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1           **Recirculating Foxp3<sup>+</sup> regulatory T cells are restimulated in the thymus under Aire control**

2

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15

16 **Running title:**

17 Recirculating Foxp3<sup>+</sup> regulatory T cells are restimulated in the thymus under Aire control

18

19 **Keywords**

20 Autoimmune regulator; Autoimmunity; Medullary thymic epithelial cells; Foxp3<sup>+</sup> Regulatory T cells; Thymus

21

22 **Abstract**

23 Thymically-derived Foxp3<sup>+</sup> regulatory T cells (T<sub>reg</sub>) critically control immunological tolerance. These cells are  
24 generated in the medulla through high affinity interactions with medullary thymic epithelial cells (mTEC)  
25 expressing the Autoimmune regulator (Aire). Recent advances have revealed that thymic T<sub>reg</sub> contain not only  
26 developing but also recirculating cells from the periphery. Although Aire is implicated in the generation of Foxp3<sup>+</sup>  
27 T<sub>reg</sub>, its role in the biology of recirculating T<sub>reg</sub> remains elusive. Here, we show that Aire regulates the suppressive  
28 signature of recirculating T<sub>reg</sub> independently of the remodeling of the medullary 3D organization throughout life  
29 where T<sub>reg</sub> reside. Accordingly, the adoptive transfer of peripheral Foxp3<sup>+</sup> T<sub>reg</sub> in *Aire*<sup>KO</sup> recipients led to an  
30 impaired suppressive signature upon their entry into the thymus. Furthermore, recirculating T<sub>reg</sub> from *Aire*<sup>KO</sup> mice  
31 failed to attenuate the severity of multiorgan autoimmunity, demonstrating that their suppressive function is  
32 altered. Using bone marrow chimeras, we reveal that mTEC-specific expression of Aire controls the suppressive  
33 signature of recirculating T<sub>reg</sub>. Finally, mature mTEC lacking *Aire* were inefficient in stimulating peripheral T<sub>reg</sub>  
34 both in polyclonal and antigen-specific co-culture assays. Overall, this study demonstrates that Aire confers to  
35 mTEC the ability to restimulate recirculating T<sub>reg</sub>, unravelling a novel function for this master regulator in T<sub>reg</sub>  
36 biology.

## 37 Introduction

38 CD25<sup>+</sup>Foxp3<sup>+</sup> regulatory T cells (T<sub>reg</sub>) constitute a distinct subset of CD4<sup>+</sup> T cells endowed with suppressive  
39 functions. By maintaining immune tolerance, T<sub>reg</sub> critically prevent the emergence of autoimmune and  
40 inflammatory diseases. The vast majority of T<sub>reg</sub> is produced in the thymic medulla where they arise from two  
41 distinct developmental programs involving CD25<sup>+</sup>Foxp3<sup>-</sup> (CD25<sup>+</sup> T<sub>reg</sub>P) and CD25<sup>-</sup>Foxp3<sup>lo</sup> (Foxp3<sup>lo</sup> T<sub>reg</sub>P)  
42 precursors (1-4). For a long-time, thymic CD25<sup>+</sup>Foxp3<sup>+</sup> T<sub>reg</sub> were thought to exclusively correspond to developing  
43 cells. However, recent studies have shown that circulating CD25<sup>+</sup>Foxp3<sup>+</sup> T<sub>reg</sub> have the ability to migrate back into  
44 the thymus (5-7). Thus, thymic CD25<sup>+</sup>Foxp3<sup>+</sup> T<sub>reg</sub> is a heterogeneous population containing both developing and  
45 recirculating cells (3). Interestingly, recirculating T<sub>reg</sub> show suppressive properties similar to their splenic  
46 counterparts (7). They exhibit an activated and differentiated phenotype and were found to negatively regulate the  
47 *de novo* generation of CD25<sup>+</sup>Foxp3<sup>+</sup> T<sub>reg</sub> through IL-2 consumption (5).

48 The recirculation of peripheral Foxp3<sup>+</sup> T<sub>reg</sub> in the thymus is mediated at least by two chemokine receptors, CCR6  
49 and CXCR4 (5, 8). Interestingly, the entry of peripheral *Ccr6*<sup>-/-</sup> T<sub>reg</sub> into the thymus is impaired, suggesting that  
50 CCR6, expressed by effector/memory T cells, is involved in the recirculation of Foxp3<sup>+</sup> T<sub>reg</sub> from the periphery to  
51 this organ (8). Furthermore, the expression of the CCR6 ligand, CCL20, is regulated by the Autoimmune regulator  
52 (Aire) (8). Aire is mainly expressed by a subset of medullary thymic epithelial cells (mTEC) commonly called  
53 mTEC<sup>hi</sup>, characterized by a CD80<sup>hi</sup>MHCII<sup>hi</sup> phenotype. Furthermore, upon recirculation into the thymus, a subset  
54 of B cells has been described to upregulate Aire (9). Independent reports have described that CD25<sup>+</sup>Foxp3<sup>+</sup> T<sub>reg</sub>  
55 are reduced in the thymus of *Aire*<sup>KO</sup> mice (10, 11). Accordingly, *Aire* expression was proposed to direct  
56 autoreactive CD4<sup>+</sup> thymocytes into the T<sub>reg</sub> cell lineage (12). Interestingly, in the perinatal life, Aire promotes the  
57 generation of a distinct population of Foxp3<sup>+</sup> T<sub>reg</sub>, which persists in adults and prevents the emergence of  
58 autoimmunity (13). In C57BL/6 mice, *Aire*-deficiency is associated with mild autoimmunity characterized by  
59 lymphocyte infiltrations and autoantibody production targeting several organs such as the pancreas, eyes, salivary  
60 glands, liver and lungs (14-17). In human, autosomal recessive mutations in the *Aire* gene induce a life threatening  
61 pathology called autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy (APECED), also known as  
62 autoimmune polyendocrine syndrome-type 1 (APS-1) (18, 19). Interestingly, numbers, activation and suppressive  
63 functions of Foxp3<sup>+</sup> T<sub>reg</sub> are altered in APECED patients (20-22).

64 Although thymic Foxp3<sup>+</sup> T<sub>reg</sub> have been found to be more heterogeneous than previously thought (3), this emerging  
65 population of recirculating peripheral T<sub>reg</sub> remains poorly described. In particular, the mechanisms that control

66 their suppressive phenotype in the thymus are unknown. In this study, we show that Aire regulates the suppressive  
67 properties of recirculating CCR6<sup>+</sup> T<sub>reg</sub>, independently of the remodeling of the 3D organization of the thymic  
68 medulla where they reside (5). High-throughput sequencing revealed that recirculating CCR6<sup>+</sup> T<sub>reg</sub> in the thymus  
69 of *Aire*<sup>KO</sup> mice have impaired expression of several genes associated with T<sub>reg</sub> suppressive functions and helper T  
70 cell polarization. Accordingly, recirculating CCR6<sup>+</sup> T<sub>reg</sub> from *Aire*<sup>KO</sup> mice failed to attenuate the severity of multi-  
71 organ autoimmunity, demonstrating that their suppressive activity is impaired. Moreover, the adoptive transfer of  
72 splenic Foxp3<sup>+</sup> T<sub>reg</sub> in *Aire*<sup>KO</sup> recipients resulted in an impaired suppressive signature upon their recirculation in  
73 the thymus. By using bone marrow (BM) chimeras, we demonstrate that *Aire* expressed by mTEC rather than by  
74 thymic B cells is responsible for the suppressive properties of recirculating CCR6<sup>+</sup> T<sub>reg</sub>. Finally, recirculating T<sub>reg</sub>  
75 were found in close contact with Aire<sup>+</sup> mTEC and restimulated in an antigen-specific manner. Altogether, our data  
76 reveal that Aire confers to mTEC the capacity to control the restimulation of recirculating CCR6<sup>+</sup> T<sub>reg</sub> in the  
77 thymus.

78

## 79 **Materials and methods**

### 80 **Mice**

81 CD45.1 WT (B6.SJL-*PtprcaPepcb*/BoyCr1, Stock n°002014, Charles River), CD45.2 WT (Stock n°000664,  
82 Charles River), CD45.1/2 WT, CD45.2 *Aire*<sup>KO</sup> (23), *Rag2*<sup>KO</sup> (24), CD45.1 Foxp3<sup>eGFP</sup> mice (25), OTII (26) and  
83 Rip-mOVA (27) were on a C57BL/6J background. Rip-mOVA x OTII mice were backcrossed on a *Rag2*<sup>KO</sup>  
84 background. All mice were maintained under specific pathogen-free conditions at the Centre d'Immunologie de  
85 Marseille-Luminy (CIML, France). Standard food and water were given *ad libitum*. Males and females were used  
86 at the age of 5 days, 9 days, 6 weeks and 1 year. Chimeras were generated between 6 to 10 weeks of age. All  
87 experiments were done in accordance with national and European laws for laboratory animal welfare (EEC  
88 Council Directive 2010/63/UE) and the Marseille Ethical Committee for Animal experimentation no. 14.

### 89 **BM chimeras**

90 BM chimeras were generated by injecting intravenously (*i.v.*) 5.10<sup>6</sup> BM cells from CD45.1 Foxp3<sup>eGFP</sup> mice into  
91 lethally irradiated CD45.2 *Aire*<sup>WT</sup> or *Aire*<sup>KO</sup> recipients (two doses of 500 rads, 8h apart, X-ray using a RS-2 000  
92 Irradiator; Rad Source Technologies). Similarly, 5.10<sup>6</sup> BM cells from either CD45.2 *Aire*<sup>WT</sup> or *Aire*<sup>KO</sup> mice were  
93 injected into lethally irradiated CD45.1/2 WT recipients. Mice were analyzed 6 weeks post-reconstitution.

### 94 **Foxp3<sup>+</sup> T<sub>reg</sub> adoptive transfer**

95 5.10<sup>5</sup> cell-sorted splenic congenic CD45.1 CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>eGFP</sup> T<sub>reg</sub> were *i.v* injected into sublethally irradiated  
96 CD45.2 *Aire*<sup>WT</sup> or *Aire*<sup>KO</sup> recipient mice (one dose of 500 rads, X-ray using a RS-2 000 Irradiator; Rad Source  
97 Technologies). Mice were analyzed 1-week post-T<sub>reg</sub> adoptive transfer.

### 98 **Multi-organ autoimmunity experiments**

99 *Rag2*<sup>KO</sup> recipients were injected *i.v.* with 3.10<sup>6</sup> CD4<sup>+</sup>CD25<sup>+</sup> T<sub>reg</sub>-depleted splenocytes purified from CD45.1 WT  
100 mice. Four weeks later, 15.10<sup>4</sup> splenic CD4<sup>+</sup>CD25<sup>hi</sup> T<sub>reg</sub> or 10.10<sup>4</sup> recirculating thymic CCR6<sup>+</sup>CD4<sup>+</sup>CD25<sup>+</sup> T<sub>reg</sub>  
101 from *Aire*<sup>WT</sup> or *Aire*<sup>KO</sup> mice were adoptively transferred. Mice that did not receive any T<sub>reg</sub> were used as controls.  
102 Peripheral tissues were harvested and analyzed three weeks later.

### 103 **Cell isolation**

104 Thymic T<sub>reg</sub>, splenic T<sub>reg</sub> and T<sub>reg</sub>-depleted splenocytes were isolated by scratching the thymus or the spleen on a  
105 70-µm mesh. Red blood cells were lysed with RBC lysis buffer (eBioscience). Thymic and splenic T<sub>reg</sub> cells were  
106 pre-enriched by depleting CD8<sup>+</sup> and CD19<sup>+</sup> cells using biotinylated anti-CD8α (clone 53.6.7; BD Biosciences)  
107 and anti-CD19 (clone 1D3; BD Bioscience) antibodies with anti-biotin microbeads by AutoMACS using the  
108 Deplete program (Miltenyi Biotec). Recirculating thymic T<sub>reg</sub> were sorted as CCR6<sup>+</sup>CD4<sup>+</sup>CD8<sup>-</sup>CD25<sup>+</sup> cells and  
109 splenic T<sub>reg</sub> as CD4<sup>+</sup>CD25<sup>hi</sup> cells using a FACSAria III cell sorter (BD Biosciences). Splenocytes were depleted  
110 of CD4<sup>+</sup>CD25<sup>+</sup> T<sub>reg</sub> using a FACSAria III cell sorter (BD Biosciences). Thymus were digested at 37°C in HBSS  
111 medium containing Liberase TM (50 µg/ml; Roche) and DNase I (100 µg/ml; Roche) until complete tissue  
112 digestion. Total mTEC (CD45<sup>-</sup>EpCAM<sup>+</sup>UEA-1<sup>+</sup>Ly51<sup>-/lo</sup>) or mTEC<sup>hi</sup> (CD45<sup>-</sup>EpCAM<sup>+</sup>UEA-1<sup>+</sup>Ly51<sup>-/lo</sup>CD80<sup>hi</sup>)  
113 were pre-enriched by depleting CD45 hematopoietic cells using anti-CD45 magnetic beads (Miltenyi Biotec) by  
114 AutoMACS with the DepleteS program and sorted using a FACSAria III cell sorter (BD Biosciences).

### 115 ***In vitro* co-culture assays**

116 3.10<sup>4</sup> cell-sorted splenic CD4<sup>+</sup>CD25<sup>hi</sup> T<sub>reg</sub> from *Aire*<sup>WT</sup> or *Aire*<sup>KO</sup> mice were co-cultured for 24h with 6.10<sup>3</sup> CD45<sup>-</sup>  
117 EpCAM<sup>+</sup>UEA-1<sup>+</sup>Ly51<sup>-/lo</sup> mTEC from *Aire*<sup>WT</sup> or *Aire*<sup>KO</sup> mice in RPMI (ThermoFisher) supplemented with 10%  
118 FBS (Sigma Aldrich), L-glutamine (2 mM, ThermoFisher), sodium pyruvate (1 mM, ThermoFisher), 2-  
119 mercaptoethanol (2×10<sup>-5</sup> M, ThermoFisher), penicillin (100 IU per ml, ThermoFisher), streptomycin (100 µg per  
120 ml, ThermoFisher) and mouse IL-2 (40 ng/ml, Immunotools). For antigen-specific co-culture assays, 5.10<sup>3</sup> cell-  
121 sorted splenic CD4<sup>+</sup>CD25<sup>hi</sup> T<sub>reg</sub> from Rip-mOVA x OTII x *Rag2*<sup>KO</sup> mice were co-cultured for 24h with 1.10<sup>3</sup> CD45<sup>-</sup>  
122 EpCAM<sup>+</sup>Ly51<sup>-/lo</sup>Aire<sup>eGFP</sup> mTEC from *Aire*<sup>het</sup> or *Aire*<sup>KO</sup> mice previously loaded with OVA<sub>323-339</sub> peptide (5µg/ml,  
123 Polypeptide group) for 2h.

124 **Flow cytometry**

125 Cells were stained with standard procedures using antibodies listed in Table S2. For intracellular staining with  
126 anti-Foxp3, anti-GITRL and anti-OX40L antibodies, cells were fixed, permeabilized and stained with the Foxp3  
127 staining kit according to the manufacturer's instructions (eBioscience). Stained cells were analyzed with  
128 FACSCanto II and LSR II (BD Biosciences) and data were analyzed using FlowJo software (Tree Star).

