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

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**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 H2-O, an MHC class II peptide editing molecular chaperon, on selection of thymic Tregs. We provide evidence 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 not only of an enrichment of effector-like Tregs but also of activated CD4 T cells in the absence of H2-O. Our data support two concepts; a) lack of H2-O expression in the 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 predispositions to autoimmunity and design of therapeutics for treatment of autoimmune diseases.

30

31 ## Introduction

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

39 Regulation of T cell activation begins during thymic development where immature
40 thymocytes are screened for self-reactivity. Broadly divided into positive and negative
41 selection, this process ensures that CD4 T cells expressing high avidity self-reactive TCRs are
42 either eliminated (1), or become CD4 regulatory T cells (Tregs) (2). Critical to the screening
43 process for identifying auto-reactive T cells is the Class II antigen processing machinery
44 expressed by medullary thymic epithelium cells (mTECs) and thymic antigen presenting cells
45 (APCs). Two chaperone proteins, H2-M (murine; human, HLA-DM), and H2-O (murine; human,
46 HLA-DO) are major components of the MHC II processing pathway. While H2-M is expressed in
47 all APCs, H2-O was originally discovered to be expressed in the thymic medulla and B cells (3). It
48 was only later that HLA-DO was reported to be expressed on cross presenting CD8αα+ DC (4),
49 then followed by identification of H2-O on murine CD8αα+ DC (5). H2-M plays a critical role in
50 MHC class II antigen processing by dissociating the Class II Invariant Chain peptide (CLIP) from
51 the newly synthesized MHC II. Dissociation of CLIP promotes a peptide-receptive MHC II

52 conformation that can readily scan denatured antigens for the best MHC II groove fitting
53 epitopes. However, a peptide-receptive MHC II conformation is highly transient (6, 7, 8, 9). We
54 have proposed that H2-O binds to H2-M (10) and works cooperatively with H2-M by stabilizing
55 the peptide-receptive MHC II conformation in order to optimize epitope selection (11).
56 Together, these two molecules can ensure that the best MHC II groove fitting epitopes are
57 presented to cognate CD4 T cells.

58 While the exact mechanism of Treg selection remains to be fully understood (12), two
59 critical requirements have been identified as necessary for successful thymic Treg development.
60 First, thymic Treg development requires relatively strong TCR signaling in the thymic medulla
61 (13), and second, Treg development relies on signaling by common γ chain (γC) cytokines,
62 mainly IL-2, for driving Foxp3 expression (14). Considering that strong TCR signaling during
63 negative selection normally leads to CD4 T cell deletion (1), when investigating Treg selection,
64 both the nature and density of medulla presented self-peptides must be considered. If epitopes
65 are in high abundance and more ubiquitously expressed, then cognate CD4 T cells will undergo
66 clonal deletion. However, if epitopes are in lower abundance and have a sparser expression,
67 leading to discontinuous TCR stimulation, then cognate CD4 T cells might undergo Treg
68 selection (12, 15, 16). This model of Treg selection relies on the level of TCR signaling that
69 medulla localized CD4 single-positive (SP) T cells receive. Should a lower density of self pMHCII
70 be presented in the thymic medulla to CD4 T cells undergoing negative selection, an increased
71 number of self-reactive CD4 T cells might escape deletion, leading to an increased frequency of
72 auto-reactive T cell clones in the periphery. Alternatively, presentation of a lower density of self
73 pMHCII could promote selection of regulatory T cells. Here, we demonstrate that loss of H2-O

74 generates a more stimulatory *in vivo* environment impacting both the thymic development and
75 peripheral activation of regulatory T cells.
76

77 ## Results

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

79 Previously, we demonstrated that loss of H2-O expression correlated with both an
80 increased B cell presentation of low-affinity MHC II peptides, and an increased frequency of a
81 $MOG_{35-55}$ specific, self-reactive CD4 T cell (17). Because of H2-O expression in the medullary
82 thymus we speculated that H2-O deficiency might lead to presentation of lower densities of
83 high-affinity self-peptides in the medulla, thereby causing altered clonal deletion. Positively
84 selected (TCR-β+ CD5+) medulla CD4 T cells (**Fig 1a/sFig1**) from the thymi of 6 week old male
85 and female H2-O WT and H2-O mice were subdivided into "Auditing" (CCR7+ CD4+ Caspase-
86 $3_{neg}$) and "Clonally Deleted" (CCR7+ CD4+ Caspase-$3_{pos}$) CD4 T cells for evaluation of negative
87 selection.

88 Loss of H2-O was found to significantly increase the expression of the activation marker
89 CD69 on CD4 T cells undergoing active self-auditing, but not those selected for clonal deletion
90 (**Fig 1a**). CD69 in combination with MHC-I defines 3 medullary maturation stages: semi-mature
91 (CD69+ MHC-I -), mature 1 (CD69+ MHC-I +), and mature 2 (CD69- MHC-I -) (18). Subdivision of
92 auditing H2-O-KO CD4 T cells identified a significant increase in CD4 T cells with a Mature 1
93 phenotype (**Fig 1b, sFig2**). Conversely, both the semi-mature and mature 2 stages were
94 decreased in H2-O KO mice (**Fig 1b left/right**). No differences in any maturation stage were
95 found in the clonally deleted CD4 T cell populations (**sFig2). Furthermore,** H2-O deficiency, did

96 not appear to alter the rate of CD4 T cells undergoing clonal deletion (**Fig 1c**). Importantly, no
97 differences were observed in thymocytes undergoing positive selection (**sFig 3**). These data
98 suggest that loss of H2-O drives a more stimulatory thymic medulla environment, but with
99 similar levels of clonal deletion. It is therefore likely that the increased peripheral frequencies of
100 MOG-specific CD4 T cells previously identified is due to increased peripheral expansion of the
101 MOG-reactive clone, not a general alteration in clonal deletion.
102
103 **H2-O KO thymi have increased frequencies of regulatory T cells**
104 With increased levels of peripheral Tregs previously identified in H2-O KO mice (17), we
105 also questioned if H2-O deficiency was affecting Treg selection. In fact, one model of thymic
106 Treg selection centers around the concept of antigen density (15, 19). Within this "mosaic"
107 model, sporadic MHC-TCR interactions with sparsely presented self-epitopes leads to Treg
108 development. Since it has been shown that peripheral loss of H2-O leads to alterations in
109 peptide presentation (17, 20, 21), we postulated that altering the level of self-epitopes present
110 in the medulla could alter Treg selection.
111 Analysis of CD4 single-positive T cells identified an increased frequency of CD25+Foxp3+
112 T cells in H2-O KO mice (**Fig 2a**). Furthermore, H2-O KO thymic Tregs (tTregs) expressed higher
113 levels of CD25, the high affinity IL-2 receptor, and Nur77, the orphan nuclear receptor (**Fig 2b**).
114 As Nur77 has been associated with the level of TCR engagement (22), increased expression of
115 Nur77 in H2-O KO Tregs suggests that they have been strongly signaled. Finally, maturation
116 state analyses uncovered that H2-O KO tTregs were enriched in the M1 stage (**Fig 2c**).

