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## CD4<sup>+</sup> regulatory T cells lacking Helios and Eos

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### ABSTRACT

The transcriptional regulators that drive regulatory T (Treg) cell development and function remain partially understood. Helios (*Ikzf2*) and Eos (*Ikzf4*) are closely-related members of the Ikaros family of transcription factors. They are highly expressed in CD4<sup>+</sup> Treg cells and functionally important for Treg cell biology, as mice deficient for either Helios or Eos are susceptible to autoimmune diseases. However, it remains unknown if these factors exhibit specific or partially redundant functions in Treg cells. Here we show that mice with germline deletions of both *Ikzf2* and *Ikzf4* are not very different from animals with single *Ikzf2* or *Ikzf4* deletions. Double knockout Treg cells differentiate normally, and efficiently suppress effector T cell proliferation in vitro. Both Helios and Eos are required for optimal Foxp3 protein expression. Surprisingly, Helios and Eos regulate different, largely non-overlapping, sets of genes. Only Helios is required for proper Treg cell aging, as Helios deficiency results in reduced Treg cell frequencies in the spleen of older animals. These results indicate that Helios and Eos are required for distinct aspects of Treg cell function.

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### 1. Introduction

CD4<sup>+</sup> regulatory T (Treg) cells are required for immunological tolerance, and to suppress the pro-inflammatory response of effector T cells [1]. They are also affected by age, and age-related changes in their function are linked to a decrease in immune fitness and development of inflammatory syndromes in older animals [2]. The molecular mechanisms that regulate Treg cell function remain only partially understood.

Treg cell differentiation and identity are driven largely by the transcription factor (TF) Foxp3 [3,4], as reduced Foxp3 levels are

associated with Treg cell instability and the appearance of inflammatory syndromes [5]. However, Foxp3 alone does not define the entire Treg cell gene signature, and other TFs have been implicated [6,7].

The Helios (*Ikzf2*) and Eos (*Ikzf4*) TFs are two closely-related Ikaros family members which are highly expressed in Treg cells [8,9]. While each is dispensable for Treg cell development [10,11], other studies have revealed that they are important for Treg cell function in autoimmune or inflammatory settings [12–16]. Helios is implicated in Treg cell maintenance, as Helios KO Treg cells are out-competed by WT Treg cells when co-transferred into adoptive recipients [16]. Helios is also required to maintain Foxp3 expression under inflammatory conditions, and prevent conversion of Treg into effector T cells [12]. Eos has been implicated in autoimmunity, as its Treg cell-specific KO leads to worsened disease in autoimmune encephalomyelitis and intestinal bowel disease models [15].

Here we investigated how CD4<sup>+</sup> Treg cells are influenced by the loss of both Helios and Eos in double KO mice. We studied the

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ability of Treg cells from young and old mutant mice to suppress effector T cell function in vitro and the effects of Helios and Eos on gene expression. Surprisingly, our data did not show any additive effect of both mutations, suggesting that both factors exhibit largely non-redundant functions in Treg cells.

## 2. Materials and methods

### 2.1. Mice

The *Ikzf2*<sup>-/-</sup> mouse line was described [10]. A floxed allele for *Ikzf4* was generated by inserting *LoxP* sites into intron 7 and exon 8 downstream of the stop codon (positions chr10:128,633,737 and 128,635,030; mm10 mouse genome). Null *Ikzf4*<sup>-/-</sup> animals were generated by breeding floxed mice with a deleter strain. *Ikzf4*<sup>-/-</sup> mice were initially generated on a C57BL/6 background, but the mice used in this study were on a mixed C57BL/6-129/Sv background, to match the background of the *Ikzf2*<sup>-/-</sup> mice. Most mice used in this study were raised in specific-pathogen-free (SPF) conditions. A limited number of dKO, *Ikzf4*<sup>-/-</sup> and WT mice, including those used for the transcriptomic analyses, were raised in a germ-free ultra-clean bio-bubble. All experiments were approved by the Com'Eth ethical committee (approval number 2012-096).

### 2.2. Transcriptome analysis

All mice used were 6–8 weeks old. Total RNA was extracted from CD4<sup>+</sup>CD25<sup>+</sup> splenic cells (Rneasy kit, Qiagen), and used for analysis with Affymetrix mouse Gene 1.0 ST microarrays, according to standard protocols. For RNA-seq, libraries were prepared with the Clontech SMART-seq Ultra Low Input RNA Kit, followed by the Illumina Nextera XT library preparation kit. Sequencing was performed with a HiSeq 4000 (Illumina) with single-end 50bp read lengths. Differential gene expression analyses were performed using DESeq2 (v1.16.1). Microarray and RNA-seq data are available under the GEO accession numbers GSE162024 and GSE180159.

