CD4⁺ and CD8⁺ regulatory T cells characterization in the rat using a unique transgenic *Foxp3-EGFP* model.

Short running title. Treg identification using *Foxp3-GFP* rats

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Abstract.

Background: CD4⁺ and CD8⁺ regulatory T cells (Treg) in diverse species include different subsets from different origins. In all species, CD8⁺ Treg have been poorly characterized. CD4⁺ and CD8⁺ Treg in rats have only partially been characterized and there is no rat model in which FOXP3⁺ Treg are genetically tagged.

Results: We generated a rat transgenic line using the CRISPR/Cas9 system in which EGFP was inserted in frame on the 3' end of the *Foxp3* gene using a 2A self-cleaving peptide. EGFP was exclusively expressed by CD4⁺ and CD8⁺ T cells in similar proportion as observed with anti-FOXP3 antibodies. CD4⁺EGFP⁺ Treg were 5-10 times more frequent than CD8⁺EGFP⁺ Treg. CD4⁺ and CD8⁺ EGFP⁺ Treg expressed both the CD25^{high}CD127^{low}CD45RC^{low/-} markers. The suppressive activity of CD4⁺ and CD8⁺ Treg was largely confined to EGFP⁺ cells. RNAseq analyses showed similarities but also differences among CD4⁺ and CD8⁺ EGFP⁺ cells and provided the first description of the natural FOXP3⁺ CD8⁺ Treg transcriptome. In vitro culture of CD4⁺ and CD8⁺ EGFP⁺ cells with TGFbeta and IL-2 resulted in the induction of EGFP⁺ Treg. Preferential expansion of CD4⁺ and CD8⁺ EGFP⁺ Treg could be detected upon in vivo administration of a low dose of IL-2.

Conclusions: This new and unique *Foxp3-EGFP* rat line constitutes a useful model to identify and isolate viable natural and induced CD4⁺ and CD8⁺ Treg. Additionally, it allows to identify new molecules expressed in CD8⁺ Treg that may allow to better define their phenotype and function not only in rats but also in other species.

Keywords: Immune tolerance; genome editing; gene knockin, Foxp3, CD4⁺ Treg, CD8⁺ Treg.

Introduction.

Regulatory T cells (Treg) play a central role in controlling immune effector mechanisms (1-3). Both, CD4⁺ and CD8⁺ Treg have been repeatedly described in humans, non-human primates and rodents (3-5). CD4⁺FOXP3⁺ Treg can be natural (nTregs), i.e. physiologically developed in the thymus and then migrate to the periphery (3). The existence of natural CD8⁺FOXP3⁺ Treg has not been yet formally demonstrated (4). At the same time, CD4⁺ and CD8⁺FOXP3⁺ Treg can be induced from FOXP3⁻ T (iTreg) in pathophysiological situations or following treatments, either *in vivo* or *in vitro* (1, 3, 4, 6, 7). Additionally, there are also CD4⁺ and CD8⁺ iTreg that are FOXP3⁻ (1, 4, 8). The FOXP3 transcription factor is essential for the function of canonical natural CD4⁺ Treg (1). CD8⁺ Treg is a heterogeneous cell population, as CD4⁺ Treg, but the phenotype and function of natural CD8⁺FOXP3⁺ Treg is less well defined than for CD4⁺FOXP3⁺ Treg (4). We and others have described in rats and humans a population of cells CD8⁺ Treg that is defined by the low or absent expression of CD45RC (CD8⁺CD45RC^{low/-}) (7, 9-12). In mice, although CD45RC has been used to purify CD4⁺ and CD8⁺ (13) Treg little characterization of these cells has been done. In rats and humans all CD4⁺ and CD8⁺ Treg suppressive activity is comprised among the CD45RC^{low/-} fraction but the FOXP3⁺ cells are only a small fraction of this population. Although CD4⁺ Treg in all species are comprised in the CD25^{high}CD127^{low} fraction of CD45RC^{low/-} cells, for CD8⁺ Treg there is no consensus on the markers useful to further refine the analysis of the CD45RC^{low/-} population and to analyze if the suppressive activity is exclusively within the FOXP3⁺ fraction (4, 7).

Mouse FOXP3⁺CD4⁺ Treg have been extensively analyzed using transgenic animals in which the *Foxp3* promoter controls reporter genes (14-20) allowing the sorting of viable CD4⁺ *Foxp3*-GFP⁺ Treg. CD8⁺FOXP3⁺ cells were described but an in dept analysis of their phenotype and function was not reported (15-18).

Rat CD4⁺ and CD8⁺ Treg are in general less well defined than human and mouse Treg and comparison of Treg among different species are very rare and only on the T CD4⁺ lineage (5). There is no description of rat transgenic lines that allow to define FOXP3⁺ cell lineages.

The immune system of rats have several characteristics that make them more similar to humans than mice, such as expression of MHC-II molecules by T cells, CD4 expression by a subset of DCs and normal levels of complement (21, 22). Additionally, certain human immune diseases are better modeled in rat than in mouse models with the same genetic mutations (22), such as for ankylosing spondylopathy (23) and autoimmune

polyendocrinopathy candidiasis ectodermal dystrophy (APECED) (24), in *HLA-B27* transgenic and in *Aire* knockout rats, respectively.

Thus, there is need to better define Treg in the rat, specially the CD8⁺ Treg population and to dispose of animals with Foxp3-tagged Treg that can be used to study Treg in different situations.

Using CRISPR/Cas9 and transgenic technologies we generated a rat line with a knockin of EGFP in the 3' of the *Foxp3* gene allowing the natural expression of FOXP3 from the targeted allele with the expression of EGFP under the control of the endogenous *Foxp3* promoter. The phenotype, suppressive function and transcriptomic of both CD4⁺ and CD8⁺ EGFP⁺ Treg were analyzed. The transcriptomic analysis of CD8⁺EGFP⁺ Treg is to the best of our knowledge the first one to be described. Using EGFP as a marker we show expansion of both CD4⁺ and CD8⁺ Treg following IL-2 treatment in vivo and generation of iTreg in vitro.

Results.

Generation of transgenic rats with EGFP knockin into the *Foxp3* gene.

To mark FOXP3-expressing cells we inserted the EGFP coding sequence in-frame and before the stop codon of the *Foxp3* gene (**Fig. 1A**). EGFP was preceded by a T2A sequence that allowed self-splicing of a monocistronic *Foxp3-EGFP* mRNA. Thus *Foxp3*-expressing cells will show simultaneous and independent expression of FOXP3 and EGFP under the control of the endogenous *Foxp3* promoter (referred to as *Foxp3-EGFP* rats below).

Rat zygotes were microinjected and were transferred (n=148) into pseudopregnant females. Newborns (n=25) were genotyped by PCR followed by sequencing and one founder was identified as a KI (**Fig. 1B**) whereas other founders (n=4) showed indels (**data not shown**).

This founder was mated with a wild-type partner and a *Foxp3-EGFP* rat line was stably generated with a mendelian segregation of the *Foxp3-EGFP* allele (data not shown).

CD8⁺ and CD4⁺ Treg are the only mononuclear cells that express EGFP.

Cytofluorimetric analysis of lymph nodes, thymus, spleen, blood and bone marrow showed that a proportion of TCR⁺CD4⁺ or TCR⁺CD8⁺ cells expressed EGFP that was equivalent to the proportion of FOXP3⁺ cells detected using an anti-FOXP3 antibody (**Fig. 2 and supplementary Fig. 1**). Of note, analysis of homozygote *Foxp3-EGFP* animals showed normal expression of FOXP3 as detected using the anti-FOXP3 antibody and all FOXP3⁺ were also EGFP⁺ (**data not shown**) indicating that the expression of FOXP3 was not impaired by the insertion of the EGFP. NK and B cells as well as monocytes/macrophages did not express EGFP and EGFP was not expressed by cells others than T cells (**data not shown**). A quantitative analysis of CD4⁺EGFP⁺ T cells in thymus and spleen ranged in percentage between 5 and 10% of the cells, respectively (**Table 1**). In absolute numbers, CD4⁺EGFP⁺ T cells ranged between 8x10⁴ and 2.2x10⁶ cells per ml of blood and in the spleen, respectively (**Table 1**). CD8⁺EGFP⁺ T cells represented roughly 10% of the CD4⁺EGFP⁺ cells and ranged between 0.6 and 1.3% of the cells in the thymus and lymph nodes, respectively (**Table 1**). In absolute numbers, CD8⁺EGFP⁺ cells ranged between 0.7 x10⁴ and 0.4x10⁶ cells per ml of blood and in lymph nodes, respectively (**Table 1**).

Thus, *Foxp3-EGFP* rats appear to be a faithful tool to identify and analyze CD4⁺ and CD8⁺ Treg.

Phenotype and lymphoid organ distribution of TCR⁺CD4⁺EGFP⁺ and TCR⁺CD8⁺EGFP⁺ Treg.

