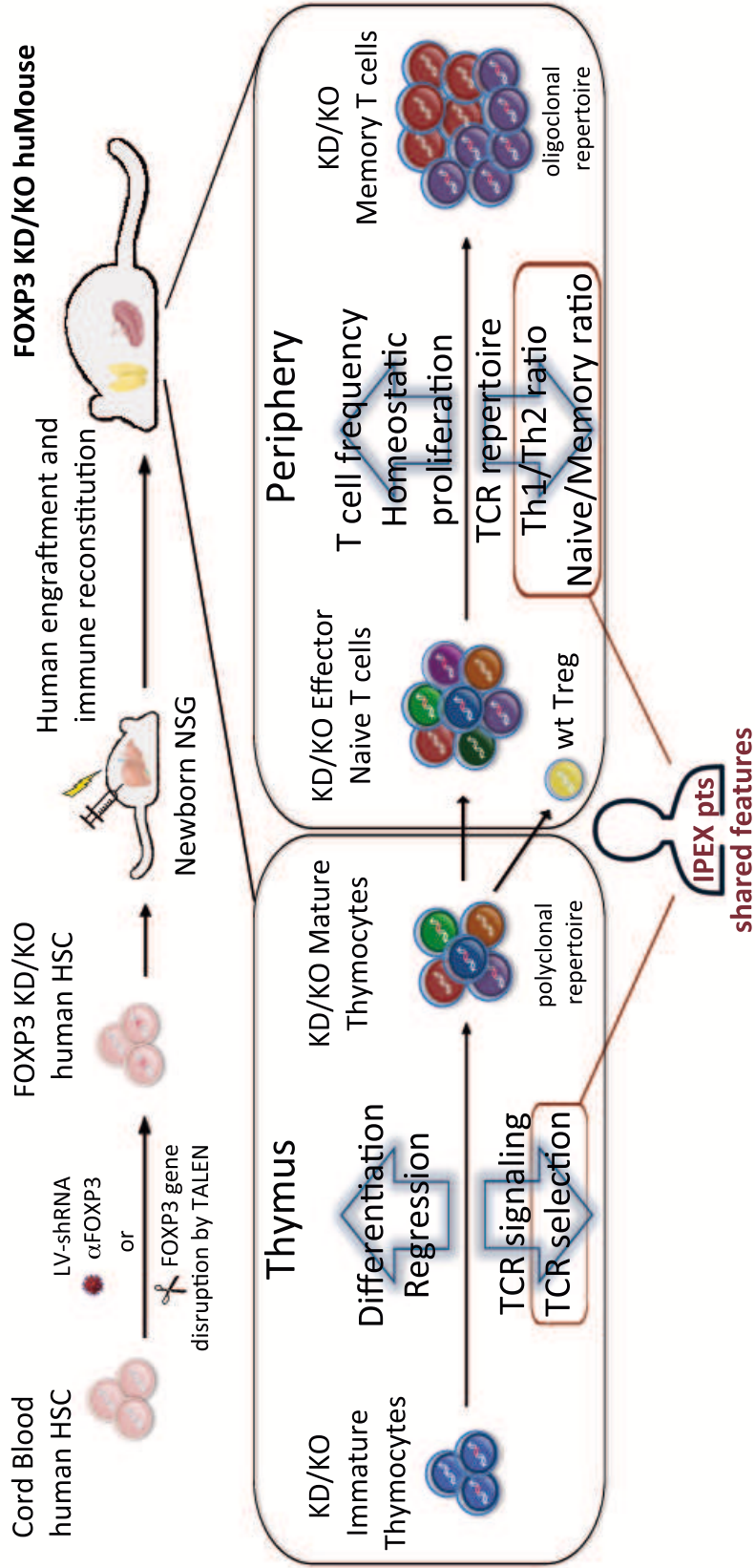


Role of human FOXP3 in early thymic maturation and peripheral T cell homeostasis



**1 Role of human FOXP3 in early thymic maturation and peripheral T cell**  
**2 homeostasis.**

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**ABSTRACT**

*Background.* FOXP3 is a key transcription factor in regulatory T cell (Treg) function. FOXP3 gene mutations cause IPEX, a fatal autoimmune syndrome. FOXP3 has also been proposed to act in effector T cells (Teff) but to date this role has not been confirmed.

*Objective.* To evaluate the effect of reduced FOXP3 expression on human Treg and Teff cell development and correlate it with the IPEX immune pathology.

*Methods.* We developed a humanized mouse model in which the human hematopoietic system is stably knocked down (KD) or knocked out (KO) for the FOXP3 gene (KD/KO huMice).

*Results.* Since FOXP3-KD/KO was not 100% effective, residual FOXP3 expression in HSPCs was sufficient to give rise to Tregs with normal expression of FOXP3. However, numerous defects in the Teff compartment appeared. Compared to controls, FOXP3-KD/KO huMice showed altered thymocyte differentiation, with KD/KO thymocytes displaying significantly reduced TCR signalling strength and increased TCR repertoire diversity. Peripheral KD/KO Teff cells were expanded and showed signs of homeostatic proliferation, such as significantly contracted TCR repertoire, severely reduced naïve compartment, decreased TERF2 expression, and a skew towards a Th2 profile, resembling an aged immune system. Consistent with FOXP3-KD/KO huMice results, analysis of IPEX patients provided evidences of defects in the Teff compartment at both thymic and peripheral levels.

*Conclusions.* These findings support an intrinsic role for human FOXP3 in controlling thymocyte maturation and peripheral expansion of Teff cells and reveal a previously not described pathogenic mechanism, through an altered Teff compartment, in IPEX syndrome.

**56 KEY MESSAGES**

57 FOXP3 controls human thymocyte development by altering TCR signaling strength  
58 and impinges peripheral T cell expansion.

59 FOXP3 plays an intrinsic role in the control of human effector T cells that accounts  
60 for the alterations found in the peripheral T cell compartment in IPEX patients.

61

**62 CAPSULE SUMMARY**

63 We identified a novel role for FOXP3 as intrinsic regulator of early maturation and  
64 peripheral homeostasis of effector T cells, which may contribute to IPEX syndrome  
65 pathology.

66

**67 KEY WORDS**

68 FOXP3, effector T cells, regulatory T cells, immune tolerance, humanized mice, T  
69 cell differentiation, IPEX syndrome, autoimmunity.

70 **ABBREVIATIONS**

|        |                                                                |
|--------|----------------------------------------------------------------|
| APECED | Autoimmune polyendocrinopathy candidiasis ectodermal dystrophy |
| AU     | Arbitrary Units                                                |
| BM     | Bone Marrow                                                    |
| CM     | Central Memory                                                 |
| DN     | Double Negative                                                |
| DP     | Double Positive                                                |
| EM     | Effector Memory                                                |
| FOXP3  | Forkhead-box P3                                                |
| HD     | Healthy Donor                                                  |
| HSPC   | Hematopoietic Stem Progenitor Cells                            |
| huMice | humanized mice                                                 |
| IPEX   | Immune dysregulation Polyendocrinopathy Enteropathy X-linked   |
| KD     | Knocked Down                                                   |
| KO     | Knocked Out                                                    |
| LV     | Lentiviral Vector                                              |
| NHEJ   | Non-Homologous End Joining                                     |
| NPC    | Non Parametric Combination                                     |
| NSG    | NOD/SCID IL2 $\gamma$ <sup>-/-</sup>                           |
| PB     | Peripheral Blood                                               |
| SP4    | Single Positive CD4 <sup>+</sup>                               |
| SP8    | Single Positive CD8 <sup>+</sup>                               |
| TALEN  | Transcription Activator-Like ENdonucelase                      |
| Teff   | T effector                                                     |
| TEMRA  | Terminally differentiated Effector Memory RA <sup>+</sup>      |
| TERF2  | Telomeric Repeat-binding Factor 2                              |
| TREC   | T cell Receptor Excised Circles                                |
| Treg   | T regulatory                                                   |
| TSDR   | Treg Specific Demethylated Region                              |
| TSS    | Transcriptional Start Site                                     |

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## INTRODUCTION

Active peripheral immune tolerance is maintained by the regulated interplay between effector (Teff) and regulatory (Treg) T cells. Amongst the latter, CD4<sup>+</sup>CD25<sup>+</sup>FOXP3<sup>+</sup> Tregs play a major role in dampening immune response and controlling auto-reactive clones escaped from thymic negative selection. The pivotal role of FOXP3 in Treg function is demonstrated by the severe autoimmune phenotype of the natural FOXP3 mutant mouse model (*Scurfy*) and by the early onset fatal polyautoimmune Immunodysregulation Polyendocrinopathy Enteropathy X-linked (IPEX) syndrome in patients with *FOXP3* mutations<sup>1-3</sup>. Moreover, in recent years, monogenic autoimmunity has been increasingly diagnosed in children and several genes identified that, when mutated, alter the Teff/Treg balance resulting in severe immunodysregulation with heterogeneous phenotypes, only partially understood pathogenesis and lack of targeted treatments<sup>4,5</sup>.

We previously showed that *FOXP3* mutations lead to Treg dysfunction but do not necessarily hamper the accumulation of circulating Tregs carrying *FOXP3* mutations in IPEX patients<sup>6,7</sup>. Nonetheless, in female carriers of *FOXP3* mutations, and in transplanted patients with low peripheral donor chimerism, only Tregs expressing wild type FOXP3 are detectable in peripheral blood, indicating a their functional competitive advantage over the mutated Tregs<sup>8,9</sup>. In the human system, FOXP3, whose highest expression is detected in Tregs, is also expressed in immature thymocytes at the early pre-double positive stage, where it may play a role in controlling the their differentiation<sup>10,11</sup>. Also, Teffs transiently express FOXP3 upon TCR-mediated activation, but only a few *in vitro* studies have investigated its function in this compartment<sup>12,13</sup>. Our *in vitro* studies on FOXP3 null Teff clones supported an intrinsic role for FOXP3 in controlling proliferation, cytokine production and TCR

97 signaling threshold in Teffs<sup>14</sup>. Another *in vitro* study has shown that FOXP3  
98 expression is associated with activation upon low antigen doses in Teffs<sup>15</sup>. However,  
99 an in-depth analysis of the intrinsic role of FOXP3 in the differentiation and function  
100 of Teffs has not been performed, limiting a full understanding of the role of FOXP3 in  
101 the development of autoimmunity.

102 This lack of knowledge on the role of FOXP3 during human Treg and Teff  
103 differentiation is likely also due to the absence of an *in vivo* model able to fully  
104 recapitulate human T cell differentiation. In the past ten years, several humanized  
105 mouse models (huMice), based on the transplantation of human hematopoietic stem  
106 progenitor cells (HSPC) into different strains of immunodeficient mice, have been  
107 developed, but showed limitations in the maturation of T cells<sup>16–18</sup>. Neonatal, instead  
108 of adult, transplantation of immunodeficient mice has been shown to improve thymic  
109 maturation up to functional Teffs and Tregs<sup>19,20</sup>. The use of this huMice model  
110 combined with genetic manipulation to alter protein expression in the transplanted  
111 HSPC, targeting immune disease causative genes, holds promise to unravel the  
112 function of these genes in different T cell subsets and developmental stages.

113 In the present work, we dissect the role of FOXP3 in human T cell development. We  
114 transplanted human HSPC genetically modified to reduce *FOXP3* gene expression  
115 into immunodeficient NOD/SCID Il2R $\gamma$ <sup>-/-</sup> (NSG) neonate mice and characterized the  
116 resulting human T cell compartments. Our findings indicate a role for FOXP3 in  
117 control of early Teff differentiation, likely by regulating TCR-signaling strength, and  
118 suggest an additional function of FOXP3 in the prevention of premature immune  
119 aging. These results elucidate the pathogenic mechanism played by the effector T cell  
120 compartment in IPEX syndrome and possibly in other autoimmune diseases,

121 indicating the importance of FOXP3 dependent control of TCR-signaling in forging  
122 the Teff compartment.