### 2.3. Flow cytometry and cell sorting

Cells were analyzed with a BD LSR II analyzer, or sorted with a BD FACSAria II cell sorter. All antibodies were from eBioscience, BioLegend or BD Biosciences. The proteins detected by the primary antibodies were: CD3 (145-2C11, KT3), CD4 (RM4-5, GK1.5), CD8 (53–7.3), CD25 (PC61), CD44 (IM78.1), CD62L (MEL-14), CD90.2 (53–2.1), Foxp3 (FJK-16S), Eos (ESB7C2), Helios (22F6). Intracellular staining was performed using the Foxp3 staining protocol (eBioscience), except for Eos where the antibody was added for 1h at RT at 8 µg/ml. Data were analyzed with FlowJo10. For transcriptome analyses, cells were sorted as Lin<sup>-</sup>CD4<sup>+</sup>CD25<sup>+</sup>, where Lin represented staining by antibodies against GR1, CD11b, TER119, CD8 and CD45R (B220). Purity of the sorted populations was >95%.

### 2.4. Treg cell suppression assays

Splenic cells were sorted into APCs (CD90.2<sup>-</sup>), CD4<sup>+</sup> effector T cells (CD4<sup>+</sup>CD25<sup>-</sup>CD44<sup>lo</sup>), or CD4<sup>+</sup> Treg cells (CD4<sup>+</sup>CD25<sup>+</sup>). In each experiment, the APCs and effector cells were sorted from the same third-party WT mouse (6- to 8-week-old), while the Treg cells came from the experimental WT or KO spleens. Suppression assays were performed in 96-well round-bottom plates, using 25 × 10<sup>3</sup> APCs, 25 × 10<sup>3</sup> CFSE-labeled effector cells, increasing numbers of Treg cells, and anti-CD3 antibodies (5 µg/ml, clone 145-2C11 Ultra-LEAF, Biolegend), in RPMI 1640 medium (supplemented with 10% FCS, 25 mM Hepes, 2 mM L-glutamine, 1x non-essential amino acids, 1 mM sodium pyruvate, 50 µM 2-mercaptoethanol and antibiotics).

Cells were analyzed after 3d.

### 2.5. In vitro generation of Treg cells

CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> cells were induced in vitro from purified CD4<sup>+</sup>CD25<sup>-</sup>CD44<sup>lo</sup> splenic cells after 3d on anti-CD3<sup>-</sup> (5 µg/ml) and anti-CD28<sup>-</sup> (2 µg/ml) coated plates in the presence of mTGFβ1 (5 ng/ml, Cell Signaling) and hIL-2 (10 ng/ml, Peprotech) in supplemented RPMI 1640 medium. CD4<sup>+</sup>CD25<sup>+</sup> cells were sorted to >95% purity for further analysis.

## 3. Results

### 3.1. Mice lacking Helios and Eos

To determine how Helios and Eos are expressed in Treg cells, we evaluated their protein expression in thymic and peripheral WT CD4<sup>+</sup>Foxp3<sup>+</sup> cells, by intracellular staining and flow cytometry (Fig. S1a). Most Treg cells in the thymus and spleen co-expressed both proteins, suggesting that Helios and Eos might cooperate in regulating their development and function.

To evaluate the effects of Helios and Eos deficiency in vivo, we generated a novel Eos KO mouse line, where the last exon of *Ikzf4*, encoding the dimerization domain, was targeted by *loxP* sites and germline-deleted (Fig. S1b), and crossed these mutants with germline Helios KO animals [10]. Eos KO mice were born at Mendelian frequencies, were fertile and phenotypically healthy in conventional or SPF conditions (not shown). In contrast, Helios KO mice required SPF conditions to be similarly viable [10,17]. Interestingly, only 25-30% of the expected double KO (dKO) animals survived the weaning age even in SPF conditions (Fig. S1c), indicating that dKO mice are more fragile than the single KO animals. Nevertheless, the dKO mice that survived the perinatal period could live up to 2 years of age without further attrition, perhaps due to the mixed C57BL/6-129/Sv background. Further, loss of Helios or Eos did not affect the expression of the other in the single KO Treg cells (Fig. S1d).