129 **Quantitative RT-PCR**

130 Total RNA was extracted with TRIzol (Invitrogen) and cDNA was synthesized with random oligo dT primers and  
131 Superscript II reverse transcriptase (Invitrogen). Quantitative PCR was performed with SYBR Premix Ex Taq  
132 Master Mix (Takara) on an ABI 7500 fast real-time PCR system (Applied Biosystem). The results were normalized  
133 to actin mRNA expression. A list of primer sequences is provided in Table S3.

134 **RNA-sequencing experiments**

135  $5.10^4$  CCR6<sup>+</sup> T<sub>reg</sub> were cell-sorted from the thymus of 6 week-old *Aire*<sup>WT</sup> and *Aire*<sup>KO</sup> mice. Two biological  
136 replicates were prepared for each condition. The total RNA was extracted using the RNeasy Micro Kit (Qiagen)  
137 and treated with DNase I. RNA-seq libraries were prepared using the TruSeq Stranded mRNA kit (Illumina) and  
138 sequenced with the Illumina HiSeq 2000 machine to generate datasets of single-end 50bp reads. The reads were  
139 mapped to the mouse reference genome (mm10) using TopHat2 (version 2.0.12) (28), then counted using Cufflinks  
140 or Cuffdiff (version 2.2.1) (29, 30) and the mm10 genome GTF gene annotation file. In addition to read counting,  
141 Cuffdiff performs between-sample normalization and was used to calculate the differential gene expression and  
142 its statistical significance in *Aire*<sup>WT</sup> vs *Aire*<sup>KO</sup> T<sub>reg</sub> by using the default "pooled" dispersion method that applies to  
143 experiments having few ( $\geq 2$ ) biological replicates per condition. Expression levels generated by Cufflinks, as  
144 fragments per kilobase of transcript per million mapped reads (FPKM), were processed by the Matrix2png program  
145 (31) to generate heatmaps of gene expression levels. The dataset generated in this study are available in the Gene  
146 Expression Omnibus (GEO) database under accession number GSE188419. The expression of *Tnfrsf4* and *Tnfrsf18*  
147 was analyzed in RNA-sequencing dataset from *Aire*<sup>KO</sup> mTEC<sup>hi</sup> (GSE87133).

148 **Immunofluorescence staining**

149 Thymi were collected from 9 day-, 6 week- and 1 year-old *Aire*<sup>WT</sup> or *Aire*<sup>KO</sup> mice and fat tissues removed to avoid  
150 any interference with the 3D reconstitution process. Organs were longitudinally included in O.C.T (Sakura  
151 Finetek), frozen at -80 °C and cut in 20- $\mu$ m-thick slices. Thymic sections were fixed with 2% paraformaldehyde,  
152 then saturated with 3% BSA and 0.01% Triton X100 in 0.1 M Tris HCl buffer. Sections were next stained for 45

153 min with rabbit anti-keratin 14 or with anti-Aire Alexa Fluor 488 (5H12; eBioscience), anti-Foxp3 PE (FJK-16s;  
154 eBioscience) and anti-CD73 Alexa Fluor 647 (TY/11.8; Biolegend) in hybridization buffer (1% BSA and 0.02%  
155 Triton X100 in 0.1 M Tris HCl, pH 7.4). Keratin 14 staining was revealed with Cy3-conjugated anti-rabbit  
156 (Invitrogen). Sections were counterstained with DAPI (1  $\mu$ L/mL, BioLegend) and mounted with Mowiol  
157 (Calbiochem). For 3D reconstruction, images were acquired with a slide scanner (Panoramic SCAN II; 3D  
158 Histech). Confocal microscopy was performed with a LSM 780 confocal microscope (Carl Zeiss Microscopy).

### 159 **Detection of immune infiltrates and autoantibodies**

160 Peripheral tissues were fixed in buffered 10% formalin solution. 4  $\mu$ m-thick paraffin-embedded sections were  
161 counterstained with hematoxylin and eosin. Autoantibody production was assessed by immunostaining organ  
162 sections from *Rag2*<sup>KO</sup> mice with the sera (1/80) of analyzed mice. Autoantibodies were revealed with FITC-  
163 conjugated goat anti-mouse IgG. Sections were counterstained with DAPI and mounted with Mowiol  
164 (Calbiochem). All images were acquired with a slide scanner (Panoramic SCAN II; 3D Histech) and analyzed with  
165 ImageJ software (National Institutes of Health) to compute the mean fluorescence intensity of each image.

### 166 **3D reconstitution**

167 For 3D reconstitution, images from the entire thymus of 9 day-, 6 week- and 1-year-old *Aire*<sup>WT</sup> and *Aire*<sup>KO</sup> mice  
168 were processed with For3D software as previously described (32, 33). Briefly, images were smoothed by median  
169 and Gaussian filtering and medulla volumes were determined using ImageJ and Matlab (The Mathworks) software.  
170 Medullary islets identified in the 3D structures were measured individually using ImageJ and were color-coded  
171 using Imaris (Bitplane).

### 172 **Statistical analysis**

173 Statistics were performed with GraphPad Prism 9.1 software. Normal distribution of the data was assessed using  
174 d'Agostino-Pearson omnibus normality test. Statistical significance was then assessed using unpaired Student's *t*  
175 test for two normal distributions, Mann-Whitney test for two non-normal distributions or Kruskal-Wallis test for  
176 more than two distributions. \**p* < 0.05; \*\**p* < 0.01; \*\*\**p* < 0.001, \*\*\*\**p* < 0.0001. All bar graphs show mean  $\pm$   
177 SEM, unless mentioned.



178 **Results**

179 **Aire regulates the recirculation of peripheral CD25<sup>+</sup>Foxp3<sup>+</sup> T<sub>reg</sub> in the thymus, independently of the**  
180 **remodeling of the 3D organization of the medulla throughout life**

181 Considering the importance of Aire in the induction of self-tolerance (34), we first assessed whether it could be  
182 involved in the topology of the thymic medulla. For this, we compared WT (*Aire*<sup>WT</sup> mice) with *Aire*-deficient mice  
183 in which the first exon of the *Aire* locus was replaced by the sequence of enhanced green fluorescent protein  
184 (eGFP) (*Aire*<sup>KO</sup> mice) (23). Thymic sections of 9 day-, 6 week- and 1-year-old, either *Aire*<sup>WT</sup> or *Aire*<sup>KO</sup> mice were  
185 stained with the keratin-14 mTEC-specific marker (35) and reconstructed with our in-house dedicated “Full organ  
186 reconstruction in 3D” (For3D) software (33) (**Fig. 1A-C and Movies S1-6**). Interestingly, the number of medullary  
187 islets in *Aire*<sup>WT</sup> mice diminished between 9 days and 6 weeks of age while it increased between 6 weeks and 1 year  
188 of age (**Fig. 1D**). Furthermore, the total and individual medullary volumes increased between 9 days and 6 weeks  
189 while it decreased between 6 weeks and 1 year (**Fig. 1E,F**). Nevertheless, the central compartment of ~1 mm<sup>3</sup> was  
190 observed at all ages analyzed (**Fig. 1F**) These observations reveal that the medulla organization is dynamic  
191 throughout life. In comparison to *Aire*<sup>WT</sup> mice, the thymi of *Aire*<sup>KO</sup> mice show a similar dynamic medulla  
192 organization characterized by normal numbers of medullary islets and total medullary volumes with the presence  
193 of a major compartment (**Fig. 1A-C,F**). These results indicate that the dynamic remodeling of the 3D organization  
194 of the thymic medulla throughout life is not regulated by Aire.

195 Because medulla formation correlates with Foxp3<sup>+</sup> T<sub>reg</sub> emergence during ontogeny (36), we next analyzed  
196 whether *Aire* deficiency could affect their development in neonates of 5 days of age. In contrast to CD25<sup>+</sup> T<sub>reg</sub>P,  
197 frequencies and numbers of Foxp3<sup>lo</sup> T<sub>reg</sub>P and mature CD25<sup>+</sup>Foxp3<sup>+</sup> T<sub>reg</sub> were diminished in the thymus of 5-day-  
198 old *Aire*<sup>KO</sup> mice compared to their respective counterparts (**Fig. 2A**). This decrease was also observed in 6-week-  
199 and 1-year-old *Aire*<sup>KO</sup> mice (**Fig. 2B,C**). Since mature CD25<sup>+</sup>Foxp3<sup>+</sup> T<sub>reg</sub> contain both developing and recirculating  
200 cells in the adult thymus, we used the key thymus-homing chemokine receptor CCR6 to distinguish developing  
201 (CCR6<sup>-</sup>) and recirculating (CCR6<sup>+</sup>) mature T<sub>reg</sub> (6). Interestingly, both numbers of CCR6<sup>-</sup> and CCR6<sup>+</sup> mature T<sub>reg</sub>  
202 were reduced in *Aire*<sup>KO</sup> mice compared to *Aire*<sup>WT</sup> mice at 6 weeks and 1 year of age (**Fig. 2D,E**). Furthermore, 1-  
203 year-old *Aire*<sup>WT</sup> mice showed a marked reduction in numbers of CCR6<sup>-</sup> and CCR6<sup>+</sup> mature CD25<sup>+</sup>Foxp3<sup>+</sup> T<sub>reg</sub>  
204 compared to 6-week-old *Aire*<sup>WT</sup> mice, which reflects the effect of thymic involution on T<sub>reg</sub> cells in normal  
205 conditions. To date, two chemokine receptors, CCR6 and CXCR4 have been implicated in the recirculation of  
206 peripheral T<sub>reg</sub> into the thymus (5, 8). Strikingly, the expression of their respective ligands, *Ccl20* and *Cxcl12*, was

207 substantially reduced in *Aire*<sup>KO</sup> mTEC<sup>hi</sup> (**Fig. 2F**), consistently with the altered recirculation of peripheral T<sub>reg</sub> in  
208 the thymus. Altogether, these results show that Aire controls both the development and recirculation of Foxp3<sup>+</sup>  
209 T<sub>reg</sub> throughout life.

### 210 **Thymic CCR6<sup>+</sup> T<sub>reg</sub> from *Aire*<sup>KO</sup> mice show an impaired effector and suppressive phenotype**

211 Because Aire controls the recirculation of CCR6<sup>+</sup> T<sub>reg</sub>, we made the hypothesis that it could also control their  
212 suppressive properties. To test this hypothesis, we first measured the expression level of several genes associated  
213 with T<sub>reg</sub> suppressive functions in purified CCR6<sup>+</sup>CD4<sup>+</sup>CD25<sup>+</sup> single-positive (SP) thymocytes that correspond to  
214 CD25<sup>+</sup>Foxp3<sup>+</sup> T<sub>reg</sub> (**Fig. S1**). Interestingly, whereas *Foxp3* level was normal, the expression of *Klrg1*, a marker of  
215 terminally differentiated T<sub>reg</sub> (37), was reduced in CCR6<sup>+</sup> T<sub>reg</sub> from 6-week-old *Aire*<sup>KO</sup> mice (**Fig. 3A**).  
216 Accordingly, the expression of genes encoding for the inhibitory cytokine *Il10*, the cytolytic molecules *Gzmb* and  
217 *Fasl*, *Lag3*-associated with dendritic cell modulation, as well as the ectoenzymes *Entpd1* (CD39) and *Nt5e* (CD73),  
218 implicated in target cell metabolic disruption, was reduced in CCR6<sup>+</sup> T<sub>reg</sub> of *Aire*<sup>KO</sup> mice. A similar altered  
219 suppressive signature was also observed in CCR6<sup>+</sup> T<sub>reg</sub> purified from the thymus of 1-year-old *Aire*<sup>KO</sup> mice (**Fig.**  
220 **S2**).

221 To further determine the impact of Aire on the functional properties of CCR6<sup>+</sup> T<sub>reg</sub>, we analyzed their gene  
222 expression profile by high-throughput RNA-sequencing (**Fig. 3B,C**). Genes showing a significant variation ( $p \leq$   
223 0.05) in gene expression between *Aire*<sup>WT</sup> and *Aire*<sup>KO</sup> CCR6<sup>+</sup> T<sub>reg</sub> with a fold change difference  $> 2$  or  $< 0.5$  were  
224 considered as up- and down-regulated, respectively. We identified a total of 2 634 upregulated genes reaching  
225 significance for 1 060 of them (Cuffdiff  $p < 0.05$ ) in *Aire*<sup>WT</sup> CCR6<sup>+</sup> T<sub>reg</sub> compared to their *Aire*<sup>KO</sup> counterparts  
226 (**Fig. 3B**). Moreover, only 1 175 genes were downregulated with 301 of them reaching significance (Cuffdiff  $p <$   
227 0.05). Thus, the expression of *Aire* in the thymus upregulates three times more genes than it downregulates in  
228 recirculating CCR6<sup>+</sup> T<sub>reg</sub>. In accordance with the altered T<sub>reg</sub> suppressive signature observed by qPCR (**Fig. 3A**),  
229 we found that *Il10*, *Gzmb*, *Fasl*, *Lag3*, *Entpd1* and *Nt5e* were downregulated in CCR6<sup>+</sup> T<sub>reg</sub> of *Aire*<sup>KO</sup> mice (**Fig.**  
230 **3C and Table S1**). Furthermore, the expression of *Prdm1* (Blimp-1), which characterizes effector Th-like T<sub>reg</sub> (38)  
231 and the terminally differentiated markers *Klrg1* and *Tigit* (37, 39) was reduced in CCR6<sup>+</sup> T<sub>reg</sub> of *Aire*<sup>KO</sup> mice  
232 compared to their *Aire*<sup>WT</sup> counterparts. Strikingly, CCR6<sup>+</sup> T<sub>reg</sub> of *Aire*<sup>KO</sup> mice also expressed lower levels of several  
233 genes associated with their suppressive signature such as *Ctla4* and *Lgals1* (galectin-1) implicated respectively in  
234 dendritic cell modulation and target cell apoptosis (40, 41). The expression of *Tbx21* and *Irf4*, encoding for  
235 transcription factors associated with Th1- and Th2-like T<sub>reg</sub>, as well as *Pparg* and *Id2* genes associated with fat-

236 resident effector T<sub>reg</sub> (42) was also diminished. Accordingly, the expression of the chemokine receptors *Cxcr3* of  
237 Th1-like, *Ccr4* and *Ccr8* of Th2-like as well as *Ccr1* and *Ccr2* of fat-resident T<sub>reg</sub> (43), implicated in effector T<sub>reg</sub>  
238 migration to the inflammatory site, was reduced in CCR6<sup>+</sup> *Aire*<sup>KO</sup> T<sub>reg</sub>. Altogether, these results indicate that *Aire*  
239 expression is crucial for the effector and suppressive properties of recirculating CCR6<sup>+</sup> T<sub>reg</sub> in the thymus.