EGFP⁺ cells, both CD4⁺ and CD8⁺ T cells were mostly CD25⁺CD127^{low} cells (**Fig. 3A**), a phenotype largely belonging to CD4⁺FOXP3⁺ Treg in humans, mice and rats (5) but not yet clearly defined for CD8⁺ Treg. EGFP⁺ cells were detected only among CD45RC^{low/-} T cells (**Fig. 3B**), a marker that has been used both for CD4⁺ and CD8⁺ Treg in rats and humans (9, 11, 12). The markers CD25, MHC- II, CD28, and CD44 were more and CD62L less expressed in both CD4⁺ and CD8⁺EGFP⁺ vs. EGFP⁻ cells (**Fig. 3C**). CD5 and CD26 were equally expressed by all cell subtypes (**data not shown**).

The proportion and absolute numbers of TCR⁺CD4⁺EGFP⁺ Treg vs. TCR⁺CD8⁺EGFP⁺ Treg were ~10-fold higher in thymus, blood, spleen and bone marrow and ~5-fold higher in lymph nodes (**Table 1**).

We compared the frequency and expression levels of TCR⁺CD4⁺EGFP⁺, TCR⁺CD8⁺EGFP⁺ Treg in rats to the ones in mice also harboring EGFP in the 3' end of the *Foxp3* gene, and thus as in *Foxp3-EGFP* rats, controlled by the *Foxp3* endogenous promoter (14). The intensity of EGFP expression was comparable (**data not shown**). A quantitative analysis of TCR⁺CD4⁺EGFP⁺ cells in rat vs. mice in different immune compartments showed similar percentage in spleen and bone marrow and higher in thymus, lymph nodes and blood (**Supplementary Table 1**). Surprisingly, TCR⁺CD8⁺EGFP⁺ cells were increased in rats vs. mice in all compartments, particularly in the thymus, lymph nodes and bone marrow (**Supplementary Table 1**).

Overall, in rats and mice CD4⁺ Treg are more abundant than CD8⁺ Treg, the phenotype of CD8⁺ Treg resemble the one of CD4⁺Treg when analyzed with a restricted panel of markers and the frequency of CD4⁺Treg and more particularly in CD8⁺Treg was higher in rats vs. mice.

In vitro suppressive function of EGFP⁺ and EGFP⁻ fractions of CD4⁺ and CD8⁺ Treg.

We then aimed to confirm the suppressive function of CD4⁺ and CD8⁺ EGFP⁺ Tregs by using a T cell proliferation assay. Present cell surface markers identifying CD8⁺ Tregs are less discriminative than for CD4⁺ Tregs. Since CD8⁺CD45RC^{low/-} and not CD8⁺CD45RC^{high} T cells contain most of the suppressive activity among CD8⁺ cells both in rats (25) and humans (7) we sorted CD8⁺CD45RC^{low/-} cells either EGFP⁺ or EGFP⁻ and compared their suppressive function. Although CD4⁺CD25⁺CD127^{low} Tregs contain a very small fraction of EGFP⁻ cells, to keep the same strategy of analysis for CD4⁺ and CD8⁺ Tregs, we sorted CD4⁺CD25^{high}CD127^{low} cells EGFP⁺ or EGFP⁻ fractions and compared their suppressive

activity. The suppressive activity was confined to the EGFP⁺ fraction for both the CD8⁺CD45RC^{low/-} and CD4⁺CD25^{high}CD127^{low} Tregs (**Fig. 4 and supplementary Figure 2**). Thus, isolation of viable CD4⁺ and CD8⁺ Treg by their expression of EGFP allows to explore their suppressive function.

Transcriptomic analyses of natural and induced EGFP⁺ CD8⁺ and CD4⁺ Treg.

To gain further insight into the molecular properties of CD4⁺ and CD8⁺ Treg we sorted CD25^{high}CD127^{low}CD4⁺EGFP⁺, CD25^{high}CD127^{low}CD4⁺EGFP⁻, CD45RC^{low/-}CD8⁺EGFP⁺ and CD45RC^{low/-}CD8⁺EGFP⁻ cells and compared their transcriptomic profiles. Principal components analysis (PCA) showed that CD4⁺EGFP⁺ vs. CD4⁺EGFP⁻ (**Fig. 5A left**) and CD8⁺EGFP⁺ vs. CD8⁺EGFP⁻ T cells (**Fig. 5A middle**) clustered separately. Notably, there was no clear distinction between the CD4⁺EGFP⁺ and CD8⁺EGFP⁺ T cells (**Fig. 5A right**).

Comparison gene expression revealed multiple differentially expressed genes (DEG) between the different cell types (FDR Q-value <0.05) (**Fig. 5B**). When comparing CD4⁺EGFP⁺ vs. CD4⁺EGFP⁻ cells, CD4⁺EGFP⁺ cells had 309 genes with higher expression. Among them were several Treg specific genes (eg: *Foxp3*, *Ikzf2*, *Tox*, *Lrrc32(GARP)*). Whereas CD4⁺EGFP⁻ cells had 290 genes upregulated with some genes with a CD4⁺ Th cell specific (eg: *Nkg7*, *Gzmb*, *Cd40lg*...) (**Fig. 5B left, supplementary table 2**). Comparison of CD8⁺EGFP⁺ vs. CD8⁺EGFP⁻ cell, revealed that CD8⁺EGFP⁺ cells had 373 genes with higher expression, again with genes that are specific to Treg such as (eg: *Foxp3*, *Lrrc32(GARP)*, *Il2ra*) but others acts as regulators of CD8⁺ (eg: *C1qa* and *C1qc*) whereas CD8⁺EGFP⁻ cells 363 genes upregulated, such as genes critical for the cytotoxic function of effector CD8⁺ T (eg : *Gzmk*, *Gzmm*...) (**Fig. 5B middle, supplementary table 3**). When comparing CD4⁺EGFP⁺ vs. CD8⁺EGFP⁺ cells, CD4⁺EGFP⁺ cells upregulated 322 genes, such as *Gm2a*, *Smpdl3a* and *Nrp1*. CD8⁺EGFP⁺ upregulated 242 genes, such as *Lag3*, *Np4*, *C1qa*, and *Tnfrsf18* (**Fig. 5B right, supplementary table 4**).

Heatmap analysis of DEG grouped together with different transcriptomic profiles all CD4⁺EGFP⁺ vs. CD4⁺EGFP⁻ cells, CD8⁺EGFP⁺ vs. CD8⁺EGFP⁻ cells, as well as CD4⁺EGFP⁺ cells vs. CD8⁺EGFP⁺ cells (**Fig. 5 C**). Among these DEG many were genes of the immune system and for CD4⁺EGFP⁺ cells with a fold change of >1-fold there were already identified key genes such as some described above as well as *Itgb8*, *Cd80* and *Tnfrsf9* (**Supplementary Table 2**) (26-30).

For CD8⁺EGFP⁺ cells with a fold change of >1-fold there were genes shared with CD4⁺EGFP⁺ Treg such as some described above as well as *Il4r*, *Fcna* and *Art2b* as well as

others previously described in human or mouse CD4⁺ Treg, such as *Il2ra*, *Lgmn* (31) and *Tnfrsf9* (**Supplementary Table 3**).

We then compared upregulated genes with immune functions in CD4⁺EGFP⁺ vs. CD8⁺EGFP⁺ Treg (**Supplementary Table 4**). In CD4⁺EGFP⁺ Treg we observed increase expression of genes such as *MHC-II (LOC688090)*, *Lrrc32*, *Ccr6*, *Selplg* and *Tnfrsf4* (**Supplementary Table 4**). In CD8⁺EGFP⁺ Treg we observed upregulation of genes with immune function not previously linked to Treg, such as *Fcna*, *Hmox1*, *Spic* and complement pathway genes (*C1qc*, *C1qa*, and *C1qb*) (**Supplementary Table 4**). Further analysis of expression levels of selected immune genes in individual samples of the four populations of cells showed that some were upregulated in both CD4⁺EGFP⁺ and CD8⁺EGFP⁺ populations vs. the EGFP⁻ counterparts, such as *Foxp3*, *Lrrc32*, *Tnfrsf1b*, *Tnfrsf9*, *Il2ra*, *Tox* and *Il4r* whereas others were only upregulated in CD4⁺EGFP⁺ Treg, such as *Stap2* and *Igtb8* or in CD8⁺EGFP⁺ Treg, such as *Timp1* and *Erc1* (**Supplementary Fig. 3A**).

Based on the genes differently expressed and the biological pathways in which they are involved we determined 4 principal processes with different pathways and with differential involvement for each cell type: immune cells activation, proliferation and adhesion as well as a miscellaneous one (**Supplementary fig 3 B**). CD4⁺EGFP⁺ vs. CD4⁺EGFP⁻ cells, expressed genes involved in immune cell activation and adhesion but not in immune cell proliferation and several of these pathways were shared with CD8⁺EGFP⁺ Treg. CD8⁺EGFP⁺ vs. CD8⁺EGFP⁻ cells also expressed genes involved in immune cell proliferation or its regulation. CD4⁺EGFP⁺ vs. CD8⁺EGFP⁺ showed genes involved in the miscellaneous pathways, such as Th1 and Th2 cell differentiation and regulation of inflammatory responses.