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## METHODS

See also supplementary methods for extended information.

### Cells and patients

Human cord blood CD34<sup>+</sup> (purity $\geq$ 95%) were purchased (Lonza) or purified by magnetic selection (Miltenyi Biotech) from healthy donors. Cord and peripheral blood were collected from healthy donors and patients after informed consent and approved protocols by Institutional Ethical Committee (TIGET02, TIGET06, PERIBLOOD), accordingly to Helsinki declaration. Clinical features of IPEX patients were described elsewhere (patients 11, 14 in<sup>7</sup>, patients 11, 14, 19, 21, 22, 24 in<sup>21</sup> and patient 25 in<sup>22</sup>). Polyautoimmune patients enrolment criteria: i) two autoimmune diseases+autoantibodies for another autoimmune disease and/or familial history positive for autoimmunity, or three autoimmune diseases, ii) not diagnosed for IPEX or APECED disease. Patients were enrolled at the Pediatric Unit/Allergology and Immunology Unit of San Raffaele Hospital, Milan and Pediatric Unit of Federico II Hospital, Naples, Italy. Detailed clinical information about human donors is described in Table S1.

### Gene modifications

HSPC were transduced in StemSpan (StemCell-Technologies) with hSCF, hTPO, hIL6 and hFlt3-L (Peprotech) at MOI 200, as previously described<sup>23</sup>. Lentiviral constructs were generated from LVamiR EF1a.(intronic)miR142siFOXP3+miR223siFOXP3. $\Delta$ LNGFR.Wpre and EF1a.(intronic)miR223siLuciferase. $\Delta$ LNGFR.Wpre vectors<sup>24</sup> by cloning the GFP instead of the  $\Delta$ LNGFR cDNA.

FOXP3 knockout was obtained by RNA transfection of TALENs designed to disrupt the transcriptional start site (TSS) of the gene (sequences provided upon request) in CD34<sup>+</sup> cells from male donors, according to published protocol<sup>25</sup>. NHEJ assessed by Cell1-nuclease based mutation assay (Surveyor Mutation Detection Kit–Transgenomic) on genomic DNA.

#### **HuMice**

2-5 days old NSG (NOD.Cg-Prkdc<sup>scid</sup> Il2rg<sup>tm1</sup>Wjl/SzJ, JAX mouse strain) mice were sublethally irradiated (1.5cGy), randomly assigned to experimental groups and injected intrahepatically with 10<sup>5</sup> CD34<sup>+</sup> 5-7 hours later. No signs of xeno-GVHD were detected (up to 20 weeks follow up of the mice). Each experiment included test and control huMice and was performed with a different CD34<sup>+</sup> donor (expressed in figure legends as N=). Mice showing <1% hCD45<sup>+</sup> cells, <0.7 LV copies per cell (KD) or <10% of NHEJ (KO) in BM were excluded. Phenotyping performed on the total human CD45<sup>+</sup> population, unless otherwise specified. All protocols involving animals followed DL116 (January 27th 1992, Italian Parliament) and have been approved by the Institutional Ethical Committee (protocols 488 and 632).

#### **In vitro proliferative assays**

Proliferation dye dilution (efluor670, eBioscience) was assessed 6 days after stimulation by anti-CD3/CD28 expansion beads (Thermo Fisher) or anti-CD3/CD28/CD2 suppression inspector beads (Miltenyi) of total splenocytes or purified CD4<sup>+</sup>/CD4<sup>+</sup>CD25<sup>-</sup> T cells (CD4-T cell isolation kit/CD25-conjugated beads, Miltenyi), in presence of IL2 (100U/ml Proleukin, Novartis) where indicated.

**Flow Cytometry and Multiplex Immunoassay**

Transduction level was assessed by direct GFP measurement. Single cell suspensions were obtained by physical disruption of mouse lymphoid organs and lysis of RBC. Analyses were gated on hCD45<sup>+</sup> fraction (details in Supplementary Methods and Tables S2-S3). CD45<sup>+</sup>GFP<sup>+</sup> cells were sorted from splenocyte and thymocyte suspensions. Acquisitions performed by FACSCantoII (BD Biosciences) and analyzed by FlowJo Software. Sorting performed by MoFloXDP (Beckman Coulter). Antibody and gating strategies are indicated in supplementary methods. Multiplex Immunoassay was performed by Bio-Plex Pro™ Human Cytokine Th1/Th2 Assay (Bio-Rad), following manufacturer's instructions, on huMice derived cell culture supernatants and plasma. Acquisition and analysis was performed on BioPlex MagPix Multiple reader (Bio-Rad). Cytokines tested: GM-CSF, IFN $\gamma$ , IL2, IL4, IL5, IL10, IL12 (p70), IL13 and TNF $\alpha$ . Cytokines displayed are the only ones above detection level (but GM-CSF that was clearly detectable but not discriminative for Th1/Th2 profiles). Th1/Th2 ratio was calculated as ratio between the total amount of detected Th1 cytokines and the total amount of detected Th2 cytokines in the same sample, both for in vitro and ex vivo conditions.

**DNA and RNA analyses**

Genomic DNA was extracted by Qiagen DNeasy kit from total splenocytes and thymocytes (for TCR and TSDR) or PB cells (for TREC).  $1-9 \times 10^5$  productive rearrangements were sequenced by Adaptive Biotechnology (Survey depth; <http://www.adaptivebiotech.com/immunoseq>) for TCR $\beta$ -locus analysis. See supplementary methods for clonality and fraction of productive rearrangement calculation. Treg-Specific Demethylated Region (TSDR) was quantified by Epiontis

Biotech, as described<sup>26</sup>. TREC analysis was performed by probe-based ddPCR analysis on 80ng of DNA by using TREC and GAPDH specific primers to standardize for DNA content<sup>27</sup>. RNA extracted from CD45+GFP+ sorted thymocytes and splenocytes by ReliaPrep RNA Miniprep kit (Promega) and retrotranscribed by High Capacity cDNA kit (Life Technologies). Gene expression was assessed on 10-20ng of RNA equivalent by ddPCR (QX200 Bio-Rad) using target gene and HPRT amplifying primers, used as normalizer (sequences provided upon request), and analyzed by QuantaSoft software.

### **Immunohistochemistry**

Thymus, spleen and liver were fixed in 4% neutral buffered formalin and embedded in paraffin. Subsequent 3 $\mu$ m paraffin sections were stained by hematoxylin/eosin and specific antibodies (details in Supplementary Methods and Table S3) by standard immunohistochemical technique using Ventana Bench Mark Ultra immunostainer. Enzymatic activity was visualized with 3,3 diaminobenzidine. Photomicrographs by AxioCam HRc/AxioVision SE64 Rel 4.9.1 (Zeiss).

### **Statistical methods**

Values are expressed as mean $\pm$ SEM in column bar and median and interquartile range in box/scatter plot graphs. In figure legends: n= number of biological replicates, N=number of independent experiments. Since the assumptions for the correct application of parametric tests were not met or not verifiable, we used nonparametric tests (two-tailed Mann-Whitney, unless stated otherwise). P values are reported in figures/figure legends only if significant ( $p < 0.05$ ). Analysis of thymocyte populations (Fig. 3C) performed by Nonparametric Combination (NPC) test<sup>28,29</sup>, a permutation-

224 based test that allows multivariate comparison of the means in groups with small  
225 number of observations. A Nonlinear Mixed Effects model was fitted for the analysis  
226 of CD3+ T cell kinetics (Fig.4A and Fig. S2B)<sup>30</sup>: we assumed an exponential dynamic  
227 in the time period 7-15 weeks and tested a model with common growth and group-  
228 specific shifts. We fitted a regression model that included indicator variables for  
229 group membership (IPEX, HD, PolyAuto), age and their interaction as covariates  
230 (Fig.6). Analyses performed with R 3.2.2<sup>31</sup> and NPC Test R10<sup>32</sup> and with Prism  
231 v5.04.

## RESULTS

### Development of *FOXP3*-KD/KO humanized mouse models

We first assessed the possibility of generating mature T cells in huMice. By intrahepatically injecting unmodified human cord blood-derived HSPCs in sublethally irradiated newborn NSG mice, we obtained very efficient and homogenous human cell engraftment ( $76\pm 6.5\%$  and  $81\pm 2.7\%$  hCD45<sup>+</sup> cells in bone marrow (BM) and peripheral blood (PB), respectively) (Fig.1A). Importantly, we observed human thymocyte development (Fig.1B) and peripheral differentiation of detectable numbers of human T cells accounting for up to 30% of PB human cells (Fig.1C), which were equally distributed between CD4<sup>+</sup> and CD8<sup>+</sup> populations, and allocated to naïve/effector/memory compartments in percentages resembling human PB T cells (Fig.1D). The CD4/CD8 ratio observed in huMice was lower than what observed in healthy subjects, and more similar to that of bone marrow transplanted (BMT) patients<sup>33</sup> (Fig. 1D). We also detected substantial proportions of Tregs in all analyzed tissues (Fig.1E). Mature spleen Teffs showed a polyclonal TCR rearrangement and proliferated in response to a TCR-mediated polyclonal stimulus *in vitro* (Fig.1F-G) and Tregs showed suppressive abilities, as depletion of CD4<sup>+</sup>CD25<sup>+</sup> Tregs resulted in increased *in vitro* proliferation of Teffs (Fig.1H). These results indicate that the huMouse model is suitable to study the effects of *FOXP3* on the maturation of functional human T cells. *FOXP3* expression was diminished by either KD or KO in HSPCs. To KD *FOXP3*, we transduced CD34<sup>+</sup> cells with miR-based lentiviral vectors (LVs) expressing a control shRNA or a *FOXP3*-targeting shRNA and GFP as reporter gene<sup>24</sup>. *FOXP3*-KO was obtained by nucleofecting CD34<sup>+</sup> with TALENs designed to disrupt the *FOXP3* TSS. Efficiency of shRNA-LV transduction ranged between 50% and 70%, and TALEN-mediated Non-Homologous End Joining

(NHEJ) averaged 20% in CD34<sup>+</sup> cells (Fig. 2A). Both KD and KO approaches reduced FOXP3 expression, as assessed by flow cytometry in T cells (40% FOXP3 KD and 55% KO on average) (Fig. 2B-C). On the basis of clinical data on IPEX carrier mothers and BMT transplanted IPEX patients<sup>8,9</sup>, we hypothesized that the reduction we obtained in FOXP3 expression could be sufficient to investigate the intrinsic role of FOXP3 in Tregs without impairing the Treg compartment, in which few Treg with normal FOXP3 expression could have a selective advantage. Nevertheless, to study a human lymphopoiesis composed mainly of *FOXP3*-KD/KO cells, we excluded huMice showing <0.7 LV copies per cell (KD) or <10% of NHEJ (KO) in the engrafted BM. Since the two approaches provided overlapping results, they were analyzed and are shown together, with individual data from each approach also provided.