#### 240 **Recirculating CCR6<sup>+</sup> T<sub>reg</sub> from *Aire*<sup>KO</sup> mice fail to attenuate the severity of multi-organ autoimmunity**

241 Because numbers and suppressive signature of recirculating CCR6<sup>+</sup> T<sub>reg</sub> were reduced in the thymus of *Aire*<sup>KO</sup>  
242 mice, we analyzed whether these defects would be also observed in the periphery. Blood and splenic CD4<sup>+</sup>Foxp3<sup>+</sup>  
243 T<sub>reg</sub> from 6-week- and 1-year-old *Aire*<sup>KO</sup> mice showed similar frequencies, numbers and expression levels of  
244 suppressive genes compared to their *Aire*<sup>WT</sup> counterparts (**Fig. S3**). We next assessed the ability of peripheral and  
245 thymic CCR6<sup>+</sup> T<sub>reg</sub> from *Aire*<sup>KO</sup> mice to dampen the severity of multiorgan autoimmunity. To this end, CD4<sup>+</sup>CD25<sup>+</sup>  
246 T<sub>reg</sub>-depleted splenocytes from CD45.1 WT mice were transferred into *Rag2*<sup>KO</sup> lymphopenic recipients. Four  
247 weeks later, splenic T<sub>reg</sub> or thymic CCR6<sup>+</sup> T<sub>reg</sub> purified from either *Aire*<sup>WT</sup> or *Aire*<sup>KO</sup> mice were adoptively  
248 transferred into these recipients (**Fig. 4A and Fig. S4A**). Signs of autoimmunity in peripheral tissues were  
249 visualized by histology and quantified by flow cytometry three weeks later. *Rag2*<sup>KO</sup> mice that did not receive any  
250 T<sub>reg</sub> were used as controls. Accordingly with the normal suppressive signature of splenic T<sub>reg</sub> from *Aire*<sup>KO</sup> mice  
251 (**Fig. S3**), *Rag2*<sup>KO</sup> mice adoptively transferred with these cells show tissue infiltration levels similar to mice  
252 transferred with *Aire*<sup>WT</sup> splenic T<sub>reg</sub> (**Fig. 4B,C**). In marked contrast to mice that received *Aire*<sup>WT</sup> thymic CCR6<sup>+</sup>  
253 T<sub>reg</sub>, mice adoptively transferred with thymic CCR6<sup>+</sup> T<sub>reg</sub> from *Aire*<sup>KO</sup> mice failed to attenuate T-cell infiltration in  
254 several peripheral tissues despite similar numbers of CD45.2 donor Foxp3<sup>+</sup> T<sub>reg</sub> in lymph nodes (**Fig. 4D,E and**  
255 **Fig. S4B,C**). Examination of CD45.1 donor cell infiltration by flow cytometry revealed that 100% of *Rag2*<sup>KO</sup> mice  
256 that received *Aire*<sup>KO</sup> thymic CCR6<sup>+</sup> T<sub>reg</sub> showed a high infiltration level in the pancreas and salivary glands, 83%  
257 in eyes, 33% in the lung and liver as well as 16% in the kidney (**Fig. 4E**). In contrast, only 16% of *Rag2*<sup>KO</sup> mice  
258 transferred with *Aire*<sup>WT</sup> thymic CCR6<sup>+</sup> T<sub>reg</sub> showed a high infiltration in the liver. Furthermore, flow cytometry  
259 analysis showed that thymic CCR6<sup>+</sup> T<sub>reg</sub> of *Aire*<sup>KO</sup> mice were unable to prevent the infiltration of CD45.1 CD4<sup>+</sup>  
260 and CD8<sup>+</sup> T cells in the pancreas and eyes as well as CD45.1 CD8<sup>+</sup> T cells in salivary glands (**Fig. S4D**). Finally,  
261 immunostaining of *Rag2*<sup>KO</sup> tissue sections with sera from these mice revealed higher levels of autoantibodies  
262 against the pancreas and salivary glands in mice transferred with *Aire*<sup>KO</sup> thymic CCR6<sup>+</sup> T<sub>reg</sub> than in mice injected  
263 with *Aire*<sup>WT</sup> thymic CCR6<sup>+</sup> T<sub>reg</sub> (**Fig. 4F,G**). Thus, thymic CCR6<sup>+</sup> T<sub>reg</sub> of *Aire*<sup>KO</sup> mice failed to attenuate the  
264 severity of multiorgan autoimmunity, demonstrating that their suppressive activity was impaired.

265 **Aire expression in the thymic stroma controls the suppressive signature of recirculating CCR6<sup>+</sup> T<sub>reg</sub>**

266 Since *Aire* expression is not restricted to mTEC but was also found in thymic B cells (9), we then investigated its  
267 respective contribution in the stromal and hematopoietic compartments to control the recirculation and suppressive  
268 properties of CCR6<sup>+</sup> T<sub>reg</sub>. To determine the role of Aire in hematopoietic cells, we generated BM chimeras by  
269 reconstituting lethally irradiated CD45.1/2 WT recipients with either CD45.2 *Aire*<sup>WT</sup> or *Aire*<sup>KO</sup> BM cells (**Fig.**  
270 **S5A**). Six weeks later, *Aire*<sup>KO</sup> BM chimeras did not show major defects in total CD19<sup>+</sup>B220<sup>+</sup> B cells, neither in  
271 the IgD<sup>-</sup> and IgD<sup>+</sup> B cell subsets, both described to express Aire (9) (**Fig. S5B,C**). Overall, frequencies and numbers  
272 of CD25<sup>+</sup> T<sub>reg</sub>P, Foxp3<sup>lo</sup> T<sub>reg</sub>P, CCR6<sup>-</sup> and CCR6<sup>+</sup> mature CD25<sup>+</sup>Foxp3<sup>+</sup> T<sub>reg</sub> were also normal (**Fig. S5D,E**). We  
273 next cell-sorted CCR6<sup>+</sup> T<sub>reg</sub> from the thymus of these BM chimeras and analyzed their suppressive signature.  
274 Recirculating T<sub>reg</sub> from both chimeras exhibited a similar expression of suppressive genes that was altered in  
275 CCR6<sup>+</sup> T<sub>reg</sub> of *Aire*<sup>KO</sup> mice (**Fig. S5F**). Thus, *Aire* expression in thymic B cells is unlikely involved in T<sub>reg</sub>  
276 development, recirculation and suppressive signature.

277 We then analyzed whether *Aire* expression in stromal cells controls CCR6<sup>+</sup> T<sub>reg</sub> functional suppressive properties.  
278 To this end, we generated BM chimeras in which lethally irradiated CD45.2 *Aire*<sup>WT</sup> or *Aire*<sup>KO</sup> mice were  
279 reconstituted with CD45.1 Foxp3<sup>eGFP</sup> BM cells (**Fig. 5A**). Six weeks later, similar numbers of CD4<sup>+</sup> SP thymocytes  
280 of CD45.1 donor origin were observed in *Aire*<sup>WT</sup> and *Aire*<sup>KO</sup> recipients (**Fig. 5B**). Although numbers of CD25<sup>+</sup>  
281 T<sub>reg</sub>P and Foxp3<sup>lo</sup> T<sub>reg</sub>P were also similar in both groups, frequencies and numbers of mature CD25<sup>+</sup>Foxp3<sup>+</sup> T<sub>reg</sub>  
282 were specifically reduced in *Aire*<sup>KO</sup> chimeras (**Fig. 5C**). This defect was attributable to diminished frequencies and  
283 numbers of CCR6<sup>+</sup> T<sub>reg</sub> (**Fig. 5D**). Recirculating CCR6<sup>+</sup> mature CD25<sup>+</sup>Foxp3<sup>+</sup> T<sub>reg</sub> of CD45.1 origin were then  
284 cell-sorted from the thymus of both chimeras and analyzed for the expression of several genes associated with T<sub>reg</sub>  
285 effector functions. Whereas *Foxp3* level was normal, the expression of *Klrg1*, *Il10*, *Tgfb1*, *Gzmb*, *Fasl*, *Lag3*,  
286 *Entpd1* and *Nt5e* was reduced in *Aire*<sup>KO</sup> chimeras compared to control chimeras (**Fig. 5E**). Altogether, these results  
287 show that whereas *Aire* in hematopoietic cells is dispensable, its specific expression in stromal cells controls both  
288 recirculation and suppressive properties of CCR6<sup>+</sup> T<sub>reg</sub>.

289 **Antigen-specific restimulation of recirculating T<sub>reg</sub> by Aire<sup>+</sup> mTEC**

290 To further decipher the impact of *Aire*-expressing mTEC in the suppressive signature of recirculating T<sub>reg</sub>, we used  
291 an *in vitro* co-culture setup of peripheral T<sub>reg</sub> and mTEC purified from *Aire*<sup>WT</sup> or *Aire*<sup>KO</sup> mice. Compared to *Aire*<sup>WT</sup>  
292 splenic T<sub>reg</sub> co-cultured with *Aire*<sup>WT</sup> mTEC, the expression of *Tgfb1*, *Gzmb*, *Fasl*, *Entpd1* and *Nt5e* was reduced  
293 in *Aire*<sup>WT</sup> splenic T<sub>reg</sub> co-cultured with *Aire*<sup>KO</sup> mTEC (**Fig. 6A**). Moreover, *Aire*<sup>KO</sup> splenic T<sub>reg</sub> co-cultured with

294 *Aire*<sup>WT</sup> mTEC upregulated the expression of these genes in contrast to *Aire*<sup>KO</sup> splenic T<sub>reg</sub> co-cultured with *Aire*<sup>KO</sup>  
295 mTEC. Thus, as compared to their *Aire*<sup>WT</sup> counterparts, *Aire*<sup>KO</sup> mTEC failed to enhance suppressive signatures of  
296 both *Aire*<sup>WT</sup> and *Aire*<sup>KO</sup> splenic T<sub>reg</sub>.

297 We then analyzed the *in vivo* effect of the *Aire*<sup>KO</sup> thymic stroma in regulating the suppressive signature of  
298 peripheral WT T<sub>reg</sub>. To this end, splenic T<sub>reg</sub> purified from CD45.1 Foxp3<sup>eGFP</sup> congenic mice were adoptively  
299 transferred into either *Aire*<sup>WT</sup> or *Aire*<sup>KO</sup> recipients (**Fig. 6B**). One week later, we observed reduced frequencies and  
300 numbers of donor CD45.1 Foxp3<sup>eGFP</sup> T<sub>reg</sub> in the thymus of *Aire*<sup>KO</sup> mice as compared to *Aire*<sup>WT</sup> mice (**Fig. 6C**).  
301 Moreover, the remaining donor Foxp3<sup>eGFP</sup> T<sub>reg</sub> that recirculated back to the thymus exhibited an impaired  
302 suppressive signature (**Fig. 6D**). These results not only confirm that Aire favors the recirculation of peripheral T<sub>reg</sub>  
303 in the thymus but also highlight its key role in enhancing their suppressive properties.

304 Thus, we made the hypothesis that recirculating T<sub>reg</sub> could be restimulated by Aire<sup>+</sup> mTEC. To test this, WT thymic  
305 sections were first stained for Aire, Foxp3 and CD73, the latter being a reliable marker of recirculating T<sub>reg</sub> (1, 44).  
306 Of note, anti-CCR6 antibody was not used in this experiment because it failed to give any signal in our hands.  
307 Interestingly, we observed that both developing CD73<sup>-</sup> and recirculating CD73<sup>+</sup> T<sub>reg</sub> were found in close proximity  
308 to Aire<sup>+</sup> mTEC (**Fig. 6E**). These results suggest that recirculating T<sub>reg</sub> could be restimulated by establishing  
309 antigen-specific contacts with Aire<sup>+</sup> mTEC. To test this hypothesis, splenic OTII T<sub>reg</sub> from Rip-mOVA x OTII  
310 mice were co-cultured with OVA<sub>323-339</sub>-loaded *Aire*<sup>het</sup> (*Aire*<sup>eGFP/WT</sup>) or *Aire*<sup>KO</sup> (*Aire*<sup>eGFP/eGFP</sup>) mTEC<sup>hi</sup> (**Figure S6**).  
311 Compared to unstimulated OVA-specific T<sub>reg</sub>, *Aire*<sup>KO</sup> mTEC<sup>hi</sup> were able to activate OTII T<sub>reg</sub> but to a lesser extent  
312 than *Aire*<sup>het</sup> mTEC<sup>hi</sup> (**Fig. 6F**). These results demonstrate that Aire<sup>+</sup> mTEC can stimulate peripheral T<sub>reg</sub> in an  
313 antigen-specific manner and this ability is impaired in the absence of Aire. Furthermore, as compared to OTII T<sub>reg</sub>  
314 co-cultured with *Aire*<sup>het</sup> mTEC<sup>hi</sup>, the expression of *Il10*, *Tgfb1*, *Gzmb*, *FasL*, *Lag3*, *Entpd1* and *Nt5e* was reduced  
315 in OTII T<sub>reg</sub> co-cultured with *Aire*<sup>KO</sup> mTEC<sup>hi</sup> (**Fig. 6G**), indicating that this antigen-specific restimulation is much  
316 less efficient. We thus analyzed MHCII expression in mTEC from *Aire*<sup>KO</sup> mice. In accordance with a previous  
317 study (45) and the pro-apoptotic role of Aire (46), we found increased frequencies of MHCII<sup>+</sup> mTEC in these mice  
318 (**Fig. S7A**). We then made the hypothesis that costimulatory signals may be implicated in the phenotype observed.  
319 Two ligands of the Tumor Necrosis Factor Superfamily (TNFSF) have been described to be constitutively  
320 expressed by mTEC and participate in thymic T<sub>reg</sub> development (47). Interestingly, we found that mTEC<sup>hi</sup>  
321 expressed reduced levels of *Tnfsf4* (OX40L, *Aire*<sup>WT</sup>: 2.32 FPKM vs *Aire*<sup>KO</sup>: 0.37) and *Tnfsf18* (GITRL, *Aire*<sup>WT</sup>:  
322 2.92 vs *Aire*<sup>KO</sup>: 0.81) in *Aire*<sup>KO</sup> mice (**Fig. S7B**). This result was also confirmed at the protein level by flow  
323 cytometry (**Fig. S7C**). These observations are consistent with the reduced cellularity of Foxp3<sup>lo</sup> T<sub>reg</sub>P and

324 CD25<sup>+</sup>Foxp3<sup>+</sup> T<sub>reg</sub> in *Aire*<sup>KO</sup> mice (**Fig. 2A-C**). Further investigations are needed to decipher the role of these two  
325 TNFSF ligands in the biology of recirculating T<sub>reg</sub> in the thymus. Altogether, our data reveal that Aire<sup>+</sup> mTEC,  
326 through antigen-specific restimulation, are responsible for the strong suppressive signature of recirculating T<sub>reg</sub>.

327

## 328 **Discussion**

329 Our study demonstrates that Aire expression by mTEC promotes the suppressive properties of recirculating CCR6<sup>+</sup>  
330 T<sub>reg</sub> independently of the dynamic remodeling of the medullary 3D organization, where recirculating T<sub>reg</sub> reside.  
331 We previously described that the medulla of 6-week-old young adult mice is complex with a large central  
332 compartment surrounded by hundreds of individual islets (32). Nevertheless, determining whether this topology  
333 varies throughout life and whether Aire is implicated remained open issues. Interestingly, we found that 9-day-  
334 and 6-week-old WT mice show ~1 000 and ~400 medullary islets, respectively. Given that individual islets arise  
335 from a single progenitor (48), these observations suggest that during thymic development, they grow and fuse  
336 together, leading to a reduced number of islets in young adult mice compared to neonates. In 1-year-old mice, the  
337 number of medullary islets increases to reach ~1 000 islets, as observed in neonates. Considering that the medulla  
338 topology is governed by crosstalk with autoreactive CD4<sup>+</sup> thymocytes (32, 49-52), the high islet number observed  
339 in aged mice could be due to a suboptimal cellular crosstalk due to reduced cellularity of CD4<sup>+</sup> thymocytes linked  
340 to age-related thymic involution. Although Aire plays multiple roles in T-cell tolerance induction (34), our results  
341 show that it does not shape the 3D organization of the thymic medulla. This is consistent with the fact that the  
342 clonal deletion of autoreactive CD4<sup>+</sup> thymocytes is impaired in *Aire*<sup>KO</sup> mice (45, 53), which consequently leads to  
343 an effective medulla organization.