Venn analyses of all genes upregulated by CD4⁺EGFP⁺ vs. CD8⁺EGFP⁺ Treg vs. their EGFP⁻ counterparts as well as between the two Treg populations allowed to define genes that were unique and common among them (**Supplementary Fig. 3C**). The genes up and down regulated in the Venn diagrams are listed in **Supplementary Table 5 and 6**, respectively.

We finally confirmed at the protein level some of the genes that were upregulated at the RNAseq level such as for CD25/*Il2ra* (**Figure 3 and Supplementary Fig. 3A, respectively**), as well as CD44/*Cd44* and ICOS/*Icos* (**Supplementary Fig. 4**).

Altogether, the transcriptome of CD4⁺EGFP⁺ and CD8⁺EGFP⁺ Treg showed common genes previously described for CD4⁺ Treg but also many others that differ and that may allow to define a CD8⁺ Treg transcriptomic signature.

Analysis of in vitro generated EGFP⁺ induced Tregs.

We tested whether EGFP⁻ Tconv cells from *Foxp3-EGFP* rats could be converted to EGFP⁺ induced Tregs. To this end, CD4⁺CD25⁻ and CD8⁺CD45RC^{low/-}EGFP⁻ T cells were isolated and cultured in the presence of anti-CD3 and CD28 mAbs, IL-2 and TGFbeta. Stimulation with IL-2 and in the absence of TGFbeta has been shown to induce FOXP3 in T cells that were FOXP3⁻ in humans (32), likely as a marker of T cell activation, but not in mice (33) whereas in the presence of IL-2 and TGFbeta FOXP3 is induced in both species (32, 33). The induction of FOXP3 in rat T cells has not been clearly addressed. In the presence of anti-CD3 and anti-CD28 and TGFbeta alone, CD4⁺CD25⁻EGFP⁻ and CD8⁺CD45RC^{low}EGFP⁻ T cells converted to 19.7 % and 7.9 % of EGFP⁺ induced Tregs, respectively, and the MFI expression of EGFP was very similar than the one of EGFP⁺ natural Tregs (for CD4⁺EGFP⁺: 9711 vs. 8394, respectively and for CD8⁺EGFP⁺ 11000 vs. 13000, respectively) (**Fig. 6 A**). The culture with IL-2 alone or IL-2 and TGF beta did not further increase the percentage of EGFP⁺ cells compared to culture medium alone or TGF-beta alone, respectively (**Fig. 6 A**).

We then evaluated the suppressive activity of the induced EGFP⁺ Tregs from the condition in the presence of TGFbeta and IL-2 vs. the T cells from the same cultures that were EGFP⁻. Induced CD4⁺ and CD8⁺ EGFP⁺ Tregs were highly suppressive of MLR proliferation and significantly more than EGFP⁻ cells from the same cultures that only suppressed MLRs marginally (**Fig. 6 B**).

In conclusion, the use of *Foxp3-EGFP* rats allowed to define in vitro conditions of generation of induced Treg and to demonstrate that both T CD4⁺ and CD8⁺ lineages could generate induced Treg.

IL-2 induced expansion of EGFP⁺ Tregs in vivo.

In vivo injection of low doses of IL-2 has been shown to preferentially expand CD4⁺ and CD8⁺ FOXP3⁺ Treg in mice (34), nonhuman primates (35) and humans (36). In rats, CD4⁺FOXP3⁺ Treg were increased upon administration of IL-2 and CD8⁺FOXP3⁺ Treg were not described (37). We aim to define whether we could detect expansion of CD4⁺ and CD8⁺ EGFP⁺ Treg upon IL-2 in vivo injection. Injection of IL-2 resulted in a significant expansion of EGFP⁺ cells as compared to untreated rats in both the CD4⁺ (from 5.6 ± 1.4 to 17.1 ± 0.6) and CD8⁺ (from 2.2 ± 0.4 to 9.8 ± 0.9) T compartments as well as a decrease of the reciprocal EGFP⁻ compartments whereas NK cells remained EGFP⁻ (**Fig. 7A**). In blood of IL-2-treated animals compared to controls increased absolute numbers of T CD4⁺EGFP⁺ (19-fold) and CD8⁺EGFP⁺ (31-fold) cells showed a higher increase vs. T CD4⁺EGFP⁻ (4.4-fold) and

CD8⁺EGFP⁻ (14-fold) cells and NK cells (10-fold) (**supplementary Table 7**). The proportions of T CD4⁺ and CD8⁺ EGFP⁺ cells were also increased in the spleen (**supplementary Fig. 5**). Phosphorylated Stat5 (**Fig. 7B**) and CD25 (**Fig. 7C**) were increased in EGFP⁺ cells but also in EGFP⁻ T cells.

Thus, *Foxp3-EGFP* rats allow to detect increases in CD4⁺ and CD8⁺ Treg induced by IL-2.

Discussion.

Foxp3-EGFP rats were generated using a strategy in which *Foxp3* gene drives the expression of EGFP placed in the 3' end of the gene. This strategy preserves *Foxp3* expression of the allele in which the transgene is inserted avoiding decrease expression in heterozygous animals and also allows the use of homozygous animals to more efficiently maintain the mutated rat line. EGFP was exclusively expressed by CD4⁺ and CD8⁺ T cells and in proportions like the ones defined using anti-FOXP3 antibodies and thus EGFP was strictly restricted to subsets of the T cell lineage. CD4⁺EGFP⁺ Treg were CD25^{high}CD127^{low/-} CD45RC^{low/-}, consistent with the what has already been previously described in rats, humans and mice. All CD8⁺EGFP⁺ Treg were also CD25^{high}CD127^{low/-}CD45RC^{low/-}. Quantitative analysis of *Foxp3-EGFP* rats showed that CD4⁺EGFP⁺ Treg were 5-10-fold more abundant than CD8⁺EGFP⁺ Treg.

Genetic labelling of *Foxp3*-expressing cells preserving *Foxp3* expression has been previously used in some mouse models (14, 16, 18-20). Other mouse *Foxp3-eGFP* models generated a knockout of the allele in which GFP was inserted (15, 17). In some of these mouse models, CD8⁺GFP⁺ Treg were described with frequencies ranging between 5 to-100 fold lower than CD4⁺GFP⁺ Treg (15-18), whereas in the others they were not described. Analysis in our experimental conditions of CD8⁺GFP⁺ Treg in one of this mouse models (14) showed 9 to 130-

fold more CD4⁺EGFP⁺ Treg vs. CD8⁺EGFP⁺ Treg. To the best of our knowledge there are no other mammalian species with transgenic lines allowing to define FOXP3⁺ cell lineages.

CD4⁺ Treg defined by the usual CD25^{high} and CD127^{low/-} markers showed a majority of EGFP⁺ cells but also the presence of EGFP⁻ cells. Analysis of their suppressive activity revealed that >90% was confined to the EGFP⁺ fraction of cells. Similarly, CD8⁺ Treg defined using CD45RC^{low/-} as a marker also showed EGFP⁺ and EGFP⁻ cells and most of the suppressive activity was observed in the EGFP⁺ vs. EGFP⁻ fraction.

Transcriptomic analysis revealed for CD4⁺EGFP⁺ Treg the upregulation of genes previously described in mouse and/or human CD4⁺ Treg as important for their differentiation, suppressive function and oligodendrocyte regeneration, such as *Itgb8*, *Tnfrsf13b*, *Foxp3*, *Ikzf2*, *Ccn3*, *Il1rl1*, *Lrrc32*, *Cd80*, *Tnfrsf9*, *Gata3*, *Il2ra*, *Il2rb*, *Tnfrsf1b* (TNFR2 or CD120b), *Tnfrsf4* (OX40 or CD134) and *Tnfrsf18* (GITR or CD357) (26-30, 38-40). CD4⁺EGFP⁺ Treg also highly upregulated genes coding with immune function not previously described for CD4⁺ Treg, such as *Stap2* (41), *Tox* (42) and *C1qtnf6* a soluble inhibitor of the alternative complement activation pathway, for which further work is needed to define their functional role. Transcriptomic analysis of CD8⁺EGFP⁺ Treg is to the best of our knowledge the first one

to be described. CD8⁺EGFP⁺ Treg shared some genes previously described in CD4⁺EGFP⁺ Treg, such as *Lrrc32*, *Il2ra*, *Tnfrsf1b*, *C1qtnf6* and *Tnfrsf9*, but not other such as *Ctla4* and *Gata3*, (26-31, 38-40). CD8⁺EGFP⁺ Treg expressed immune genes that need to be analyzed for their functional role in Treg, such as complement genes (*C1qc*, *C1qa*, *C1qb*, *Cfd*) and at the same time *C1qtnf6* (a secreted molecule that inhibits complement activation), *Fcna* (a secreted molecule with elastin-binding activity), *Hmox1* (enzyme that degrades heme), *Spic* (an enhancer of transcription in lymphoid cells), and *Vipr2* (one of the receptors for vaso intestinal peptide).