### **Reduced FOXP3 expression accelerates thymocyte maturation and thymic involution**

We first analyzed the thymic compartment. At 12 weeks post-transplantation, we observed increased thymic size in *FOXP3*-KD/KO huMice compared to controls (1.3 range:1.1-1.7 fold the control), which showed a median thymic size very similar to the thymic rudiment of the untransplanted NSG (Fig.3A). Accordingly, human differentiating thymocytes were detected in 4/5 *FOXP3*-KO huMice, and in only 1/5 control huMouse at this time point (Fig.3A). At 14-17 weeks, *FOXP3*-KD/KO showed a reduced thymus size compared to control huMice (0.45 fold on average) (Fig. 3B). This suggested an accelerated thymocyte differentiation and premature thymic regression. Altered thymocyte differentiation was confirmed by immunophenotyping. *FOXP3*-KD/KO thymocytes showed a significantly different

composition at 14-17 weeks, with decreased percentages of immature DP ( $50\pm14\%$  vs  $73\pm4.3\%$ , KD/KO vs Ctrl) and increased percentages of DN and SP cells (DN:  $1.9\pm1.0\%$  vs  $0.54\pm0.19\%$ , SP4:  $19\pm8.5\%$  vs  $6.5\pm2.1\%$ , SP8:  $11\pm2.9\%$  vs  $5.7\pm1.2\%$ , KD/KO vs Ctrl), when compared to control huMice (Fig.3C and Fig.S1A). This altered thymocyte distribution observed in *FOXP3*-KD/KO huMice was not the effect but likely the cause of the premature thymic involution at the analyzed time point, as a very similar composition was already detectable at 12 weeks, when thymic size was bigger in KD/KO (Fig.S1B-C).

Treg frequencies were comparable in *FOXP3*-KD/KO and control thymi (Fig.3D). We found a 24% and 42% reduction on average in Ki67+ proliferating SP4 and SP8, respectively, thymocytes in *FOXP3*-KD/KO compared to their control counterpart ( $26\pm6.7$  vs  $34\pm6.0$  CD4+/Ki67+ and  $21\pm3.8\%$  vs  $36\pm4.4\%$  CD8+/Ki67+, KD/KO vs Ctrl) (Fig.3E), while apoptotic rate was not affected (Fig.S1D). Thymocyte differentiation and proliferation are ruled by TCR selection and signaling<sup>34</sup>. Since both processes were altered in KD/KO huMice, we assessed the efficiency of TCR signaling in *FOXP3*-KD/KO thymocyte by evaluating clonal distribution and gene expression patterns. High throughput sequencing of the TCR $\beta$  locus showed lower clonality (higher diversity) in *FOXP3*-KD/KO thymocytes (0.06 range:0.04-0.08 vs 0.21 range:0.04-0.38, KD/KO vs Ctrl) (Fig.3F), suggesting altered thymic selection when FOXP3 was depleted. To directly assess dampened TCR signaling strength upon FOXP3 down-regulation, we analyzed the expression of TCR induced genes in GFP-sorted *FOXP3*-KD and control thymocytes. Consistent with the clonality data, the expression of *Nur77/NR4A1*, which is an early TCR response gene used to directly measure the strength of TCR signaling<sup>35,36</sup>, was significantly reduced in *FOXP3*-KD thymocytes ( $0.045\pm0.01$  KD vs  $0.14\pm0.04$  Ctrl arbitrary units (a.u.)). Similarly, the

expression of transcription factors controlled by TCR signaling and regulating thymocyte differentiation were significantly reduced in *FOXP3*-KD when compared to control thymocytes (Fig.3G)<sup>37</sup>. We conclude that *FOXP3*-KD/KO huMice display faster thymocyte differentiation and thymic regression, coupled to reduced TCR signaling. These results indicate that FOXP3 is necessary for proper thymocyte maturation, likely by affecting TCR-mediated negative selection.

### ***FOXP3*-KD/KO huMice show early increased thymic output and peripheral T cell expansion**

We next evaluated peripheral lymphoid compartments. When assessing the kinetic of human T cell appearance in PB, we observed higher T cell percentages in KD/KO compared to control huMice (27±15% vs 9.3±5.3% at 10-11 weeks, 34±14% vs 5.9±1.0% at 12-13 weeks; 47±13% vs 25±6.4% at 15-16 weeks huCD45+/CD3+, KD/KO vs Ctrl) (Fig.4A, Fig.S2A). By fitting a statistical model for nonlinear mixed effects on the PB T cell kinetics, we found a significant difference in the dynamics, suggesting that T cells appeared at an earlier time point in *FOXP3*-KD/KO huMice when compared to control mice (Fig.S2B). This, together with the observed faster thymic development (see Fig.3A-C), suggested enhanced thymic output at early time points in KD/KO huMice. To directly assess thymic output, we evaluated T cell Receptor Excised Circles (TREC), which directly measure recent thymic emigrants<sup>27</sup>, in PB cells. At 7-9 weeks post-transplant, TREC were significantly increased in *FOXP3*-KO compared to control PB cells (0.013±0.0050 vs 0.0019±0.0015 TREC/GAPDH copies, KO vs Ctrl) (Fig.4B), indicating enhanced thymic output at early time points when FOXP3 expression is reduced. In line with the faster thymic regression observed at 14-17 weeks in KD/KO huMice (see Fig.3A-C), KD/KO PB

cells showed reduced TREC when compared to their counterpart at 12-15 weeks post-transplant ( $0,017 \pm 0,009$  vs  $0,063 \pm 0,023$  TREC/GAPDH copies, KD/KO vs Ctrl) (Fig.4B).

Interestingly, T cells were found expanded in the periphery also at late time points (14-17 weeks), when the thymus already regressed, in KD/KO huMice. KD/KO huMice showed increased T cell number ( $2.8$  range:  $1.0-7.4 \times 10^5$  vs  $1.8$  range:  $0.35-2.3 \times 10^5$  CD3+ cell count, KD/KO vs Ctrl) (Fig.4C) and significantly higher CD4+ and CD8+ cell percentages (17 range:  $1.6-46\%$  vs  $3.6$  range:  $0.42-21\%$  CD4+ and  $8.7$  range:  $1.7-21\%$  vs  $2.8$  range:  $0.03-17\%$  CD8+, KD/KO vs Ctrl) (Fig.4D) in the spleen, compared to controls. Thus, both CD4+ and CD8+ Teff cells were found expanded and both characterized by significantly enlarged memory compartments (EM:  $12 \pm 3.5\%$  vs  $2.4 \pm 0.7$  and CM:  $4.9 \pm 1.4$  vs  $0.86 \pm 0.24$ , KD/KO vs Ctrl CD4+; EM:  $5.2 \pm 1.2\%$  vs  $1.7 \pm 0.48$  and CM:  $1.5 \pm 0.46$  vs  $0.37 \pm 0.09$ , KD/KO vs Ctrl CD8+) in KD/KO huMice compared to controls (Fig.4E). Likely reflecting the intrinsic different outcomes of the KO (absence of FOXP3 expression) and the KD (reduced FOXP3 expression) approaches in the gene-modified cells, the reduced naïve/memory ratio was more pronounced in KO compared to KD T cells (Fig.S2C). According to a model where the phenotypic effect is proportional to the level of FOXP3 expression, the expansion of gene-modified (GFP+) cells was evident only in the T cell compartment and significantly correlated with T cell expansion exclusively in KD huMice and not in controls (Fig.S2D). The reduced naïve compartment and the peripheral Teff enlargement observed in KD/KO huMice when thymus was already shrunk suggested that T cell expansion was not only due to an initial increased thymic output, but also to peripheral expansion. Consistently, immunohistochemistry analysis of fixed organ sections showed enlarged T-B spleen follicles in KO compared to

control huMice. As well, KO livers showed consistent T cell infiltrations, which were absent in controls (Fig.4F).

We next assessed the regulatory compartment and found no difference in Treg percentages between KD/KO and control huMice either by flow cytometry (0.90% range:0.76%-4.85% vs 2.2% range:0.88%-4.5% CD4<sup>+</sup>/CD25<sup>hi</sup>CD127<sup>lo</sup>HEL<sup>+</sup>FOXP3<sup>+</sup> cells, KD/KO vs Ctrl) or by TSDR quantification (1.4% range:0.51%-1.6% vs 0.78% range:0.35%-1.0% CD3<sup>+</sup>/TSDR<sup>+</sup> cells, KD/KO vs Ctrl) (Fig.4G). Tregs from KO huMice also showed suppressive abilities, as indicated by the higher proliferation index of KO CD4<sup>+</sup>CD25<sup>-</sup> compared to total KO CD4<sup>+</sup> T cells *in vitro* (Fig.4H) (see Fig. 1H as reference). In line with the results obtained in humans<sup>8,9</sup>, this finding indicates that wild type FOXP3 Tregs outcompete Tregs depleted for FOXP3 in this partial KD/KO model, as confirmed by similar FOXP3 expression levels in CD4<sup>+</sup>CD25<sup>+</sup> Treg splenocytes isolated from KD/KO and control huMice (Fig.S2E). Thus, Teff expansion observed in the FOXP3 KD/KO huMice model is not due to defective Treg control but rather to a Teff intrinsic FOXP3-dependent dysregulation.

### **Impaired FOXP3 expression alters peripheral effector T cell homeostasis in huMice**

Peripheral expansion and reduced naïve/memory ratios of T cells after thymic involution in KD/KO huMice suggested aberrant homeostatic proliferation taking place upon reduction of FOXP3 expression. Thus, in order to assess the possible impact of aberrant homeostatic proliferation on peripheral T cell fitness, we tested proliferative response of T cell in FOXP3-KD/KO and control huMice. We did not observe major differences in the percentage of proliferating T cells, between KD/KO

and control splenocytes at steady state (Fig.5A). Instead, activated KD/KO CD4+ cells showed a significantly reduced proliferation compared to the control counterpart (65% range:48%-86% vs 83% range:77%-89%, CD4+/proliferating cells, KD vs Ctrl) (Fig.5B) exclusively when in the presence of IL2 (Fig.S3A), suggesting an impaired proliferation in response to IL2. Proliferation of KD CD8+ cell remained unaltered. This impairment was not due to altered expression of the high affinity IL2 receptor, which was similar between KD/KO and Ctrl CD4+ cells (Fig. S3B). Aberrant homeostatic proliferation can lead to clonal contraction and telomere shortening<sup>38</sup>. In order to test clonal contraction in FOXP3-KD/KO peripheral T cells, we performed high-throughput sequencing of the TCR $\beta$  locus in splenocytes and observed significantly increased clonality (reduced clonal diversity) in KD/KO T cells, when compared to controls (0.34 range:0.27-0.36 vs 0.17 range:0.1-0.18 clonality, KD/KO vs ctrl) (Fig.5C), indicating uneven homeostatic proliferation. The clonal contraction took place in the periphery because clonality was lower (higher diversity) in KD/KO thymocytes compared to the control counterpart (see Fig.3F). In order to assess possible telomere damage, we evaluated the expression of the Telomeric Repeat-binding Factor 2 (TERF2), which is needed for correct telomere capping and maintenance<sup>39</sup>. TERF2 expression was lower in FOXP3-KD/KO compared to control cells ( $1.7 \pm 0.92$  vs  $3.0 \pm 1.4$  a.u., KD/KO vs Ctrl), suggesting increased telomere attrition (Fig.5D). The expression of FOXP3 and a panel of FOXP3-target genes<sup>40</sup> were also altered as expected, confirming the specificity of the gene modification obtained in the FOXP3-KD/KO huMice model (Fig.S3C). Uneven homeostatic proliferation may also result in skewed cytokine production towards a Th2 predominance, as it has been described in IPEX patients<sup>41,42</sup>. Therefore, we assessed cytokine concentration in the supernatant of *in vitro* activated

T cells derived from the spleen of huMice to test a possible Th2 skewing in *FOXP3*-KD/KO huMice. Although all detectable cytokines tested were increased in KD/KO samples compared to controls, we observed a more pronounced increase in the concentration of Th2 cytokines when compared to Th1, resulting in significant reduction of the Th1/Th2 ratio in *FOXP3*-KD/KO samples (Fig. 5E). Human cytokines in the plasma of huMice were undetectable but the Th1 cytokine IL12, whose level was similar between KD/KO and controls, and the Th2/regulatory cytokine IL10, whose expression was increased in KD/KO huMice (Fig. S3D). Altogether these results indicate that peripheral T cell homeostasis and differentiation are severely compromised upon reduction of *FOXP3* expression in huMice.