### 3.2. CD4<sup>+</sup> Treg cell phenotype in single and double KO mice

To evaluate the role of Helios and Eos in CD4<sup>+</sup> T cell homeostasis, we analyzed the thymus and spleen of Eos KO and dKO mice, and compared them with those of the Helios KO mice previously described (Fig. S2a; [10]). All mutants displayed normal frequencies of CD4<sup>+</sup> (and CD8<sup>+</sup>) T cells compared with WT controls, regardless of age (Fig. S2, b and c). We analyzed the activation status of splenic CD4<sup>+</sup> T cells in young and old mutants according to CD62L and CD44 expression (Fig. S2, c and d). CD4<sup>+</sup> T cells were mainly CD62L<sup>hi</sup>CD44<sup>lo</sup> in young mice (6-9 weeks of age) of all genotypes, suggesting a naive phenotype. However, increased frequencies of CD62L<sup>lo</sup>CD44<sup>hi</sup> effector memory T cells were detected in some Helios KO mice as early as 6 weeks of age, which were not observed in Eos KO or dKO animals until 40 weeks of age, though their frequencies were still significantly higher in old Helios KO mice. Thus Helios deficiency correlates with the appearance of activated T cells, which is somewhat corrected in dKO mice when Eos is also lost.

To assess the role of Helios and Eos in Treg cells, we studied the CD4<sup>+</sup>Foxp3<sup>+</sup> cells from young single KO and dKO mice, which revealed similar frequencies in mice of all genotypes (Fig. 1, a and b, and S2a). Further, CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> cells, in vitro-generated from naive CD4<sup>+</sup>CD25<sup>-</sup>CD44<sup>lo</sup> T cells stimulated with anti-CD3 + anti-CD28 antibodies in the presence of TGFβ1 and IL-2, were similar in phenotype, yield and the ability to suppress the proliferation of WT effector T cells across genotypes (Fig. S3, and not shown). These results indicated that Helios and Eos are not required for Treg cell

generation and homeostasis in young animals.

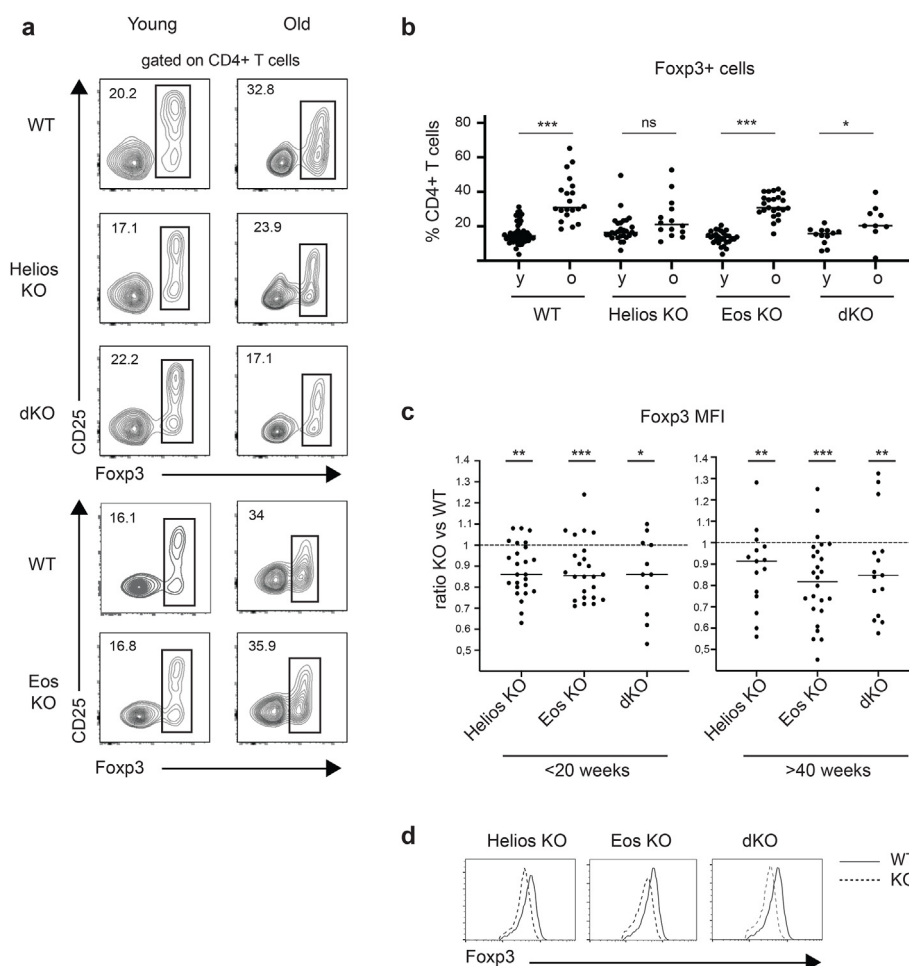
To determine if Helios and Eos are important for maintaining Treg cells with age, we analyzed splenic CD4<sup>+</sup>Foxp3<sup>+</sup> cells of old mice (>40 weeks of age). The proportion of Treg cells increased in old WT animals compared with young adults, as expected (Fig. 1, a and b) [2,18]. Similar results were observed in Eos KO and dKO mice. Interestingly, the percentage of CD4<sup>+</sup>Foxp3<sup>+</sup> cells did not significantly increase in old Helios KO mice compared with young animals. None of the KO mice showed consistent signs of autoimmunity with age, though enlarged spleens were observed in about half of the old Helios KO and dKO animals, compared with age-matched controls (Fig. S4). No splenomegalies were detected in the Eos KO mice.