Since there is need to identify cell membrane markers that could allow to purify CD45RC^{low/-} CD8⁺FOXP3⁺ Treg, several genes that code for membrane markers that were upregulated in CD45RC^{low/-}CD8⁺EGFP⁺ Treg or in CD45RC^{low/-}CD8⁺EGFP⁻ Treg were identified that could be used to identify and isolate CD8⁺EGFP⁺ Treg by positive or negative selection, respectively. Genes upregulated in CD8⁺EGFP⁺ Treg included *Vcam1* (*CD106*), *Cdh1* (*CD324*), *CD163*, *Mrc1* (*CD206*), *Trpm2*, *Kdr* (*CD309*), *FcmR*, *Itgb5*, *Il9r* (*CD129*), *Il2ra* (*CD25*), *Cd79b*, *Ly49s7*, *Jag1* (*CD339*), *Il6st* (*CD130*), *IL4r* (*CD124*), *Vipr2*, *Tfrc* (*CD71*), *Il6r* (*CD126*) and *Tnfrsf1b* (*TNFR2*). In CD8⁺EGFP⁻ Treg genes encoding for cell membrane molecules that were upregulated included *Ly49s3*, *Nkg7*, *Ly49s3*, *Fcnb*, several *RT1* (*MHC-II*) molecules, *Nrc1* (*CD335*), *Klrk1* (*CD314*), *CD38*, *CD59* and *CD174*. For this, the RNAseq data need to be confirmed by protein cytometry analyses, as done for CD25, CD44 and ICOS, since not only mRNA levels may not translate into higher protein levels but also because the RNAseq analysis on whole cell populations do not allow to conclude whether it is a large or a small fraction of the cells that upregulate a given gene. Thus, these cell membrane markers could potentially be used to identify and purify CD8⁺ nTreg in wild-type rats and even in other species. On this regard, human CD8⁺CD45RC^{low/-} cells contain the CD8⁺ Treg suppressive activity but further enrichment of cells positives for CD25 or negatives for CD127 did not increase the suppressive activity compared to the total CD8⁺CD45RC^{low/-} population (7). This suggest a difference between human and rat nCD8⁺ Treg for these markers but human CD8⁺CD45RC^{low/-}FOXP3⁺ cells did express higher levels of GITR than CD8⁺CD45RC^{low/-}FOXP3⁻ cells and were more suppressive than the GITR⁻ fraction of CD8⁺CD45RC^{low/-} cells (7) showing a concordance with the transcriptomic data presented in this manuscript.

Despite that most suppressive activity was confined in nTreg to the rat EGFP⁺ fraction for both CD4⁺ and CD8⁺ T cells, in vitro and in vivo iTreg have been described (1, 3, 4, 6, 7). In vitro culture Tconv CD4⁺CD25⁻ EGFP⁻ or CD8⁺CD45RC^{low/-} GFP⁻ cells in the presence of

TGFbeta and IL-2 but not of IL-2 alone resulted in the induction of EGFP in a fraction of cells. The suppressive activity of the cultured cells was restricted to the EGFP⁺ fraction of cells in both CD4⁺ and CD8⁺ T cell compartments and these are thus truly iTreg. This suggests that *Foxp3-EGFP* rats could be used to analyze iTreg in different situations in vitro and in vivo. Generation of FOXP3⁺ cells from CD4⁺FOXP3⁻ in humans can be obtained with IL-2 in the absence of TGFbeta but these cells may not be truly Treg since expression of FOXP3 by human cells can be a marker of activation (33). On the contrary, IL-2 alone in mice does not induces FOXP3⁺ cells (32). In both species, mice and human, TGFbeta induces the expression of FOXP3 (32). Thus, induction of expression of FOXP3 in rat Treg cells could be similar to the one observed in mice and different from the one in human Treg but this needs further experiments with additional stimuli.

Low dose IL-2 treatment was shown to preferentially expand both CD4⁺ and CD8⁺ FOXP3⁺ Treg in mice (34, 43), non-human primates (35) and humans (36). In mice, the doses used ranged between 5x10³ and 5x10⁴ IU/g of body weight (34, 43). The dose of IL-2 used in this study also (10⁴ IU/g of body weight) was the same used in a previous study in rats that showed increase in CD4⁺ Treg but in which CD8⁺ Treg were not described (37). Although with this dose of IL-2 we observed a preferential expansion and activation of both CD4⁺ and CD8⁺ EGFP⁺ Treg, we also observed increased CD4⁺ and CD8⁺ Tconv and NK cells and thus future experiments could analyze lower doses to define a more restricted effect on Treg. It should be noted that doses of 2x10³ IU/rat in HLA-B27 with spondyloarthritis had a weak effect on CD4⁺ Treg induction (44).

Similar percentages of CD4⁺FOXP3⁺ Treg have been described in different rat strains and CD4⁺ and CD8⁺ Treg in rats have been studied in a variety of pathophysiological situations (5, 6). Although the role of CD8⁺ Treg is less studied than for CD4⁺ Treg, there is solid evidence that this subset of Treg plays an important role in immune responses not only in rats but also in all other species analyzed (4, 7, 9, 10, 45).

Conclusions.

Foxp3-EGFP rats are a useful model to identify CD4⁺ and CD8⁺ Treg and to define new molecules expressed in by CD4⁺ but particularly CD8⁺ Treg that will allow to better define their phenotype and function.

The immune system of rats have several characteristics that make them more similar to humans than mice (21) and the recent generation of many gene edited rats (22) will allow to cross *Foxp3-EGFP* rats with other mutated strains to analyze the role of Treg in these models.

Materials and Methods.

Animals.

Wild type Sprague-Dawley (SD/Crl) rats were from Charles River (L'Arbresle, France). *Foxp3-GFP* founder reporter mice were kindly provided by Bernard Malissen (14). All the animal care and procedures performed in this study were approved by the Animal Experimentation Ethics Committee of the Pays de la Loire region, France, in accordance with the guidelines from the French National Research Council for the Care and Use of Laboratory Animals (Permit Number : Apafis 692). All efforts were made to minimize suffering. The rats were housed in a controlled environment (temperature 21±1°C, 12-h light/dark cycle).

Generation and genotyping of *Foxp3-EGFP* animals.

Several CRISPR sgRNA sequences cleaving immediately 3' of the stop codon of *Foxp3* were designed using the CRISPOR software. The in vitro transcribed sgRNA were purified and one selected for highest cleavage in the rat C6 cell line, as previously described in detail (46, 47). Fertilized one-cell stage embryos were collected for subsequent microinjection using a previously published procedure (48). Briefly, a mixture of Cas9 protein (50 ng/μl), sgRNA (GCAGGGGTTGGAGCACTTGC) (10 ng/μl) and donor DNA (2 ng/μl) encoding the 2A self-cleaving peptide and EGFP sequences flanked by homology arms (1 kb each) 5' and 3' from the DNA cleavage point (**Fig. 1A**) was microinjected both into the male pronucleus and into the cytoplasm of fertilized one-cell stage embryos. Microinjected zygotes were maintained under 5% CO₂ at 37°C for 2h. Surviving embryos were implanted on the same day in the oviduct of pseudo-pregnant females (0.5 dpc) and allowed to develop to full term. For genotyping rats, DNA from tail biopsy from 8- to 10-day-old rats were digested in 500 μL of tissue digestion buffer (Tris-HCl 0.1 mol/L pH 8.3, EDTA 5 mmol/L, SDS 0.2%, NaCl 0.2 mol/L, PK 100 μg/mL) in a 1.5 mL tube at 56°C overnight and genotyped by PCR using the following primers: *Foxp3*-up; 5'- AAC CTG GGG CTA AAT GTG TG -3'; *Foxp3*-low; 5'- TAG GGT TTG GGT TGA GTC CA -3'; *EGFP*-up; 5'- CCT CGT GAC CAC CCT GAC CT -3'; *EGFP* -low; 5'- TCC ATG CCG AGA GTG ATC CC -3'. PCR amplicons were analyzed by capillary electrophoresis as described (49), followed by Sanger sequencing in *Foxp3*-mutated founders.

Cytofluorimetry and antibodies.

Single-cell suspensions from the spleen, thymus, bone marrow and lymph nodes were prepared as described previously (50). Cell suspensions were analyzed using antibodies (antibodies used are listed in supplementary Table 8). The incubation period was 30 min at 4°C, and the analysis was performed with a FACSVerse system (BD Biosciences, Franklin Lakes, NJ) and FlowJo software (Tree Star, Ashland, OR).

In vitro suppressive assays.