#### **Impaired thymic and peripheral homeostasis of effector T cells in patients with IPEX syndrome**

To test the relevance of our findings for the human pathology, we assessed whether alterations similar to the ones observed in *FOXP3* KD/KO huMice were found in IPEX patients. We retrospectively analyzed a cohort of autoimmune and healthy donors and fitted a regression model plotting percentage of PB CD4<sup>+</sup>/CD45RA<sup>+</sup> naïve cells against age. While slopes were similar between groups, the intercept obtained from the regression curve of IPEX patients was significantly lower than the one obtained from healthy donors, indicating a significantly reduced naïve cell fraction in IPEX patients (Fig.6A). To verify if this reduction was a direct consequence of *FOXP3* mutation rather than a secondary effect of the autoimmune processes, we also plotted data from patients with early onset autoimmunity against different organs but no detectable mutations in *FOXP3*. Interestingly, the regression curve obtained from these *FOXP3*-competent polyautoimmune patients was not

different from the one obtained from healthy donors (Fig.6A). Thus, IPEX patients, like *FOXP3*-KD/KO huMice, have a contracted naïve compartment in the periphery as a consequence of the expression of mutant *FOXP3* alleles within the overall T cell compartment. We next sequenced TCR $\beta$  locus in PBMC from the same experimental groups. As opposed to what observed in *FOXP3* KD/KO huMice, we did not find an increased clonality in the analyzed IPEX patients, when compared to healthy donors (Fig.S4). This was likely due to the immunosuppressive regimen, to which patients were subjected, that limited Teff clonal expansion in the periphery. Instead, when we quantified the fraction of productive TCR $\beta$  rearrangements and plotted it against age of the donors, we found that IPEX patients, but not polyautoimmunity patients displayed a significantly lower intercept of the regression line in comparison to the HD counterpart (Fig. 6B). This finding indicates a reduced fraction of productive TCR rearrangement in IPEX patients, suggestive of a defect during thymocyte selection. We tested if the reduction in the fraction of TCR productive rearrangements was also displayed by KD/KO huMice. We found a significantly lower fraction of productive rearrangements in KD/KO thymocytes when compared to controls, confirming that the defect was *FOXP3*-dependent (Fig.6C). The reduction was less evident in huMice periphery, where, most likely, the peripheral expansion of some clones masked the non-productive ones (Fig. 6C; see also Fig.5C). Overall, these data indicate a relevant role for *FOXP3* in both thymocytes and peripheral Teff cells in the human system.

**DISCUSSION**

In order to assess the role of FOXP3 in the differentiation of human Teff cells, we developed a huMouse displaying a hematopoietic system generated from human HSPCs genetically modified to impair FOXP3 expression. *FOXP3*-KD/KO huMice showed defects in both early and late stages of Teff differentiation, in line with the defects observed in the natural and experimental *FOXP3* KO mouse models<sup>43,44</sup>. Indeed, *FOXP3*-KD/KO huMice displayed accelerated thymocyte differentiation that resulted in enhanced thymic output at early time points, as indicated by the earlier appearance of T cells and the increased fraction of recent thymic emigrants in PB, and in premature thymic regression at later time points. Nevertheless, KD/KO huMice showed peripheral T cell expansion at both early and late time points, when thymus already regressed, suggesting that T cell expansion resulted from a wave of enhanced thymic output coupled to peripheral homeostatic proliferation. *FOXP3*-KD/KO thymocytes showed decreased TCR-signaling strength and higher TCR repertoire diversity compared to controls, suggesting an altered negative selection and unveiling a direct effect of FOXP3 in thymopoiesis. Several studies have documented FOXP3 expression in human thymocytes, including a recent one that has clearly identified the thymus as the tissue displaying the highest FOXP3 expression (NCBI PRJNA280600)<sup>10,11,45</sup>. Further supporting a role for FOXP3 in thymopoiesis, FOXP3 is activated downstream of TCR engagement in human Teffs<sup>14</sup> and can modify TCR signaling threshold during thymic selection in murine Tregs<sup>46</sup>. By suggesting a role for FOXP3 in controlling TCR-signaling strength, our data propose that FOXP3 contributes to immune regulation at different levels. Indeed, TCR signaling and the expression of the downstream gene *Nur77*, rule central and peripheral immune tolerance by regulating auto-reactive clones neglection and

Treg/Teff functions, respectively<sup>47,48</sup>. By directly impacting these pathways and on the basis of the results obtained by sequencing the TCR $\beta$  locus in IPEX patients (see below), we thus propose FOXP3 to control immune tolerance at both central and peripheral levels.

In the periphery, the clonal contraction, the reduced naïve/memory ratio and the decreased TREC amount at late time points in KD/KO T cells further support uneven homeostatic proliferation<sup>38</sup>, which likely occurred to compensate the premature thymic regression and might be led by the higher fraction of auto-reactive clones escaping negative selection. These features, together with the expansion of peripheral T cells displaying hypo-responsiveness to IL2 and the skew towards a Th2 cytokine profile, indicate a severe impairment of the peripheral T cell compartment. Only further experiments will define if the cytokines we detected, and that we showed as Th1/Th2 ratio for sake of clarity, are co-expressed within the same cell or whether they are expressed individually in each cell. Both central and peripheral defects observed in KD/KO-FOXP3 huMice were reminiscent of an aged immune system<sup>49</sup>. On the same line, the observed reduction in TERF2 expression suggested increased telomere attrition in KD/KO cells, although further functional demonstrations are needed to formally prove it. In light of the demonstrated association between reduced TCR signaling and immune aging<sup>50</sup>, we thus hypothesize that FOXP3 expression down-regulation results in an immune aged-like phenotype, as consequence of the reduced control of TCR signaling. Although further studies are needed to formally prove the link between FOXP3 and immune aging, this hypothesis is intriguing in light of the well-known association between aging and autoimmunity<sup>38</sup>.

In contrast to the altered Teff compartment, wild type FOXP3-Tregs outcompeted Tregs expressing reduced FOXP3, resembling what observed in chimeric human

systems<sup>8,9</sup>. Indeed, Tregs expressed normal levels of FOXP3 and were functional *in vitro* in our partial *FOXP3*-KD/KO models. This indicates that the T cell phenotype observed was not due to dysfunctional Tregs but rather Teff intrinsic. On the other hand, since the phenotypic defects were evident at the level of Teff bulk population, composed by both unmodified and gene-modified cells, we postulate that *FOXP3*-KD/KO have a paracrine effect on FOXP3-competent cells.

Although the phenotype of KD/KO huMice might have been exacerbated by the recipient mouse lymphopenia, we found that IPEX patients, who are not lymphopenic, also show a *FOXP3*-dependent decrease in the naïve pool of peripheral CD4<sup>+</sup> T cells. Similarly to what we observed in *FOXP3*-KD/KO huMice, IPEX patients also show a Th2-skewed cytokine profile in CD4<sup>+</sup> T cells<sup>41,42</sup>. In contrast to the data we obtained in *FOXP3*-KD/KO huMice, we did not detect increased peripheral T cell clonality in IPEX patients, likely due to the fact that T cell expansion in patients was kept under control by the immunosuppressive therapies (see Table S1 for detailed clinical information about human donors). Nevertheless, IPEX patients, as well as *FOXP3*-KD/KO huMice, displayed an increased fraction of non-productive TCR rearranged clones, suggesting a defect at the level of  $\beta$  selection in the thymus upon decreased FOXP3 expression. We could thus speculate that, when functional FOXP3 expression is reduced, as in both the huMice model and IPEX patients, thymocytes fated to apoptosis because of a non-productive TCR rearrangement, are rescued because the molecular pathways inducing programmed death, partially controlled by FOXP3<sup>10,37,51,52</sup>, are not properly working. Future investigations will be required to determine whether an early defect in thymocyte differentiation could occur also in other autoimmune diseases, such as IPEX-like syndromes, in which Treg/Teff balance is impaired as a consequence of mutations in other genes<sup>4,5</sup>.

Our studies demonstrate the feasibility of using the huMouse model, complemented by gene transfer technologies, to investigate the role of different genes in the human immune system and better understand pathogenesis of immune mediated diseases, often hindered by the limited availability of patients' cells. Overall our data call for a previously not described role for FOXP3 as intrinsic regulator of early maturation and peripheral homeostasis of effector T cells, which we identified in the presented huMouse and detected also in human autoimmune pathology. Indeed, our findings support a new pathogenic model for IPEX, where FOXP3 mutations intrinsically affect not only regulatory but also effector T compartment at different levels.

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**AUTHOR CONTRIBUTIONS**

F.R.S.d.S. designed and performed experiments, analyzed data, and wrote the manuscript; L.P. helped in designing experiments and analyzing data; S.R. and M.M.V. performed experiments; A.P. performed statistical analyses; M.E.M. analyzed patients' data; F.S. analyzed immunohistochemistry data; M.G.R. discussed data; M. Porteus helped in designing KO experiments and provided reagents; R.B. supervised the whole project and experimental plan, analyzed data, and wrote the manuscript.

The authors declare no conflicts of interest.

**562 ONLINE MATERIAL**

563 The following supplementary information can be found in the online repository  
564 material:

- 565 - Figures S1-S4
- 566 - Supplementary methods
- 567 - Table S1-S3

568 High-resolution versions of IHC panels shown in Figure 4F for use with the Virtual  
569 Microscope are available as eSlides: VM0456, VM04558, VM04559, VM04560,  
570 VM04557, VM04551, VM04548, VM04549, VM04550, VM04547, VM04556,  
571 VM04553, VM04554, VM04555, VM04552, VM04546, VM04543, VM04544,  
572 VM04545, VM04542.

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ACCEPTED MANUSCRIPT

## FIGURE LEGENDS

**Figure 1. huMouse model employed in the study.** A) Percentage of hCD45<sup>+</sup> cells in bone marrow (BM) and peripheral blood (PB) 9-12 weeks post-transplant (n=6-7). B) Thymocyte composition at 9 weeks (9w) and 12 weeks (12w) post-transplant (n=3-5). C) Left, hematopoietic lineage composition in PB (n=8); right, absolute number of CD3<sup>+</sup>/ml of PB, dashed line: range in healthy human donors (HD), (n=10). D) Peripheral Teff populations in the indicated tissues (n=6). Left, values shown as fraction of the CD3<sup>+</sup> population (far left) or as fraction of the CD4<sup>+</sup>/CD8<sup>+</sup> (top/bottom middle left) populations. Right, CD4<sup>+</sup>/CD8<sup>+</sup> ratio (n=5). E) Regulatory T cells (n=10-12). D-E) Relative T cells populations in human peripheral blood from adult and pediatric HD provided as reference. B-E) Flow cytometry plots showing gating strategies are provided. F) Representative proliferation dye dilution 6 days after in vitro stimulation of CD4<sup>+</sup> cells in presence of IL2 (n=3 out of 6 performed). G) Clonality of TCR $\beta$  in mature T cells. Left, representative clonal composition pie chart; right, clonality index (n=6), dashed line: reference HD PBMC value. H) Median proliferation index (left) and representative proliferation dye dilution histogram (right) of mature CD4<sup>+</sup> and CD4<sup>+</sup>CD25<sup>-</sup> cells upon stimulation by anti-CD3/CD28/CD2 beads. A-H) N=2-3.

**Figure 2. Efficiency of FOXP3 KD and KO.** A) CB-derived CD34<sup>+</sup> cell transduced by LV expressing anti-FOXP3 (LV-shFOX) or anti-luciferase (LV-shCtrl) shRNA, or nucleofected by TALEN targeting the transcription start site (TSS) of *FOXP3*. 4-7 days later, %gene modification assessed by reporter gene expression (%GFP<sup>+</sup> or % $\Delta$ NGFR) (N=6, n=6) or by non-homologous end joining (NHEJ) at the *FOXP3* locus (N=5, n=5). B) FOXP3 expression by flow cytometry in untransduced

CD4+CD25<sup>-</sup> (Teff), as negative control, and CD4+CD25<sup>+</sup> (Treg) cells transduced by the indicated LV; representative plots (left) and average knockdown level (right) (N=2, n=4). C) Percentage of genomic NHEJ and protein KO in CD4<sup>+</sup> cells nucleofected by TALEN (left) and representative flow cytometry plots of FOXP3 expression upon activation (right) (N=2, n=3-5).