344 Several studies have shown that mTEC are implicated in Foxp3<sup>+</sup> T<sub>reg</sub> development (54-56). Nevertheless, the  
345 specific role of Aire in thymic T<sub>reg</sub> heterogeneity throughout life remained to be defined. Interestingly, we found  
346 that Aire controls the cellularity of Foxp3<sup>lo</sup> T<sub>reg</sub>P and mature CD25<sup>+</sup>Foxp3<sup>+</sup> T<sub>reg</sub> in 5-day-, 6-week- and 1-year-old  
347 mice. This is illustrated by decreased numbers of Foxp3<sup>lo</sup> T<sub>reg</sub>P and CD25<sup>+</sup>Foxp3<sup>+</sup> T<sub>reg</sub> in *Aire*<sup>KO</sup> mice, which could  
348 not be due to impaired medulla organization since no defect was observed at this level. Given that CD25<sup>+</sup> T<sub>reg</sub>P  
349 and Foxp3<sup>lo</sup> T<sub>reg</sub>P show distinct developmental pathways that give rise to CD25<sup>+</sup>Foxp3<sup>+</sup> T<sub>reg</sub> with non-overlapping  
350 regulatory activities (1), our results provide new insights in the role of Aire in the emergence of Foxp3<sup>lo</sup> T<sub>reg</sub>P.  
351 Furthermore, Aire regulates the pool of recirculating CCR6<sup>+</sup> T<sub>reg</sub> throughout life. Interestingly, CCR6<sup>+</sup> T<sub>reg</sub> from  
352 *Aire*<sup>KO</sup> mice expressed reduced levels of several genes associated with their polarization and suppressive functions.

353 Importantly, they express normal levels of *Foxp3*, indicating that they remain engaged in the T<sub>reg</sub> cell lineage. In  
354 accordance with the defective suppressive signature of CCR6<sup>+</sup> T<sub>reg</sub> from *Aire*<sup>KO</sup> mice, we found that the adoptive  
355 transfer of these cells failed to attenuate the severity of multiorgan autoimmunity. In contrast to their thymic  
356 counterparts, splenic T<sub>reg</sub> of *Aire*<sup>KO</sup> mice show a protection similar to *Aire*<sup>WT</sup> splenic T<sub>reg</sub>, consistently with their  
357 normal suppressive signature. Altogether, these results indicate that Aire is crucial for the suppressive functions  
358 of recirculating CCR6<sup>+</sup> T<sub>reg</sub> in the thymus.

359 Although Aire is expressed by a subset of recirculating thymic B cells (9), we found that its absence in  
360 hematopoietic cells had no impact neither in T<sub>reg</sub> development nor in CCR6<sup>+</sup> T<sub>reg</sub> recirculation and suppressive  
361 signature. Considering the weak expression of *Aire* in thymic B cells compared to mTEC, it is not surprising that  
362 *Aire* deficiency in hematopoietic cells does not control the cellularity and suppressive signature of CCR6<sup>+</sup> T<sub>reg</sub>. In  
363 marked contrast, beyond controlling the recirculation of CCR6<sup>+</sup> T<sub>reg</sub>, BM chimeras in *Aire*<sup>KO</sup> recipients revealed  
364 that *Aire* expression in stromal cells is responsible for their highly suppressive phenotype.

365 Interestingly, recirculating Foxp3<sup>+</sup> T<sub>reg</sub> were observed in close proximity to Aire<sup>+</sup> mTEC, similarly to developing  
366 Foxp3<sup>+</sup> T<sub>reg</sub>. The reduced expression of *Ccl20* and *Cxcl12*, likely responsible for the lower amount of recirculating  
367 T<sub>reg</sub> in these mice as previously reported (5, 8), could contribute to a lesser stimulation of recirculating T<sub>reg</sub> by  
368 mTEC. However, *in vitro* co-culture experiments suggest that defective chemoattraction is unlikely responsible  
369 for the phenotype observed in *Aire*<sup>KO</sup> mice. Furthermore, *in vitro* co-culture assays revealed that Aire<sup>+</sup> mTEC were  
370 capable to activate peripheral T<sub>reg</sub> in an antigen-specific manner. Our results suggest that peripheral T<sub>reg</sub> could  
371 regulate *de novo* T<sub>reg</sub> development not only by competing for IL-2 (5, 57) but also for the cognate self-antigen. In  
372 the absence of Aire, *in vitro* co-culture assays and *in vivo* adoptive transfer experiments demonstrate that  
373 recirculating T<sub>reg</sub> activation and suppressive signature were altered. Therefore, our results unravel that Aire<sup>+</sup> mTEC  
374 control the activated and differentiated phenotype of recirculating T<sub>reg</sub> upon their entry into the thymus. A possible  
375 explanation could be that Aire controls co-stimulation signals that are responsible for the effector phenotype of  
376 recirculating T<sub>reg</sub>. In addition, Aire could also modulate indirectly CCR6<sup>+</sup> T<sub>reg</sub> suppressive properties through other  
377 mechanisms such as the medullary positioning of XCR1<sup>+</sup> type 1 conventional dendritic cells by controlling the  
378 production of the chemokine XCL1 (11). Moreover, further investigations are required to determine the fate of  
379 CCR6<sup>+</sup> T<sub>reg</sub> in the thymic medulla. Three possibilities can be envisaged: CCR6<sup>+</sup> T<sub>reg</sub> (1) become long-term resident  
380 cells, (2) migrate back to the periphery or (3) die by apoptosis.

381 This study ameliorates our understanding on recirculating T<sub>reg</sub> in the thymus, which remain poorly described to  
382 date. In summary, it identifies that Aire controls the suppressive properties of recirculating CCR6<sup>+</sup> T<sub>reg</sub> in the  
383 thymus. It also assigns a new role for Aire in conferring to mTEC the aptitude to restimulate recirculating T<sub>reg</sub>.  
384 Thus, this study furthers our understanding on the mechanisms allowing recirculating T<sub>reg</sub> to fine-tune *de novo* T<sub>reg</sub>  
385 production. Finally, our results are expected to contribute to a better understanding in T<sub>reg</sub> deficiencies observed  
386 in the human pathology APECED.

387

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393

### 394 **Author contributions**

395 JC, AB, JCS, LC, and MI conducted the experiments, analyzed and interpreted the data. MG and AS analyzed the  
396 data. JC, AB, JCS and MI wrote the manuscript. MI initiated, supervised and conceived the study.

397

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### 407 **Data availability**



408 All data generated or analyzed during this study are included in this published article and its supplementary  
409 information files. The dataset generated in this study are available in the Gene Expression Omnibus (GEO)  
410 database under accession number GSE188419.

411 **Code availability**

412 Not applicable

413

414 **Declarations**

415 **Conflict of interests**

416 The authors declare that they have no conflict of interest.

417 **Ethical approval**

418 All experiments were done in accordance with national and European laws for laboratory animal welfare (EEC  
419 Council Directive 2010/63/UE) and the Marseille Ethical Committee for Animal experimentation.

420 **Consent to participate**

421 Not applicable.

422 **Consent to publication**

423 Not applicable.

424

425 **References**

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565

## 566 Legends

567 **Fig. 1 The medullary topology is dynamic throughout life, independently of *Aire* expression.**

568 **A-C** Representative images of thymic sections stained for keratin 14 (red) and counterstained with DAPI (blue)  
569 (upper panel). For3D reconstruction of thymic lobes from 9-day- (**A**), 6-week- (**B**) and 1-year-old (**C**) *Aire*<sup>WT</sup> and  
570 *Aire*<sup>KO</sup> mice, using Matlab (middle panel) and Imaris to depict medullary regions according to their volumes from  
571 cyan (smallest) to magenta (largest) (lower panel). Axes are graduated in millimeters (mm). Scale bar, 1 mm. The  
572 asterisk denotes the central medulla.

573 **D,E** Histograms show the number of medullary islets (**D**) and the total medullary volume (**E**) derived from two  
574 thymic lobes for each condition.

575 **F** The graph shows the volumes of each medullary islet derived from two thymic lobes for each condition from  
576 individual mice measured by For3D. The dashed circle denotes the central medulla of each lobe. Horizontal lines  
577 represent the geometric mean and SD. \*\*\*\* $p < 0.0001$  using Kruskal-Wallis test for (**F**).

578

579 **Fig. 2 Developing CCR6<sup>-</sup> and recirculating CCR6<sup>+</sup> T<sub>reg</sub> are reduced in *Aire*<sup>KO</sup> mice throughout life.**

580 **A** Flow cytometry profiles, frequencies and numbers of CD25<sup>+</sup> T<sub>reg</sub>P, Foxp3<sup>lo</sup> T<sub>reg</sub>P and CD25<sup>+</sup>Foxp3<sup>+</sup> T<sub>reg</sub>  
581 analyzed in CD4<sup>+</sup> SP thymocytes from the thymus of 5-day-old *Aire*<sup>WT</sup> and *Aire*<sup>KO</sup> mice. The data are derived from  
582 3 independent experiments (n=2-4 mice per group and per experiment).

583 **B,C** Frequencies and numbers of CD25<sup>+</sup> T<sub>reg</sub>P, Foxp3<sup>lo</sup> T<sub>reg</sub>P and CD25<sup>+</sup>Foxp3<sup>+</sup> T<sub>reg</sub> in the thymus of 6-week- (**B**)  
584 and 1-year- (**C**) old *Aire*<sup>WT</sup> and *Aire*<sup>KO</sup> mice.

585 **D,E** Flow cytometry profiles (**D**), frequencies and numbers (**E**) of CCR6<sup>-</sup> and CCR6<sup>+</sup> cells in CD25<sup>+</sup>Foxp3<sup>+</sup> T<sub>reg</sub>  
586 from 6-week- and 1-year-old *Aire*<sup>WT</sup> and *Aire*<sup>KO</sup> mice. The data are derived from at least 3 independent experiments  
587 (n=3-4 mice per group and per experiment).

588 (**F**) The expression of *Ccl20* and *Cxcl12* was measured by qPCR in purified mTEC<sup>hi</sup> (EpCAM<sup>+</sup>UEA-1<sup>+</sup>Ly51<sup>-</sup>  
589 <sup>lo</sup>CD80<sup>hi</sup>) from 6-week-old *Aire*<sup>WT</sup> and *Aire*<sup>KO</sup> mice.

590 Bar graphs show  $\pm$  SEM, \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ , \*\*\*\* $p < 0.0001$  using unpaired Student's t-test (**A**,  
591 **B, C, E**) and two-tailed Mann-Whitney test for (**F**).

592 **Fig. 3 The suppressive signature of recirculating CCR6<sup>+</sup> T<sub>reg</sub> is impaired in the thymus of *Aire*<sup>KO</sup> mice.**

593 **A** The expression level of *Foxp3*, *Klrg1*, *Il10*, *Gzmb*, *Fasl*, *Lag3*, *Entpd1* and *Nt5e* was measured by qPCR in  
594 thymic CCR6<sup>+</sup> T<sub>reg</sub> from 6-week-old *Aire*<sup>WT</sup> (n=6) and *Aire*<sup>KO</sup> (n=9) mice. Bar graphs show mean  $\pm$  SEM,  
595 \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$  using two-tailed Mann-Whitney test.

596 **B** Scatterplot representations of log10 gene expression levels (FPKM) in recirculating CCR6<sup>+</sup> T<sub>reg</sub> from *Aire*<sup>WT</sup>  
597 and *Aire*<sup>KO</sup> mice. For representation purposes, expression values of genes below 0.01 were assigned to 0.01. Genes  
598 with fold difference  $> 2$  and  $p$ -adj  $< 0.05$  were considered as up- or down-regulated genes (red and green dots,  
599 respectively). RNA-seq was performed on 2 independent biological replicates derived from two mice.

600 **C** Heatmap of selected genes down-regulated in recirculating CCR6<sup>+</sup> *Aire*<sup>KO</sup> T<sub>reg</sub> (Fold Change  $> 2$ ) compared to  
601 their CCR6<sup>+</sup> *Aire*<sup>WT</sup> counterparts and involved in T<sub>reg</sub> suppressive functions, T<sub>reg</sub> Th-like, helper T cell polarization,  
602 fat and muscle T<sub>reg</sub>. Two biological replicates are shown for each condition.

603 **Fig. 4 The adoptive transfer of recirculating CCR6<sup>+</sup> T<sub>reg</sub> of the *Aire*<sup>KO</sup> thymus fails to protect from multi-**  
604 **organ autoimmunity.**

605 **A** Experimental setup: *Rag2*<sup>KO</sup> recipients were adoptively transferred with CD4<sup>+</sup>CD25<sup>+</sup> T<sub>reg</sub>-depleted CD45.1 WT  
606 splenocytes. Four weeks later, they were injected with splenic T<sub>reg</sub> or thymic CCR6<sup>+</sup> T<sub>reg</sub> derived from *Aire*<sup>WT</sup> or  
607 *Aire*<sup>KO</sup> mice. Three weeks after T<sub>reg</sub> adoptive transfer, peripheral tissues were examined for immune infiltration by  
608 histology and quantified by flow cytometry. *Rag2*<sup>KO</sup> recipients injected only with CD4<sup>+</sup>CD25<sup>+</sup> T<sub>reg</sub>-depleted  
609 CD45.1 WT splenocytes were used as controls.

610 **B-E** Representative photographs of peripheral tissue sections derived from mice transferred with splenic T<sub>reg</sub> (**B**)  
611 or thymic CCR6<sup>+</sup> T<sub>reg</sub> (**D**) and counterstained with hematoxylin/eosin. Diagrams represent organ infiltration levels  
612 by CD45.1 donor cells measured by flow cytometry upon splenic T<sub>reg</sub> (**C**) or thymic CCR6<sup>+</sup> T<sub>reg</sub> (**E**) transfer.  
613 Infiltration levels were normalized to the infiltration observed in controls. Dark and light violet in diagram  
614 represent high and low infiltrations. Each diagram represents one individual mouse. Scale bar, 150μm for (**B**),  
615 500μm for (**D**).

616 **F,G** Sera from mice transferred with CCR6<sup>+</sup> T<sub>reg</sub> were tested for the presence of autoantibodies (green) against  
617 pancreas (**F**) and salivary glands (**G**) of *Rag2*<sup>KO</sup> mice. Nuclei were counterstained with DAPI (blue). Secondary  
618 antibodies (II Abs) alone were used as controls. Scale bar, 1 mm. Histograms show mean fluorescence intensity  
619 for each condition. Data are derived from 2 to 3 independent experiments (n=3-5 mice per group and per  
620 experiment). Bar graphs show mean ± SEM, \*\*\*\*p < 0.0001 using unpaired Student's *t* test for (**F**, **G**).