117 Peripheral Tregs are known to recirculate back to the thymus (23, 24). We, therefore
118 assessed the percentages of recirculating Tregs in H2-O Wt and H2-O KO thymi. **Figure 2d**
119 shows that similar levels of recirculating (CCR6+ CD73+) Tregs were present in both H2-O WT
120 and H2-O KO thymi. The results shown in Fig 2D are in agreement with the notion that
121 increased Mature Tregs are of de novo origin rather than recirculating.
122
123 **Loss of H2-O correlates with increased peripheral CD4 T cell activation**
124 Considering the observation that lack of H2-O did not appear to alter clonal deletion
125 frequencies but did affect the level of thymic CD4 T cell activation, we next evaluated whether
126 peripheral loss of H2-O also increased CD4 T cells activation. Unimmunized H2-O KO spleens
127 had an increased frequency of CD4 T cells co-expressing the key activation markers CD44+ and
128 CD69+ (**Fig 3a**) and the tissue homing marker CCR7 (**Fig 3b**). We further assessed the levels of
129 "non-activated" (CCR7+ CD62L+) versus "activated" (CCR7- CD62L-) CD4 T cells (25), and found
130 lower frequencies of non-activated CD4 T cells in H2-O KO mice (**Fig 3c, left**). Importantly, this
131 correlated with an increase in percentage of activated CD4 T cells (**Fig 3c, right**). Collectively,
132 these phenotypic analyses support the idea that loss of H2-O leads to increased basal levels of
133 activated CD4 T cells in unimmunized H2-O KO mice.
134 As discussed above, H2-O KO thymi promoted Tregs selection. Consistent with these
135 observations and our previously published data (17, 26), H2-O KO spleens had an increased
136 frequency of CD25+ Foxp3+ Tregs (**Fig 3d**). Furthermore, H2-O KO Tregs showed decreased
137 levels of CD62L (**Fig 3e**) and increased levels of Nur77 (**Fig 3f)** indicating these cells are likely
138 more activated and circulating through the periphery.

139

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

141 Based upon the strong FACS data above exhibiting increased numbers of Tregs, and
142 more activated CD4 T cells, we attempted single-cell RNA-sequencing (scRNA-seq) to gain a
143 more holistic unbiased characterization. CD3+ CD4+ NK1.1- CD19- cells were sorted from
144 spleens of 3 unimmunized H2-O WT and 3 H2-O KO mice and subjected to 10x Genomics
145 scRNA-seq analyses. In total, 11 distinct CD4 T cell clusters were identified (**Fig 4a & sFig4**).
146 Separation of the clusters based upon H2-O WT or H2-O KO genotypes identified a dramatic
147 increase in cluster 0, cluster 2 and cluster 3 in the H2-O KO samples. Conversely, clusters 1 and
148 4 were significantly increased in H2-O WT samples (**Fig 4b**).

149 Comparing the genes from Clusters 0, 1 and 5 to the published CD4 T cell datasets using
150 the "My Geneset" function on the Immunological Genome Project (www.imgen.org) suggested
151 that upregulated genes ($Log_2$Avg Fold-change (FC) $\geq$ 0.5) were present in both "naïve" and
152 "activated" CD4 T cell datasets. Based upon the high expression of CCR7 however, these
153 clusters were labeled as "non-activated". Clusters 3, 6, 7, and 11 aligned with mainly an
154 "activated" CD4 T phenotype. Cluster 3 was found to have increased expression of the
155 activation marker CD44, while Cluster 11 expressed high Ki67. Clusters 2 and 4 contained the
156 known Treg genes Foxp3 and Il2ra (CD25) (**Fig 4a & Sup Table 1**). Clusters 8, 9, and 10 were
157 small populations of Macrophages and NKT cell (**Sup Table 1**). Condensing the cluster analysis
158 further supported our initial FACS data that unimmunized H2-O-KO mice have a decreased
159 frequency of "Non-activated" CD4 T cells (68.90% avg H2-O KO vs 74.17% avg H2-O WT with a

160 coinciding increase in "Activated" (14.14% avg H2-O KO vs 8.53% avg H2-O WT) CD4 T cells (**Fig**
161 **4b)**.
162 To test whether the higher activated state in H2-O KO T cells were due to the H2-O KO
163 CD4 T cells themselves, or possibly induced by the *in vivo* environment, we performed an
164 adoptive transfer experiment of dye labeled naïve (CD4+ CD44- CD25-) Thy1.1+ CD4 T cells from
165 H2-O WT into either unimmunized Thy1.2+ H2-O WT, or Thy1.2+ H2-O KO hosts. As shown in
166 **Fig 4d**, H2-O KO hosts induced significantly more *in vivo* proliferation of the Thy1.1+ WT cells
167 than the H2-O WT hosts. These data additionally support the idea that loss of H2-O creates a
168 more activating CD4 T cell environment by presenting a wider range of self-epitopes (17).
169
170 **H2-O KO Tregs have an activated phenotype**
171 Analyses of the two Treg clusters revealed that the frequency of Clusters 2 and 4 were
172 roughly equal in H2-O WT samples, whereas H2-O KO samples had an overrepresentation of
173 Cluster 2 (**Fig 5a, left**). Both cluster 2 and 4 expressed the classic Treg genes Foxp3, IL2ra
174 [CD25], CTLA4, and Pdcd1 [PD-1] (**Fig 5a, right**; **Sup Table 1**). Also identified was the
175 transcription factor Ikzf2 [Helios], a marker of stable Treg lineage commitment in inflammatory
176 conditions (27) and debated marker of thymic Tregs (28, 29, 30, 31). Supporting our scRNA-Seq
177 analysis, Helios protein expression was found in both H2-O WT and H2-O KO CD4 Tregs (**Fig 5b**).
178 Comparison of the cluster 2 genetic signature with a published Treg data set indicated that
179 these cells are likely effector-like Tregs. Furthermore, breaking down Cluster 2 by genotype
180 showed that H2-O KO Tregs had a larger fold change in expression of the core effector genes
181 signature (32) (**Fig 5c**). We hypothesize that this activated Treg state is likely driven by the

182 increased basal CD4 T cell activation. Expansion of this Treg population could certainly be a
183 mechanism controlling spontaneous autoimmunity in H2-O KO mice.
184 In summary, both scRNA-seq and FACs analyses showed that H2-O KO mice have an
185 increased frequency of both conventional and regulatory CD4 T cells in the spleens of
186 unimmunized mice. Loss of thymic H2-O expression correlated with an increased tTreg
187 population that was not from peripheral recirculation and had received increased TCR
188 stimulation, which suggests an increased autoreactivity. Furthermore, increased activation of
189 conventional splenic CD4 T cells is likely a driving factor for the increased effector-like Treg
190 status that was identified by scRNA-seq analyses of H2-O-KO CD4 cells.
191
192
193 **Discussion**
194 In this study, we have refined our understanding of how loss of H2-O alters CD4 T cell
195 activity *in vivo*. Thymic analysis of medulla localized CD4 T cells pointed to a novel role for H2-O
196 on CD4 T cells undergoing self-auditing. Importantly, lack of H2-O expression led to an
197 increased frequency of both thymic and peripheral Tregs.
198 Tregs play a fundamental role in maintenance of homeostasis, hence a better
199 understanding of the mechanism of their development is highly desirable and the subject of
200 numerous studies (15, 19). Our studies here are the first attempt in demonstrating that H2-O
201 plays a role in Treg development. While the exact mechanism by which thymic Treg cells are
202 selected remains somewhat uncertain, recent studies support the idea that intermittent TCR
203 signaling along with cytokine signaling drive Treg development (16). These findings and others

