0, 1 and 5 to the published CD4 T cell datasets using the "My Geneset" function on the Immunological Genome Project (www.imgen.org) suggested that upregulated genes (Log_2Avg Fold-change (FC) ≥ 0.5) were present in both "naïve" and "activated" CD4 T cell datasets. Based upon the high expression of CCR7 however, these

163 clusters were labeled as “non-activated”. Clusters 3, 6, 7, and 11 aligned with mainly an
164 “activated” CD4 T phenotype. Cluster 3 was found to have increased expression of the
165 activation marker CD44, while Cluster 11 expressed high Ki67. Clusters 2 and 4 contained the
166 known Treg genes Foxp3 and Il2ra (CD25) (**Fig 5a & Sup Table 1**). Clusters 8, 9, and 10 were
167 small populations of Macrophages and NKT cell (**Sup Table 1**). Condensing the cluster analysis
168 further supported our initial FACS data that unimmunized H2-O-KO mice have a decreased
169 frequency of “Non-activated” CD4 T cells (68.90% H2-O KO vs 74.17% H2-O WT with a
170 coinciding increase in “Activated” (14.14% H2-O KO vs 8.53% H2-O WT) CD4 T cells (**Fig 5b**). To
171 further test whether the higher activated state in H2-O KO T cells were due to the H2-O KO CD4
172 T cells themselves, or the environment, we performed an adoptive transfer experiment of dye
173 labeled naïve (CD4+ CD44- CD25-) Thy1.1+ CD4 T cells from H2-O WT into either unimmunized
174 Thy1.2+ H2-O WT, or Thy1.2+ H2-O KO hosts. As shown in **Fig 5d**, H2-O KO hosts induced
175 significantly more *in vivo* proliferation of the WT cells than the H2-O WT hosts. These data
176 additionally confirms that loss of H2-O creates a more activating CD4 T cell environment by
177 presenting a wider range of self-epitopes (15).
178
179 **H2-O KO Tregs have a more activated phenotype**
180 Analyses of the two Treg clusters revealed that the frequency of Clusters 2 and 4 were
181 roughly equal in H2-O WT samples, whereas H2-O KO samples had an overrepresentation of
182 Cluster 2 (**Fig 6a, left**). Both cluster 2 and 4 expressed the classic Treg genes Foxp3, IL2ra
183 [CD25], CTLA4, and Pdcd1 [PD-1] (**Fig 6a, right**; **Sup Table 1**). Also identified was the
184 transcription factor Ikzf2 [Helios], a marker of stable Treg lineage commitment in inflammatory
185 conditions (22) and debated marker of thymic Tregs (23, 24, 25, 26). Supporting our scRNA-Seq
186 analysis, Helios protein expression was found in both H2-O WT and H2-O KO CD4 Tregs (**Fig 6b**).
187 The data also showed significant Helios expression in H2-O KO Foxp3- CD4 T cells supporting
188 that loss of H2-O increases CD4 T cell activation. Interestingly, comparison of the cluster 2
189 genetic signature with a published Treg data set indicated that these cells are likely effector-like
190 Tregs. Furthermore, breaking down Cluster 2 by genotype showed that H2-O KO Tregs had a
191 larger fold change in expression of the core effector genes signature (27) (**Fig 6c**). We
192 hypothesize that this activated Treg state is likely driven by the increased basal CD4 T cell
193 activation. Expansion of this Treg population could certainly be a mechanism controlling
194 spontaneous autoimmunity in H2-O KO mice.
195 In summary, both scRNA-seq and FACs analyses showed that H2-O KO mice have an
196 increased frequency of both conventional and regulatory CD4 T cells in the spleens of
197 unimmunized mice. Loss of thymic H2-O expression correlated with an increased tTreg
198 population that have received increased TCR stimulation which suggests increased
199 autoreactivity. Furthermore, increased activation of conventional splenic CD4 T cells is likely a
200 driving factor for the increased effector-like Treg status that was identified by scRNA-seq
201 analyses of H2-O-KO CD4 cells.
202
203 ## Discussion
204 In this study, we have refined our understanding of how loss of H2-O alters CD4 T cell
205 activity *in vivo*. Thymic analysis of medulla localized CD4 T cells pointed to a novel role for H2-O

206 on CD4 T cells undergoing self-auditing. Importantly, lack of H2-O expression led to an
207 increased frequency of both thymic and peripheral Tregs.
208 Tregs play a fundamental role in maintenance of homeostasis, hence a better
209 understanding of the mechanism of their development is highly desirable and the subject of
210 numerous studies (13, 18). Our studies here are the first attempt in demonstrating that H2-O is
211 a critical player in Treg development. While the exact mechanism by which thymic Treg cells
212 are selected remains somewhat uncertain, recent studies support the idea that intermittent
213 TCR signaling along with cytokine signaling drive Treg development (14). These findings and
214 others (28, 29, 30, 31, 32) support a more avidity based model of Treg selection, in which
215 alterations to the density of self-ligands present in the medulla will have differential effects on
216 CD4 T cell selection. Intermittent TCR signaling as the driving force in Treg selection is very
217 relevant to our understanding of how HLA-DO contributes to epitope selection during antigen
218 processing. HLA-DO in complex with HLA-DM leads to better refinement of the epitopes from
219 denatured antigens, promoting selection of the best fitting epitopes in the groove of MHC II
220 molecules (9, 33). As such, when DO is present, higher affinity peptides are more likely to be
221 presented. Conversely, in the absence of DO a larger percent of lower affinity epitopes are
222 selected. Indeed, we have recently reported that peptides eluted from H2-O KO mice
223 expressing either murine I-A^b (15) or human HLA-DR1 (Welsh et al, unpublished data) were of a
224 lower general affinity. Accordingly, presentation of a larger portion of lower affinity peptides in
225 the thymic medulla of H2-O KO mice is expected. Intermittent TCR signaling is typically
226 generated by either pMHC II that are less stable (34, 35, 36, 37), or TCR/pMHC of lower affinity
227 (38). Our findings here support the former. We associate increased Treg development as a
228 consequence of the presentation of lower affinity peptides to self-auditing CD4 T cells.
229 Due to the continuous egress of mature CD4 T cells from the thymus, we also analyzed
230 splenic CD4 T cells from unimmunized H2-O WT and H2-O KO mice. H2-O KO splenic CD4 T cells
231 in unimmunized mice revealed an increased frequency of CD44hiCD69+ antigen experienced
232 CD4 T cells, which suggests an increased level of antigen-specific TCR signaling in H2-O KO mice.
233 As previously implied (39), a simple explanation for this activated state is incomplete thymic
234 deletion in H2-O KO mice. While we were able to show that H2-O KO mice failed to delete
235 specific CD4 clonal populations (15), detection of differences at the global levels did not show
236 significant changes between the two genotypes. Nonetheless, we suggest that presentations of
237 different arrays of self-antigens as well as their lower densities on thymic medulla leads to a
238 less than optimal thymic deletion of self-reactive CD4 T cells and their routing to the periphery.
239 Similar to activated CD4 T cells in periphery, H2-O KO Treg cells also had a more effector-like
240 phenotype, indicating enhanced Treg activation in unimmunized mice (27, 40).
241 In conclusion, our studies add a new dimension to our understanding of the role of H2-O
242 in both CD4 T cell selection and activation. For the first time, we report on thymic negative
243 selection in H2-O KO mice and demonstrate that loss of H2-O enhances thymic selection of
244 regulatory T cells. Once in the periphery, an increased proportion of H2-O KO Tregs appear to
245 be activated in a TCR dependent manner. These effector-like Tregs will likely help control
246 increased basal CD4 T cell activation. However, the exact mechanism by which increased Treg
247 cells are selected in the H2-O KO thymus remains to be determined. While we propose that
248 alterations in medulla pMHCII-TCR avidity interactions could lead to enhanced Treg selection,