621 **Fig. 5 *Aire* deficiency in the thymic stroma impairs the recirculation and the suppressive signature of CCR6<sup>+</sup>**  
622 **T<sub>reg</sub>.**

623 **A** Experimental setup: lethally irradiated CD45.2 *Aire*<sup>WT</sup> or *Aire*<sup>KO</sup> recipients were reconstituted with BM cells  
624 from CD45.1 Foxp3<sup>eGFP</sup> mice. Six weeks later, thymic T<sub>reg</sub> subsets of CD45.1 origin were analyzed by flow  
625 cytometry. CCR6<sup>+</sup> T<sub>reg</sub> were cell-sorted to measure the expression levels of genes associated with their suppressive  
626 functions.

627 **B** Flow cytometry profiles, frequencies and numbers of CD4<sup>+</sup> SP thymocytes of CD45.1 origin in the thymus of  
628 BM chimeric mice.

629 **C, D** Flow cytometry profiles and numbers of CD25<sup>+</sup> T<sub>reg</sub>P, Foxp3<sup>lo</sup> T<sub>reg</sub>P and total CD25<sup>+</sup>Foxp3<sup>+</sup> cells (**C**) as well  
630 as of CCR6<sup>-</sup> and CCR6<sup>+</sup> cells in CD25<sup>+</sup>Foxp3<sup>+</sup> T<sub>reg</sub> (**D**) of CD45.1 origin in the thymus of BM chimeras. Data are  
631 derived from 2 independent experiments (n=4 mice per group and per experiment).

632 **E** The expression level of *Foxp3*, *Klrg1*, *Il10*, *Tgfb1*, *Gzmb*, *Fasl*, *Lag3*, *Entpd1* and *Nt5e* was measured by qPCR  
633 in purified CCR6<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> T<sub>reg</sub> from CD45.1 Foxp3<sup>eGFP</sup> → *Aire*<sup>WT</sup> (n=8) and CD45.1 Foxp3<sup>eGFP</sup> → *Aire*<sup>KO</sup>  
634 (n=9) chimeras. Data are derived from 2 independent experiments (n=4-5 mice per group and per experiment).  
635 Bar graphs show mean ± SEM, \*p < 0.05, \*\*p < 0.01, \*\*\*p < 0.001, \*\*\*\*p < 0.0001 using unpaired Student's *t* test  
636 for (**B-D**) and two-tailed Mann-Whitney test for (**E**).

637 **Fig. 6. Aire<sup>+</sup> mTEC control the activation and suppressive signature of recirculating T<sub>reg</sub> through antigen-**  
638 **dependent contact.**

639 **A** The expression level of *Tgfb1*, *Gzmb*, *Fasl*, *Entpd1* and *Nt5e* was measured by qPCR in splenic T<sub>reg</sub> from *Aire*<sup>WT</sup>  
640 or *Aire*<sup>KO</sup> mice co-cultured with *Aire*<sup>WT</sup> or *Aire*<sup>KO</sup> mTEC. Data are derived from 2 independent experiments.

641 **B** Experimental setup: purified splenic T<sub>reg</sub> from CD45.1 Foxp3<sup>eGFP</sup> mice were adoptively transferred *i.v.* into  
642 sublethally irradiated CD45.2 *Aire*<sup>WT</sup> or *Aire*<sup>KO</sup> recipients. Adoptively transferred CD45.1 Foxp3<sup>eGFP</sup> T<sub>reg</sub> were  
643 cell-sorted from the thymus of recipient mice one week later and their suppressive signature was analyzed by  
644 qPCR.

645 **C** Flow cytometry profiles, frequencies and numbers of CD45.1 Foxp3<sup>eGFP</sup> donor T<sub>reg</sub> observed in the thymus of  
646 CD45.2 *Aire*<sup>WT</sup> or *Aire*<sup>KO</sup> recipients.

647 **D** The expression level of *Il10*, *Tgfb1*, *Gzmb*, *Fasl*, *Lag3* and *Nt5e* was measured by qPCR in CD45.1 Foxp3<sup>eGFP</sup>  
648 donor T<sub>reg</sub> transferred into *Aire*<sup>WT</sup> (n=4) or *Aire*<sup>KO</sup> (n=4) recipients.

649 **E** Representative images of WT thymic sections stained for Aire (green), Foxp3 (red) and CD73 (white). Yellow  
650 and white arrowheads denote developing CD73<sup>-</sup> and recirculating CD73<sup>+</sup> T<sub>reg</sub>, respectively. Scale bar, 30µm.

651 **F** Flow cytometry profiles of CD69 activation of splenic OTII T<sub>reg</sub> from Rip-mOVA x OTII x *Rag2*<sup>KO</sup> mice co-  
652 cultured with OVA<sub>323-339</sub>-loaded *Aire*<sup>het</sup> (*Aire*<sup>eGFP/WT</sup>) or *Aire*<sup>KO</sup> (*Aire*<sup>eGFP/eGFP</sup>) mTEC<sup>hi</sup> after 24h later. The  
653 histogram shows the frequencies of CD69<sup>+</sup> T<sub>reg</sub> normalized to the activation of OTII T<sub>reg</sub> co-cultured with *Aire*<sup>het</sup>  
654 mTEC<sup>hi</sup>.

655 **G** The expression level of *Il10*, *Tgfb1*, *Gzmb*, *Fasl*, *Lag3*, *Entpd1* and *Nt5e* was measured by qPCR in splenic OTII  
656 T<sub>reg</sub> from Rip-mOVA x OTII x *Rag2*<sup>KO</sup> mice co-cultured with OVA<sub>323-339</sub>-loaded *Aire*<sup>het</sup> (*Aire*<sup>eGFP/WT</sup>; n=6) or  
657 *Aire*<sup>KO</sup> (*Aire*<sup>eGFP/eGFP</sup>; n=6) mTEC<sup>hi</sup>.

658 Data are derived from 2 independent experiments. Bar graphs show mean ± SEM, \*p < 0.05, \*\*p < 0.01,  
659 \*\*\*p < 0.001 and \*\*\*\*p < 0.0001 using two-tailed Mann-Whitney test for (**A, C, D, F, G**).

660



661 **Fig. S1 Gating strategy used to purify recirculating CCR6<sup>+</sup> T<sub>reg</sub> in the thymus.**

662 CD4<sup>+</sup>CD25<sup>+</sup> cells were identified in CCR6<sup>+</sup>CD4<sup>+</sup> T cells and analyzed for Foxp3 expression by flow cytometry.

663 **Fig. S2 The suppressive signature of thymic CCR6<sup>+</sup> T<sub>reg</sub> from 1-year-old *Aire*<sup>KO</sup> mice is altered.**

664 The expression level of *Foxp3*, *Klrg1*, *Il10*, *Gzmb*, *Fasl*, *Entpd1* and *Nt5e* was measured by qPCR in thymic  
665 CCR6<sup>+</sup> T<sub>reg</sub> from 1-year-old *Aire*<sup>WT</sup> (n=5-6) and *Aire*<sup>KO</sup> (n=9) mice. Bar graphs show mean ± SEM, ns>0.05,  
666 \*\*\*p<0.001, \*\*\*\*p<0.0001 using two-tailed Mann-Whitney test.

667 **Fig. S3 Splenic *Aire*<sup>KO</sup> T<sub>reg</sub> show a normal suppressive signature throughout life.**

668 **A, B** Flow cytometry profiles, frequencies and numbers of CD4<sup>+</sup>Foxp3<sup>+</sup> T<sub>reg</sub> in the blood of 6 week- (**A**) and 1-  
669 year-old (**B**) *Aire*<sup>WT</sup> and *Aire*<sup>KO</sup> mice.

670 **C, D** Flow cytometry profiles, frequencies and numbers of CD4<sup>+</sup>Foxp3<sup>+</sup> T<sub>reg</sub> in the spleen of 6-week- (**C**) and 1-  
671 year- old (**D**) *Aire*<sup>WT</sup> or *Aire*<sup>KO</sup> mice. Data are derived from 2 independent experiments (n=2-5 mice per group and  
672 per experiment).

673 **E, F** The expression level of *Foxp3*, *Il10*, *Tgfb1*, *Gzmb*, *Fasl*, *Lag3*, *Entpd1* and *Nt5e* was measured by qPCR in  
674 splenic T<sub>reg</sub> from 6-week- (**E**) and 1-year- (**F**) old *Aire*<sup>WT</sup> (n=4-9 for 6 wk and n=8-13 for 1 yr) and *Aire*<sup>KO</sup> (n=5-9  
675 for 6 wk and n=8-13 for 1 yr) mice. Bar graphs show mean ± SEM, \*p<0.05 and \*\*p<0.01 using unpaired Student's  
676 *t* test for (**C, D**).

677 **Fig. S4 The adoptive transfer of thymic CCR6<sup>+</sup> T<sub>reg</sub> from *Aire*<sup>KO</sup> mice fail to attenuate peripheral tissue**  
678 **infiltration.**

679 **A** Gating strategy used to sort CCR6<sup>+</sup>CD4<sup>+</sup>CD8<sup>-</sup>CD25<sup>+</sup> cells, corresponding to CCR6<sup>+</sup> T<sub>reg</sub> from the thymus of 6-  
680 week-old *Aire*<sup>WT</sup> and *Aire*<sup>KO</sup> mice.

681 **B** Flow cytometry profiles and numbers of CD45.2 donor T<sub>reg</sub> in inguinal lymph nodes.

682 **C** Flow cytometry profiles, frequencies and numbers of CD45.1 infiltrating cells in the pancreas, eyes and salivary  
683 glands.

684 **D** Flow cytometry profiles and numbers of CD4<sup>+</sup> and CD8<sup>+</sup> T cells of CD45.1 origin infiltrating the pancreas, eyes  
685 and salivary glands. Data are derived from 3 independent experiments (n=2-5 mice per group and per experiment).

686 Bar graphs show mean  $\pm$  SEM, \* $p$ <0.05, \*\* $p$ <0.01, \*\*\* $p$ <0.001 and \*\*\*\* $p$ <0.0001 using two-tailed Mann-  
687 Whitney test for (B) or using unpaired Student's  $t$  test for (C,D).

688 **Fig. S5 *Aire* expression in hematopoietic cells does not control the recirculation and suppressive signature**  
689 **of thymic CCR6<sup>+</sup> T<sub>reg</sub>.**

690 **A** Experimental setup: Lethally irradiated CD45.1/2 WT recipients were reconstituted with CD45.2 *Aire*<sup>WT</sup> or  
691 *Aire*<sup>KO</sup> BM cells. Six weeks later, the recirculation and the suppressive signature of thymic CCR6<sup>+</sup> T<sub>reg</sub> of CD45.2  
692 origin were analyzed by flow cytometry and qPCR, respectively.

693 **B,C** Flow cytometry profiles, frequencies and numbers of total B220<sup>+</sup>CD19<sup>+</sup> B cells (**B**) and of IgD<sup>-</sup> or IgD<sup>+</sup> cells  
694 in B220<sup>+</sup>CD19<sup>+</sup> B cells (**C**).

695 **D,E** Flow cytometry profiles, frequencies and numbers of CD25<sup>+</sup> T<sub>reg</sub>P, Foxp3<sup>lo</sup> T<sub>reg</sub>P and CD25<sup>+</sup>Foxp3<sup>+</sup> T<sub>reg</sub> (**D**)  
696 as well as CCR6<sup>-</sup> and CCR6<sup>+</sup> cells in total CD25<sup>+</sup>Foxp3<sup>+</sup> T<sub>reg</sub> (**E**).

697 **F** The expression level of *Foxp3*, *Klrg1*, *Il10*, *Gzmb*, *Fasl*, *Lag3*, *Entpd1* and *Nt5e* was measured by qPCR in  
698 CCR6<sup>+</sup> T<sub>reg</sub> of CD45.2 origin purified from the thymus of *Aire*<sup>WT</sup> (n=8) and *Aire*<sup>KO</sup> (n=8) BM chimeric mice. Data  
699 are derived from 2 independent experiments (n=4 mice per group and per experiment). Bar graphs show  
700 mean  $\pm$  SEM, \* $p$ <0.05 using two-tailed Mann-Whitney test.

701 **Fig. S6 Gating strategy used to purify *Aire*<sup>+</sup> mTEC<sup>hi</sup>.**

702 *Aire*<sup>+</sup> mTEC<sup>hi</sup> were identified as EpCAM<sup>+</sup>Ly51<sup>-/lo</sup>CD80<sup>+</sup>*Aire*<sup>eGFP</sup> cells and purified from *Aire*<sup>het</sup> (*Aire*<sup>eGFP/WT</sup>) and  
703 *Aire*<sup>KO</sup> (*Aire*<sup>eGFP/eGFP</sup>) mice.

704 **Fig. S7 *Aire*<sup>KO</sup> mTEC express reduced levels of OX40L and GITRL.**

705 **A** Representative flow cytometry profiles of MHCII in mTEC from *Aire*<sup>WT</sup> and *Aire*<sup>KO</sup> mice. The histogram shows  
706 the frequency of MCHII<sup>+</sup> mTEC. Bar graphs show mean  $\pm$  SEM, \*\* $p$ <0.001 using two-tailed Mann-Whitney.

707 **B-C** *Aire*<sup>KO</sup> mTEC express reduced levels of OX40L and GITRL. Expression levels of *Tnfsf4* (OX40L) and *Tnfsf18*  
708 (GITRL) measured by RNA-seq (**B**) and flow cytometry (**C**) in *Aire*<sup>WT</sup> and *Aire*<sup>KO</sup> mTEC<sup>hi</sup>. Bar graphs show  
709 mean  $\pm$  SEM, \*\* $p$ <0.01 using two-tailed Mann-Whitney test.

710

711 **Table S1. FPKM values of RNA-seq data derived from thymic CCR6<sup>+</sup> Treg from *Aire*<sup>WT</sup> and *Aire*<sup>KO</sup> mice.**

712 **Table S2. List of antibodies used for flow cytometry.**

713 **Table S3. List of primers used for RT-qPCR.**

714 **Movie S1. 3D rotation of *Aire*<sup>WT</sup> thymic lobe of 9-day-old mouse, with medullary compartment colored**  
715 **according to their volume.**

716 *Aire*<sup>WT</sup> thymic lobe (DAPI, blue) of a 9-day-old mouse is rendered in 3D with medullary compartments (Keratin  
717 14, pseudo-colors) encoded according to medullary volumes from cyan (smallest medullae) to magenta (largest  
718 medullae). All axes are graduated with 500- $\mu$ m grid spacing.

719 **Movie S2. 3D rotation of *Aire*<sup>KO</sup> thymic lobe of 9-day-old mouse, with medullary compartment colored**  
720 **according to their volume.**

721 *Aire*<sup>KO</sup> thymic lobe (DAPI, blue) of a 9-day-old mouse is rendered in 3D with medullary compartments (Keratin  
722 14, pseudo-colors) encoded according to medullary volumes from cyan (smallest medullae) to magenta (largest  
723 medullae). All axes are graduated with 500- $\mu$ m grid spacing.

724 **Movie S3. 3D rotation of *Aire*<sup>WT</sup> thymic lobe of 6-week-old mouse, with medullary compartment colored**  
725 **according to their volume.**

726 *Aire*<sup>WT</sup> thymic lobe (DAPI, blue) of a 6-week-old mouse is rendered in 3D with medullary compartments (Keratin  
727 14, pseudo-colors) encoded according to medullary volumes from cyan (smallest medullae) to magenta (largest  
728 medullae). All axes are graduated with 500- $\mu$ m grid spacing.