Suppressive assays were performed as previously described in detail (25, 45). Briefly, CD4⁺CD25⁻ naive T cells, CD4⁺CD25⁺CD127^{low} and CD8⁺CD45RC^{low/-} T cells expressing or not FOXP3 were FACS-sorted from *Foxp3-EGFP* or WT rats (haplotype RT-1u). CD4⁺CD25⁻ naive T cells were CPD-670-labeled. Naïve T cells and CD4⁺CD25⁺CD127^{low} (both labeled with CPD-450) and CD8⁺CD45RC^{low/-} T cells were co-cultured at different ratios with spleen DCs from Lew.1A rats (haplotype RT-1a) for 6 d at 37°C in 5% CO₂. Proliferation of CPD 670-labeled CD4⁺CD25⁻ naive T cells was analyzed by gating on TCR⁺CD4⁺ cells among live cells and analyzing CPD-670 signal dilution.

Transcriptomic analysis of CD8⁺ and CD4⁺ EGFP⁺ cells.

We isolated TCR⁺CD25^{high}CD127^{low}CD4⁺EGFP⁺, TCR⁺CD25^{high}CD127^{low}CD4⁺EGFP⁻, TCR⁺CD45RC^{low/-}CD8⁺EGFP⁺ and TCR⁺CD45RC^{low/-}CD8⁺EGFP⁻ cells and performed total RNAseq by performing 3'Digital Gene Expression (3'DGE) RNA-sequencing. A RNeasy-Mini Kit (Qiagen) were used to isolate total RNA that was then processed for RNA sequencing. Protocol of 3'digital gene expression (3'DGE) RNA-sequencing was performed as previously described (7). Briefly, the libraries were prepared from 10ng of total RNA. The mRNA poly(A) tail is tagged with well specific barcodes and unique molecular identifier (UMI) and flanked by universal sequencing adapters during the template-switching reverse transcriptions.

Barcoded cDNAs from multiple samples are then pooled, amplified and tagmented using a transposon-fragmentation approach which enriches for 3'ends of cDNA. A library of 350-800bp is sequenced on a S1 flowcell on a NovaSeq 6000 (Illumina). Digital gene expression (DGE) profiles are generated by counting for each sample, the number of unique UMIs associated with each RefSeq genes. Counts matrix was normalized by a linear model with the R package DESeq2 (51). Differential gene expression was determined Wald test and corrected with the False Discovery Rate (FDR) multiple testing method. Genes were considered

differentially expressed if the FDR ≤ 0.05 and the absolute value of $\log_2(\text{Fold Change})$ greater than 0.5. Heatmaps were generated with the R package gplots (<https://cran.r-project.org/web/packages/gplots/index.html>) Volcano plots were drawn by plotting $-\log_{10}(\text{FDR})$ in function of $\log_2(\text{Fold-Change})$.

Genes were highlighted when absolute value of $\log_2(\text{Fold-Change})$ was superior to 1 and FDR ≤ 0.05 . Principal component analyses (PCA) were performed on all expressed genes with the R package FactoMineR (52). Both pathways from the Kyoto Encyclopedia of genes and genomes (KEGG)(53) as well as gene ontologies (GO) (54) were tested for enrichment among differentially expressed genes with the R package cluster Profiler (55). The significant GO terms and their enrichment scores were filtered with a corrected p-value ≤ 0.05 (Benjamini-Hochberg method).

In vitro generated EGFP⁺ induced Tregs.

EGFP⁻CD4⁺CD25⁻ and EGFP⁻CD8⁺CD45RC^{low/-} T cells were FACS-sorted from *Foxp3-EGFP* rats, labeled with CPD 670 and cultured for 3 days in the presence of anti-CD3 and anti-CD28 MAbs with different doses of hTGFbeta (0, 5, 20 $\mu\text{g/ml}$) and with or without 100 IU of hIL-2 at 37°C in 5% CO₂. Proliferation of CPD-labeled T cells measured by dilution of the CPD signal and EGFP expression were analyzed using a FACSVerse system (BD Biosciences, Franklin Lakes, NJ) and FlowJo software (Tree Star, Ashland, OR). EGFP⁺ CD4⁺ and CD8⁺ cells as well as EGFP⁻ CD4⁺ and CD8⁺ cells were sorted at the end of the culture period and used in suppressive assays as described in a section above.

IL-2 expansion of EGFP⁺ Tregs in vivo.

Human IL-2 (Proleukin) was diluted with NaCl for iv injection, a new dilution was used for each day of treatment, at a dose (10^4 IU/0.62 $\mu\text{g/g}$ of body weight for 4 consecutive days) previously described to expand Treg in rats (37) and analyzed at day 5 the proportion and absolute numbers of NK, CD4⁺EGFP⁻ Tconv, CD4⁺EGFP⁺ Treg, CD8⁺EGFP⁻ Tconv and CD8⁺EGFP⁺ Treg cells in blood and spleen as well as the expression levels of Stat-5 phosphorylation (clone pY694, BD Biosciences) and CD25 (clone OX39) compared to those of these cells populations from untreated *Foxp3-EGFP* rats.

Statistical analysis

Results are presented as means $\pm$ SD. Statistical analysis between samples was performed by a Mann-Whitney test using GraphPad Prism 4 software (GraphPad Software, San Diego, CA,

USA). Differences associated with probability values of ^a P<0.05, ^b P<0.005, ^c P<0.0002 and ^d<0.0001 were considered statistically significant.

Abbreviations

APC: Antigen Presenting Cell

APECED : autoimmune polyendocrinopathy candidiasis ectodermal dystrophy

BM: Bone Marrow

CPD: Cell Proliferation Dye

DEG: Differentially Expressed Genes

DGE: Digital Gene Expression

DNA: deoxyribonucleic acid

EDTA: Ethylenediaminetetraacetic acid

EGFP: Enhanced Green Fluorescent Protein

FDR: False Discovery Rate

FSC: forward-scattered light

GO : Gene ontologies

HCl: Hydrochloric acid

iTreg : induced regulatory T cells

iv: intravenous injection

KEGG: Kyoto Encyclopedia of Genes and Genomes

KI: Knock-In

LN: axillar Lymph Node

mAbs: monoclonal Antibody

MFI: Mean Fluorescence Intensity

MLR: Mixte Lymphocyte Reaction

NaCl: Sodium chloride

NK cells: Natural Killer cells

nTreg : natural regulatory T cells

PCA: Principal Component Analyses

PK: Proteinase K

RNA: Ribonucleic acid

SD/Crl : Sprague-Dawley

SDS: Sodium Dodecyl Sulfate

SEM: Standard Error of Mean

sgRNA: single guide RNA

SSC: side-scattered light

Tconv: Conventional T cells

TCR: T Cell Receptor

Treg: regulatory T cells

UMI: Unique Molecular Identifier

WT: Wild-Type

Declarations

Ethics approval and consent to participate. Not applicable.

Consent for publication. Not applicable

Availability of data and materials. The sequencing data have been available from the NCBI GEO database, under accession GSE189797. Graphical representations were created with R packages (35).

Competing interests. The authors declare that they have no competing interests.

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Author contributions. SM designed and performed research, analyzed data and edited the manuscript. LT performed research, analyzed data and edited the manuscript. SR, VG, CS, CU, AG, VC, L-HO, MG, J-MH and CF performed research and analyzed data. CG and JP analyzed data and edited the manuscript. IA designed research, provided funding, analyzed data and wrote the manuscript.

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Table 1. Quantification of CD4⁺ and CD8⁺ Treg in different immune compartments.

	units	thymus	LN	spleen	BM	blood/ml
CD4⁺EGFP⁺ (n=5)	% *	5.2 ± 0.9	7.8 ± 1.7	10 ± 0.2	7.2 ± 3	7.9 ± 0.6
	cellsx10 ⁶	2.24 ± 1	0.4 ± 0.1	2.2 ± 0.4	0.19 ± 0.004	0.083±0.03
CD8⁺EGFP⁺ (n=5)	% **	0.6 ± 0.15	1.3 ± 0.4	0.7 ± 0.01	0.8 ± 0.45	0.7 ± 0.1
	cellsx10 ⁶	0.28 ± 0.2	0.4 ± 0.6	0.16 ± 0.02	0.003 ± 0.002	0.007 ± 0.002

LN; axillar lymph nodes; BM, bone marrow from 1 femur/animal. * among TCR⁺CD4⁺;

**among TCR⁺CD8⁺

Figure Legends.

Figure 1. Schematic representation of the GFP KI strategy and genotyping of potential *Foxp3-EGFP* founders.

A. (upper diagram) A double stranded DNA donor was generated containing a EGFP sequence preceded by a self-splicing T2A sequence both flanked in 5' and 3' homology arms for the indicated *Foxp3* sequences. (lower diagram). Upon microinjection of a sgRNA designed to recognize a DNA sequence immediately 3' of the stop codon, Cas9 protein and donor DNA, cleavage of the *Foxp3* gene favors homologous-recombination of the donor DNA sequences. **B.** (upper diagram) Schematic representation of EGFP under the transcriptional control of an intact *Foxp3* gene. Both, FOXP3 and EGFP will be generated as independent proteins through self-splicing imposed by the T2A sequence. (lower diagram) Sanger sequences of a founder in which in-frame junctions are present between the 5' and 3' homology arms of the DNA donor and the *Foxp3* gene as well as the entire donor DNA sequence is conserved with conserved junctions between exon 11-T2A, T2A-EGFP and EGFP-stop sequences. Primers used for the different PCR sequences are outlined.