204 (33, 34, 35, 36, 37) support a more avidity based model of Treg selection, in which alterations to
205 the density of self-ligands present in the medulla will have differential effects on CD4 T cell
206 selection. Intermittent TCR signaling as a driving force in Treg selection is very relevant to our
207 understanding of how HLA-DO contributes to epitope selection during antigen processing. We
208 have proposed that HLA-DO in complex with HLA-DM leads to better refinement of the
209 epitopes from denatured antigens, promoting selection of the best fitting epitopes in the
210 groove of MHC II molecules (11, 38). While this idea might appear in conflict with the multiple
211 earlier interpretations of DO function (39, 40, 41), recent studies using human HLA-DO variants
212 showed that certain variants enhanced DM activity (42).

213 Understanding of how DO works has been tumultuous over the past decades (5, 43, 44,
214 45, 46, 47, 48, 49). DO has been proposed to be an inhibitor of DM, or a helper of DM. Most
215 reports acknowledge that understanding of DO requires further research. Our model that is
216 based on studying kinetics of interactions of DO with DM and MHC class II using a variety of
217 peptides of different binding characteristics is the most direct investigation of the biochemical
218 mechanism of DO function. The most important evidence for our model come from data
219 showing that DO or DO/DM binds to DR1 in a peptide receptive conformation: DO does not
220 bind to the DR1 in complex with tightly bound peptides. Based on this model, when DO is
221 present, higher affinity peptides are more likely to be presented. Conversely, in the absence of
222 DO a larger percent of lower affinity epitopes are selected. Indeed, our in vivo peptide elution
223 studies supports this biochemical mechanism as peptides eluted from H2-O KO mice expressing
224 murine I-$A^b$ (17) were of a lower general affinity. Accordingly, presentation of a larger portion
225 of lower affinity peptides in the thymic medulla of H2-O KO mice is possible. Intermittent TCR

226 signaling is typically generated by either pMHCII that are less stable (50, 51, 52, 53), or
227 TCR/pMHC of lower affinity (54). Our findings here support the former. We associate increased
228 Treg development as a consequence of the presentation of lower affinity peptides to self-
229 auditing CD4 T cells.

230 Due to the continuous egress of mature CD4 T cells from the thymus, we also analyzed
231 splenic CD4 T cells from unimmunized H2-O WT and H2-O KO mice. H2-O KO splenic CD4 T cells
232 in unimmunized mice revealed an increased frequency of CD44hiCD69+ antigen experienced
233 CD4 T cells, which suggests an increased level of antigen-specific TCR signaling in H2-O KO mice.
234 As previously implied (55), a simple explanation for this activated state is incomplete thymic
235 deletion in H2-O KO mice. While we were able to show that H2-O KO mice failed to delete
236 specific CD4 clonal populations (17), detection of differences at the global levels did not show
237 significant changes between the two genotypes. Nonetheless, we suggest that presentations of
238 different arrays of self-antigens as well as their lower densities on thymic medulla leads to a
239 less than optimal thymic deletion of self-reactive CD4 T cells and their routing to the periphery.
240 Similar to activated CD4 T cells in the periphery, H2-O KO Treg cells also had a more effector-
241 like phenotype, indicating enhanced Treg activation in unimmunized mice (32, 56).

242 In conclusion, our studies add a new dimension to our understanding of the role of H2-O
243 in both CD4 T cell selection and activation. For the first time, we report on thymic negative
244 selection in H2-O KO mice and demonstrate that loss of H2-O enhances thymic selection of
245 regulatory T cells. Once in the periphery, an increased proportion of H2-O KO Tregs appear to
246 be activated in a TCR dependent manner. These effector-like Tregs will likely help control
247 increased basal CD4 T cell activation. However, the exact mechanism by which increased Treg

248 cells are selected in the H2-O KO thymus remains to be determined. While we propose that
249 alterations in medulla pMHCII-TCR avidity interactions could lead to enhanced Treg selection,
250 increased presentation of self-antigens in a more tissue restricted antigenic (TRA) manner could
251 also be possible (15).
252
253

# Methods

## Mice

Male and female C57BL/6J (H2-O WT, stock no: 000664), Female B6.PL-Thy1a/CyJ (stock no: 000406) were purchased from Jackson Laboratories and bred in house. Generation of the H2-O knock-out mice has been previously described (57) and mice bred in house. Unless otherwise stated analyzed mice were 6-8 weeks. All mouse procedures were approved by the Johns Hopkins University Animal Care and Use Committee and were following relevant ethical regulations.

## Antibodies/reagents

**Flow Cytometry**: anti-CD3e (17A2), anti-CD4 (RM4-5), anti-CD5 (53-7.3), anti-CD8α (53-6.7), CD11c (N418), anti-CD19 (ID3), anti-CD25 (PC61), anti-CD44 (IM7), anti-CD45R/B220 (RA3-6B2), anti-CD62L (MEL-14), anti-CD69 (H1.2F3), anti-CD197/CCR7 (4B12), anti-NK1.1 (PK136), anti-TCRβ (H57-597), anti-TCRγδ (GL3), anti-Cleaved Caspase 3 (D3E9) Cell signaling (Danvers, MA); Foxp3 (150D), anti-Helios (22F6), anti-Nur77 (12.14), Fixable viability dye eFluor 780 (eBioscience).

**FACs Sorting**: anti-CD3e, anti-CD4, anti-CD19, anti-NK1.1, Propidium iodide. Briefly, 30,000 live CD4 T cells [Gating: CD3+, CD4, CD19-, NK1.1-, PI-] were FACs sorted from the spleens of 3 unimmunized 10 week-old H2-O WT and 3 unimmunized 10 week-old unimmunized H2-O KO female mice.

## Cell staining

For cleaved caspase 3 staining (18) homogenized mice thymocyte cells were stained with anti-CCR7/CD197 at a final dilution of 1:50 for 30 min at 37˚C prior to additional surface stains. Following surface staining, cells were fixed with Cytofix/Cytoperm (BD Biosciences) for 20 min at 4˚C. Cells were then washed with Perm/Wash buffer (BD Biosciences) twice. Cells were stained with anti–cleaved caspase 3 at a 1:50 dilution at 23˚C for 30 min.

For transcription factor staining, cells were incubated with surface antibody at 4˚C for 20 min, permeabilized at 4˚C for 30 min using a Foxp3/Transcription factor buffer set (Invitrogen, ThermoFisher Scientific), and then stained with anti–Foxp3 and/or anti-Helios at 23˚C for 30 min.

### Adoptive Cell Transfer

Naïve CD4 T cells were isolated from the pooled spleens and LNs of 4 week old Thy1.1 expressing C57BL/6J mice using the EasySep Mouse Naive CD4 T cell Isolation Kit (StemCell Technologies). Isolated cells were stained with eFluor 450 viability dye (eBiosciences) according to manufactures directions. $3x10^6$ dye labeled Thy1.1 cells were IV injected into 10 week old H2-O WT or H2-O KO hosts. Transferred cells were recovered 7 days post-transfer.