Since Helios has been reported to regulate Foxp3 expression in Treg cells [12,19], we measured Foxp3 levels in Helios KO, Eos KO and dKO splenic CD4<sup>+</sup>Foxp3<sup>+</sup> cells by flow cytometry. Foxp3 expression was reduced approximately 10% in the cells of all KO animals, young and old (Fig. 1, c and d). The effect was not cumulative in dKO cells. Thus, both Helios and Eos are important for Foxp3 expression but they do not cooperate in its regulation.

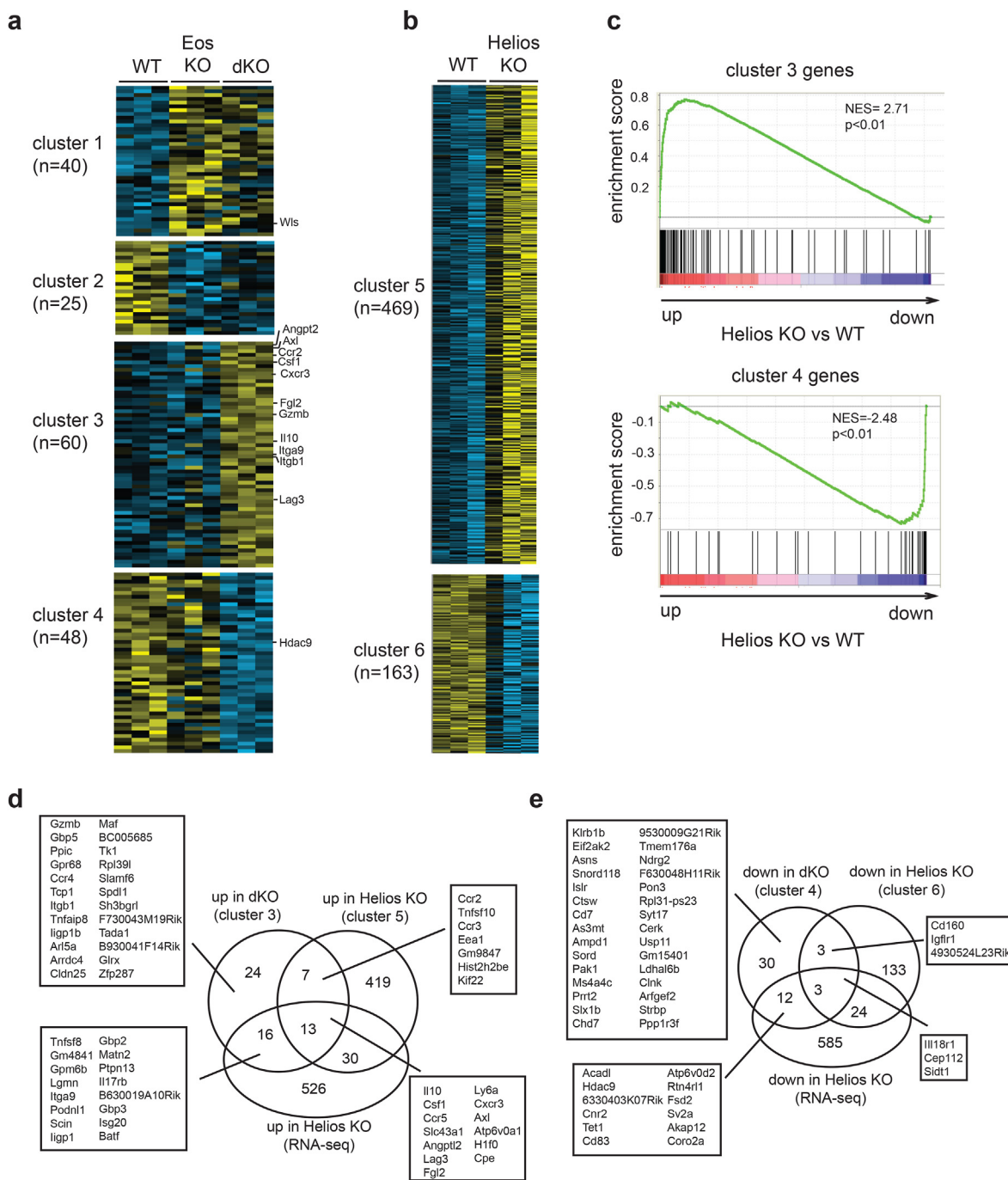
### 3.3. Helios and Eos regulate separate gene sets in CD4<sup>+</sup> Treg cells