**Figure 3. Reduced FOXP3 expression alters thymocyte differentiation.** Thymocytes from FOXP3 KD/KO and control huMice analyzed 12 weeks (A, N=1) and 14-17 weeks (B-G, N=6) post-transplant. A) Left, thymic weight normalized to the mean of the controls (set as 1); right, number of mice showing human differentiating thymocytes (n=5). B) Thymic size measured by weight or cell count, normalized to the mean of the controls (set as 1) (n=8 KD/KO, n=18 Ctrl). C) Thymocyte composition (n=7 KD/KO, n=14 Ctrl); Left, p value by non-parametric combination test (p=0.0401; see methods). Right, representative plots of CD45<sup>+</sup> thymocytes (CD3<sup>-</sup>CD1a<sup>-</sup> cells excluded). D) Percentage of Treg cells (n=9 KD/KO, n=18 ctrl). E) Percentage of Ki67<sup>+</sup> proliferating cells in the indicated populations (n=7 KD/KO, n=14 ctrl). F) Clonality index assessed by next generation sequencing of the TCR $\beta$  locus (n=3 KD/KO, n=4 ctrl). G) ddPCR analysis on sorted GFP<sup>+</sup> thymocytes cDNA. Arbitrary units: #copies/microliter of the test gene / #copies/microliter of HPRT. \*\*p=0.0052 Nur77, p=0.0057 GATA3, p=0.0034 MYB, p=0.0034 RORC, by one-tail Mann-Whitney (n=8 KD/KO, n=19 ctrl). Single data from the KD/KO approaches are either shown as circles (KD) and squares (KO) (B, D, E, F) or provided as supplementary information (C).

**Figure 4. Reduced FOXP3 expression induces peripheral Teff cell expansion.**

PB (A-B), spleen (C-E, G-H) and liver-infiltrating (F) analysis, 14-17 weeks, otherwise indicated, post-transplant. A) Percentage of CD3<sup>+</sup> T cells at different time points post-transplant (n=11 KD/KO, n=20 ctrl, N=6). B) TREC detection at 7-9 (N=2, n=8) and 12-15 (N=3, n=4-5) weeks post-transplant. C) Absolute numbers of CD3<sup>+</sup> T cells. D) Percentages of CD4<sup>+</sup> and CD8<sup>+</sup> splenocytes; \*\*p=0.0030, \*p=0.0475 by Mann-Whitney test (n=11 KD/KO, n=20 ctrl, N=6). E) Percentages (left) and representative plots (right, CD4<sup>+</sup>) of T subpopulations. CD4<sup>+</sup>EM \*\*p=0.0098, CD4<sup>+</sup>CM \*\*p=0.0098, CD8<sup>+</sup>EM \*p=0.0272, CD8<sup>+</sup>CM \*p=0.0350 by Mann-Whitney test (n=11 KD/KO, n=20 ctrl, N=6). F) Representative immunohistochemistry of subsequent sections of fixed tissues. Spleen (10x magnification, bar:100mm), circle identifies a follicle; liver (5x magnification, bar:50mm), transversal section of portal vein with hepatic duct(s) (n=3). High-resolution slides available with the Virtual Microscope (see online material). G) Percentage of CD25<sup>hi</sup>CD127<sup>lo</sup>FOXP3<sup>+</sup> (n=11 KD/KO, n=16 Ctrl, N=6) and TSDR<sup>+</sup> (n=3, N=2) thymic-derived Tregs among CD3<sup>+</sup> splenocytes. H) Mean proliferation index of KO CD4<sup>+</sup> and CD4<sup>+</sup>CD25<sup>-</sup> cells upon stimulation by anti-CD3/CD28/CD2 beads (n=6, N=1). Data from KD/KO approaches are either shown as circles (KD) and squares (KO) (B-D,G) or provided as supplementary information (A,E).

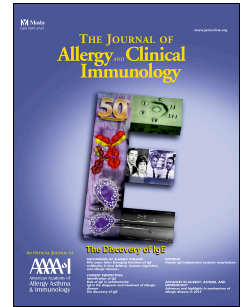
**Figure 5. Impaired FOXP3 expression affects peripheral Teff cell homeostasis.**

A) Percentage of Ki67<sup>+</sup> CD4<sup>+</sup> and CD8<sup>+</sup> splenic (n=11 KD/KO, n=20 ctrl, N=6). B) In vitro proliferation dye dilution upon stimulation with anti-CD3/CD28 beads in presence of exogenous IL2. \*p=0.0303 by Mann-Whitney test (n=6 KD, n=5 ctrl, N=2). C) Clonality index assessed by next generation sequencing of the TCR $\beta$  locus.

# Accepted Manuscript

Role of human FOXP3 in early thymic maturation and peripheral T cell homeostasis

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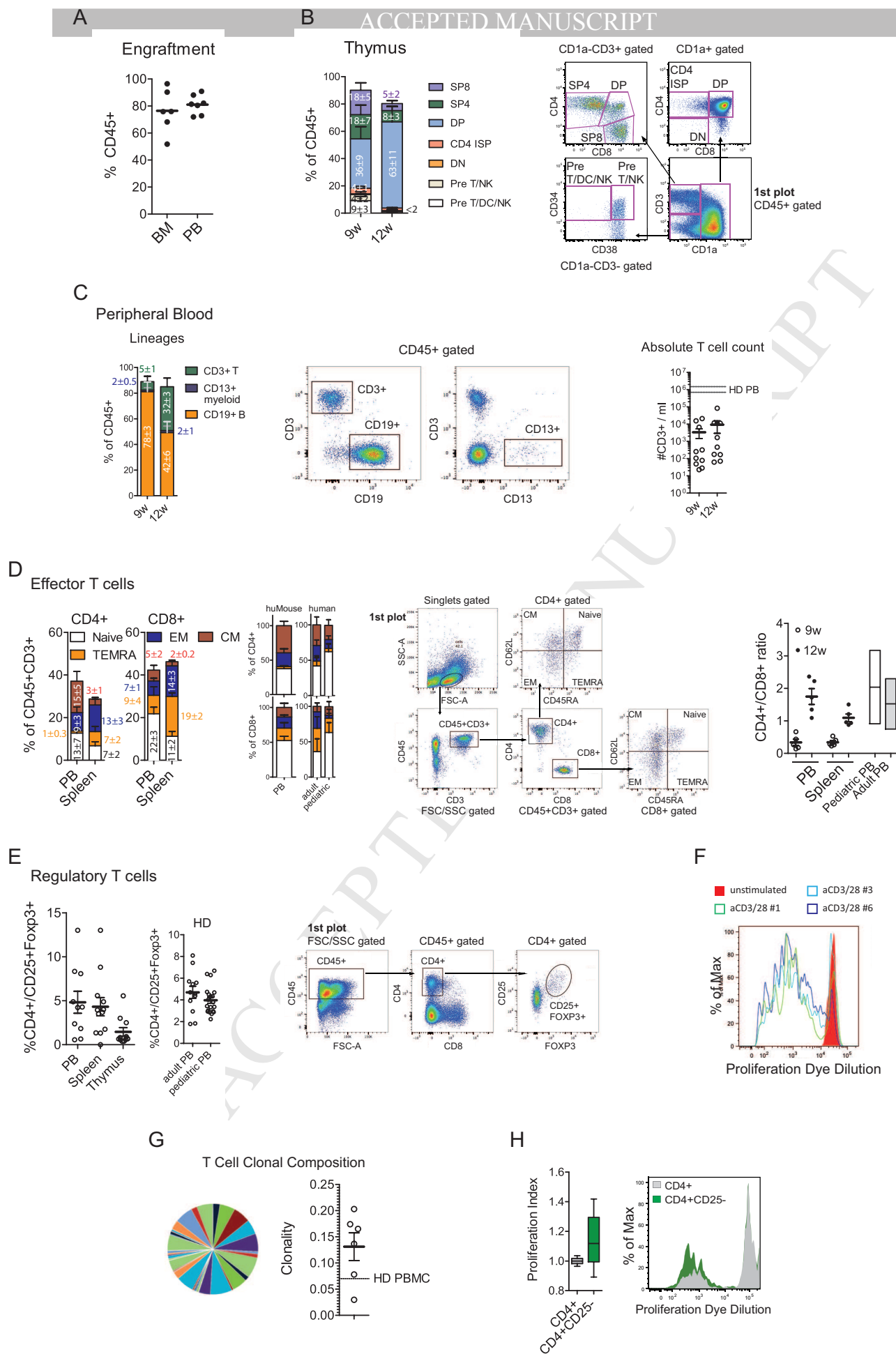
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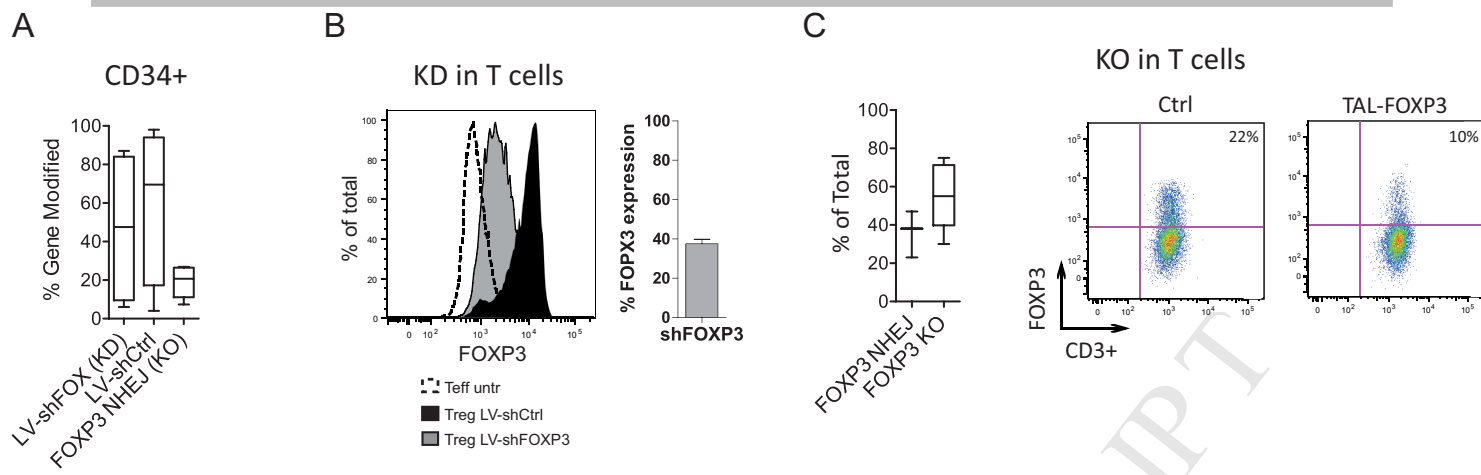
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\*p=0.0466 by one-tail Mann-Whitney test (n=5 KD/KO, n=8 ctrl, N=3). D) TERF2 expression assed by ddPCR analysis on sorted GFP+ (for KD) or CD4+ (for KO) splenocytes. Arbitrary units: #copies/microliter of the TERF2 gene / #copies/microliter of HPRT (n=4 KD/KO, n=9 ctrl, N=3). E) Th1 and Th2 cytokine concentration in the supernatant of *in vitro* cultured human cells from spleen (left, n=6 KD, n=5 Ctrl, N=2) from KD/KO and control huMice. Right, Th1/Th2 ratio in KD/KO and Ctrl samples. \*p=0.0287 by one-tail Mann-Whitney test (n=10 KD/KO, n=16 Ctrl), each symbol represents a mouse sample. Data from KD/KO approaches are shown as circles (KD) and squares (KO) (A, C-E).