## scRNA-Sequencing

### Library & sequencing

The samples were prepared using the 10x Genomics Chromium Next GEM Single Cell 5' Library

297 and Gel Bead Kit v1.1, Chromium Next GEM Chip and Dual Index Kit TN Set A. They were run on
298 the Illumina NovaSeq6000 with a run configuration of 28bp x 10bp x 10bp x 91bp.
299
300 **Analysis**
301 Analyses of T-cell scRNA-seq were performed with the package Seurat (58), as follows. Data was
302 filtered to remove cells with low gene count (<200), large number of UMIs (>12,000) and high
303 (>5%) fraction of mitochondrial reads. Expression levels of genes were log-normalized, and the
304 most variable 2000 genes were selected for linear dimensionality reduction with Principal
305 Component Analysis (PCA). The first 15 principal components were then used to performed
306 unsupervised clustering using the Seurat SNN clustering package, with a resolution of 0.2. To
307 identify cell types, potential markers for each cluster were calculated as the set of genes
308 significantly differentially expressed in each cluster compared to all others, using the function
309 FindAllMarkers, and by searching existing literature and marker databases. Lastly, differentially
310 expressed genes for each cell type between conditions were determined using the function
311 FindMarkers with the default function (bimod).
312
313 Defining clusters – Genes with an average fold-change (avgFC) >0.5 and an adjusted p-value
314 <0.05 were run against CD4 T cell data sets available on the Immunological Genome Project
315 (https://www.immgen.org/) using the "My Geneset" data browser function.
316
317 **Statistical testing**
318 GraphPad Prism was used for all statistical analyses. A standard Student T-test was used for
319 estimation of statistical significance. Data is shown as mean ± SEM. *p<0.05, **p<0.01,
320 ***p<0.001, ****p<0.0001.
321
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330
331

## Figure Legends

**Figure 1**: Loss of thymic H2-O increases the activation state of auditing CD4 T cells

**A**. **Left**: representative contour plots showing total CD69 expression in auditing (Caspase-3 negative) signaled (TCR-B+CD5+) CCR7+CD4+ T cells from 6-week-old H2-O WT (Top) and H2-O KO (Bottom) thymi.

**Right**: Combined CD69 expression data from 5 repeat experiments. N= 18 mice per genotype

**B**. **Right**: representative contour plots showing total CD69 expression in Clonally deleted (Caspase-3 positive) signaled (TCR-B+CD5+) CCR7+CD4+ T cells from 6-week-old H2-O WT (Top) and H2-O KO (Bottom) thymi.

**Left**: Combined CD69 expression data from 5 repeat experiments. N= 18 mice per genotype

**C**. **Top**: representative contour plots showing the subdivision of auditing (Caspase-3 negative) signaled (TCR-B+CD5+) CCR7+CD4+ T cells from 6-week-old H2-O WT (Left) and H2-O KO (Right) thymi into three maturation stages: Semi-Mature (SM), Mature 1 (M1), and Mature 2 (M2).

**Bottom**: Cumulative maturation state data from 5 repeat experiments, N = 18 mice per genotype.

Expression has been normalized to the average H2-O WT levels within each experiment to allow for comparison across experiments. Raw percentage data can be found in Supplemental Figure 3.

**D**. 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: unpaired student T-test

**Figure 2: H2-O KO thymi have increased selection of regulatory T cells**

**A**. **Left**: representative contour plots showing the frequency of Tregs (CD25+ Foxp3+) cells in the CD4 single-positive thymus population.

**Right**: cumulative 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, N= 16 mice per genotype.

**B**. Geometric mean fluorescence intensity (gMFI) of CD25 (left), and Nur77 (right) 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). Data from 2 replicate experiments.

**D**. **Left**: representative contour plots showing the frequency of Naive (CCR6- CD73-), Mature (CCR6- CD73+), and Recirculating (CCR6+ CD73+) Foxp3+ CD25+ Tregs in 6-week-old H2-O WT and H2-O KO thymi.

374 **Right**: Summary plots showing the frequency of Naive (CCR6- CD73-), Mature (CCR6- CD73+),
375 and Recirculating (CCR6+ CD73+) Foxp3+ CD25+ Tregs from 2 independent repeat experiments,
376 N= 6 mice per group.
377
378 **Figure 3: H2-O KO mice have an increased activated peripheral CD4 T cell population**
379 **A**. Basal levels of CD44 and CD69 expressed by H2-O WT (white) and H2-O KO (red) splenic CD4
380 T cells. Increased CD69 expression, a marker of recent activation showed increased levels on
381 H2-O KO CD4 T cells
382 **B.** Percentage of CD4+ T cells expressing the lymphoid tissue homing receptor CCR7
383 **C**. CCR7 and CD62L expression levels in unimmunized splenic CD4 T cells
384 **D.** Frequency of Foxp3+CD25+ cells in splenic CD4+ T cell population of unimmunized mice
385 **E.** H2-O KO peripheral Tregs express decreased levels of naïve (CD44+ CD62L+) expressing cells
386 **F.** Normalized Nur77 gMFI levels in Foxp3+CD25+ Treg cells in H2-O WT (white) and H2-O KO
387 (red) cells. To account for experimental variation the average Nur77 gMFI level in H2-O WT
388 samples was calculated. gMFI levels in both H2-O WT and H2-O KO samples were then divided
389 by the calculated H2-O WT average. An increased Nur77 ratio indicates increased Nur77 gMFI
390 levels. Summary of 3 repeat experiments.
391
392 * = <0.05
393 ** = <0.001
394 *** = <0.0001
395 **** = <0.00001
396
397 **Figure 4**: **Loss of H2-O function causes increased basal CD4 cell activation**
398 **A.** scRNA-seq clustering of CD4 T cells after Seurat analysis. Data represents the average of of 3
399 biological replicates per genotype.
400 **B.** Breakdown of clusters in H2-O WT (Left) or H2-O KO (Right) samples. Clusters are grouped
401 based upon, (1) known CD4 T cell subset markers and (2) gene comparison to published CD4 T
402 cell data sets available on the Immunological Genome Project (www.immgen.org). Identified
403 CD4 Cell phenotypes were: Non-activated, Activated, and Regulatory T cells. ”Other” refers to a
404 minor macrophage and NKT cell contamination from the sorting process.
405 **C.** Distribution of the for the Non-activated and Activated phenotypes across the H2-O WT and
406 H2-O KO biological replicates (N = 3 mice per genotype).
407 **D.** In vivo proliferation of adoptively transferred naïve Thy1.1+ CD4 T cells (Thy1.1+ CD3+ CD4+
408 CD44- CD25-) after 7 days in either Thy1.2 H2-O WT (white) or Thy1.2 H2-O KO (red) hosts.
409 Pooled data from 2 independent experiments (N= 6 H2-O WT, 5 H2-O KO)
410
411 **Figure 5**: **The H2-O KO Treg population has a more effector like phenotype**
412 **A.** (Left) distribution of the two Treg populations identified by scRNA-seq clustering in either
413 H2-O WT (left) or H2-O KO (right) samples. (Right) Expression of key Treg phenotypic markers:
414 Foxp3 (Top) and CD25 [Il2ra] (Bottom)
415 **B.** Subdivision of the splenic CD4+ T cell population by Foxp3 and Helios expression
416 **C.** Comparison of cluster 2 upregulated genes ($Log_2$Avg FC >0.5) in H2-O WT and H2-KO samples
417 to a published (Miragaia *et al*.) splenic effector Treg genetic profile