249 increased presentation of self-antigens in a more tissue restricted antigenic (TRA) manner could
250 also be possible (13).
251
252
253 # Methods
254 ## Mice
255 Male and female C57BL/6J (H2-O WT, stock no: 000664), Female B6.PL-Thy1a/CyJ (stock
256 no: 000406) were purchased from Jackson Laboratories and bred in house. Generation of the
257 H2-O knock-out mice has been previously described (41) and mice bred in house. All mouse
258 procedures were approved by the Johns Hopkins University Animal Care and Use Committee
259 and were following relevant ethical regulations.
260
261 ## Antibodies/reagents
262 **Flow Cytometry**: anti-CD3e (17A2), anti-CD4 (RM4-5), anti-CD5 (53-7.3), anti-CD8α (53-6.7),
263 CD11c (N418), anti-CD19 (ID3), anti-CD25 (PC61), anti-CD44 (IM7), anti-CD45R/B220 (RA3-6B2),
264 anti-CD62L (MEL-14), anti-CD69 (H1.2F3), anti-CD197/CCR7 (4B12), anti-NK1.1 (PK136), anti-
265 TCRβ (H57-597), anti-TCRγδ (GL3), anti-Cleaved Caspase 3 (D3E9) Cell signaling (Danvers, MA);
266 Foxp3 (150D), anti-Helios (22F6), anti-Nur77 (12.14), Fixable viability dye eFluor 780
267 (eBioscience).
268
269 **FACs Sorting**: anti-CD3e, anti-CD4, anti-CD19, anti-NK1.1, Propidium iodide. Briefly, 30,000 live
270 CD4 T cells [Gating: CD3+, CD4, CD19-, NK1.1-, PI-] were FACs sorted from the spleens of 3
271 unimmunized 10 week-old H2-O WT and 3 unimmunized 10 week-old unimmunized H2-O KO
272 female mice.
273
274 ## Cell staining
275 For cleaved caspase 3 staining (17) homogenized mice thymocyte cells were stained
276 with anti-CCR7/CD197 at a final dilution of 1:50 for 30 min at 37˚C prior to additional surface
277 stains. Following surface staining, cells were fixed with Cytofix/Cytoperm (BD Biosciences) for
278 20 min at 4˚C. Cells were then washed with Perm/Wash buffer (BD Biosciences) twice. Cells
279 were stained with anti–cleaved caspase 3 at a 1:50 dilution at 23˚C for 30 min.
280 For transcription factor staining, cells were incubated with surface antibody at 4˚C for 20
281 min, permeabilized at 4˚C for 30 min using a Foxp3/Transcription factor buffer set (Invitrogen,
282 ThermoFisher Scientific), and then stained with anti–Foxp3 and/or anti-Helios at 23˚C for 30
283 min.
284
285 **Adoptive Cell Transfer**
286 Naïve CD4 T cells were isolated from the pooled spleens and LNs of 4 week old Thy1.1
287 expressing C57BL/6J mice using the EasySep Mouse Naive CD4 T cell Isolation Kit (StemCell
288 Technologies). Isolated cells were stained with eFluor 450 viability dye (eBiosciences) according
289 to manufactures directions. $3x10^6$ dye labeled Thy1.1 cells were IV injected into 10 week old
290 H2-O WT or H2-O KO hosts. Transferred cells were recovered 7 days post-transfer.
291

scRNA-Sequencing

Library & sequencing

The samples were prepared using the 10x Genomics Chromium Next GEM Single Cell 5' Library and Gel Bead Kit v1.1, Chromium Next GEM Chip and Dual Index Kit TN Set A. They were run on the Illumina NovaSeq6000 with a run configuration of 28bp x 10bp x 10bp x 91bp.

Analysis

Analyses of T-cell scRNA-seq were performed with the package Seurat (42), as follows. Data was filtered to remove cells with low gene count (<200), large number of UMIs (>12,000) and high (>5%) fraction of mitochondrial reads. Expression levels of genes were log-normalized, and the most variable 2000 genes were selected for linear dimensionality reduction with Principal Component Analysis (PCA). The first 15 principal components were then used to performed unsupervised clustering using the Seurat SNN clustering package, with a resolution of 0.2. To identify cell types, potential markers for each cluster were calculated as the set of genes significantly differentially expressed in each cluster compared to all others, using the function FindAllMarkers, and by searching existing literature and marker databases. Lastly, differentially expressed genes for each cell type between conditions were determined using the function FindMarkers with the default function (bimod).

Defining clusters – Genes with an average fold-change (avgFC) >0.5 and an adjusted p-value <0.05 were run against CD4 T cell data sets available on the Immunological Genome Project (https://www.immgen.org/) using the “My Geneset” data browser function.

Statistical testing

GraphPad Prism was used for all statistical analyses. A standard Student T-test was used for estimation of statistical significance. Data is shown as mean ± SEM. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

Acknowledgments

Supported by grants from NIAID, R01AI063764, R21AI101987, and R01AI120634, to SS-N. FACs analysis was performed in the BD Immunology and Flow Cytometry Laboratory at the Johns Hopkins School of Public Health.

326

327 **Bibliography**

328 1. Murphy K, Weaver C. Janeway's Immunobiology. New York & London: Garland Science;
329 2016 2016.

330 2. Nishizuka Y, Sakakura T. Thymus and reproduction: sex-linked dysgenesia of the gonad
331 after neonatal thymectomy in mice. Science. 1969;166(3906):753-5.

332 3. Karlsson L, Surh CD, Sprent J, Peterson PA. A novel class II MHC molecule with unusual
333 tissue distribution. Nature. 1991;351(6326):485-8.

334 4. Narayan K, Su KW, Chou CL, Khoruzhenko S, Sadegh-Nasseri S. HLA-DM mediates
335 peptide exchange by interacting transiently and repeatedly with HLA-DR1. Mol Immunol.
336 2009;46(15):3157-62.

337 5. Chou CL, Mirshahidi S, Su KW, Kim A, Narayan K, Khoruzhenko S, et al. Short peptide
338 sequences mimic HLA-DM functions. Mol Immunol. 2008;45(7):1935-43.

339 6. Natarajan SK, Assadi M, Sadegh-Nasseri S. Stable peptide binding to MHC class II
340 molecule is rapid and is determined by a receptive conformation shaped by prior association
341 with low affinity peptides. J Immunol. 1999;162(7):4030-6.

342 7. Sadegh-Nasseri S. A step-by-step overview of the dynamic process of epitope selection
343 by major histocompatibility complex class II for presentation to helper T cells. F1000Res.
344 2016;5.