**Figure 6. Teff defects observed in FOXP3 KD/KO huMice are also displayed by IPEX patients.** A) Percentage of peripheral blood CD4+/CD45RA+ cells plotted against age of healthy donors (HD, n=28), IPEX patients (n=7), polyautoimmune (n=11) and APECED patients (n=2). B) Fraction of productive TCR $\beta$  rearrangements in PBMC plotted against age of HD (n=32), IPEX (n=5) and polyautoimmune (n=5) donors. A-B) An estimated regression line for each group and statistical analysis is shown in bottom panels. °p-values indicate statistical significant difference of the estimated coefficient from 0. For the remaining rows, p values indicate statistical difference from the relative baseline HD. C) Fraction of productive TCR $\beta$  rearrangements in human thymocyte (Thy, n=3-4) and peripheral cells (Peri, n=5-8) from KD (circles) and KO (squares) and control huMice (N=3). \*p=0.0286 by one-tail Mann-Whitney test.

Figure 1





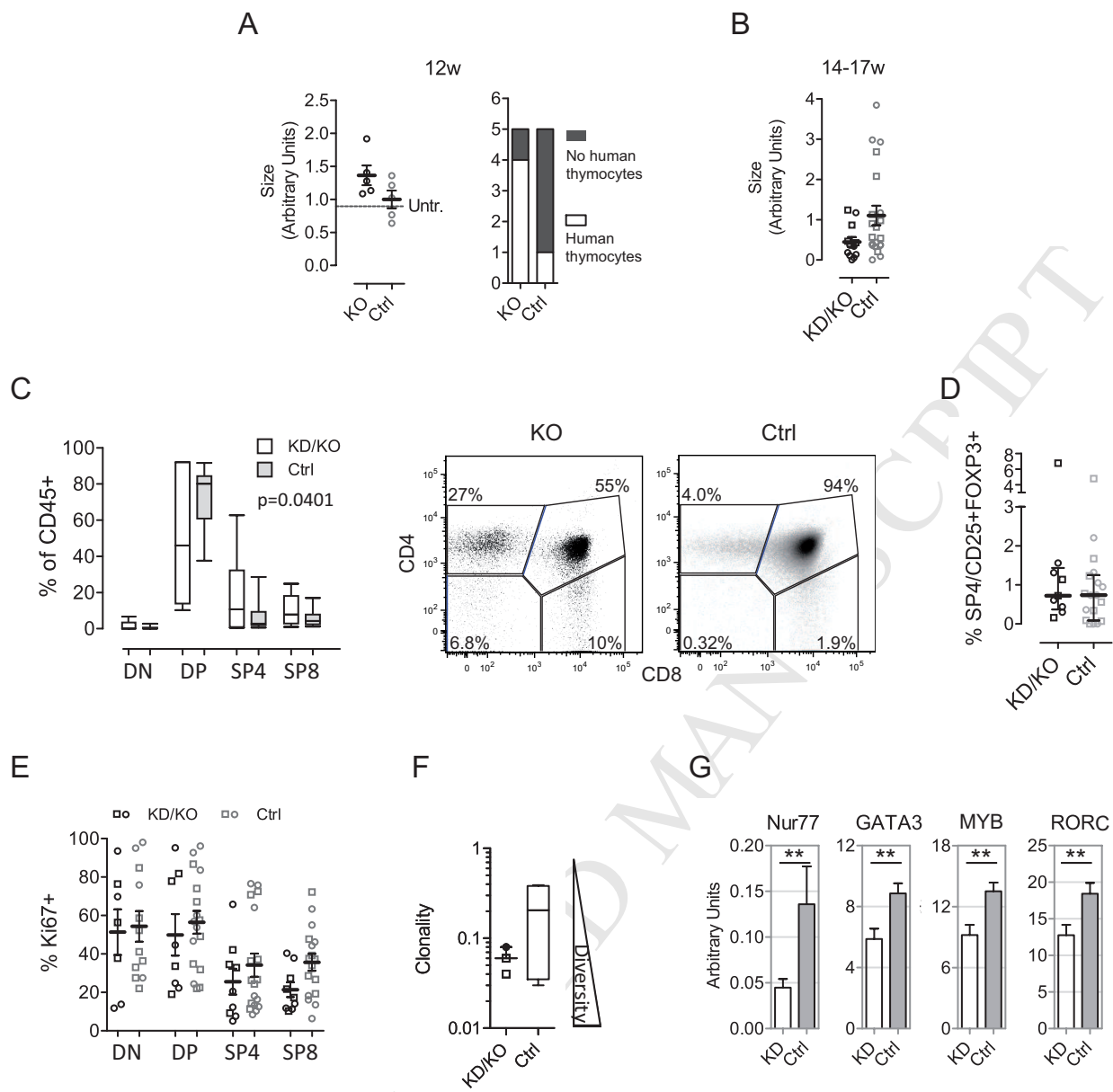


Figure 4

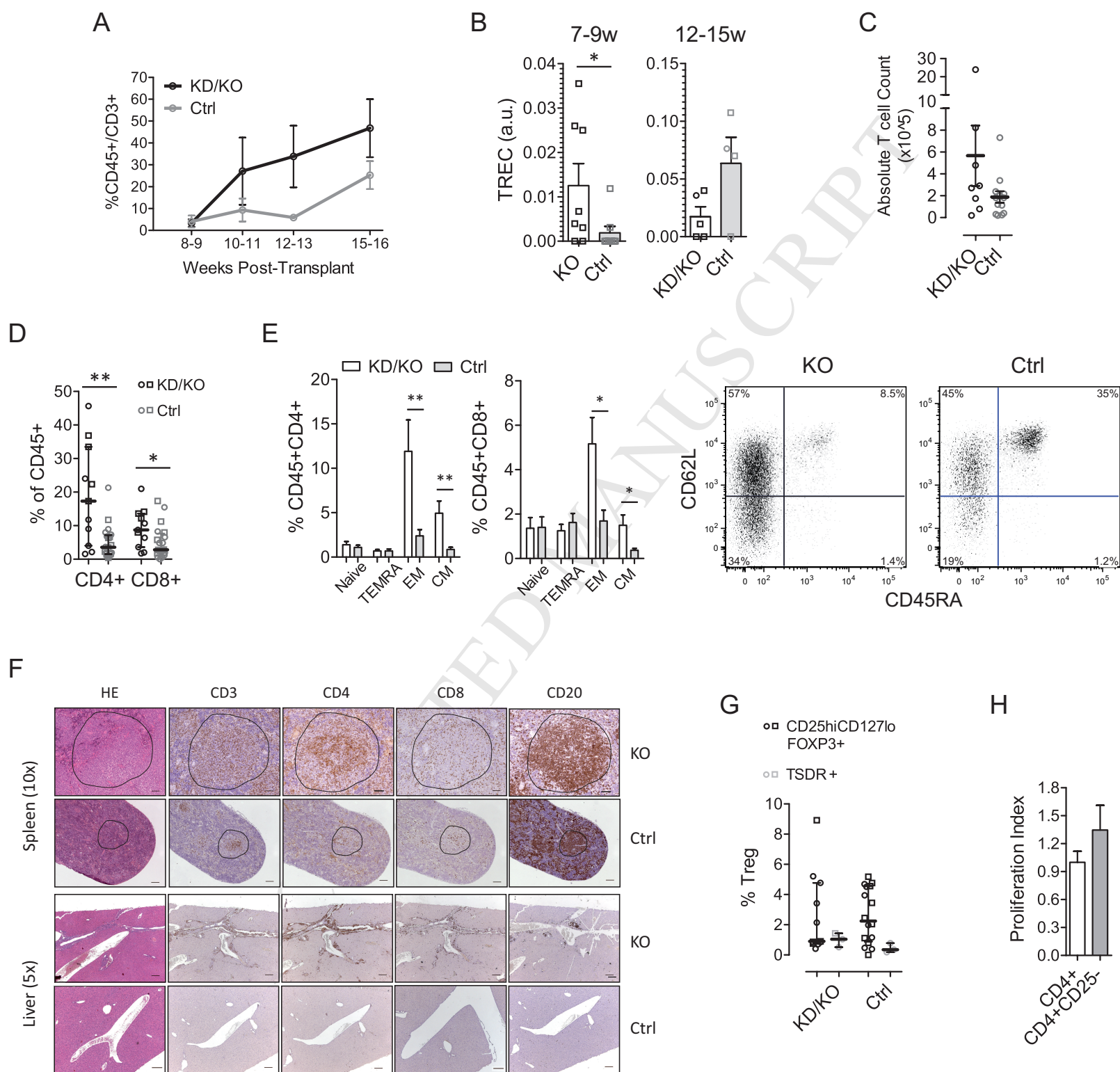
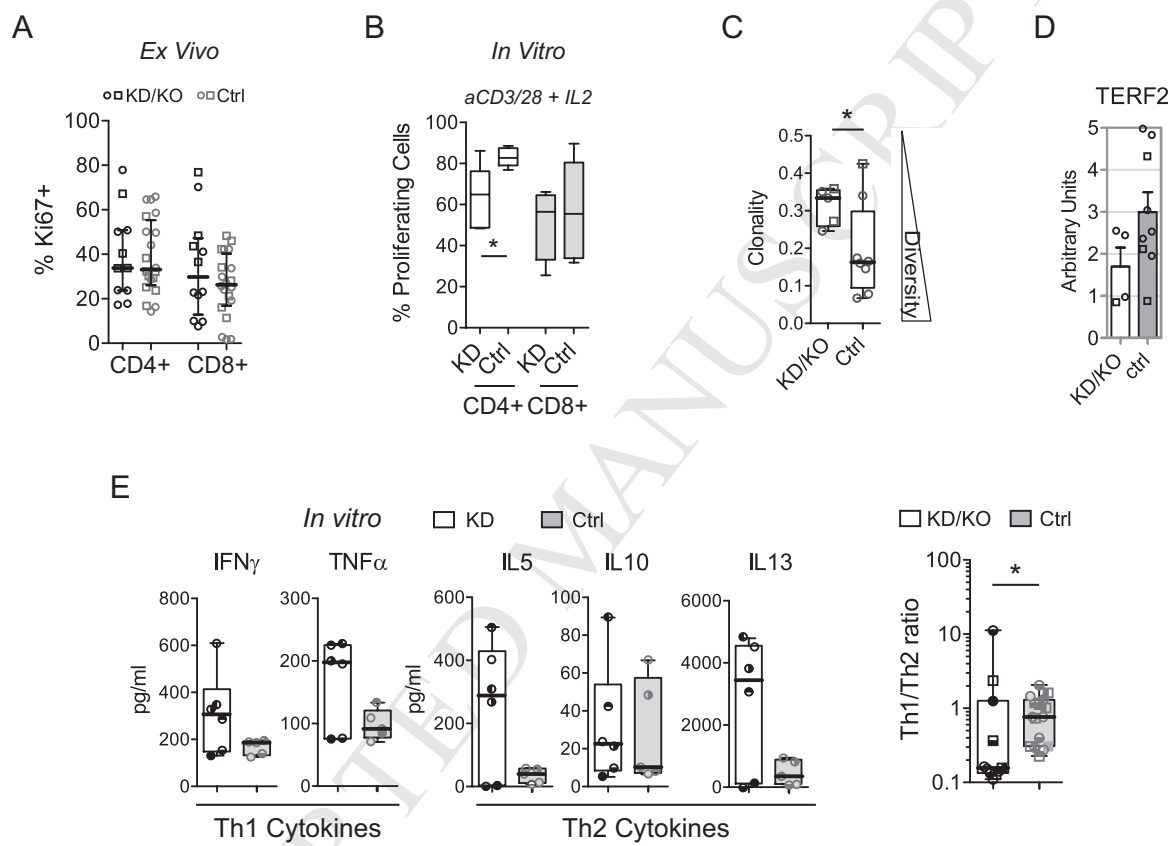