418
419 **Supplemental Figure 1: Gating strategy used for detection of Auditing vs Clonally Deleted**
420 **Medulla CD4 T cells**
421 Red arrows indicate how Auditing vs Clonal Deletion was identified in 6-week-old H2-O WT and
422 H2-O KO thymi.
423 Blue arrows indicate additional gating corresponding to Figure 1a.
424 Black Arrows indicate additional gating corresponding to Figure 1b.
425
426 **Supplemental Figure 2: The frequency of auditing Mature 1 CD4 T cells was increased in H2-O**
427 **KO thymi**
428 **A**. Percentage of auditing (Caspase-3 negative) CD4 T cells in the Semi-mature (CD69+ MHC-I
429 neg), Mature 1 (CD69+ MHC-1+), and Mature 2 (CD69- MHC-I+) in H2-O WT (white) and H2-O
430 KO (red) mice N= 18 mice per group
431 **B**. Percentage of clonally deleted (Caspase-3+) CD4 T cells in the Semi-mature (CD69+ MHC-I
432 neg), Mature 1 (CD69+ MHC-1+), and Mature 2 (CD69- MHC-I+) in H2-O WT (white) and H2-O
433 KO (red) mice. N= 18 mice per group
434 Statistical Testing: unpaired student T-test
435
436 **Supplemental Figure 3: Positive Selection is not affected by loss of H2-O**
437 A. Representative plots showing CD4, CD8 and Double Positive (DP) percentages in H2-O WT
438 (Top) and H2-O KO (Bottom)
439 B. Summary plots of the single-positive CD4 (Top) and CD8 (Bottom) percentages from 5 repeat
440 experiments.
441 C. (Left) representative flow plots showing the "Signaled" vs "Non-signaled" thymocytes in H2-
442 O WT (Top) or H2-O KO (Bottom).
443 (Right) Summary plots of >5 repeat experiments.
444 D. Frequency of signaled CCR7+ medulla CD4 T cells in H2-O WT (white) and H2-O KO (red) mice
445
446 **Supplemental Figure 4**:
447 Top: breakdown of H2-O KO (right) and H2-O WT (Left) Seurat clustering
448 Bottom: Seurat clustering for each individual biological replicate
449
450