Figure 2. Flow cytometry analysis of peripheral blood lymphocyte and lymph node cells from *Foxp3-EGFP* rats.

A) Blood and **B)** axillary lymph nodes were harvested from 12-week-old *Foxp3-EGFP* or wild-type (WT) rats and single cell suspensions were gated by SSC and FSC on lymphocytes followed by the identification with mAbs of major cell populations; TCR⁺ cells, TCR⁺CD4⁺, TCR⁺CD8⁺ and CD161^{high} for NK cells. These populations were then analyzed for FOXP3 expression by EGFP expression and by using an anti-FOXP3 mAb. Contour plots from one animal representative of 6 analyzed in the same conditions.

Figure 3. Phenotype of EGFP⁺ cells.

Single cell suspensions were obtained from spleen from a *Foxp3-EGFP* rat, lymphocytes gated by SSC/FSC and analyzed for the phenotype of CD4⁺ and CD8⁺ EGFP⁺ cells. **A)** TCR⁺ cells were labelled with an anti-CD8alpha mAb to identify CD8⁺ and CD8⁻/CD4⁺ and analyzed for CD25 and CD127 expression followed by EGFP detection. **B)** TCR⁺CD4⁺ or TCR⁺CD8⁺ cells were analyzed for CD45RC expression, levels identifying high (H), low (L) and negative (N) cells, and EGFP expression in these populations. **C)** Histograms of TCR⁺CD4⁺EGFP⁺, TCR⁺CD4⁺EGFP⁻, TCR⁺CD8⁺EGFP⁺ and TCR⁺CD8⁺EGFP⁻ cells analyzed with the indicate mAbs. Percentages above the traits correspond to the number of positive cells above staining with isotype control mAbs. In A, B and C one experiment representative of 5 performed in the same conditions.

Figure 4. Suppressive activity of TCR⁺CD4⁺EGFP⁺ and TCR⁺CD8⁺EGFP⁺ cells in an MLR. An MLR was performed by co-culturing in a 1:1 ratio spleen CD4⁺CD25⁻ Tconv cells from SD/Crl rats (MHC haplotype u) with enriched spleen APCs from Lewis 1A (MHC haplotype a) rats. Spleen Tregs from wild-type (WT) or *Foxp3-EGFP* SD/Crl rats were sorted based on their surface phenotype using previously defined markers. For CD4⁺ Tregs, TCR⁺CD4⁺CD25⁺CD127^{low/-} and for CD8⁺ Tregs, TCR⁺CD8⁺CD45RC^{low/-}. CD4⁺ and CD8⁺ Tregs were then sorted based on the expression or not of EGFP. The four populations of Tregs, CD4⁺EGFP⁺ and EGFP⁻ as well as CD8⁺EGFP⁺ and EGFP⁻ were added to the MLR in a 1:1 or 1:2 Tregs to CDP-670 labelled Tconv cells. Values are expressed as mean % +/- SEM of suppression normalized to MLR proliferation in the absence of Tregs (0 % inhibition of proliferation). n=4. *p<0.05, **p<0.01, ***p<0.001.

Figure 5. RNAseq analyses of EGFP⁺ and EGFP⁻ within CD4⁺ and CD8⁺ T cells. TCR⁺CD25^{high}CD127^{low}CD4⁺ and TCR⁺CD45RC^{low/-} EGFP⁺ and EGFP⁻ T cells were cell sorted and RNAseq analyses were performed comparing CD4⁺EGFP⁺ vs. CD4⁺EGFP⁻ cells (left column), and CD8⁺EGFP⁺ vs. CD8⁺EGFP⁻ cells (middle column) and CD8⁺EGFP⁺ vs. CD4⁺EGFP⁺ cells right column. **A.** principal components analysis (PCA) of all samples and of expressed genes. **B.** Volcano plots. (Left) Light green indicates overexpression in CD4⁺EGFP⁺ vs. CD4⁺EGFP⁻ cells and black of CD4⁺EGFP⁺ vs. CD4⁺EGFP⁺ cells. (middle) Dark green indicates overexpression in CD8⁺EGFP⁺ vs. CD8⁺EGFP⁻ cells and black of CD8⁺EGFP⁺ vs. CD8⁺EGFP⁺ cells. (right) Light green indicates overexpression in CD4⁺EGFP⁺ vs. CD8⁺EGFP⁺ cells and dark green of CD8⁺EGFP⁺ vs. CD4⁺EGFP⁺ cells. **C.** Heatmaps of all differentially expressed genes. Low expression levels are in blue, mean expression levels are in white and high expression levels are in red. Each sample number is depicted on the top.

Figure 6. Conversion of EGFP⁻ T cells in EGFP⁺ induced Treg. Spleen cells from *Foxp3-EGFP* animals were used to sort Tconv CD4⁺CD25⁻ Treg and T CD8⁺CD45RC^{low/-}EGFP⁻ cells, labelled them with CPD-670 and cultured for 3 days in the presence of anti-CD3/CD28 mAbs, IL-2 and in the absence or presence of hTGFbeta. **A.** Cells that proliferated (CPD-670low/neg) were then analyzed for EGFP expression. Left graphs show mean +/- SEM of 3 different experiments and right histograms show one representative analysis. **B.** Suppressive assays using EGFP⁺ (CD4⁺ or CD8⁺) vs. EGFP⁻ cells (CD4⁺ or CD8⁺) from the same cultures at different ratios with CD4⁺ Tconv responder cells. n=3, mean +/- SEM.

Figure 7. IL-2 induce expansion of EGFP⁺ Tregs in vivo. *Foxp3*-EGFP rats received or not hIL-2 and the percent of the indicated cell populations was analyzed in blood. **A.** Percentages of cell EGFP⁺ cells in the indicated cell populations of IL-2-treated and untreated rats. Left graph shows mean $\pm$ SEM, n=3, and right contour plots show a representative animal. **B.** Percentage and MFI (left and middle histograms, respectively) of pStat5⁺ cells in the indicated cell populations in IL-2-treated and untreated rats. (mean $\pm$ SEM, n=3). Middle contour plots show a representative animal. **C.** Percentage and MFI (left and middle histograms, respectively) of CD25⁺ cells in the indicated cell populations of IL-2-treated and untreated rats. (mean $\pm$ SEM, n=3). Middle contour plots show a representative animal.

Supplementary figure legends.

Supplementary figure 1. Flow cytometry analyses of spleen and thymus cells from *Foxp3*-EGFP rats. **A)** Spleen and **B)** thymus were harvested from 12 weeks-old *Foxp3*-EGFP or wild-type (WT) rats and single cell suspensions were gated by SSC and FSC on lymphocytes followed by the identification with mAbs of major cell populations such as TCR⁺ cells, TCR⁺CD4⁺, TCR⁺CD8⁺ and CD161⁺ for NK cells. These populations were then analyzed for FOXP3 expression by EGFP expression and by using an anti-FOXP3 mAb. Contour plots from one animal representative of 6 analyzed in the same conditions.

Supplementary figure 2. Suppression assay using T CD4⁺EGFP⁺ and CD8⁺EGFP⁺ cells. Representative histograms of a suppressive assay using spleen cells from 12 weeks-old *Foxp3*-EGFP rats. TCR⁺CD4⁺CD25⁺CD127^{low}EGFP⁺ (labeled with CPD-450) and TCR⁺CD8⁺CD45RC^{low/-}EGFP⁺ cells were sorted and added to an MLR in a 1:1 or 1:2 Tregs to Tconv cells. The MLR was performed by co-culturing in a 1:1 ratio spleen CD4⁺CD25⁻ Tconv cells from SPD rats (MHC haplotype u) labelled with CPD-670 along with enriched spleen APCs from Lewis 1A (MHC haplotype a) rats. Decrease in the percentage of CPD-670 bright cells and increase in the percentages of CPD-670 low cells denotes dilution of CPD-670 with cell proliferation. The left histogram shows the CPD-670 dilution of proliferating T cells in the absence of Treg and the others show less proliferation in the presence of the EGFP⁺ Treg. Representative experiment out of 4 performed.