345 8. Alfonso C, Williams GS, Han JO, Westberg JA, Winqvist O, Karlsson L. Analysis of H2-O
346 influence on antigen presentation by B cells. J Immunol. 2003;171(5):2331-7.

347 9. Poluektov YO, Kim A, Hartman IZ, Sadegh-Nasseri S. HLA-DO as the optimizer of epitope
348 selection for MHC class II antigen presentation. PloS One. 2013;8(8):e71228.

349 10. Savage PA, Klawon DEJ, Miller CH. Regulatory T Cell Development. Annu Rev Immunol.
350 2020;38:421-53.

351 11. Hsieh CS, Lee HM, Lio CW. Selection of regulatory T cells in the thymus. Nat Rev
352 Immunol. 2012;12(3):157-67.

353 12. Tai X, Erman B, Alag A, Mu J, Kimura M, Katz G, et al. Foxp3 transcription factor is
354 proapoptotic and lethal to developing regulatory T cells unless counterbalanced by cytokine
355 survival signals. Immunity. 2013;38(6):1116-28.

356 13. Klein L, Robey EA, Hsieh CS. Central CD4(+) T cell tolerance: deletion versus regulatory T
357 cell differentiation. Nat Rev Immunol. 2019;19(1):7-18.

358 14. Tai X, Indart A, Rojano M, Guo J, Apenes N, Kadakia T, et al. How autoreactive
359 thymocytes differentiate into regulatory versus effector CD4(+) T cells after avoiding clonal
360 deletion. Nat Immunol. 2023;24(4):637-51.

361 15. Welsh RA, Song N, Foss CA, Boronina T, Cole RN, Sadegh-Nasseri S. Lack of the MHC
362 class II chaperone H2-O causes susceptibility to autoimmune diseases. PLoS Biol.
363 2020;18(2):e3000590.

364 16. Nanaware PP, Jurewicz MM, Leszyk J, Shaffer SA, Stern LJ. HLA-DO modulates the
365 diversity of the MHC-II self-peptidome. Mol Cell Proteomics. 2018.

366 17. Breed ER, Watanabe M, Hogquist KA. Measuring Thymic Clonal Deletion at the
367 Population Level. J Immunol. 2019;202(11):3226-33.

368 18. Li MO, Rudensky AY. T cell receptor signalling in the control of regulatory T cell
369 differentiation and function. Nat Rev Immunol. 2016;16(4):220-33.

370 19. Moran AE, Holzapfel KL, Xing Y, Cunningham NR, Maltzman JS, Punt J, et al. T cell
371 receptor signal strength in Treg and iNKT cell development demonstrated by a novel
372 fluorescent reporter mouse. J Exp Med. 2011;208(6):1279-89.

373 20. Golubovskaya V, Wu L. Different Subsets of T Cells, Memory, Effector Functions, and
374 CAR-T Immunotherapy. Cancers (Basel). 2016;8(3).

375 21. Welsh R, Sadegh-nasseri S. The love and hate relationship of HLA-DM/DO in the
376 selection of immunodominant epitopes. Curr Opin Immunol. 2020;64:117–23.

377 22. Lam AJ, Uday P, Gillies JK, Levings MK. Helios is a marker, not a driver, of human Treg
378 stability. Eur J Immunol. 2022;52(1):75-84.

379 23. Thornton AM, Korty PE, Tran DQ, Wohlfert EA, Murray PE, Belkaid Y, et al. Expression of
380 Helios, an Ikaros transcription factor family member, differentiates thymic-derived from
381 peripherally induced Foxp3+ T regulatory cells. J Immunol. 2010;184(7):3433-41.

382 24. Thornton AM, Lu J, Korty PE, Kim YC, Martens C, Sun PD, et al. Helios(+) and Helios(-)
383 Treg subpopulations are phenotypically and functionally distinct and express dissimilar TCR
384 repertoires. Eur J Immunol. 2019;49(3):398-412.

385 25. Serre K, Benezech C, Desanti G, Bobat S, Toellner KM, Bird R, et al. Helios is associated
386 with CD4 T cells differentiating to T helper 2 and follicular helper T cells in vivo independently of
387 Foxp3 expression. PLoS One. 2011;6(6):e20731.

388 26. Akimova T, Beier UH, Wang L, Levine MH, Hancock WW. Helios expression is a marker of
389 T cell activation and proliferation. PLoS One. 2011;6(8):e24226.

390 27. Miragaia RJ, Gomes T, Chomka A, Jardine L, Riedel A, Hegazy AN, et al. Single-Cell
391 Transcriptomics of Regulatory T Cells Reveals Trajectories of Tissue Adaptation. Immunity.
392 2019;50(2):493-504 e7.

393 28. Simons DM, Picca CC, Oh S, Perng OA, Aitken M, Erikson J, et al. How specificity for self-
394 peptides shapes the development and function of regulatory T cells. J Leukoc Biol.
395 2010;88(6):1099-107.

396 29. Relland LM, Mishra MK, Haribhai D, Edwards B, Ziegelbauer J, Williams CB. Affinity-
397 based selection of regulatory T cells occurs independent of agonist-mediated induction of
398 Foxp3 expression. J Immunol. 2009;182(3):1341-50.

399 30. Picca CC, Oh S, Panarey L, Aitken M, Basehoar A, Caton AJ. Thymocyte deletion can bias
400 Treg formation toward low-abundance self-peptide. Eur J Immunol. 2009;39(12):3301-6.

401 31. Feuerer M, Jiang W, Holler PD, Satpathy A, Campbell C, Bogue M, et al. Enhanced thymic
402 selection of FoxP3+ regulatory T cells in the NOD mouse model of autoimmune diabetes. Proc
403 Natl Acad Sci U S A. 2007;104(46):18181-6.

404 32. Atibalentja DF, Murphy KM, Unanue ER. Functional Redundancy between Thymic
405 CD8{alpha}+ and Sirp{alpha}+ Conventional Dendritic Cells in Presentation of Blood-Derived
406 Lysozyme by MHC Class II Proteins. J Immunol. 2011;186(3):1421-31.

407 33. Poluektov YO, Kim A, Sadegh-Nasseri S. HLA-DO and Its Role in MHC Class II Antigen
408 Presentation. Frontiers in immunology. 2013;4:260.

409 34. Mirshahidi S, Ferris LC, Sadegh-Nasseri S. The magnitude of TCR engagement is a critical
410 predictor of T cell anergy or activation. J Immunol. 2004;172(9):5346-55.

411 35. Sadegh-Nasseri S, Dalai SK, Korb Ferris LC, Mirshahidi S. Suboptimal engagement of the
412 T-cell receptor by a variety of peptide-MHC ligands triggers T-cell anergy. Immunology.
413 2010;129(1):1-7.

414 36. Baumgartner CK, Ferrante A, Nagaoka M, Gorski J, Malherbe LP. Peptide-MHC class II
415 complex stability governs CD4 T cell clonal selection. J Immunol. 2010;184(2):573-81.

416 37. Smyth LA, Williams O, Huby RDJ, Norton T, Acuto O, Ley SC, et al. Altered peptide
417 ligands induce quantitatively but not qualitatively different intracellular signals in primary
418 thymocytes. PNAS. 1998;95(14):8193-8.

419 38. Lucas B, Stefanova I, Yasutomo K, Dautigny N, Germain RN. Divergent changes in the
420 sensitivity of maturing T cells to structurally related ligands underlies formation of a useful T
421 cell repertoire. Immunity. 1999;10(3):367-76.