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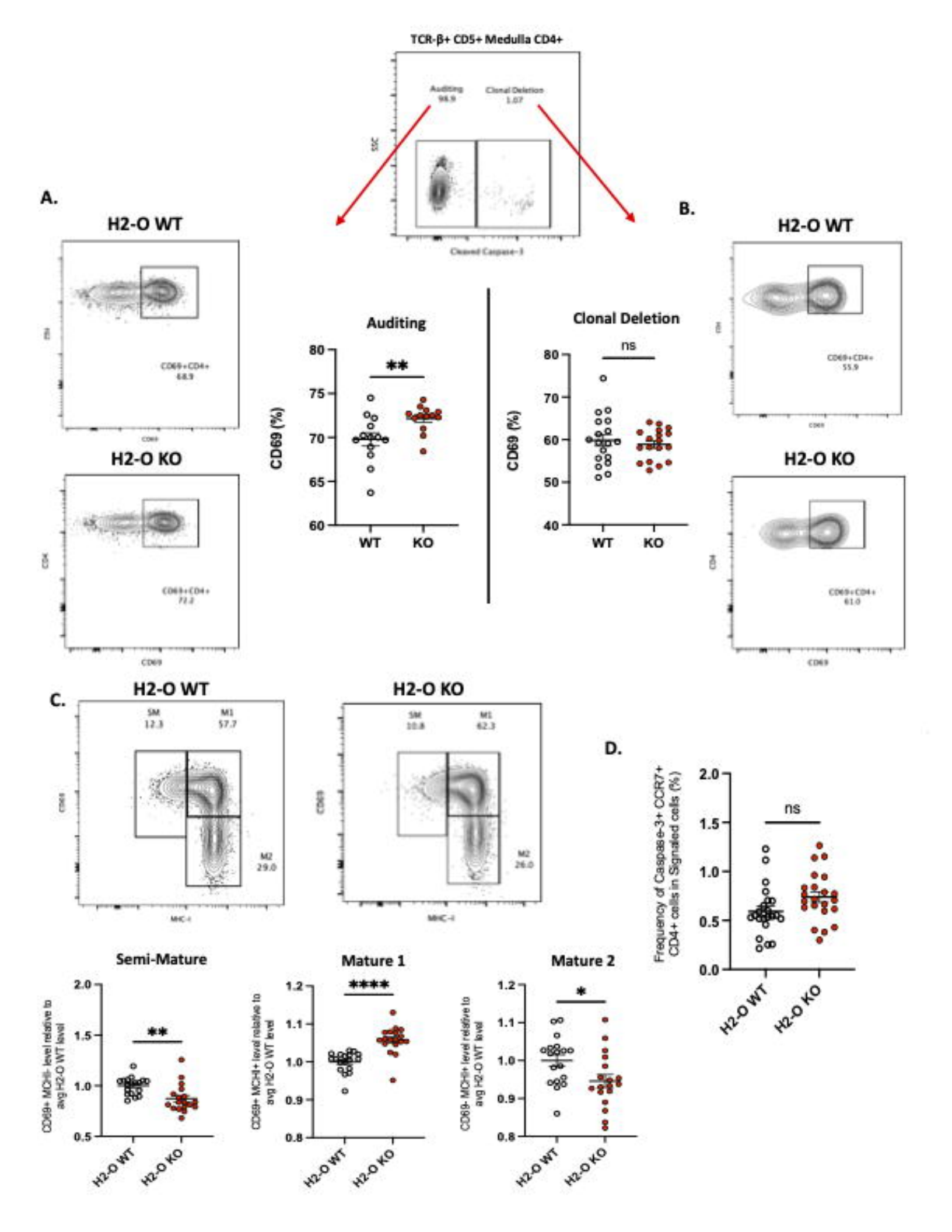
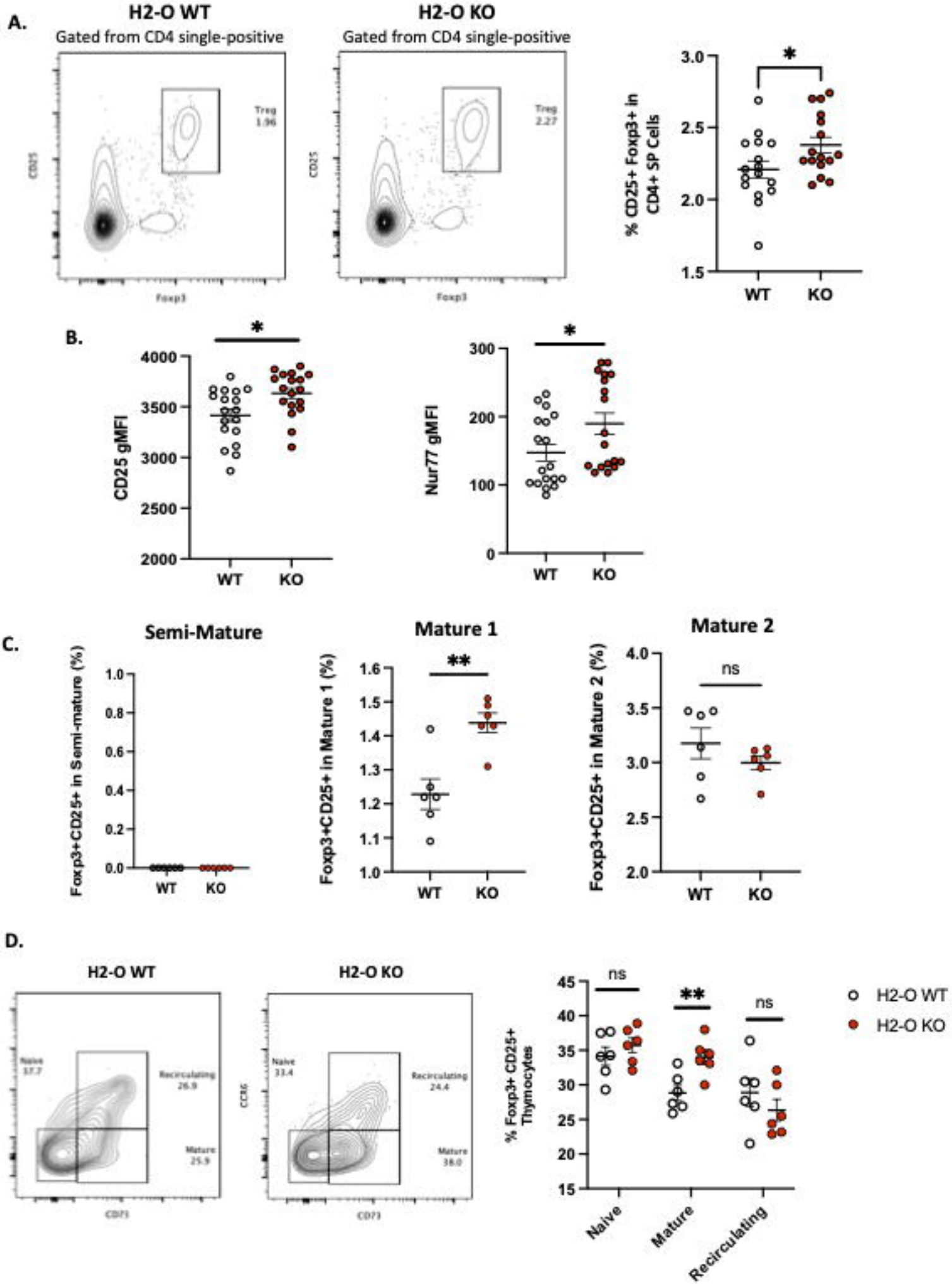
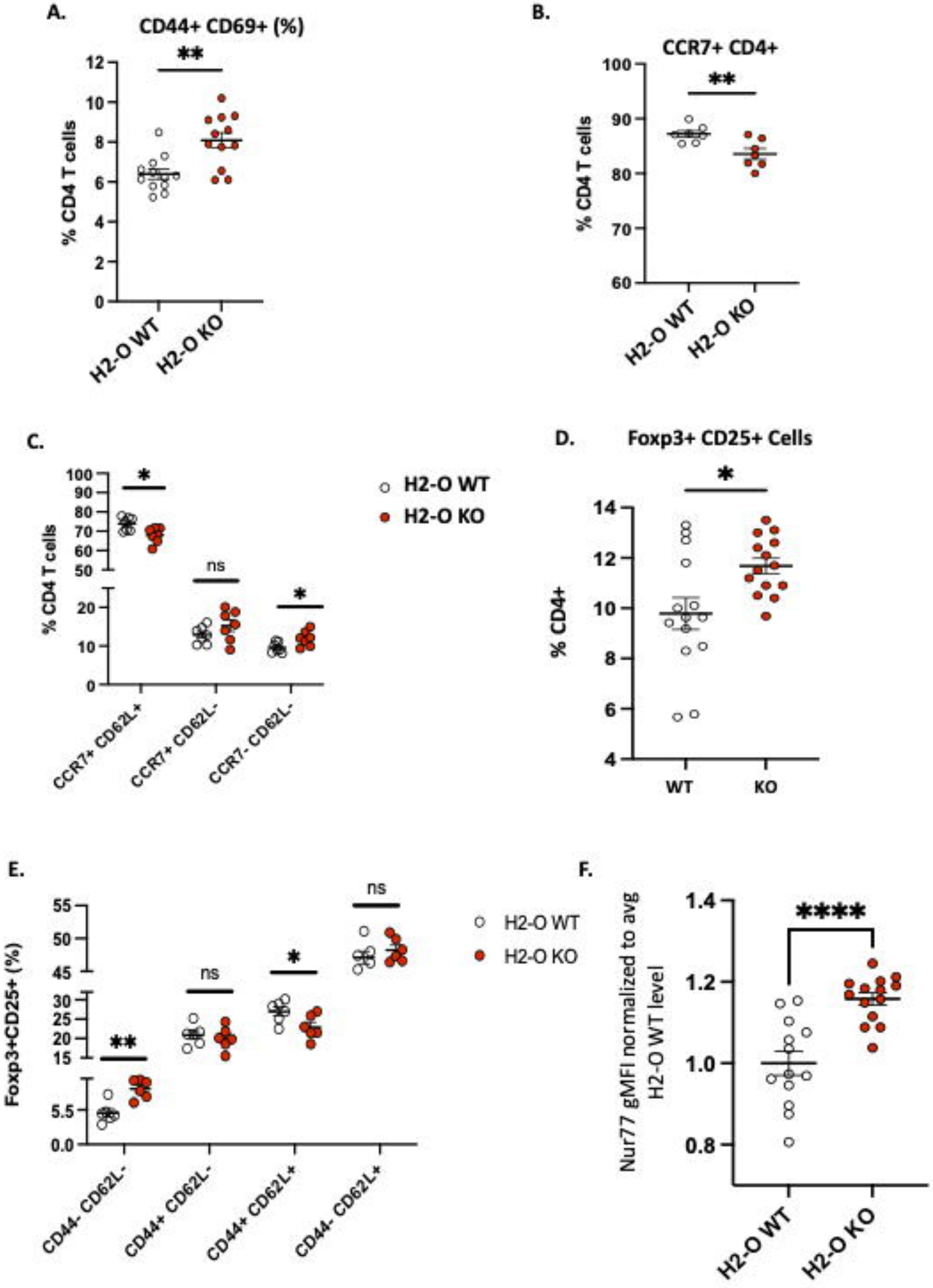
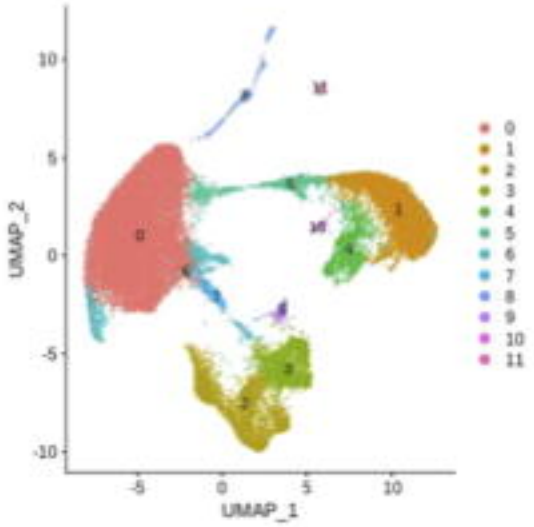