729 **Movie S4. 3D rotation of *Aire*<sup>KO</sup> thymic lobe of 6-week-old mouse, with medullary compartment colored**  
730 **according to their volume.**

731 *Aire*<sup>KO</sup> thymic lobe (DAPI, blue) of a 6-week-old mouse is rendered in 3D with medullary compartments (Keratin  
732 14, pseudo-colors) encoded according to medullary volumes from cyan (smallest medullae) to magenta (largest  
733 medullae). All axes are graduated with 500- $\mu$ m grid spacing.

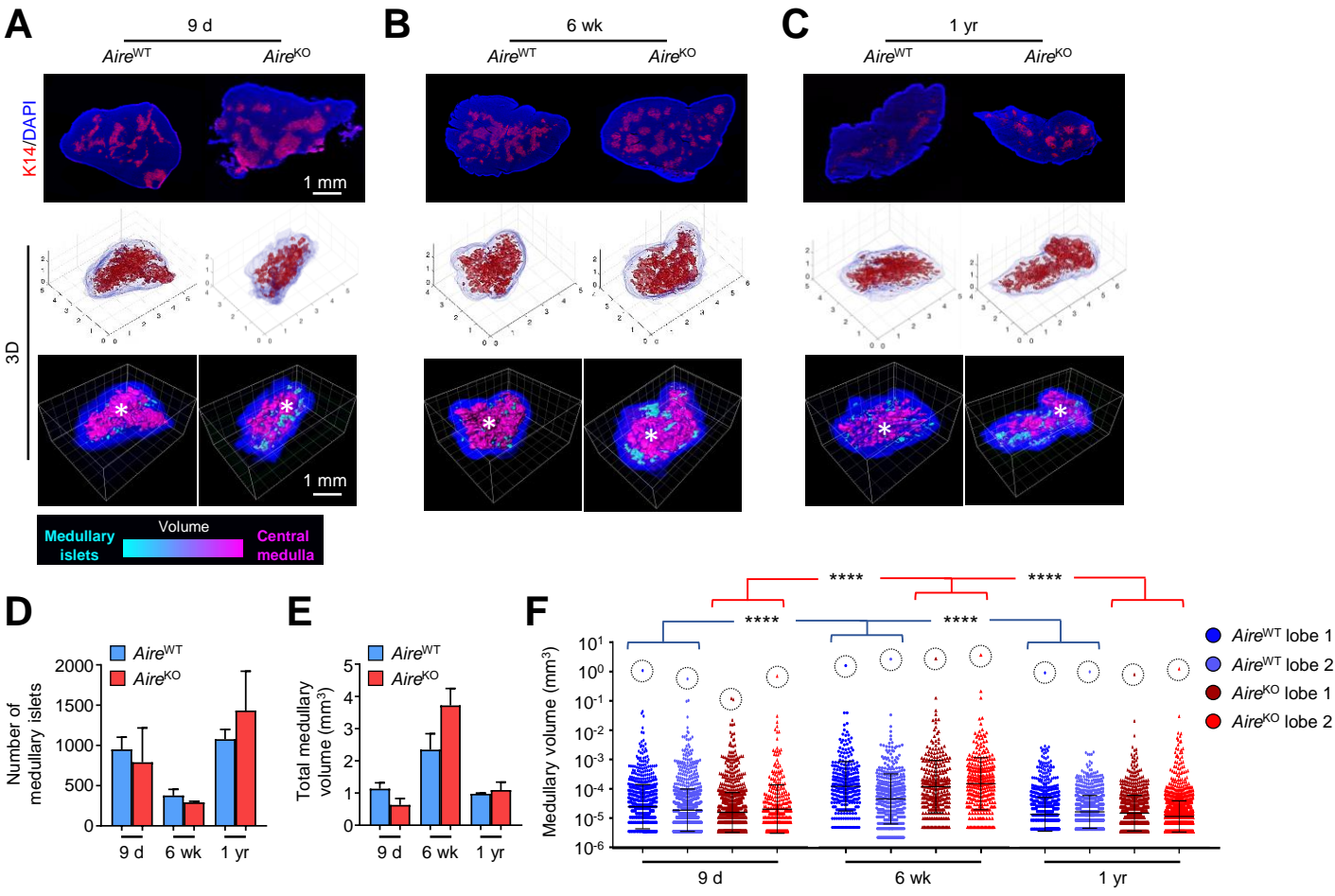
734 **Movie S5. 3D rotation of *Aire*<sup>WT</sup> thymic lobe of 1-year-old mouse, with medullary compartment colored**  
735 **according to their volume.**

736 *Aire*<sup>WT</sup> thymic lobe (DAPI, blue) of a 1-year-old mouse is rendered in 3D with medullary compartments (Keratin  
737 14, pseudo-colors) encoded according to medullary volumes from cyan (smallest medullae) to magenta (largest  
738 medullae). All axes are graduated with 500- $\mu$ m grid spacing.

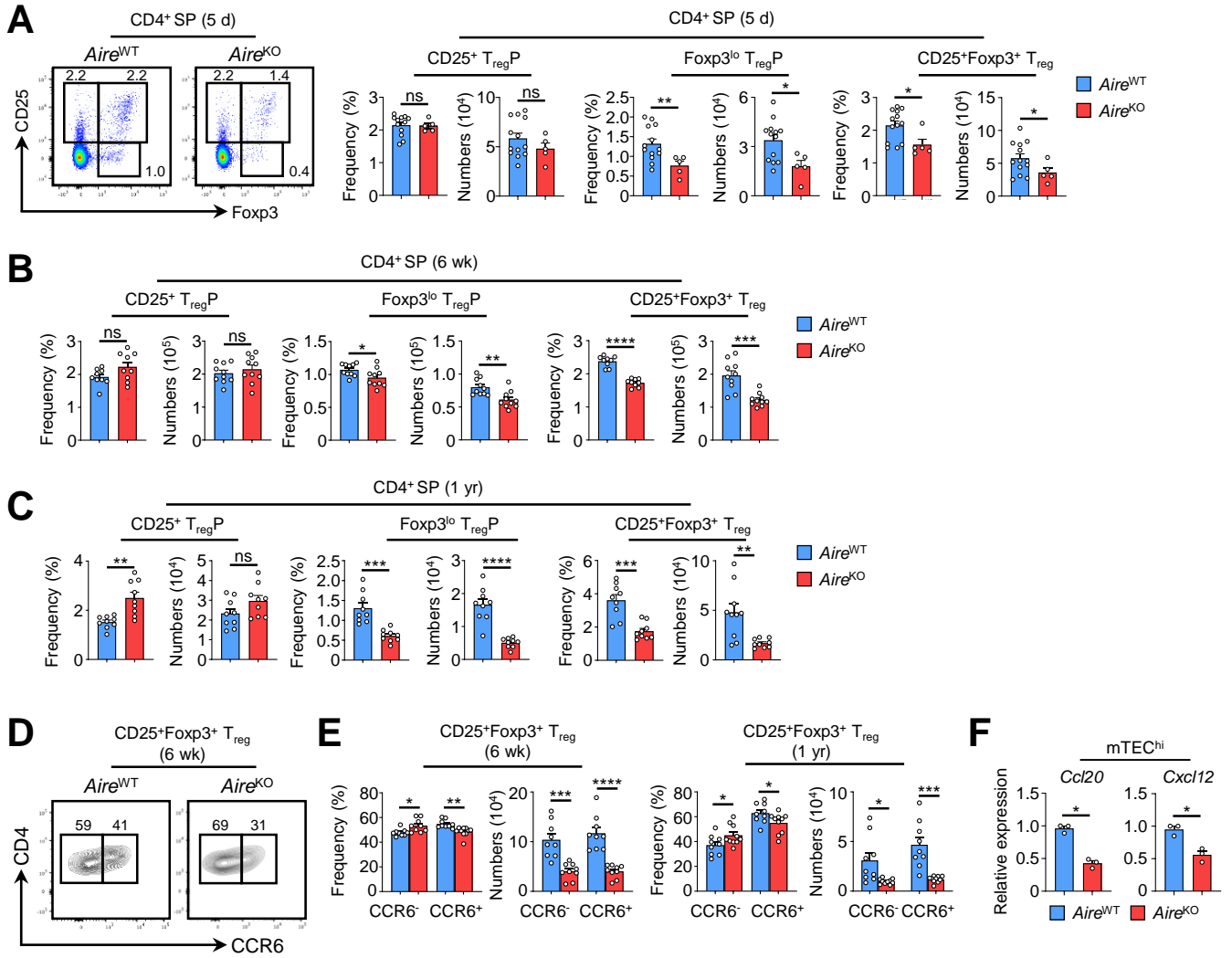
739 **Movie S6. 3D rotation of *Aire*<sup>KO</sup> thymic lobe of 1-year-old mouse, with medullary compartment colored**  
740 **according to their volume.**

741 *Aire*<sup>KO</sup> thymic lobe (DAPI, blue) of a 1-year-old mouse is rendered in 3D with medullary compartments (Keratin  
742 14, pseudo-colors) encoded according to medullary volumes from cyan (smallest medullae) to magenta (largest  
743 medullae). All axes are graduated with 500- $\mu$ m grid spacing.