422 39. Gu Y, Jensen PE, Chen X. Immunodeficiency and autoimmunity in H2-O-deficient mice.
423 Journal of immunology. 2013;190(1):126-37.

424 40. Shevyrev D, Tereshchenko V. Treg Heterogeneity, Function, and Homeostasis. Front
425 Immunol. 2019;10:3100.

426 41. Liljedahl M, Winqvist O, Surh CD, Wong P, Ngo K, Teyton L, et al. Altered antigen
427 presentation in mice lacking H2-O. Immunity. 1998;8(2):233-43.

428 42. Stuart T, Butler A, Hoffman P, Hafemeister C, Papalexi E, Mauck WM, 3rd, et al.
429 Comprehensive Integration of Single-Cell Data. Cell. 2019;177(7):1888-902 e21.

430

431

Figure Legends

Figure 1: Loss of H2-O function supports increased thymic Regulatory T cell development
A. Left: representative gating showing how "Auditing" vs "Clonal Deleted" CCR7+CD4+ T cells are defined. Middle: Frequency of the activation marker CD69 in either "auditing" medulla CD4+ T cells or (Right) "clonally deleted" medulla CD4+ T cells.
B. Subdivision of auditing CD4 T cells into three maturation stages: Semi-Mature (Left), Mature 1 (Middle), and Mature 2 (Right). Expression has been normalized to the average H2-O WT levels within each experiment to allow for comparison across experiments. Data from 5 replicate experiments.
C. Frequency of medulla specific (CCR7+) CD4 T cells selected for clonal deleted (Caspase-3+)
ns = not significant
* = <0.05
** = <0.001
***= <0.0001
**** = <0.00001
Statistics: simple student T-test

Figure 2: DO-KO thymi have an increased proportion of medulla CD4 T cells at the Mature 1 stage
A. The percentage of Foxp3+ CD25+ cells within the CD4 single-positive thymus population in H2-O WT (white) or H2-O KO (red) cells. Data from 3 replicate experiments
B. Geometric mean fluorescence intensity (gMFI) of CD25 (left), CD5 (right) and Nur77 (Bottom) expressed by Foxp3+ CD4+ T cells in the thymus of H2-O WT (white) or H2-O KO (red) mice.
C. Subdivision of Treg cells into three maturation stages: Semi-Mature (Left), Mature 1 (Middle), and Mature 2 (Right). Expression has been normalized to the average H2-O WT levels within each experiment to allow for comparison across experiments. Data from 2 replicate experiments.

Figure 3: **H2-O KO mice have an increased activated peripheral CD4 T cell population**
A. Percentage of CD4+ T cells within the splenic CD3+ population of unimmunized 6-10-week-old H2-O WT (white) and H2-O KO (red) mice. Representative of >4 independent experiments, N = 16 mice per group.
B. Percentage of CD4+ T cells expressing the lymphoid tissue homing receptor CCR7
C. CCR7 and CD62L expression levels in unimmunized splenic CD4 T cells
D. Unimmunized levels of CD44 and CD69 expressed by splenic CD4 T cells. Increased CD69 expression, a marker of recent activation showed increased levels on H2-O KO CD4 T cells
* = <0.05
** = <0.001
*** = <0.0001
**** = <0.00001

Figure 4: **Peripheral H2-O KO Tregs have increased TCR stimulation**
A. Frequency of Foxp3+CD25+ cells in splenic CD4+ T cell population of unimmunized mice
B. H2-O KO peripheral Tregs express decreased levels of naïve (CD44+ CD62L+) expressing cells
C. Normalized Nur77 gMFI levels in Foxp3+CD25+ Treg cells in H2-O WT (white) and H2-O KO (red) cells. To account for experimental variation the average Nur77 gMFI level in H2-O WT samples was calculated. gMFI levels in both H2-O WT and H2-O KO samples were then divided by the calculated H2-O WT average. An increased Nur77 ratio indicates increased Nur77 gMFI levels. Summary of 3 repeat experiments.

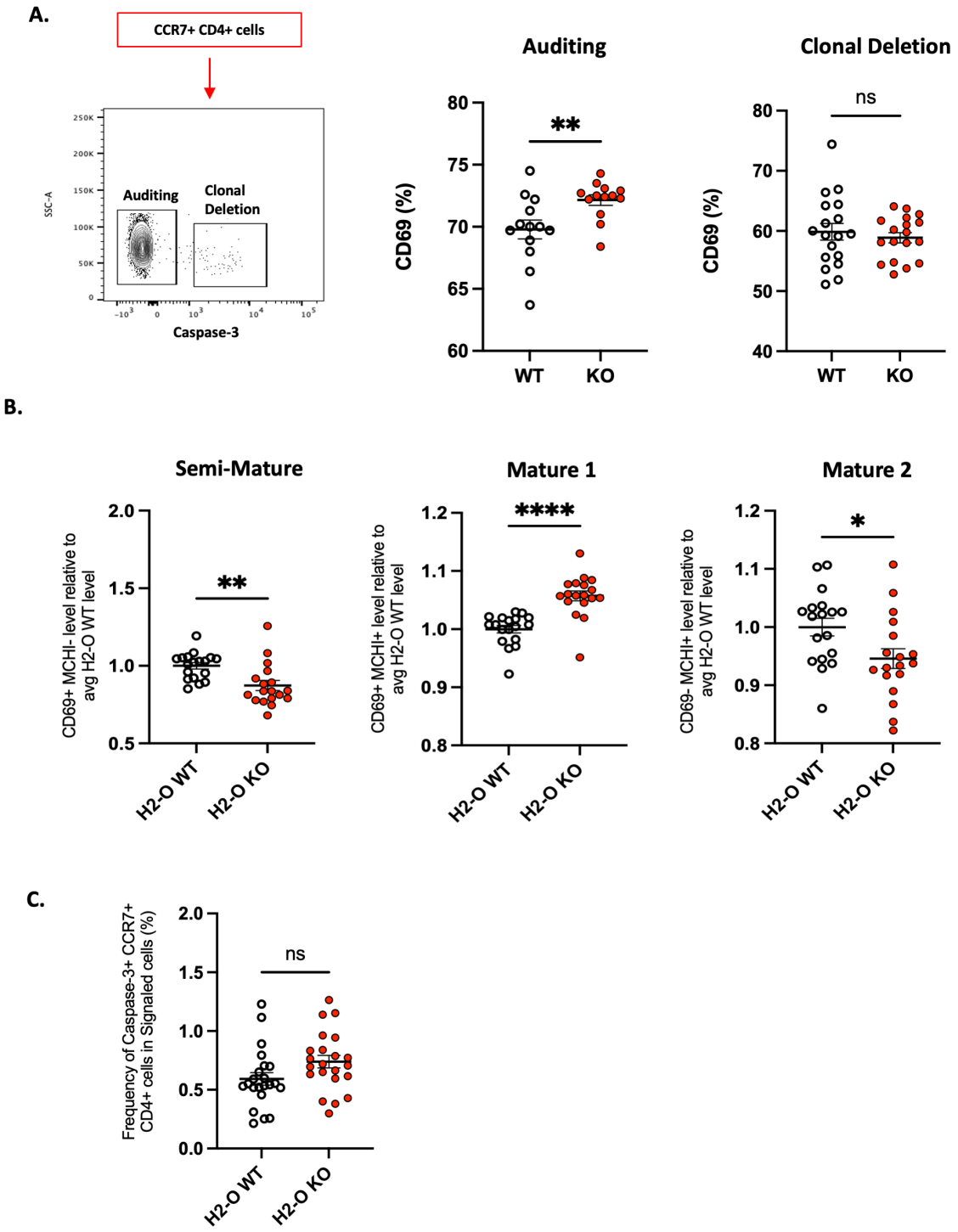
Figure 5: **Loss of H2-O function causes increased basal CD4 cell activation**
A. scRNA-seq clustering of CD4 T cells after Seurat analysis. Data represents the average of of 3 biological replicates per genotype.
B. Breakdown of clusters in H2-O WT (Left) or H2-O KO (Right) samples. Clusters are grouped based upon, (1) known CD4 T cell subset markers and (2) gene comparison to published CD4 T cell data sets available on the Immunological Genome Project (www.immgen.org). Identified CD4 Cell phenotypes were: Non-activated, Activated, and Regulatory T cells. ”Other” refers to a minor macrophage and NKT cell contamination from the sorting process.
C. Distribution of the for the Non-activated and Activated phenotypes across the H2-O WT and H2-O KO biological replicates (N = 3 mice per genotype).
D. In vivo proliferation of adoptively transferred naïve Thy1.1+ CD4 T cells (Thy1.1+ CD3+ CD4+ CD44- CD25-) after 7 days in either Thy1.2 H2-O WT (white) or Thy1.2 H2-O KO (red) hosts. Pooled data from 2 independent experiments (N= 6 H2-O WT, 5 H2-O KO)