To investigate the molecular impact of Helios and Eos deficiency, we analyzed the transcriptomes of WT and mutant Treg cells (CD4<sup>+</sup>CD25<sup>+</sup>) from young mice (6 weeks old). In the first analysis, Eos KO or dKO cells were compared with WT cells. This revealed two main gene expression patterns where (i) 65 genes were similarly deregulated in Eos KO and dKO cells compared with WT (Fig. 2a, clusters 1 and 2, and Table S1), and (ii) 108 genes were deregulated only in the dKO cells (clusters 3 and 4). In the second analysis, we compared the transcriptomes of WT and Helios KO Treg cells, which revealed that 632 genes were differentially expressed (Fig. 2b and Table S1). Almost all of the dKO-specific genes from clusters 3 and 4 were also observed in the Helios KO cells (Fig. 2c), indicating a Helios-dependent effect. Some proliferation-associated genes were upregulated in the Helios KO cells compared with dKO in the microarray analysis (Table S1). However, these genes were not confirmed in a subsequent independent RNA-seq analysis (Fig. 2d), suggesting that their upregulation may be due to a difference in housing conditions (see Materials and Methods).

Among the genes commonly deregulated in Helios KO



**Fig. 1.** - Splenic Treg cells in young and old single and double KO mice. (a) Representative analysis of CD25 and Foxp3 expression on splenic CD4<sup>+</sup> T cells from the indicated young (<20 weeks old) and old (>40 weeks old) mice. The Eos KO mice were analyzed with their WT controls. (b) Percent of Foxp3<sup>+</sup> cells among splenic CD4<sup>+</sup> T cells from young (y) and old (o) mice. \*p < 0.05, \*\*\*p < 0.001 (Mann-Whitney test). (c) Ratio of the mean fluorescence intensities (MFI) of Foxp3 staining on splenic CD4<sup>+</sup>Foxp3<sup>+</sup> T cells from Helios KO, Eos KO, or dKO vs WT mice, young and old. Ratios were calculated as a function of the average MFI values on WT cells analyzed concomitantly. The statistical significance of the difference in the MFI was calculated with the paired Student's t-test, using MFIs from pairs of WT/KO samples analyzed in the same experiment. \*p < 0.05, \*\*p < 0.01, \*\*\*p < 0.001. (d) Representative histograms of Foxp3 expression in old mice of the indicated genotypes.



**Fig. 2.** - Gene expression of Treg cells from single and double KO mice. (a) Genes up- or downregulated in splenic Eos KO or dKO CD4<sup>+</sup>CD25<sup>+</sup> cells compared with WT. Probesets up- or downregulated were selected with the following criteria: mean log<sub>2</sub>FC > (or <) 0.4, and log<sub>2</sub>FC > (or <) 0 for all pairs of WT/mutant samples. Clusters of selected probesets were generated with K-means clustering. (b) Genes up- or downregulated in Helios KO CD4<sup>+</sup>CD25<sup>+</sup> cells compared with WT (selected as in (a); K-means clustering). (c) GSEA of clusters 3 and 4 genes deregulated in the dKO samples in (a), versus a ranked gene list corresponding to all probesets from the microarray according to their Helios KO vs WT FC. NES: net enrichment score. The p-value was calculated by GSEA on the basis of 100 random permutations of the ranked gene list. (d and e) Venn diagram showing the intersection of genes upregulated (d) and downregulated (e) in dKO and Helios KO Treg cells, which were analyzed with the microarrays and in an independent RNA-seq experiment. Genes selected in the RNA-seq experiment met the criteria log<sub>2</sub>FC > 0.2 (or < -0.2) for separate comparisons of each Helios KO sample to both WT samples.