TCR-β+ CD5+ Medulla CD4+
Auditing
98.9
Clonal Deletion
1.07
SSC
Cleaved Caspase-3
A.
H2-O WT
CD69+CD4+
68.9
H2-O KO
CD69+CD4+
72.2
CD69
CD4
Auditing
CD69 (%)
80
75
70
65
60
**
WT
KO
Clonal Deletion
ns
80
70
60
50
40
B.
H2-O WT
CD69+CD4+
55.9
H2-O KO
CD69+CD4+
61.0
C.
H2-O WT
SM
12.3
M1
57.7
M2
29.0
H2-O KO
SM
10.8
M1
62.3
M2
26.0
MHC-I
D.
Frequency of Caspase-3+ CCR7+ CD4+ cells in Signaled cells (%)
2.0
1.5
1.0
0.5
0.0
ns
H2-O WT
H2-O KO
Semi-Mature
CD69+ MCHI- level relative to avg H2-O WT level
2.0
1.5
1.0
0.5
**
Mature 1
CD69+ MCHI+ level relative to avg H2-O WT level
1.2
1.1
1.0
0.9
0.8
****
Mature 2
CD69- MCHI+ level relative to avg H2-O WT level
1.2
1.1
1.0
0.9
0.8
*

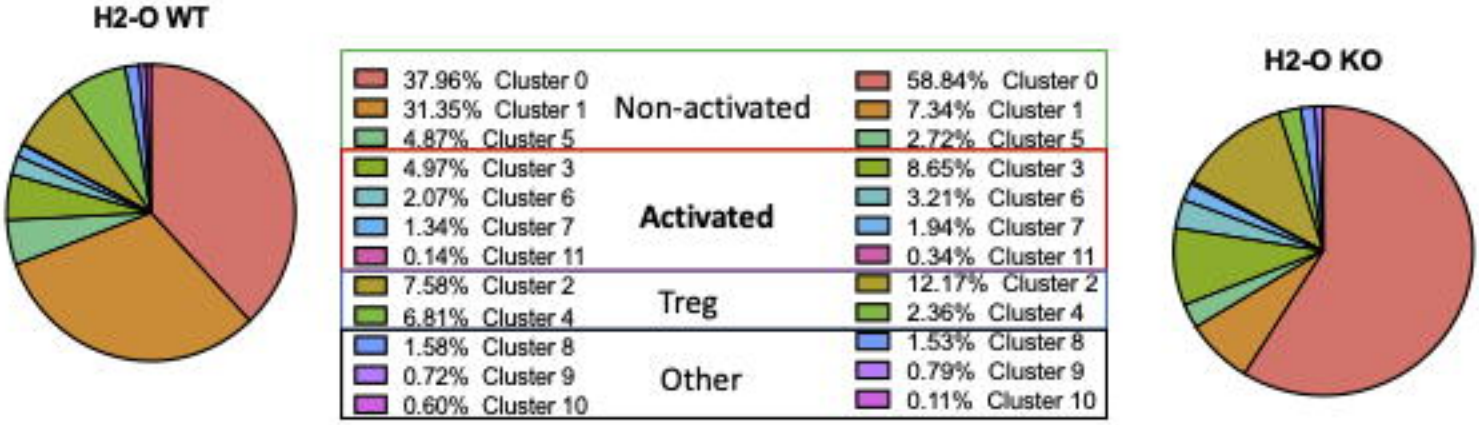

A.
H2-O WT
Gated from CD4 single-positive
Treg
1.96
CD25
Foxp3
H2-O KO
Gated from CD4 single-positive
Treg
2.27
CD25
Foxp3
% CD25+ Foxp3+ in CD4+ SP Cells
3.0
2.5
2.0
1.5
*
WT
KO
B.
CD25 gMFI
4000
3500
3000
2500
2000
*
WT
KO
Nur77 gMFI
300
200
100
0
*
WT
KO
C.
Semi-Mature
Foxp3+CD25+ in Semi-mature (%)
1.0
0.8
0.6
0.4
0.2
0.0
WT
KO
Mature 1
Foxp3+CD25+ in Mature 1 (%)
1.6
1.5
1.4
1.3
1.2
1.1
1.0
**
WT
KO
Mature 2
Foxp3+CD25+ in Mature 2 (%)
4.0
3.5
3.0
2.5
2.0
ns
WT
KO
D.
H2-O WT
Naive
37.7
Recirculating
26.9
Mature
25.9
CCR6
CD73
H2-O KO
Naive
33.4
Recirculating
24.4
Mature
38.0
CCR6
CD73
% Foxp3+ CD25+ Thymocytes
45
40
35
30
25
20
15
ns
**
ns
Naive
Mature
Recirculating
H2-O WT
H2-O KO


A.
CD44+ CD69+ (%)
**
% CD4 T cells
H2-O WT
H2-O KO
B.
CCR7+ CD4+
**
% CD4 T cells
H2-O WT
H2-O KO
C.
*
ns
*
% CD4 T cells
CCR7+ CD62L+
CCR7+ CD62L-
CCR7- CD62L-
H2-O WT
H2-O KO
D.
Foxp3+ CD25+ Cells
*
% CD4+
WT
KO
E.
ns
ns
*
**
Foxp3+CD25+ (%)
CD44- CD62L-
CD44+ CD62L-
CD44+ CD62L+
CD44- CD62L+
H2-O WT
H2-O KO
F.
****
Nur77 gMFI normalized to avg H2-O WT level
H2-O WT
H2-O KO

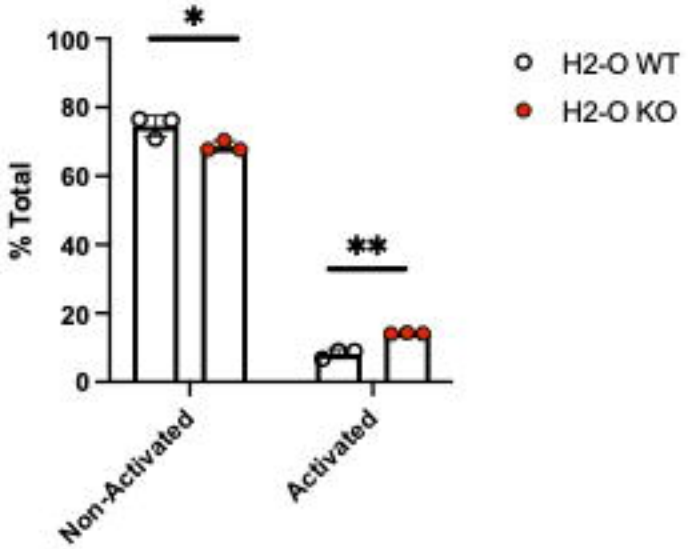
A.