Figure 6: **The H2-O KO Treg population has a more effector like phenotype**
A. (Left) distribution of the two Treg populations identified by scRNA-seq clustering in either H2-O WT (left) or H2-O KO (right) samples. (Right) Expression of key Treg phenotypic markers: Foxp3 (Top) and CD25 [Il2ra] (Bottom)
B. Subdivision of the splenic CD4+ T cell population by Foxp3 and Helios expression
C. Comparison of cluster 2 upregulated genes (Log_2Avg FC >0.5) in H2-O WT and H2-KO samples to a published (Miragaia *et al*.) splenic effector Treg genetic profile

Supplemental Figure 1
A. Representative plots showing CD4, CD8 and Double Positive (DP) percentages in H2-O WT (Top) and H2-O KO (Bottom)
B. Summary plots of the single-positive CD4 (Top) and CD8 (Bottom) percentages from 5 repeat experiments.
C. (Left) representative flow plots showing the “Signaled” vs “Non-signaled” thymocytes in H2-O WT (Top) or H2-O KO (Bottom).
(Right) Summary plots of >5 repeat experiments.
D. Frequency of signaled CCR7+ medulla CD4 T cells in H2-O WT (white) and H2-O KO (red) mice

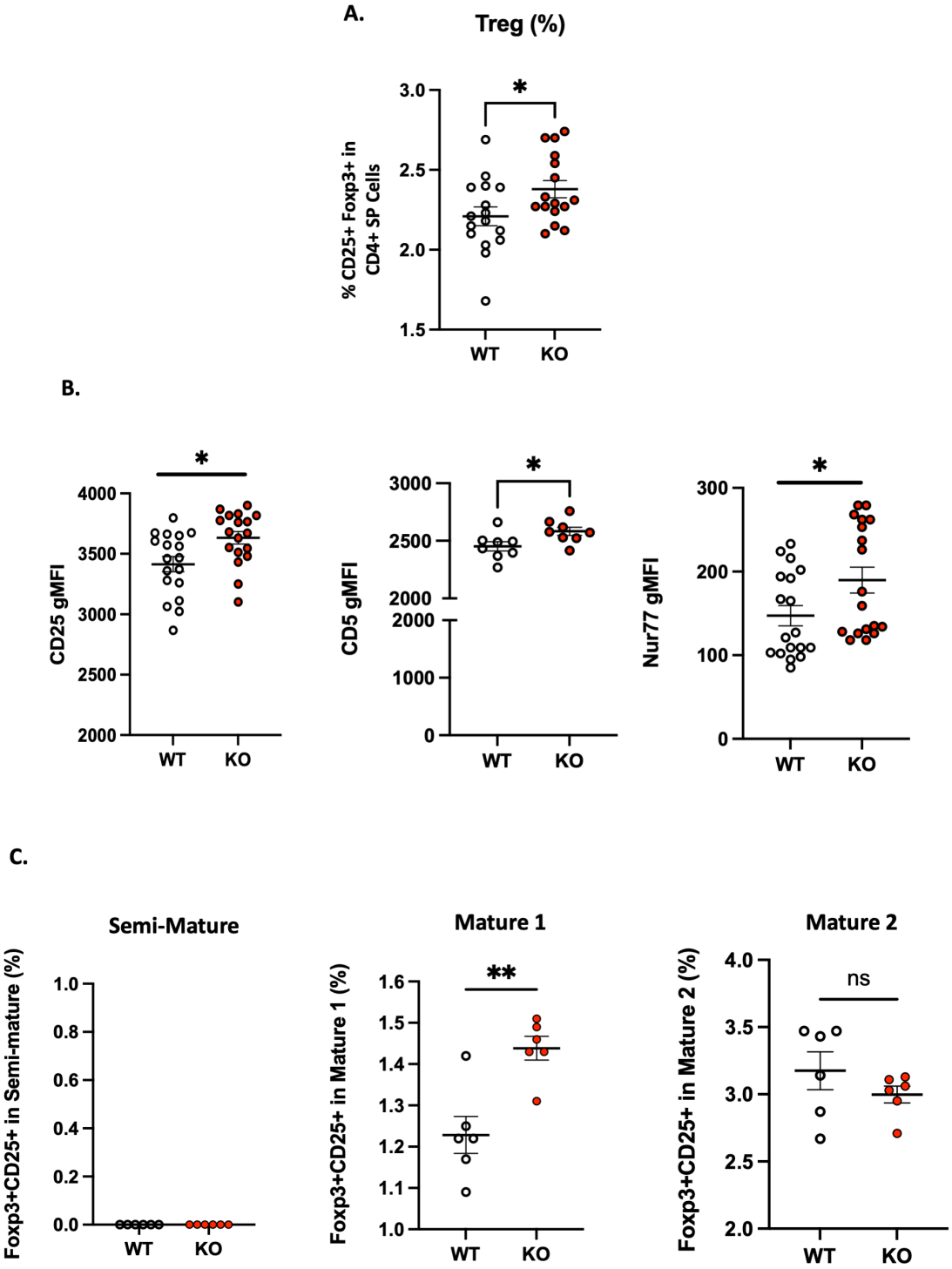
Supplemental Figure 2:
Top: breakdown of H2-O KO (right) and H2-O WT (Left) Seurat clustering
Bottom: Seurat clustering for each individual biological replicate