Supplementary figure 3. RNAseq analyses of EGFP⁺ and EGFP⁻ within CD4⁺ and CD8⁺ T cells. TCR⁺CD25^{high}CD127^{low}CD4⁺ and TCR⁺CD45RC^{low/-}CD8⁺ EGFP⁺ and EGFP⁻ T cells were cell sorted and RNAseq analyses were performed comparing CD4⁺EGFP⁺ vs. CD4⁺EGFP⁻ cells, and CD8⁺EGFP⁺ vs. CD8⁺EGFP⁻ cells and CD8⁺EGFP⁺ vs. CD4⁺EGFP⁺ cells. **A.** R-Shiny representations of RNAseq levels for individual samples of the indicated genes with immune functions expressed in units per million reads. **B.** GO analyses of analysis of functional pathways, Cell component analysis showed that the proteins encoded by comparison of TCR⁺CD25^{high}CD127^{low}CD4⁺EGFP⁺ vs. TCR⁺CD25^{high}CD127^{low}CD4⁺EGFP⁻, TCR⁺CD45RC^{low/-}CD8⁺EGFP⁺ vs. TCR⁺CD45RC^{low/-}CD8⁺EGFP⁻ and TCR⁺CD25^{high}CD127^{low}CD4⁺EGFP⁺ vs. TCR⁺CD45RC^{low/-}CD8⁺EGFP⁺ cells. **(a)** Comparison of 5 principal pathways in immune cell activation **(b)** Comparison of 3 principal pathway in immune cell proliferation. **(c)** Comparison of 4 principal pathway in immune cell adhesion. **(d)** Comparison of 11 pathways in miscellaneous process. **C.** Venn diagrams showing the overlap in genes that were significantly (adjusted p value, 0.05 and absolute log2 fold-change of genes differentially upregulated (left) or down regulated (right).

Supplementary figure 4. Confirmation of some RNAseq results on CD4+EGFP+ and CD8+EGFP+ cells by FACS. **A.** Histograms of TCR+CD4+EGFP+, TCR+CD4+EGFP-, TCR+CD8+EGFP+and TCR+CD8+EGFP- cells analyzed with the indicate mAbs. Percentages above the traits correspond to the number of positive cells above staining with isotype control mAbs. One experiment representative of 3 performed in the same conditions. **B.** R-Shiny representation of RNAseq expression levels expressed in individual sample in units per million reads for *Il2ra* (*Cd25*), *Cd44* and *Icos*.

Supplementary figure 5. IL-2 induce expansion of EGFP⁺ Tregs in spleen. *Foxp3-EGFP* rats received or not hIL-2 at low doses and the percent of the indicated cell populations was analyzed in spleen. Percentages of cells in the indicated cell populations of IL-2-treated and untreated rats. Left graph shows mean +/- SEM, n=3, and right contour plots show a representative animal.

Figure 1

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Figure 2

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Figure 3

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Figure 4

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Figure 5

Figure 6

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Figure 7

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Supplementary table 1. Frequency of CD4⁺EGFP⁺ and CD8⁺EGFP⁺ in *Foxp3-EGFP* rats and *Foxp3-EGFP* mice.

		thymus	LN	spleen	BM	blood/ml
Rat**	TCR⁺CD4⁺EGFP⁺	5.2 ± 0.9**	7.8 ± 1.7	10 ± 0.2	7.2 ± 3	7.9 ± 0.6
	TCR⁺CD8⁺EGFP⁺	0.6 ± 0.15	1.3 ± 0.4	0.7 ± 0.01	0.8 ± 0.45	0.7 ± 0.1
Mouse**	TCR⁺CD4⁺EGFP⁺	0.18 ± 0.06	3.9 ± 3.3	9.7 ± 2.5	7.9 ± 4.1	3.7 ± 1.0
	TCR⁺CD8⁺EGFP⁺	0.003 ± 0.005	0.3 ± 0.6	0.3 ± 0.2	0.06± 0.09	0.4 ± 0.3

*N=3 for rats and n=5 for mice

**% of total cells

Supplementary table 2. Immune genes upregulated in TCD4+EGFP+ vs. TCD4+EGFP- cells.

TCD4+EGFP+

TCD4+EGFP-

Name of gene	Log 2 fold change	Name of gene	Log 2 fold change
<i>Itgb8</i>	4.7371	<i>Il17rc</i>	-4.8380
<i>Tnfrsf13b</i>	4.2417	<i>Il17re</i>	-4.1888
<i>Foxp3</i>	3.5868	<i>Sphk1</i>	-3.6848
<i>Lrrc32</i>	3.2384	<i>Ccr6</i>	-2.3299
<i>Cd80</i>	2.8669	<i>Klrc1</i>	-2.0664
<i>Ccn3</i>	2.8574	<i>Cd40lg</i>	-1.8672
<i>Ikzf2</i>	2.8187	<i>Map3k6</i>	-1.7279
<i>Stap2</i>	2.5447	<i>Lgals3</i>	-1.6431
<i>Il1rl1</i>	2.4686	<i>Tnf</i>	-1.4869
<i>Ptafr</i>	2.3347	<i>Gzmc</i>	-1.2828
<i>C1qtnf6</i>	2.2820	<i>Tnfrsf21</i>	-1.2513
<i>Cbx6</i>	2.1543	<i>Il17ra</i>	-1.2335
<i>Tox</i>	1.9271	<i>Foxm1</i>	-1.2177
<i>Prf1</i>	1.9247	<i>Cd226</i>	-1.2115
<i>Fcmmr</i>	1.9183	<i>Cd7</i>	-1.1829
<i>Lbh</i>	1.8573	<i>Il3ra</i>	-1.1256
<i>Gpr15</i>	1.7579	<i>Klrd1</i>	-1.0954
<i>Slamf7</i>	1.7443	<i>Il17f</i>	-1.0861
<i>Il2rb</i>	1.7114	<i>Mcoln2</i>	-1.0473
<i>Vipr1</i>	1.6450	<i>Prr5l</i>	-1.0335
<i>Nrp1</i>	1.5586		
<i>Art2b</i>	1.5327		
<i>Peli1</i>	1.5056		
<i>Aff3</i>	1.4599		
<i>Tnfrsf9</i>	1.3500		
<i>Igf1r</i>	1.3340		
<i>Cradd</i>	1.3260		
<i>Map3k1</i>	1.2793		
<i>Cd9</i>	1.2674		
<i>Prkcz</i>	1.2653		
<i>Gata3</i>	1.2224		
<i>Rab20</i>	1.2077		
<i>Irak3</i>	1.1809		
<i>Cd38</i>	1.1486		
<i>Sell</i>	1.1299		
<i>Adora2a</i>	1.1244		
<i>Pvr</i>	1.0878		
<i>Fas</i>	1.0522		
<i>Il4r</i>	1.0304		
<i>Il18</i>	1.0003		

adjusted p-value<0.05

Supplementary table 3. Immune genes upregulated in TCD8+EGFP+ vs. TCD8+EGFP- cells.

TCD8+EGFP+		TCD8+EGFP-	
Name of gene	Log 2 fold change	Name of gene	Log2 fold change
<i>Clqc</i>	4.4393	<i>Gzmk</i>	-4.7022
<i>Foxp3</i>	4.1674	<i>Ly49s3</i>	-4.6432
<i>Clqa</i>	3.7324	<i>Ccl5</i>	-3.3444
<i>Fcna</i>	3.7230	<i>Cx3cr1</i>	-3.1771
<i>Spic</i>	3.3882	<i>LOC688090</i>	-2.9252
<i>Pla2g2d</i>	3.2813	<i>Nkg7</i>	-2.6903
<i>Hmox1</i>	3.2730	<i>Ccl6</i>	-2.5610
<i>Clqb</i>	3.2507	<i>Gzma</i>	-2.5162
<i>Lrrc32</i>	3.2369	<i>Ly49s6</i>	-2.3763
<i>Vcam1</i>	3.1587	<i>Gzmm</i>	-2.2315
<i>Cdh1</i>	3.0837	<i>Gzmc</i>	-2.2299
<i>Laptn4b</i>	2.9295	<i>Slpr5</i>	-2.2168
<i>Cd163</i>	2.8150	<i>Slamf7</i>	-2.0638
<i>Erc1</i>	2.8017	<i>Def6</i>	-2.0232
<i>Mrc1</i>	2.8002	<i>Susd2</i>	-1.9913
<i>Dnase1l3</i>	2.7207	<i>Cst7</i>	-1.9721
<i>Kdr</i>	2.7139	<i>Dapk2</i>	-1.9215
<i>Maf</i>	2.6682	<i>Klre1</i>	-1.9205
<i>Trpm2</i>	2.3964	<i>Pycard</i>	-1.7903
<i>Fcmm</i>	2.3651	<i>Ctsw</i>	-1.7737
<i>Lgmn</i>	2.3513	<i>Serpinb9</i>	-1.7531
<i>Itgb5</i>	2.3448	<i>Fcnb</i>	-1.7273
<i>Blmk</i>	2.2912	<i>Cxcr6</i>	-1.6758
<i>Cadm4</i>	2.2339	<i>Myadm</i>	-1.6118
<i>Cd51</i>	2.1863	<i>RT1-DMa</i>	-1.5824
<i>Mzb1</i>	2.1551	<i>Faslg</i>	-1.5775
<i>Cfd</i>	2.1034	<i>Plek</i>	-1.5573
<i>Rarg</i>	2.0133	<i>Klrb1a</i>	-1.5202
<i>Anxa3</i>	1.9852	<i>S100a9</i>	-1.4528
<i>Il9r</i>	1.9399	<i>Lgals3bp</i>	-1.4337
<i>Aff3</i>	1.8397	<i>Ncr1 (CD335)</i>	-1.4086
<i>Il2ra</i>	1.8367	<i>Klra22</i>	-1.3510
<i>Tnfrsf11b</i>	1.7848	<i>RT1-Da</i>	-1.3030
<i>Phlda1</i>	1.7846	<i>Klrl1 (KKG2-D)</i>	-1.2803
<i>Cd79b</i>	1.7722	<i>Cd38</i>	-1.2502
<i>Ly49s7</i>	1.7662	<i>Klrd1</i>	-1.2018
<i>Jag1</i>	1.7608	<i>RT1-Db1</i>	-1.1809
<i>Pou2f2</i>	1.7478	<i>Cd59</i>	-1.1539