(microarray and/or RNA-seq) and dKO Treg cells, 36 were up- and 18 were downregulated (Fig. 2, d and e). Upregulated genes included those encoding proteins specific to Treg cells (eg. *Angptl2*, *Axl*, *Lgmn*, *Csf1*, *Atp6v0a1*; according to Immgen), or important for Treg cell function, like cytokine production (*Il10*), cell migration and adhesion (*Ccr5*, *Cxcr3*, *Itga9*) and suppression (*Lag3*, *Fgl2*) (Fig. 2). Downregulated genes included negative regulators of Treg cell

activity (eg. *Hdac9*, *Cd83*, Fig. 2e) [20,21]. Among the genes deregulated in Eos KO and dKO Treg cells, the most consistently upregulated gene was *Wls* (2x in both the Eos KO and dKO cells; Table S1), which encodes the Wntless regulator of Wnt protein secretion, an important modulator of Treg cell function [22]. Few genes were exclusively deregulated in the dKO Treg cells (Fig. 2, d and e). Helios- and Eos-dependent genes were also distinct from



those targeted by Foxp3 [3], as most of the previously identified Foxp3-dependent genes were not perturbed in the mutant cells.

The gene expression changes in the Helios KO and dKO Treg cells suggested an activated state. Indeed, gene set enrichment analysis (GSEA) revealed that genes from a known activated Treg cell signature [23] were significantly enriched among genes upregulated in Helios KO and dKO Tregs (Fig. 3). Genes repressed in activated Treg cells were not significantly deregulated in the Helios KO cells, but were enriched among genes downregulated in the dKO cells, suggesting a possible contribution by Eos.

Collectively, these results showed that Helios and Eos affect different genes in Treg cells, and that Helios has more influence on gene expression than Eos.

### 3.4. CD4<sup>+</sup> Treg cells without Helios or Eos suppress efficiently *in vitro*

To determine how Helios and Eos affect Treg cell function, we studied the ability of single and double KO Treg cells, from young and old mice, to suppress T cell proliferation *in vitro*. Increasing numbers of splenic Treg cells (sorted as CD4<sup>+</sup>CD25<sup>+</sup> cells) were cultured with CFSE-labeled effector T cells (CD4<sup>+</sup>CD25<sup>-</sup>CD44<sup>-</sup>) from a young, third-party WT mouse, in the presence of CD90.2<sup>-</sup> APCs from the same WT, and anti-CD3 + anti-CD28 antibodies. After 3 days, the effector T cells were measured for their proliferation index and frequency of non-dividing (CFSE<sup>hi</sup>) cells, both measured as ratios of KO/WT cells in the different samples.

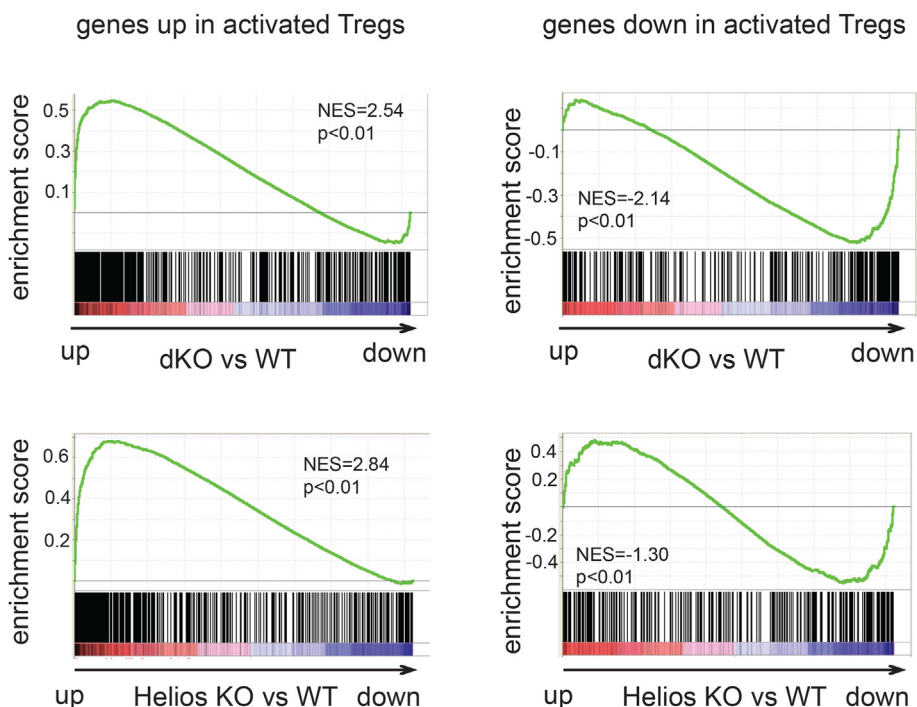
Treg cells from all mice efficiently suppressed the proliferation of effector T cells (Fig. 4, a-c). Interestingly, the Treg cells from both young and old Helios KO or dKO animals were more efficient than WT or Eos KO cells, as seen by a lower KO/WT ratio in the proliferation index and a higher corresponding KO/WT ratio in the percentage of non-dividing cells (Fig. 4d). These results suggested that Helios limits the intrinsic, suppressive ability of Treg cells.