520 **Figure 1**



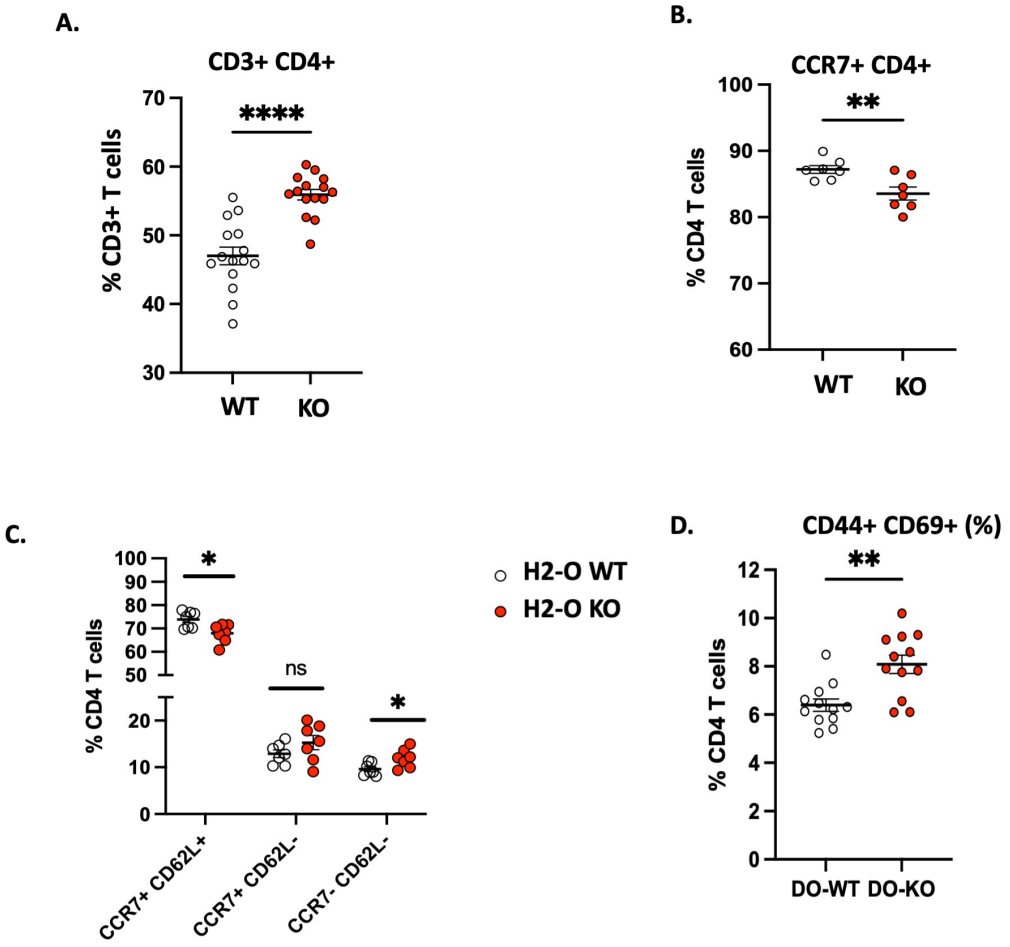
521

522 **Figure 2**



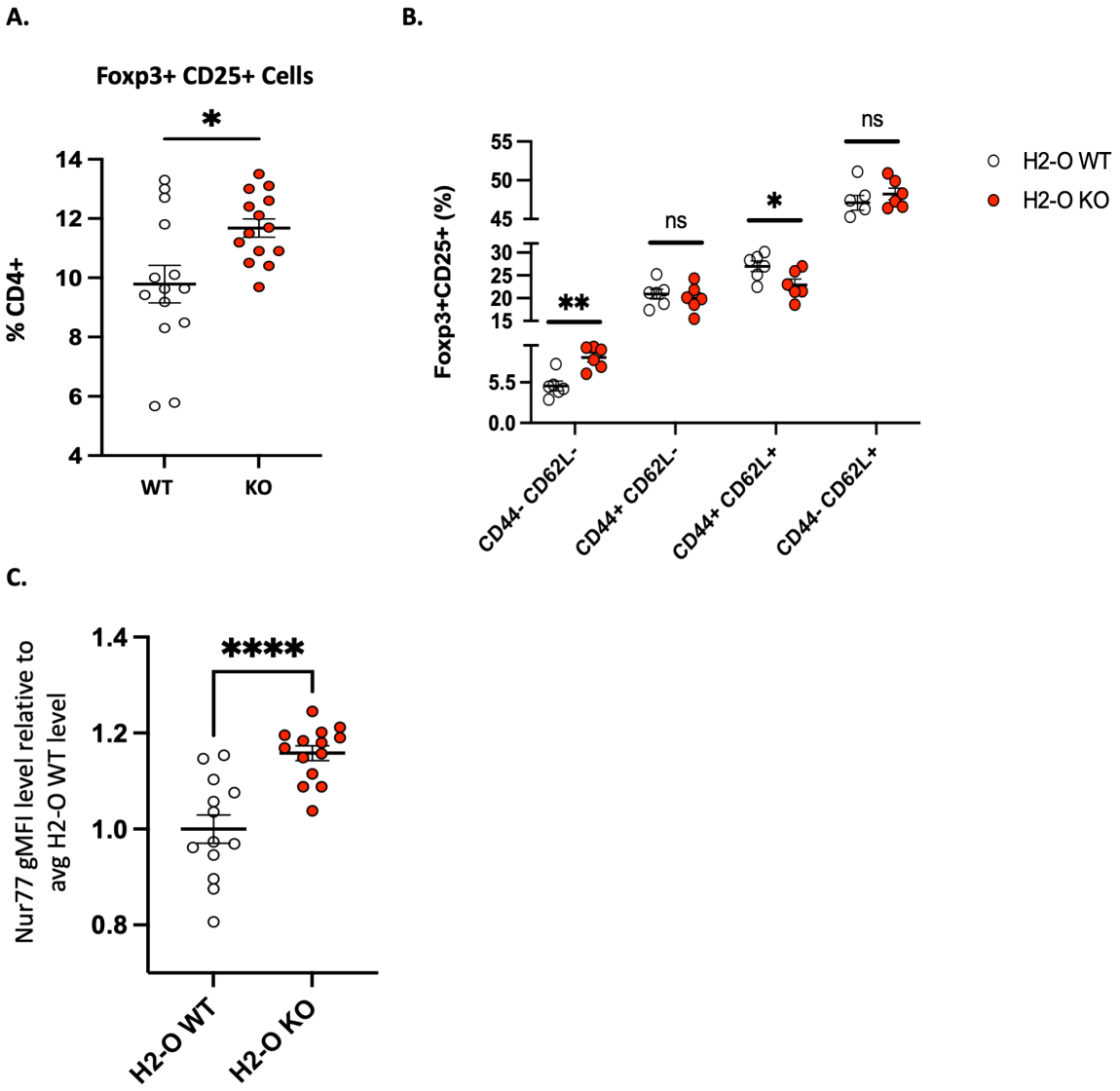
523

524 **Figure 3**