UMAP_2
UMAP_1
10
5
0
-5
-10
-5
0
5
10
0
1
2
3
4
5
6
7
8
9
10
11

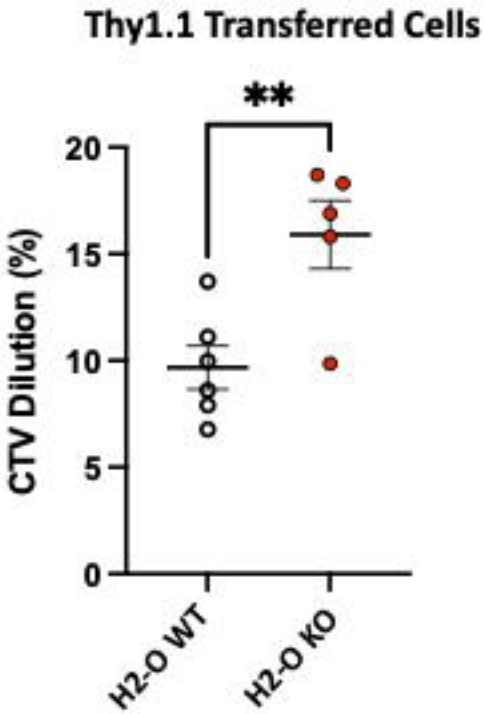
B.

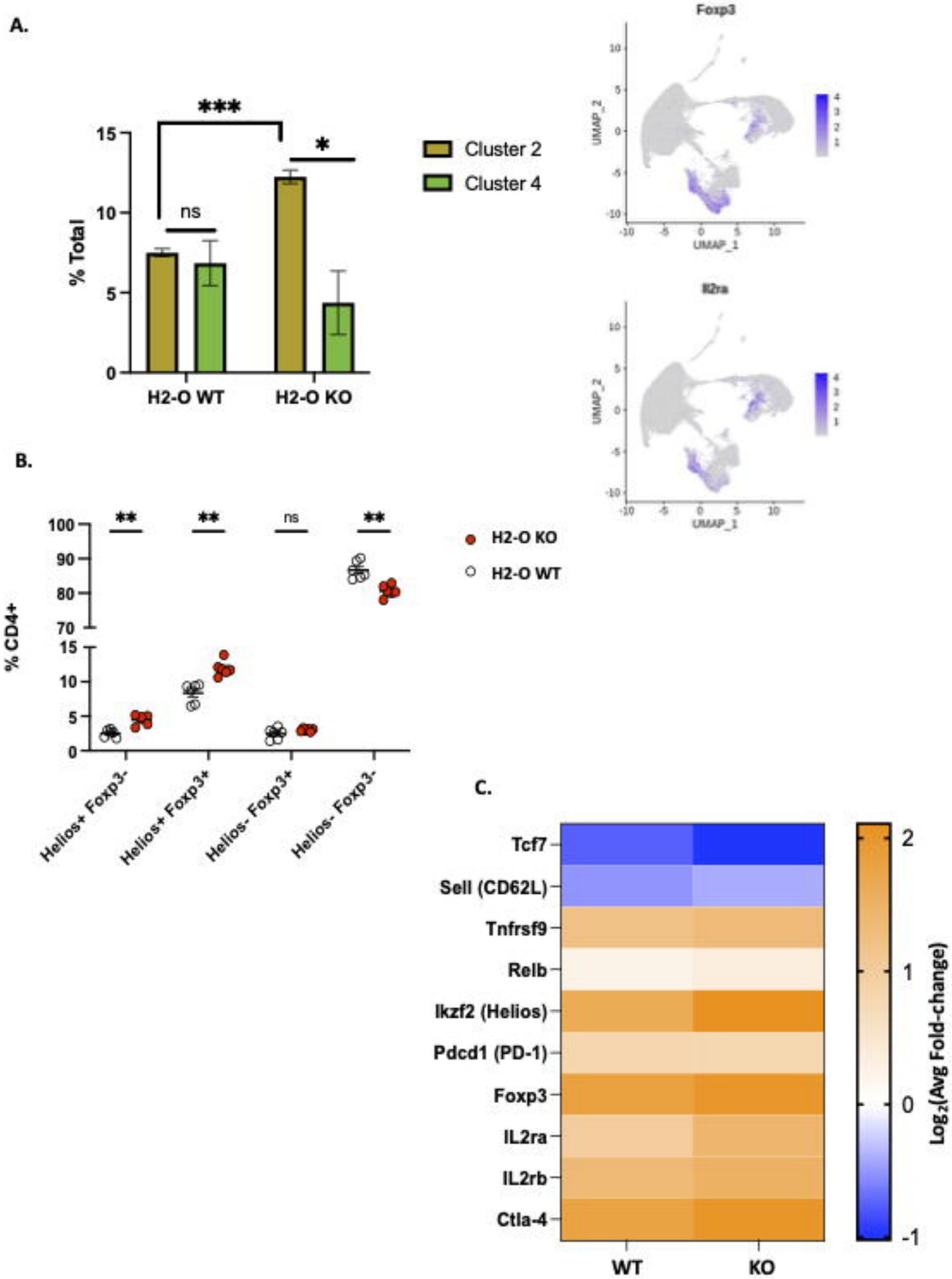

H2-O WT
37.96% Cluster 0
31.35% Cluster 1
4.87% Cluster 5
Non-activated
4.97% Cluster 3
2.07% Cluster 6
1.34% Cluster 7
0.14% Cluster 11
Activated
7.58% Cluster 2
6.81% Cluster 4
Treg
1.58% Cluster 8
0.72% Cluster 9
0.60% Cluster 10
Other
58.84% Cluster 0
7.34% Cluster 1
2.72% Cluster 5
8.65% Cluster 3
3.21% Cluster 6
1.94% Cluster 7
0.34% Cluster 11
12.17% Cluster 2
2.36% Cluster 4
1.53% Cluster 8
0.79% Cluster 9
0.11% Cluster 10
H2-O KO

C.


*
**
100
80
60
40
20
0
% Total
Non-Activated
Activated
H2-O WT
H2-O KO

D.


Thy1.1 Transferred Cells
**
20
15
10
5
0
CTV Dilution (%)
H2-O WT
H2-O KO


A.
***
*
ns
% Total
15
10
5
0
H2-O WT
H2-O KO
Cluster 2
Cluster 4
Foxp3
UMAP_2
UMAP_1
Il2ra
B.
**
**
ns
**
% CD4+
100
90
80
70
15
10
5
0
H2-O KO
H2-O WT
Helios+ Foxp3-
Helios+ Foxp3+
Helios- Foxp3+
Helios- Foxp3-
C.
Tcf7
Sell (CD62L)
Tnfrsf9
Relb
Ikzf2 (Helios)
Pdcd1 (PD-1)
Foxp3
IL2ra
IL2rb
Ctla-4
WT
KO
2
1
0
-1
Log2(Avg Fold-change)