## 4. Discussion

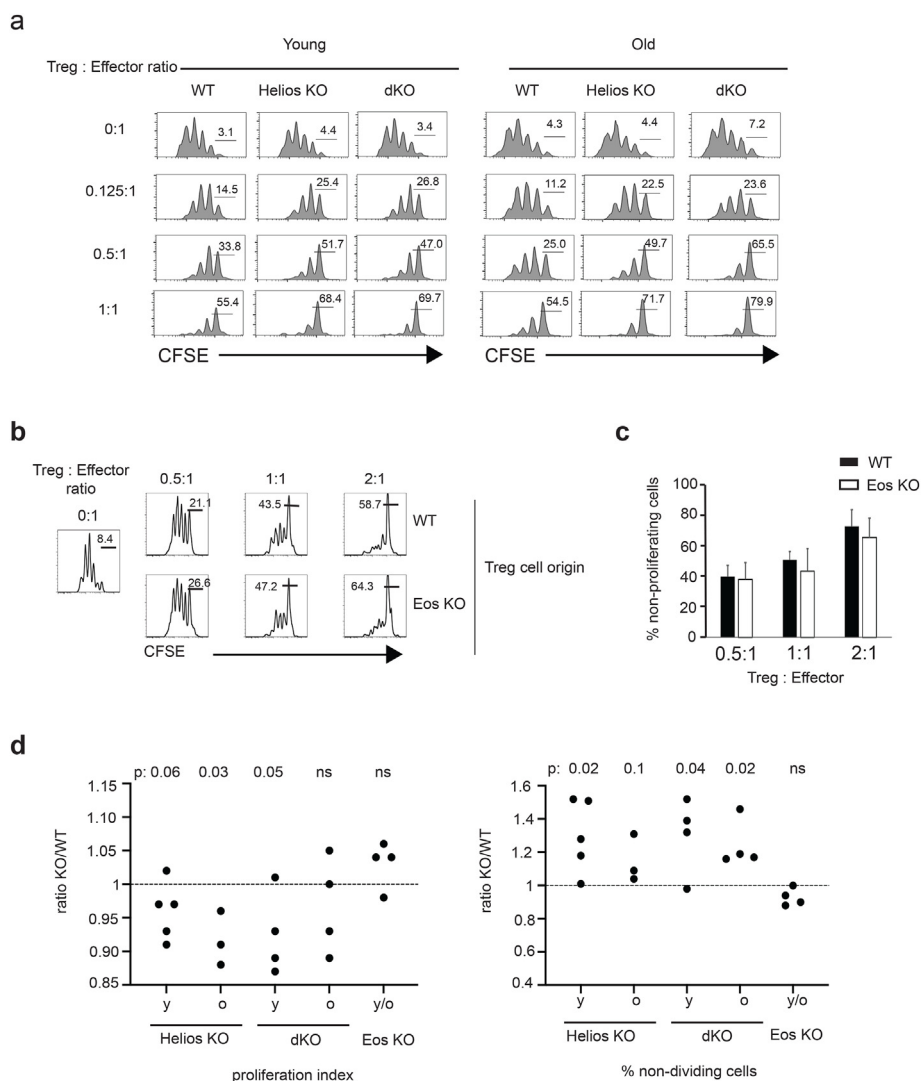
Here we studied the effects of Helios and Eos on CD4<sup>+</sup> Treg cell homeostasis and function. We show that deletion of one or both genes in the germline has a minor impact on Treg cell homeostasis in the thymus or spleen, and that mutant Treg cells still exhibit considerable, if not more, suppressive activity *in vitro* compared with WT. Helios has a stronger effect than Eos, and mice lacking both proteins exhibit a milder phenotype than animals lacking only Helios, suggesting that Eos partially antagonizes Helios. At the transcriptomic level, loss of Helios or Eos results in deregulated gene sets that are largely exclusive. Helios and Eos also do not synergize to regulate gene expression, as most of the genes deregulated in dKO cells are already deregulated to similar levels in cells lacking either Helios or Eos.