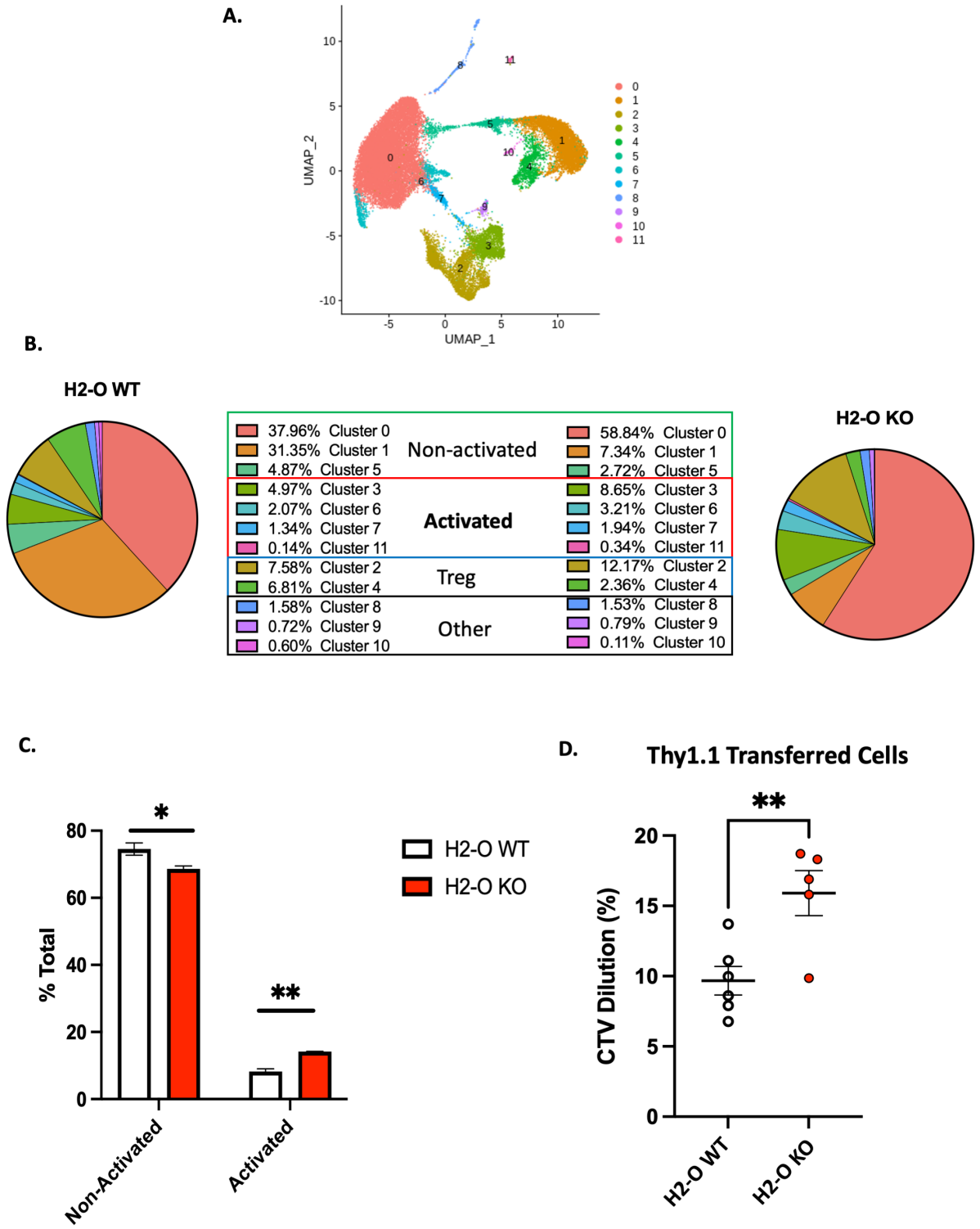
525

526 **Figure 4**



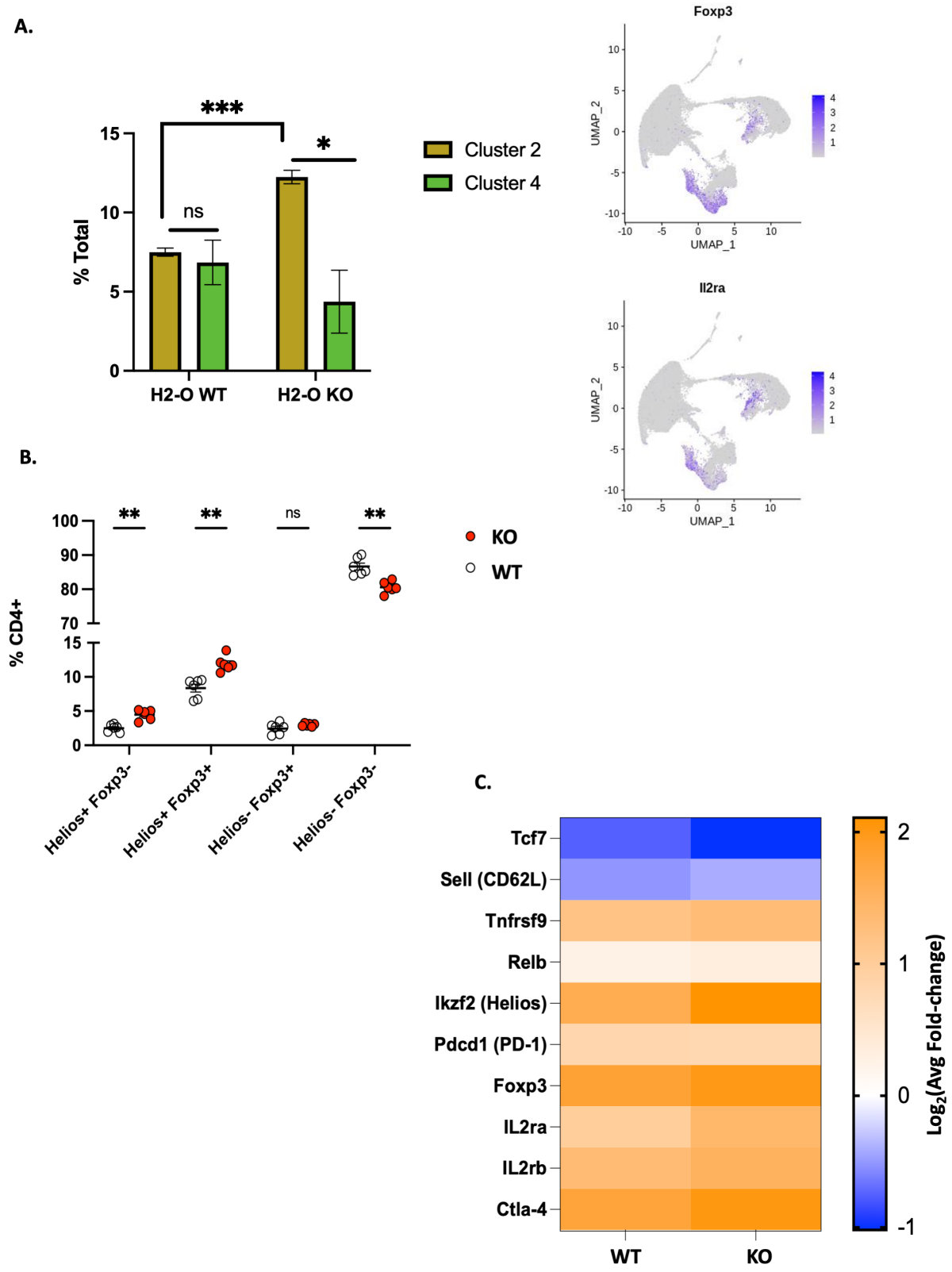
527

528 **Figure 5**



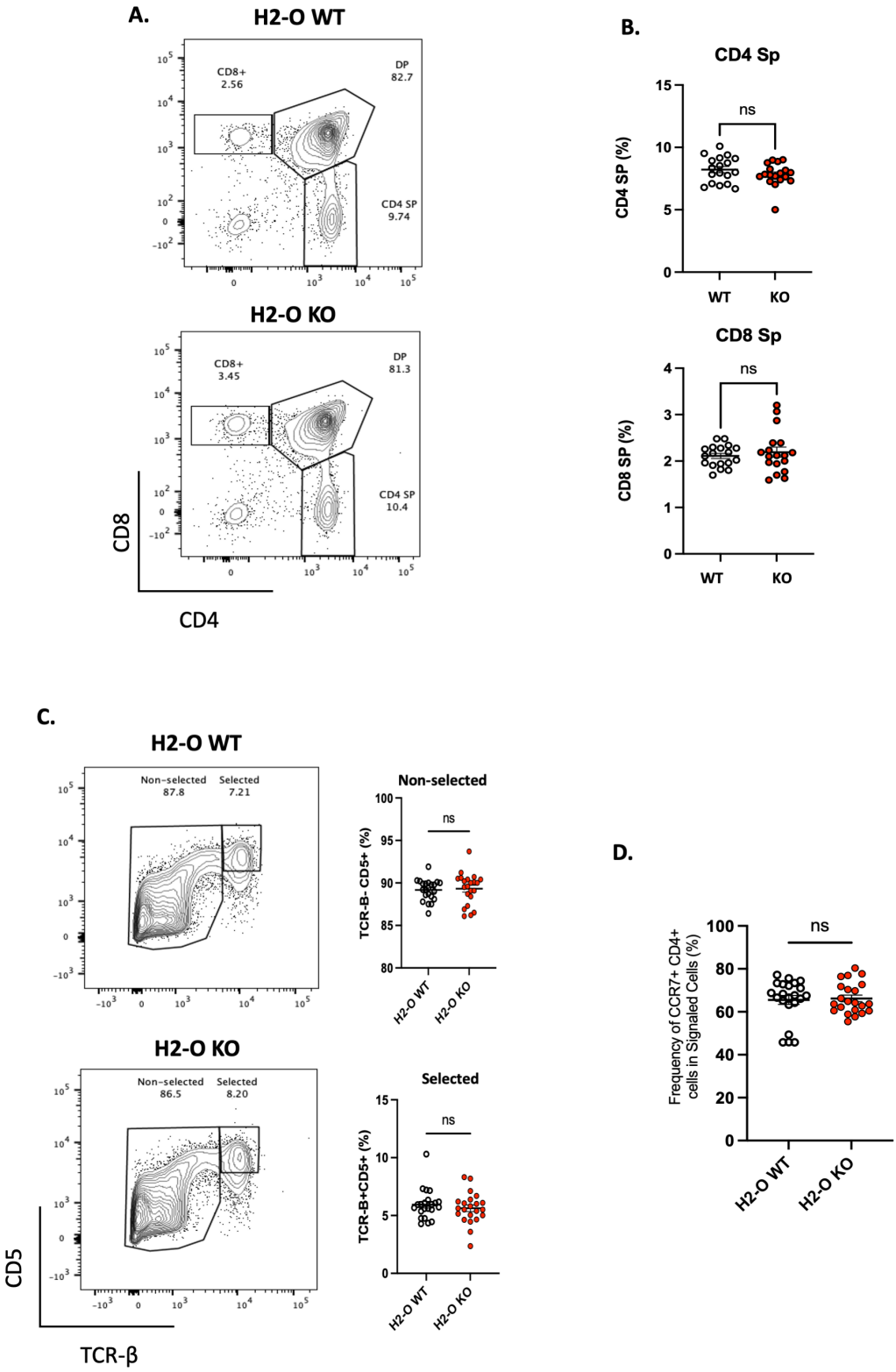
529

530 **Figure 6**