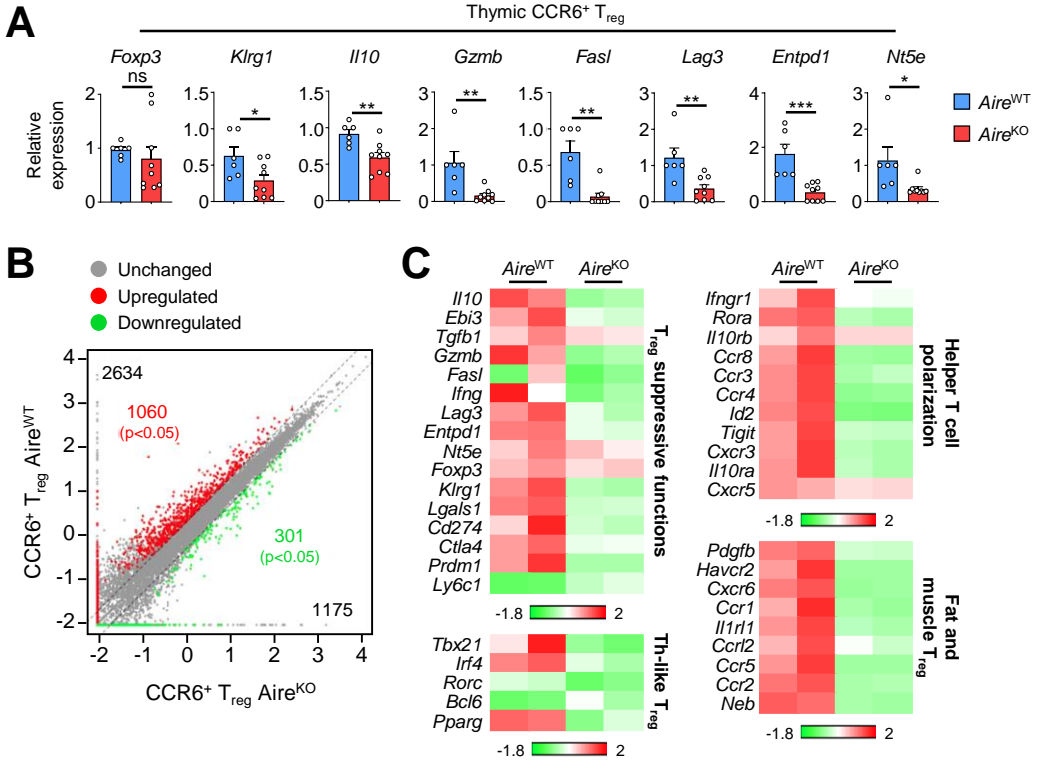
# Figure 1



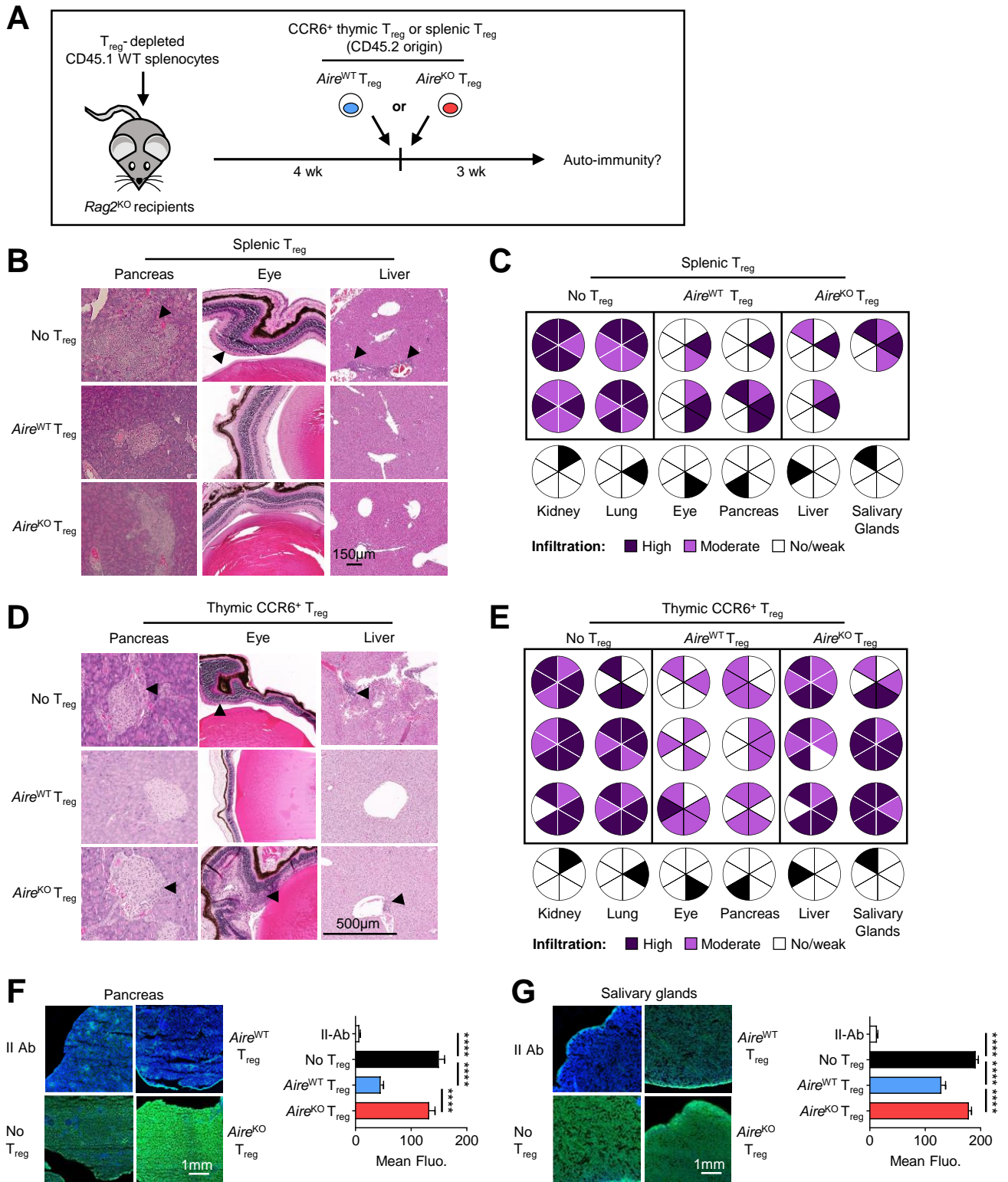
# Figure 2



**Figure 3**

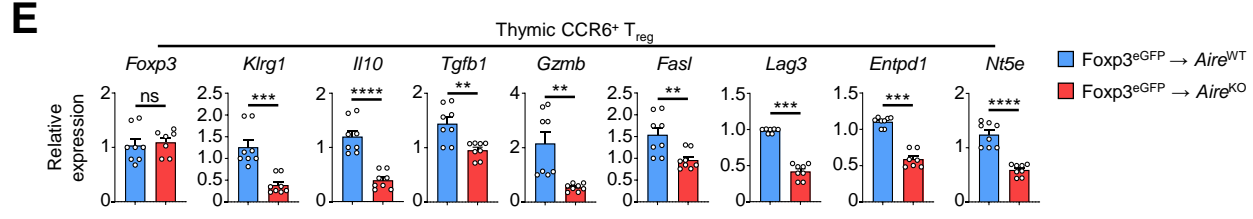
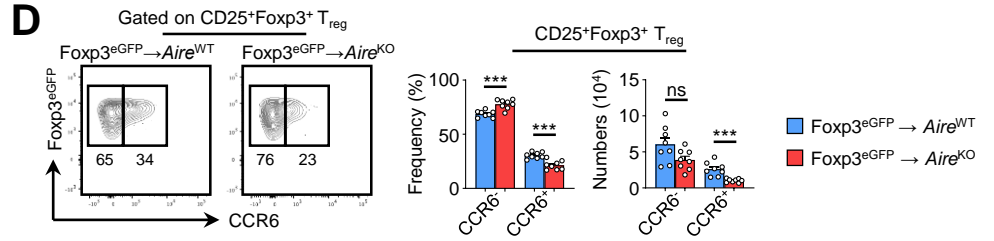
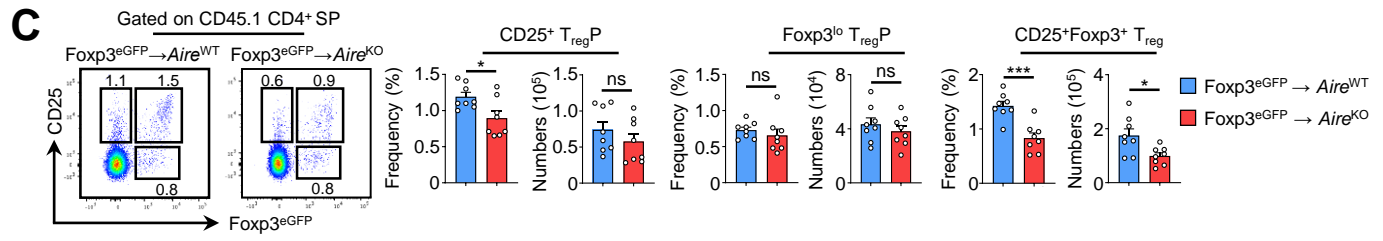
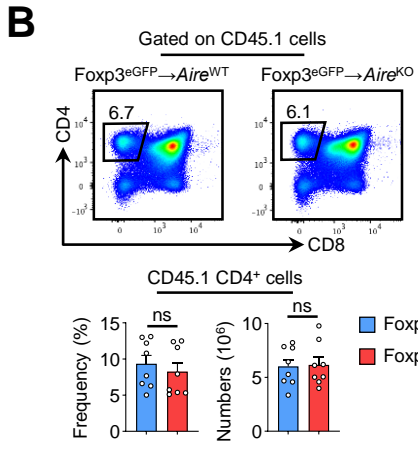
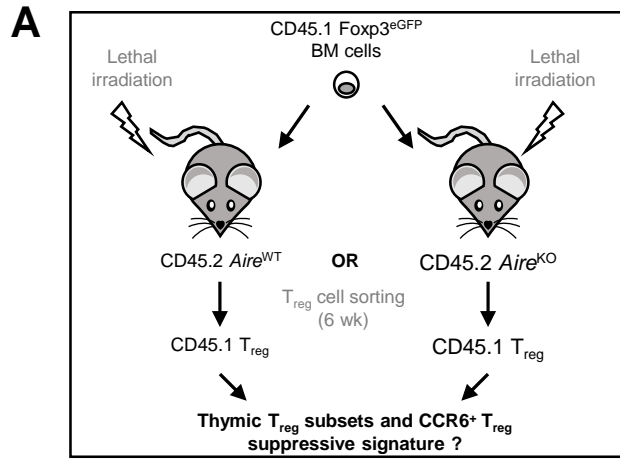