Our results suggest that Helios is required to maintain a functional CD4<sup>+</sup> Treg cell pool with age. Previously, mice with a Treg cell-specific deletion of Helios were reported to possess Treg cells that were unstable in phenotype and/or fitness [12,14,16]. Because these mice also displayed a generalized inflammation, it was unclear if the unstable phenotype came from Helios loss and/or inflammation [12,14]. We show that Treg cells from 6-week-old Helios KO mice already exhibit a transcriptomic signature resembling activated cells, well before the appearance of inflammation that occurs only sometimes in older mutants (>30 weeks of age). These results indicate that Helios negatively regulates the activation and suppressive qualities of Treg cells. However, Helios appears to do so indirectly, as no Helios binding was seen at most of the differentially expressed genes in published ChIP-seq data [12].

Both Helios and Eos are required for Foxp3 expression, as Foxp3 levels decrease ~10% in Helios KO, Eos KO and dKO Treg cells. This phenotype was already observed in young mice, and did not change with age. How Helios and Eos contribute to Foxp3 expression remains unknown. Our transcriptome analyses did not reveal



**Fig. 3.** - Helios KO and dKO Tregs exhibit a gene expression profile of activated Tregs. GSEA of published genesets up- or downregulated in activated Treg cells, versus ranked gene lists corresponding to all probesets from the microarray according to their dKO vs WT, or Helios KO vs WT, FC. NES: net enrichment score.



**Fig. 4.** - Treg cells from young and old single or dKO mice suppress efficiently in vitro. (a) Representative suppression assays of CFSE-labeled WT effector T cells ( $CD4^+CD25^-$ ) cultured in the presence of anti-CD3 Abs and WT APCs, with increasing numbers of splenic Treg cells from mice of the indicated ages and genotypes. Cell division was measured as a function of CFSE dilution after 3d. The gated population indicates non-dividing cells. Young: <20 weeks old; old: >50 weeks old. (b) Representative suppression assay performed with splenic Treg cells from WT or Eos KO mice. (c) Graph representing the mean of the % of non-proliferating cells at various Treg:effector cell ratios in suppression assays performed with Tregs cells from WT or Eos KO mice. Error bars represent standard deviation (two independent experiments). (d) Graphs representing the ratios of proliferation (left), and non-dividing cells (right) of a given mutant sample compared with that of the WT sample from the same experiment. The Treg:effector cell ratio chosen for these calculations was the same for all samples of a given experiment, where the proportion of non-dividing WT cells was 30–50%. P-values were calculated with the paired t-test.

decreased *Foxp3* mRNA levels in mutant Treg cells, suggesting that Helios or Eos do not regulate *Foxp3* at the transcriptional or post-transcriptional levels. Furthermore, no Helios binding is seen at the *Foxp3* locus in published ChIP-seq data [12]. Helios and Eos probably regulate *Foxp3* expression by promoting the activity of unknown factors that control *Foxp3* translation or degradation, or through direct protein-protein interaction with *Foxp3* to increase the latter's stability [7,8]. Since adequate *Foxp3* levels are important for Treg cell maintenance [5], its reduced expression in KO Treg cells may explain the observed Treg cell instability in these cells.

The ability of Helios and Eos to repress autoimmunity has been previously investigated in single KO mice (Treg cell-specific or germline), using murine models of scurfy, inflammatory bowel disease and multiple sclerosis [12,14,15]. There are differences between our study and those studies, including one that used the same Helios KO line that we generated and studied here [12]. In our hands, the germline Helios mutants, single and double KO, selectively develop splenomegaly with age, while the germline Eos KO

animals do not show overt signs of autoimmunity up to 1.5 years of age and age as well as WT littermates. The reasons for these differences are unclear, and could be due to differences in the microbiome, which are strongly affected by the sanitary conditions of the mice. Indeed, we observed that germline Helios KO Treg cells are more activated in mice from specific pathogen-free vs germ-free colonies, and that Helios KO and dKO animals do not survive well in conventional settings.

**Declaration of competing interest**

All authors declare that they have no competing interest.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.bbrc.2023.06.087>.

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