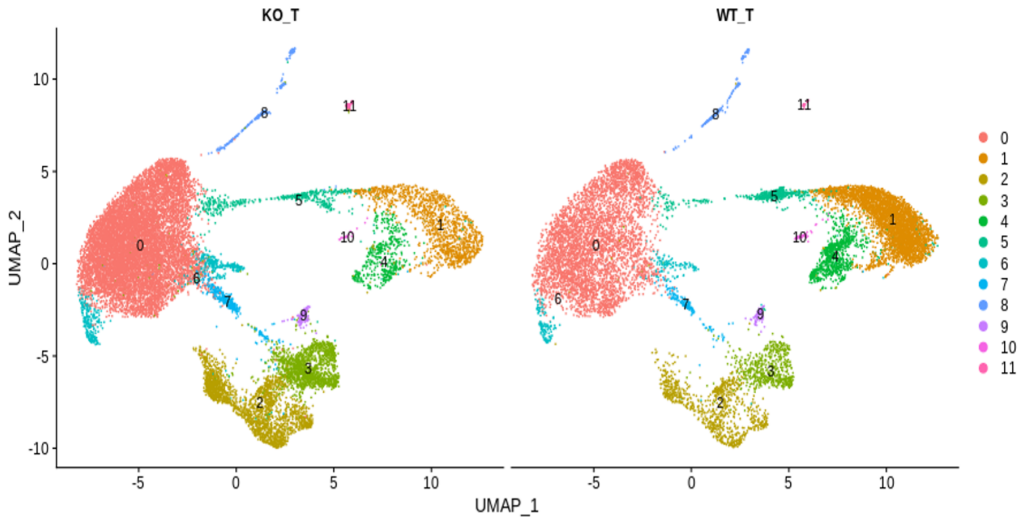
531

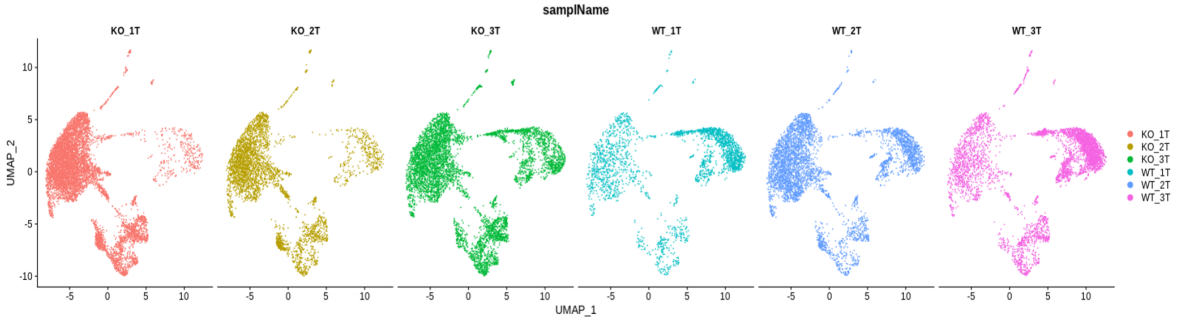
532 **Supplemental Figure 1**



533

534 **Supplemental Figure 2**





535