# Figure 4

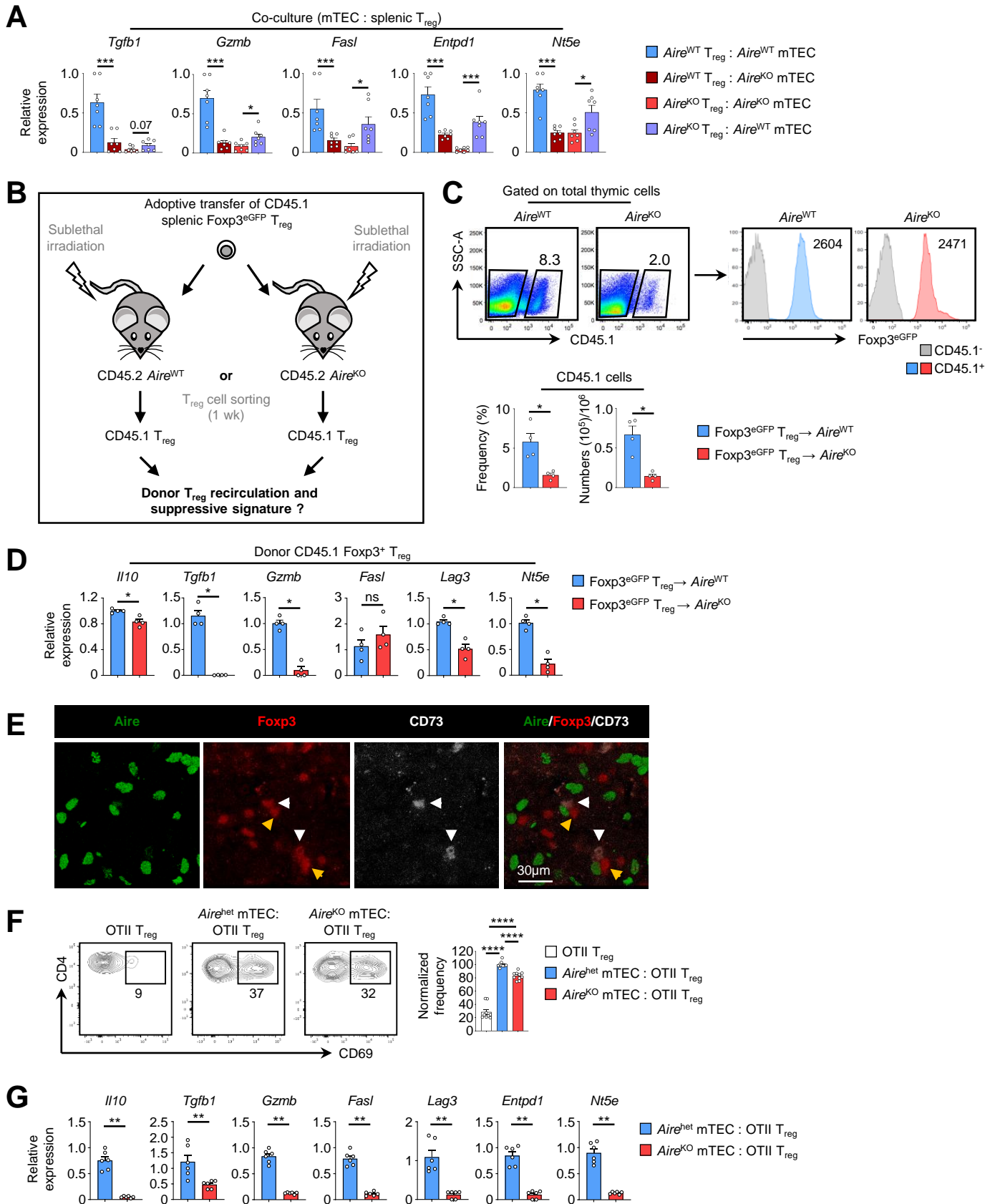




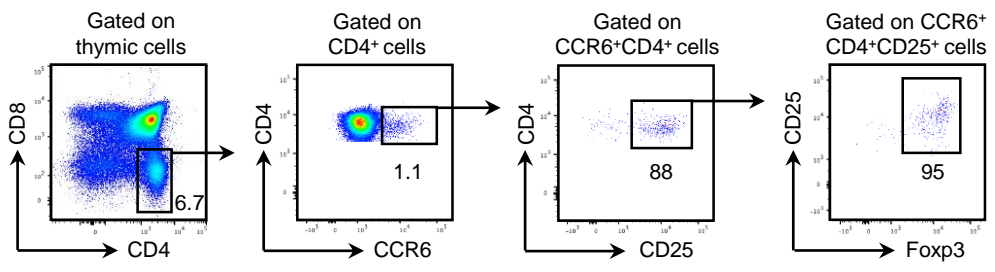
# Figure 5



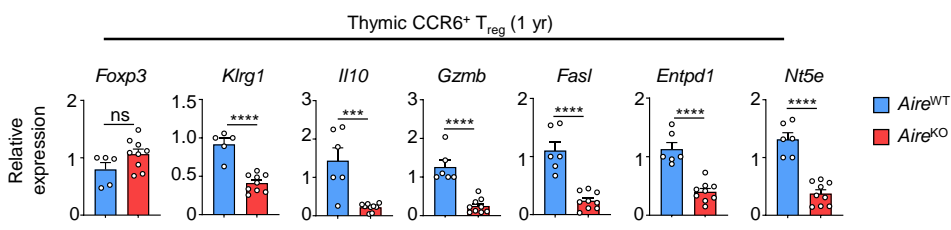
# Figure 6

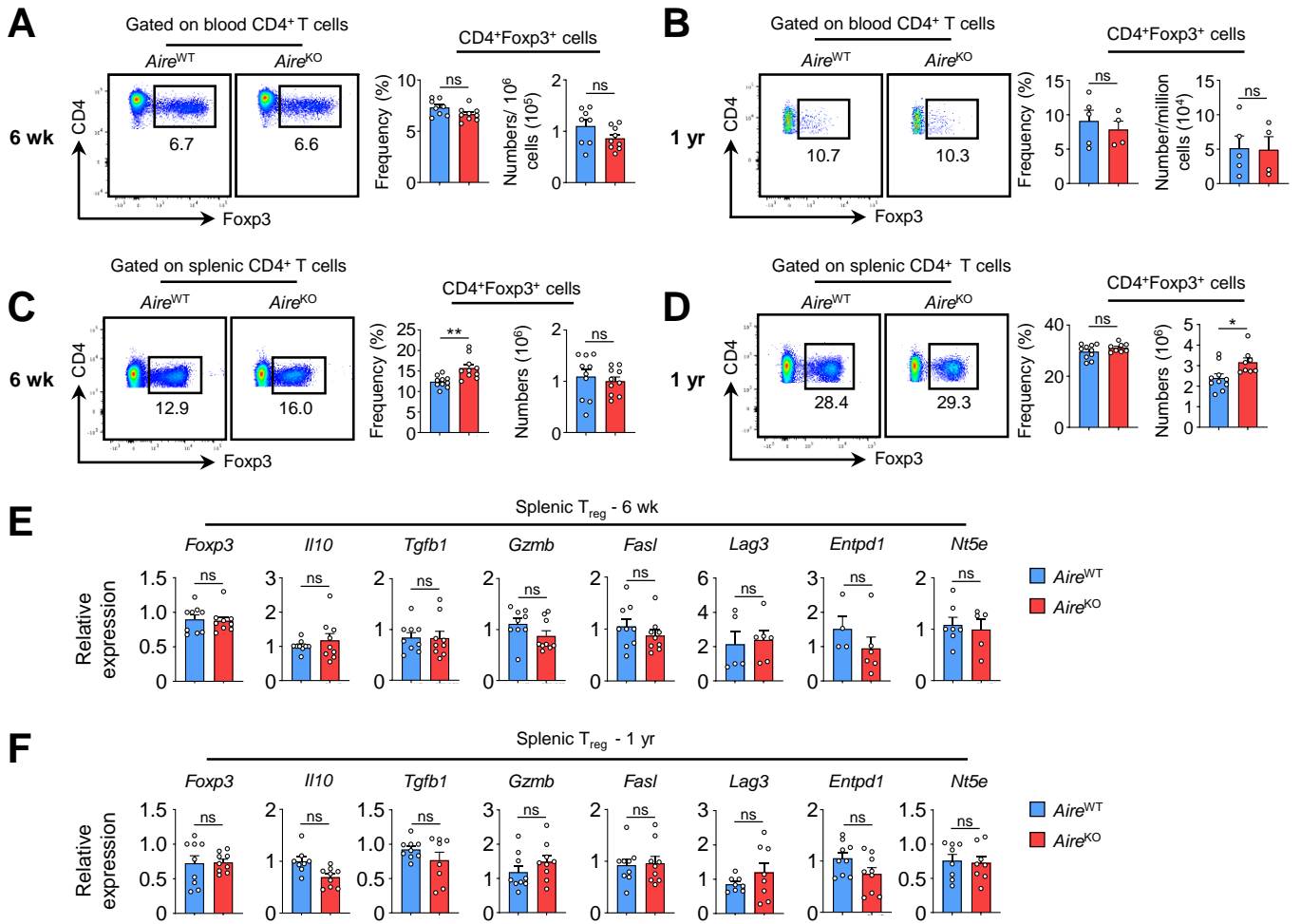


**Fig. S1**

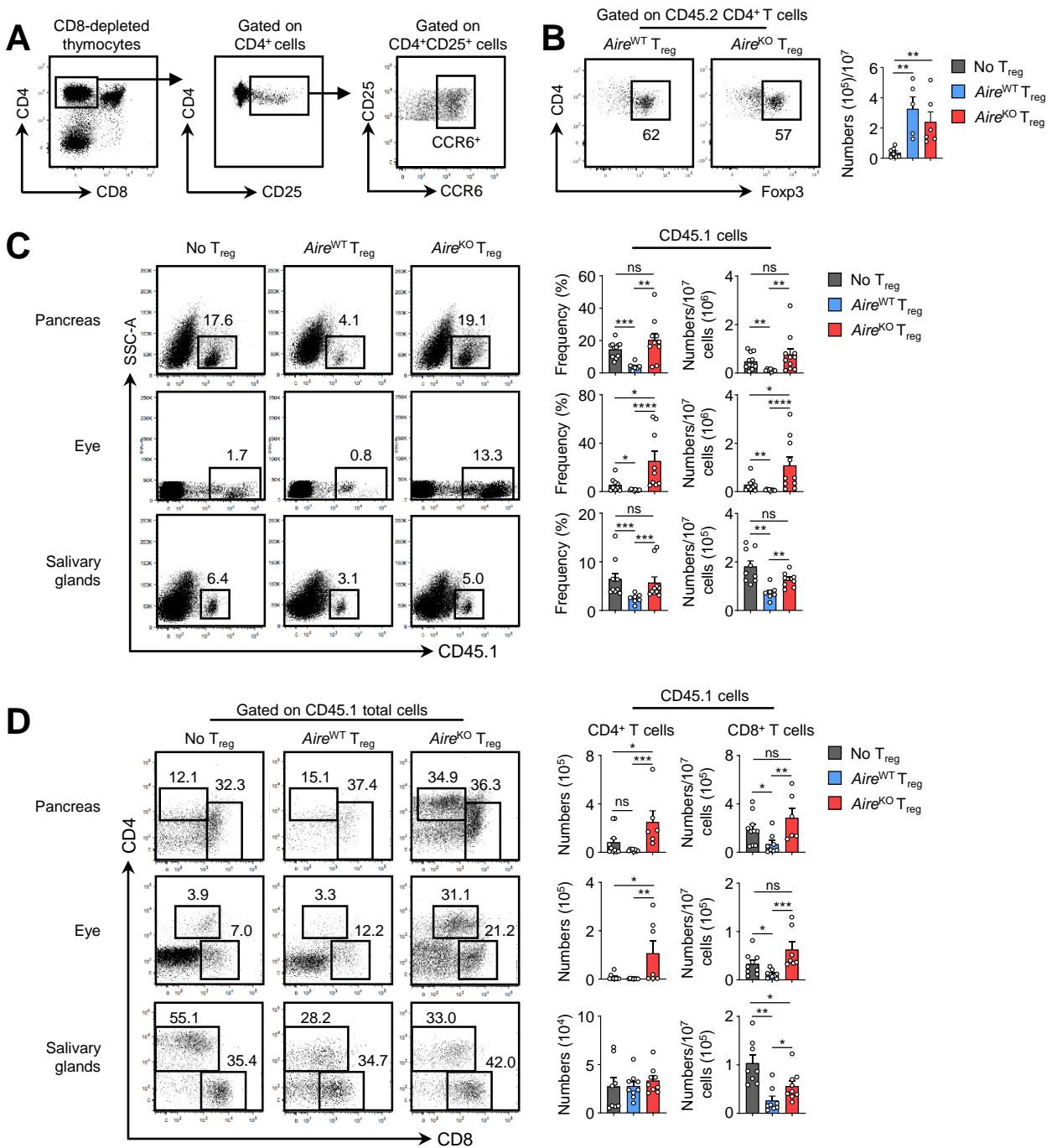


**Fig. S2**



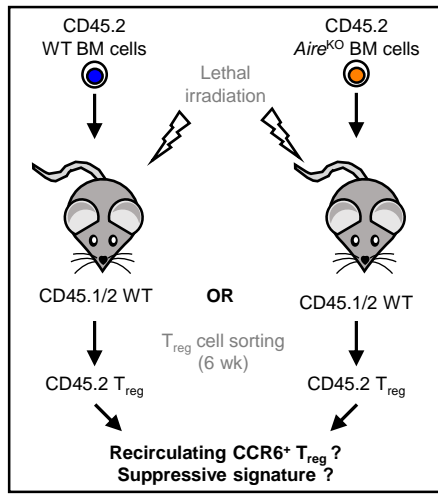
**Fig. S3**

**Fig. S4**

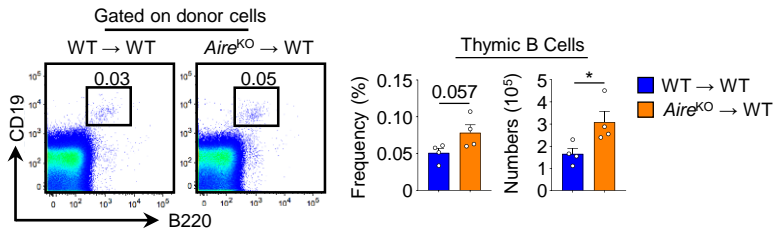


# Fig. S5

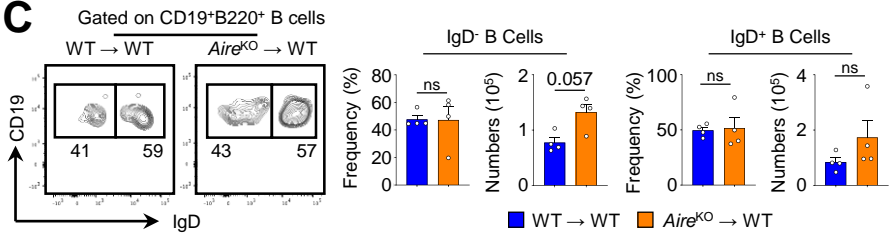
## A



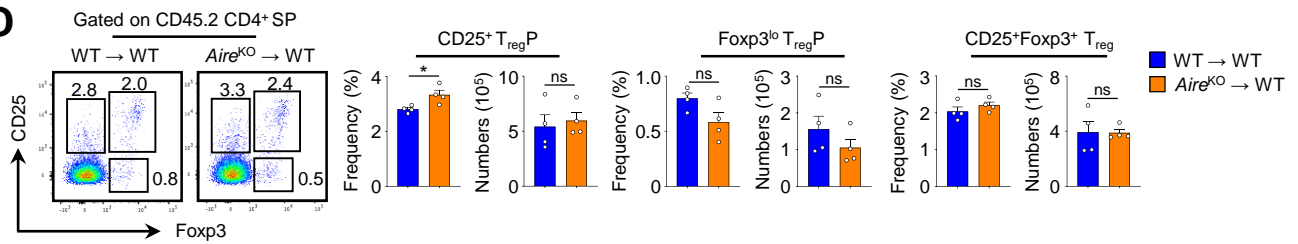
## B



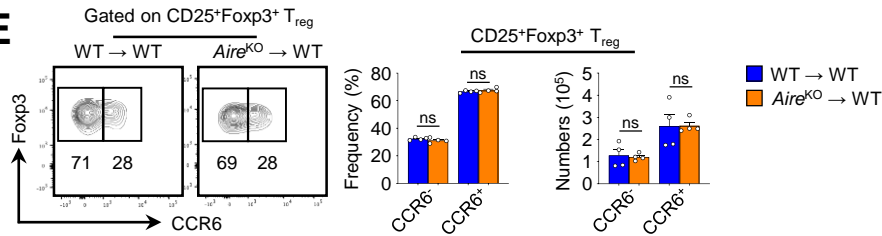
## C



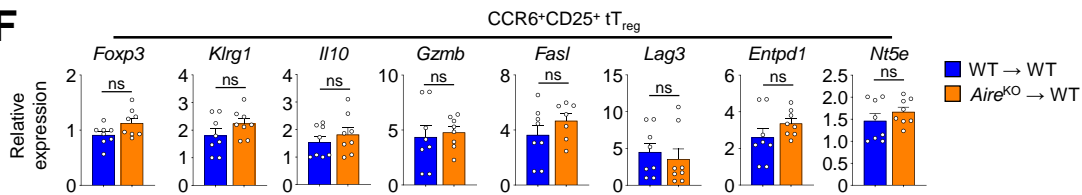
## D



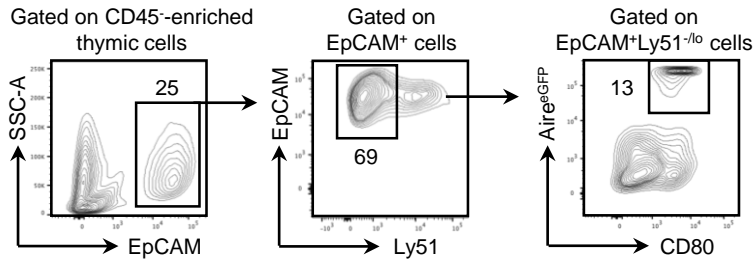
## E



## F

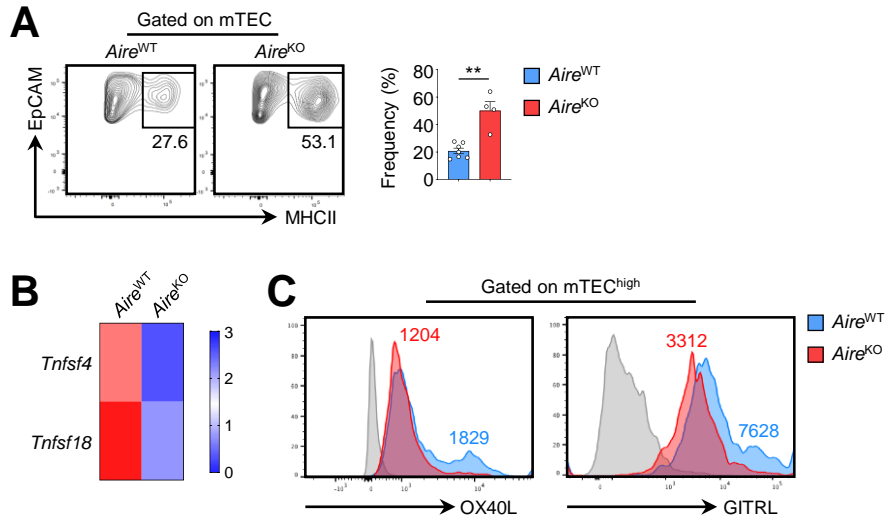


**Fig. S6**





**Fig. S7**



**Table S1**

<b>Gene</b>	<b><i>Aire</i><sup>WT</sup> mice</b>	<b><i>Aire</i><sup>KO</sup> mice</b>
<i>Il10</i>	3.09 ± 0.44	0.31 ± 0.19
<i>Gzmb</i>	25.57 ± 6.90	4.83 ± 1.84
<i>Fasl</i>	1.35 ± 0.66	0.92 ± 0.15
<i>Lag3</i>	4.29 ± 0.53	2.07 ± 0.45
<i>Entpd1</i>	10.57 ± 0.19	5.43 ± 0.38
<i>Nt5e</i>	69.74 ± 11.42	59.41 ± 7.12
<i>Prdm1</i>	18.78 ± 3.56	2.84 ± 0.06
<i>Klrg1</i>	60.16 ± 9.26	12.96 ± 2.67
<i>Tigit</i>	77.14 ± 16.33	23.54 ± 1.83
<i>Ctla4</i>	259.10 ± 19.43	156.50 ± 2.15
<i>Lgals1</i>	421.5 ± 20.12	244.0 ± 3.59
<i>Tbx21</i>	5.22 ± 1.64	2.2 ± 0.32
<i>Irf4</i>	20.71 ± 1.10	13.22 ± 1.40
<i>Pparg</i>	2.54 ± 0.09	0.87 ± 0.39
<i>Id2</i>	192.5 ± 25.10	34.59 ± 2.99
<i>Cxcr3</i>	58.30 ± 14.08	14.94 ± 2.71
<i>Ccr4</i>	35.47 ± 5.00	11.08 ± 0.12
<i>Ccr8</i>	256.6 ± 35.04	136.7 ± 3.35
<i>Ccr1</i>	3.67 ± 1.31	0.34 ± 0.13
<i>Ccr2</i>	39.94 ± 2.88	7.26 ± 0.64

**Table S2**

<b>Antibody</b>	<b>Clone</b>	<b>Fluorochrome</b>	<b>Supplier</b>	<b>Dilution</b>
B220	RA3-6B2	PerCP	Biolegend	1/200
CD45.1	A20	BV421	Biolegend	1/600
CD45.1	A20	PerCP Cy5.5	BD Biosciences	1/200
CD45.2	104	AF488	Biolegend	1/200
CD4	RM4.5	FITC	BD Biosciences	1/200
CD4	RM4.5	APC	Biolegend	1/200
CD4	GK1.5	APC Cy7	Invitrogen	1/200
CD4	RM4.5	BV421	BD Biosciences	1/200
CD8 $\alpha$	53.6.7	PeCy7	BD Biosciences	1/600
CD8 $\alpha$	53.6.7	PB	BD Biosciences	1/200
CD25	PC61	PerCP Cy5.5	BD Biosciences	1/200
CD25	PC61	APC	Biolegend	1/200
CD25	PC61	PeCy7	Biolegend	1/400
CCR6	29-2L17	PE	Biolegend	1/300
CCR6	29-2L17	PerCP Cy5.5	Biolegend	1/200
CD44	IM7	PerCP Cy5.5	eBioscience	1/200
CD19	1D3	PeCy7	BD Biosciences	1/300
CD69	H1.2F3	PE	Biolegend	1/300
IA/IE	M5/114.15.2	BV605	BD Biosciences	1/1000
GITRL	YGL386	PE	Biolegend	1/200
OX40L	RM134L	APC	eBioscience	1/200
CD80	16-10A1	PerCP Cy5.5	Biolegend	1/200
EpCAM	G8.8	PeCy7	Biolegend	1/3000
Foxp3	FKJ-16s	PE	eBioscience	1/200
Foxp3	FKJ-16s	APC	Invitrogen	1/200
Foxp3	FKJ-16s	AF488	Invitrogen	1/150
IgD	11-26c.2a	PE	BD Biosciences	1/300
Ly51	6C3	PE	Biolegend	1/3000
UEA1		FITC	Vector Laboratories	1/800

**Table S3**

Primers	Sequence	T <sub>m</sub> (°C)	Amplicon size (bp)	Accession number
<i>Actin</i> - Forward	CAGAAGGAGATTACTGCTCTGGCT	58.2	93	NM_007393.5
<i>Actin</i> - Reverse	GGAGCCACCGATCCACACA	59.9		
<i>Foxp3</i> - Forward	CCCACCTACAGGCCCTTCTC	61.1	71	NM_001199347.1 NM_001199348.1 NM_054039.2
<i>Foxp3</i> - Reverse	GGCATGGGCATCCACAGT	58.4		
<i>Klrg1</i> - Forward	CGAGGAATGGTAGCCACTGTTAC	57.3	163	NM_016970
<i>Klrg1</i> - Reverse	CCGATCCAGTAAAAGTCCTGACC	57.1		
<i>Il10</i> - Forward	GAATTCCCTGGGTGAGAAGC	55.7	105	NM_010548.2
<i>Il10</i> - Reverse	CTCTTCACCTGCTCCACTGC	58.1		
<i>Gzmb</i> - Forward	GAAAACAATGAAAAGCAGCTAACTACA	56.9	69	NM_013542.3
<i>Gzmb</i> - Reverse	TCTAGGGACGATGGGTAATCAGA	56.3		
<i>Fasl</i> - Forward	TCAGTCTTGCAACAACCAGC	55.9	310	NM_001205243.1 NM_010177
<i>Fasl</i> - Reverse	AGACAATATTCCTGGTGCCC	54.7		
<i>Lag3</i> - Forward	CTGGGACTGCTTTGGGAAG	55.9	167	NM_008479.2
<i>Lag3</i> - Reverse	GGTTGATGTTGCCAGATAACCC	56.2		
<i>Entpd1</i> - Forward	TACCACCCCATCTGGTCATT	55.7	168	NM_001304721.1 NM_009848.4
<i>Entpd1</i> - Reverse	GGACGTTTTGTTGGTTGGT	56.0		
<i>Nt5e</i> - Forward	CAAATCCCACACAACCACTG	54.3	158	NM_011851.4
<i>Nt5e</i> - Reverse	TGCTCACTTGGTCACAGGAC	56.9		
<i>Tgfb1</i> - Forward	CAGACATTCGGGAAGCAGTG	57.9	145	NM_011577.2
<i>Tgfb1</i> - Reverse	AGCCGGTTACCAAGGTAACG	57.3		