Figure 5



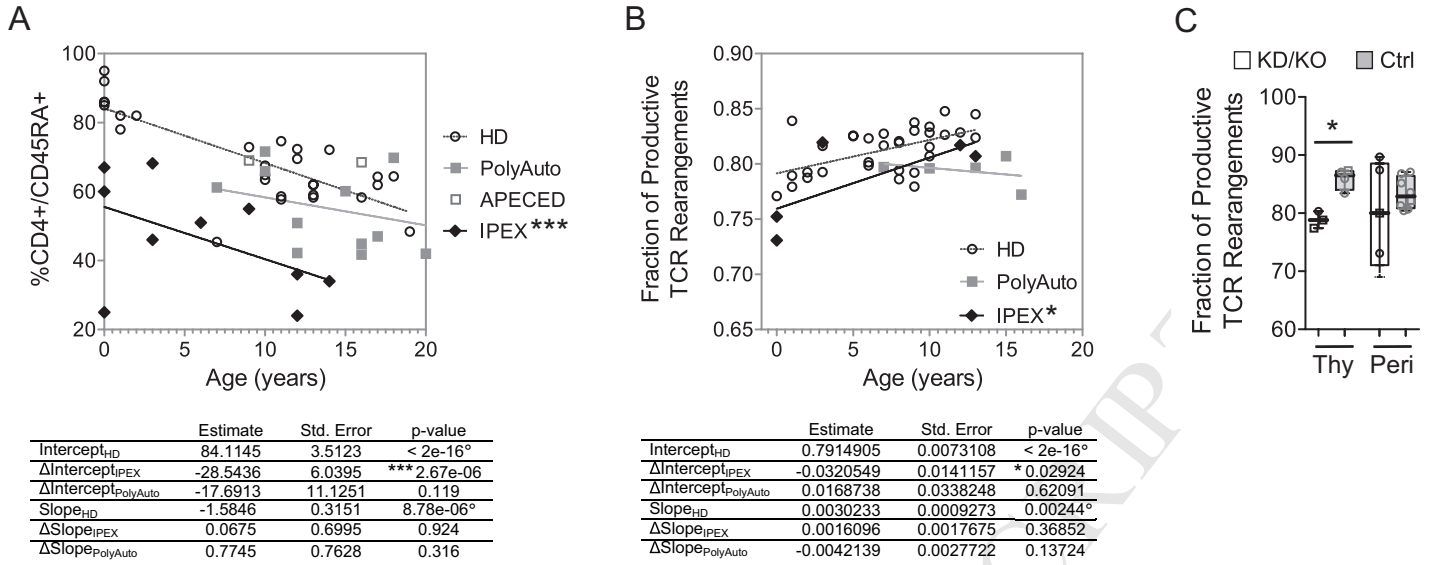


**Table S1.** Clinical details of human donors analyzed in the study

| Donor ID            | Age at time of FC analysis (years) | Age at time of TCR $\beta$ analysis (years) | Diagnosis | Autoimmune manifestations *                                     | Immune suppressive Therapy § | Genetic Mutation |
|---------------------|------------------------------------|---------------------------------------------|-----------|-----------------------------------------------------------------|------------------------------|------------------|
| PA1                 | 15                                 | 15                                          | Polyauto  | T1D/AITD/Gastritis/Fam                                          | NO                           | AIRE HET         |
| PA2                 | 7                                  | 7                                           | Polyauto  | SP/Alopecia/HYP/Fam                                             | NO                           | NO               |
| PA3                 | 10                                 | 10                                          | Polyauto  | T1D/AITD/Vitiligo/Fam                                           | NO                           | ND               |
| PA4                 | 16                                 | 16                                          | Polyauto  | AITD/CD/Fam                                                     | NO                           | ND               |
| PA5                 | 13                                 | 13                                          | Polyauto  | T1D/Vitiligo/Fam                                                | NO                           | ND               |
| PA6                 | 10                                 | -                                           | Polyauto  | AITD/Alopecia/Fam                                               | Topic steroids               | NO               |
| APE1                | 9                                  | -                                           | APECED    | AD/HYP/Fam                                                      | NO                           | AIRE             |
| PA7                 | 20                                 | -                                           | Polyauto  | T1D/AITD/AD                                                     | NO                           | NO               |
| PA8                 | 12                                 | -                                           | Polyauto  | T1D/SP/UC                                                       | NO                           | NO               |
| PA9                 | 17                                 | -                                           | Polyauto  | T1D/CD/SP                                                       | NO                           | ND               |
| PA10                | 17                                 | -                                           | Polyauto  | T1D/AITD/CD                                                     | NO                           | ND               |
| PA11                | 16                                 | -                                           | Polyauto  | AITD/AC/Psoriasis/Alopecia/CD/Fam                               | NO                           | ND               |
| PA12                | 18                                 | -                                           | Polyauto  | T1D/AITD/RA/Fam                                                 | NO                           | ND               |
| PA13                | 12                                 | -                                           | Polyauto  | AITDA/Alopecia/Fam                                              | NO                           | ND               |
| PA15                | 16                                 | -                                           | Polyauto  | T1D/AITD/AC/Fam                                                 | NO                           | NO               |
| APE3                | 16                                 | -                                           | APECED    | HYP/AD/Keratitis                                                | NO                           | AIRE             |
| PA16                | 12                                 | -                                           | Polyauto  | T1D/AITD/SP/CD/Fam                                              | NO                           | ND               |
| IP11 <sup>o\$</sup> | 14                                 | -                                           | IPEX      | Partially controlled disease (T1D, AITD, eczema, alopecia, AHE) | FK506, steroids              | FOXP3 c.1150G>A  |
| IP14 <sup>o\$</sup> | 12                                 | 12                                          | IPEX      | Clinical and histological remission                             | FK506                        | FOXP3 c.725T>C   |

**Table S1.** Clinical details of human donors analyzed in the study

| Donor ID      | Age at time of FC analysis (years) | Age at time of TCR $\beta$ analysis (years) | Diagnosis | Autoimmune manifestations *                                                 | Immune suppressive Therapy §   | Genetic Mutation   |
|---------------|------------------------------------|---------------------------------------------|-----------|-----------------------------------------------------------------------------|--------------------------------|--------------------|
| IP19°         | 0                                  | -                                           | IPEX      | Active disease (diarrhea, lymphadenopathy, hepatitis)                       | Rapamycin, MPD, MMF            | FOXP3 c.1078C>T    |
| IP21°         | 3                                  | -                                           | IPEX      | Active disease (diarrhea)                                                   | CP                             | FOXP3 c.750_752del |
| IP22°         | 0                                  | 0                                           | IPEX      | Active disease (diarrhea, AC, nephropathy)                                  | CSA, PDN                       | FOXP3 c.1110G>A    |
| IP24°         | 9                                  |                                             | IPEX      | Active disease (severe gastric inflammation, ulcers and immune infiltrates) | NO                             | FOXP3 c.210+1G>C   |
|               |                                    | 13                                          |           | Well controlled disease                                                     | Rapamycin                      |                    |
| IP25#         | 12                                 | -                                           | IPEX      | Improving disease (diarrhea)                                                | Rapamycin                      | FOXP3 c.1040G > A  |
| IP28          | 3                                  | 3                                           | IPEX      | Well controlled disease                                                     | Rapamycin                      | FOXP3 c.1190G>A    |
| IP29          | 6                                  | -                                           | IPEX      | No disease                                                                  | NO                             | FOXP3 c.1190G>A    |
| IP31          | 0                                  | 0                                           | IPEX      | Acute disease (T1D, eczema, diarrhea, infections, nephropathy)              | Rapamycin, steroids, rituximab | FOXP3 c.1010G>A    |
| HD35-39 (n=5) | -                                  | range: 0-9                                  | HEALTHY   | NO                                                                          | NO                             | ND                 |
| HIP-HD (n=27) | -                                  | range: 1-13                                 | HEALTHY   | NO                                                                          | NO                             | ND                 |
| HD4-31 (n=28) | range:0-19                         | -                                           | HEALTHY   | NO                                                                          | NO                             | ND                 |

**Table S1.** Clinical details of human donors analyzed in the study

FC: Flow Cytometry

ND: Not determined

\* AC: autoimmune cytopenia

AD: Addison disease

AHA: autoimmune hemolytic anemia

AITD: autoimmune thyroiditis

CD: celiac disease

Fam: familiar history

HYP: hypoparathyroidism

RA: rheumatoid arthritis

SP: skin pathologies

T1D: type 1 diabetes

UC: ulcerative colitis

§ CP: cyclophosphamide

CSA: cyclosporin A

MDP: mycophenolic acid prodrug

MMF: mycophenolate mofetil

PDN: prednisone

° Lampasona et al., PLoSOne 2013 and Barzaghi et al., Front Immunol 2012

# Zama et al., Ital J Pediatr. 2014

HIP-HD from Dean et al., Genome Med 2015

**Table S2.** Flow Cytometry markers used to identify hematopoietic populations

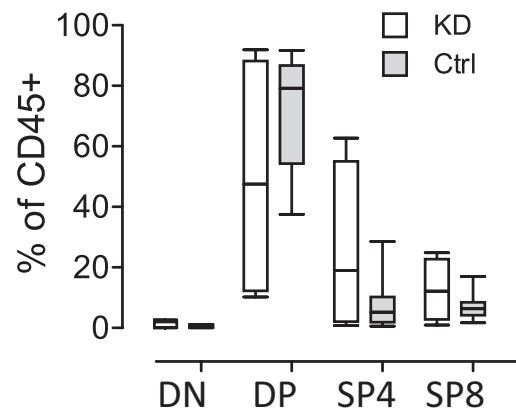
|            | Population  | Markers                              |
|------------|-------------|--------------------------------------|
| Thymocytes | Pre T/DC/NK | CD1a-CD3-CD34+CD38lo                 |
|            | Pre T/NK    | CD1a-CD3-CD34+CD38+                  |
|            | DN          | CD1a+CD4-CD8-                        |
|            | CD4 ISP     | CD1a+CD4+CD8-                        |
|            | DP          | CD3+/-CD4+CD8+                       |
|            | SP4         | CD3+CD4+CD8-                         |
|            | SP8         | CD3+CD4-CD8+                         |
| Periphery  | B           | CD19+                                |
|            | T           | CD3+                                 |
|            | Myeloid     | CD13+                                |
|            | T helper    | CD3+CD4+CD8-                         |
|            | T cytotoxic | CD3+CD4-CD8+                         |
|            | Naive T     | CD4+CD8-/- or CD4-CD8+/CD62L+CD45RA+ |
|            | TEMRA       | CD4+CD8-/- or CD4-CD8+/CD62L-CD45RA+ |
|            | EM T        | CD4+CD8-/- or CD4-CD8+/CD62L-CD45RA- |
|            | CM T        | CD4+CD8-/- or CD4-CD8+/CD62L+CD45RA- |
|            | Treg        | CD4+CD8-/-CD25+CD127loFOXP3+         |

**Table S3.** Antibodies used in this work

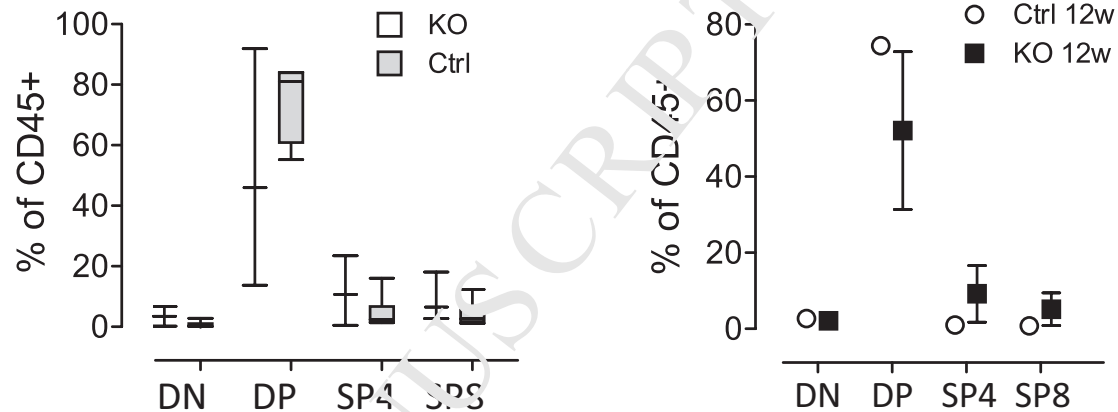
| <b>Flow Cytometry</b>       | <b>Code number</b> | <b>Company</b>         |
|-----------------------------|--------------------|------------------------|
| CD1a-PE                     | 555807             | BD                     |
| CD3-APC                     | 345767             | BD                     |
| CD3-PerCP/Cy5.5             | 300328             | Biolegend              |
| CD3-V500                    | 561416             | BD                     |
| CD4-APC                     | 345771             | BD                     |
| CD4-PECy7                   | 557852             | BD                     |
| CD8-APCH7                   | 641400             | BD                     |
| CD25-BV421                  | 562442             | BD                     |
| CD34-PB                     | MAB-136PB          | Immunological Sciences |
| CD34-PECy7                  | 25-0349-42         | eBiosciences           |
| CD38-PerCP/Cy5.5            | 551400             | BD                     |
| CD45-PB                     | 304029             | Biolegend              |
| CD45-V500                   | 560777             | BD                     |
| CD45RA-PB                   | 304123             | Biolegend              |
| CD62L-PE                    | 341012             | BD                     |
| CD127-PerCP/Cy5.5           | 351322             | Biolegend              |
| FOXP3-Alexa647              | 320214             | Biolegend              |
| Helios-PE                   | 137206             | Biolegend              |
| Ki67-FITC                   | 556026             | BD                     |
| <b>Immunohistochemistry</b> | <b>Clone</b>       | <b>Company</b>         |
| Rabbit monoclonal CD3       | 2GV6               | Ventana                |
| Mouse monoclonal CD4        | SP-35              | Ventana                |
| Rabbit monoclonal CD8       | SP-57              | Ventana                |
| Mouse monoclonal CD20       | L26                | Ventana                |

Figure S1

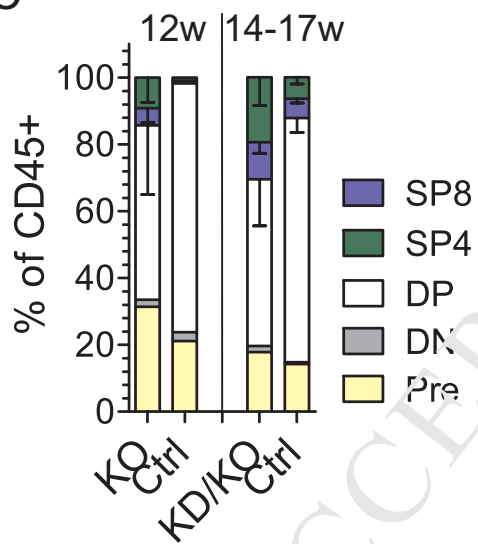
A



B



C



D

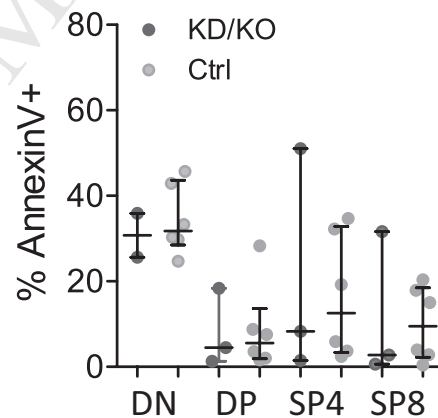


Figure S2

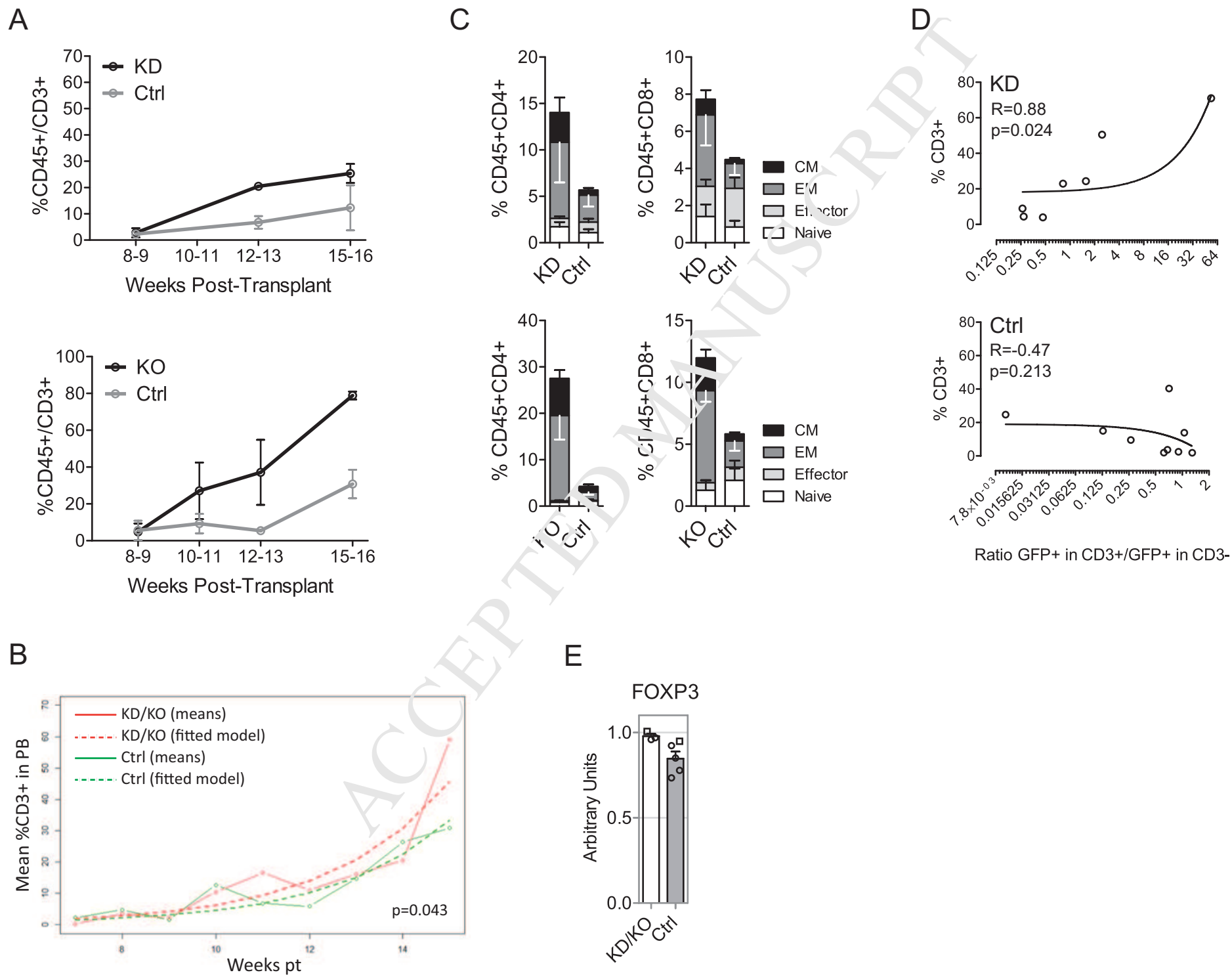
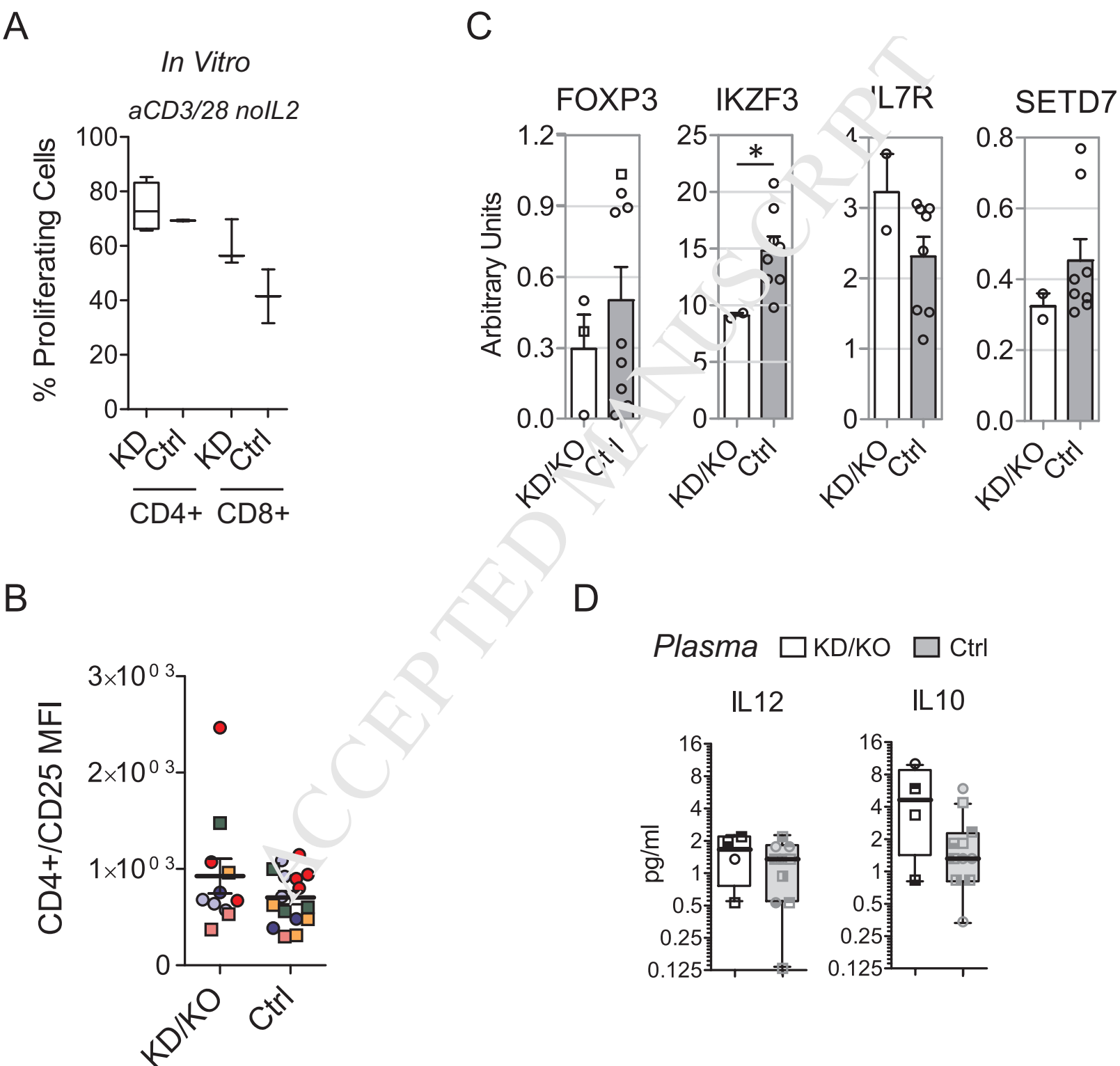


Figure S3



A