<i>Dclre1c</i>	1.7469	<i>Cxcr3</i>	-1.1520
<i>Clqtnf6</i>	1.7104	<i>RT1-DMb</i>	-1.1539
<i>Tmem176b</i>	1.6492	<i>Fyn</i>	-1.1043
<i>Timp1</i>	1.6283	<i>Nmi</i>	-1.0948
<i>Tmem176a</i>	1.6218	<i>Cd3g</i>	-1.0827
<i>Irak3</i>	1.6048	<i>Cd8b</i>	-1.0764
<i>Ctsl</i>	1.5348	<i>Tnfrsf9</i>	-1.0733
<i>Cfp</i>	1.5120	<i>Sh2d1a</i>	-1.0703
<i>Il6st</i>	1.4227	<i>Cd2</i>	-1.0583
<i>Tnfrsf9</i>	1.3808	<i>Casp3</i>	-1.0473
<i>Art2b</i>	1.3502	<i>Cd74</i>	-1.0352
<i>Lta</i>	1.3047		
<i>Il4r</i>	1.2441		
<i>Bcl3</i>	1.2323		
<i>Socs1</i>	1.1844		
<i>Vipr2</i>	1.1346		
<i>Tfrc (CD71)</i>	1.1160		
<i>Il6r</i>	1.0884		
<i>Bcl2</i>	1.0220		
<i>Ikbke</i>	1.0057		

adjusted p-value <0.05

Supplementary table 4. Immune genes upregulated in CD4+EGFP+ vs. CD8+EGFP+ cells.

TCD4+eGFP+		TCD8+eGFP+	
Name of gene	Log2 fold change	Name of gene	Log2 fold change
<i>LOC688090</i>	3.4725	<i>Fcna</i>	-2.5401
<i>Cd4</i>	3.2752	<i>Klri1</i>	-2.4753
<i>Ms4a1</i>	3.0906	<i>Cd7</i>	-2.2708
<i>Cd300c2</i>	2.7454	<i>Cd8b</i>	-2.0966
<i>Ccl6</i>	2.5616	<i>Hmox1</i>	-2.0702
<i>Selplg</i>	2.4561	<i>Clqc</i>	-2.0702
<i>Nrp1</i>	2.2765	<i>Clqa</i>	-2.0194
<i>Gata3</i>	2.1798	<i>Cd8a</i>	-1.9326
<i>Ptafr</i>	2.1467	<i>Spic</i>	-1.9273
<i>Fcmmr</i>	2.0031	<i>Cdh1</i>	-1.8467
<i>Lbh</i>	1.9287	<i>Clqb</i>	-1.8234
<i>Fcnb</i>	1.8856	<i>Dnase1l3</i>	-1.7839
<i>Cd79b</i>	1.8688	<i>Lag3</i>	-1.4806
<i>Spib</i>	1.8052	<i>Cxcl17</i>	-1.4593
<i>Ly86</i>	1.7394	<i>Cfd</i>	-1.4120
<i>Ccr6</i>	1.6931	<i>Tbx21</i>	-1.3325
<i>RGD1559482</i>	1.6797	<i>Mcoln2</i>	-1.2776
<i>Cd19</i>	1.5346	<i>Il17ra</i>	-1.2213
<i>Gpx1</i>	1.3639	<i>Cxcr5</i>	-1.2136
<i>Rab25</i>	1.3533	<i>Gzmm</i>	-1.1978
<i>Ctsc</i>	1.2477	<i>Tnk2</i>	-1.1217
<i>Cd74</i>	1.2387	<i>Ptms</i>	-1.0519
<i>Tnfrsf4</i>	1.1885		
<i>Isg15</i>	1.1781		
<i>Pvr</i>	1.1735		
<i>Anxa1</i>	1.1715		
<i>Lrrc32</i>	1.1595		
<i>Tapbp</i>	1.1323		
<i>Skap2</i>	1.1203		
<i>Icos</i>	1.0982		
<i>Nmi</i>	1.0799		
<i>RT1-Da</i>	1.0756		
<i>Ctsl</i>	1.0504		
<i>Cd9</i>	1.0235		
<i>Tnfaip8l2</i>	1.0051		

adjusted p-value <0.05

Supplementary table 5. Genes that are upregulated in Venn diagrams.

Unique genes (199) in CD4+EGFP+ vs. CD4+EGFP- cells

Abcg1	Gid8	Ms4a4c	Rnaset2	Zbtb9
Abtb2	Gimap1	Ms4a8	Rnf216	Zc3hav1
Acot2	Glcci1	Msl1	S1pr1	Zfp281
Agpat5	Glg1	Mtdh	Sbk1	Zfp3611
Aig1	Gpcpd1	Mtmr3	Scml4	Zfp830
Aldh2	Gpr15	Myh14	Sell	Znrf2
Ap1s3	Gpr171	Mylip	Sesn2	Zswim4
Arid5b	Gpr174	Mynn	Sh2d3c	
As3mt	Gsn	Myo10	Sh3tc1	
Bex4	Hebp2	Neil1	Siah1	
Btrc	Herpud2	Nfatc1	Sipal	
Castor2	Hexim1	Nsd3	Sipal11	
Cblb	Hspb11	Nupr2	Slamf7	
Cbx6	Iah1	Ocln	Slc23a3	
Ccn3	Id3	Oga	Slc43a2	
Ccr5	Ifnar1	Pacc1	Slc5a3	
Cd27	Ift80	Parp12	Smpd1	
Cd38	Igf1r	Pbx4	Snn	
Cd47	Ikzf2	Peli1	Son	
Cd80	Il16	Pgm2l1	Specc11	
Cd96	Il18	Phf20	Stap2	
Cd99l2	Il1rl1	Phf8	Stbd1	
Cerk	Il2rb	Phyh	Taf7	
Chn1	Ing1	Pias1	Taok2	
Clta	Itgb8	Pias2	Tbcc	
Cnn3	Itm2a	Pja2	Tbl1xr1	
Cradd	Jak1	Plac8	Tent5c	
Crebrf	Kcnk6	Plagl2	Tgif1	
Cyfip1	Kdsr	Plpp2	Tigd2	
Dag1	Klf7	Prf1	Tmem130	
Daglb	Krt18	Prkcq	Tmem71	
Decr2	Lamp1	Prkcz	Tnfrsf13b	
Dnmt3a	Ldlrad4	Psen2	Tnfrsf1b	
Dph2	Limd1	Ptbp3	Tnip1	
Dtnb	LOC302022	Ptpn7	Tnnt2	
Dtnbp1	Lrp11	Qprt	Tut4	
Dusp10	Lsr	Rab11fip1	Ube2r2	
Eif2ak1	Ly6al	Rab19	Ube3a	
Eif4e3	Lynx1	Rab20	Upb1	
Epsti1	Map3k1	Rab5b	Usp18	
Eva1b	Mbd2	Rabgap11	Usp3	
Fas	Mbnl2	Rap1gds1	Vamp5	
Fbxo46	Mbtps1	Rapgef6	Vnn1	
Foxn3	Mcart1	Rbl2	Was	
Foxp1	Mfap3	Rdh16	Wipf1	
Frmd6	Mfge8	RGD1309104	Zbtb2	
Gadd45g	Mmrn2	RGD1563888	Zbtb4	
Gbp4	Mns1	Rin3	Zbtb7a	

Unique genes (306) in CD8+EGFP+ vs. CD8+EGFP- Treg

Acdb3	Chd6	Gadd45b	LOC100911177	Ppp1r12b	Snnp48	Vav3
Acin1	Chkb	Gapt	LOC100912312	Ppp2r3b	Socs1	Vcam1
Acrbp	Clasrp	Gdi1	LOC361985	Prickle3	Spep	Vipr2
Adams10	Clcf1	Gfra2	LOC682870	Prkd2	Spic	Vom2r34
Adck5	Clk1	Gna15	Lpin2	Prr22	Spindoc	Vps13d
Ahsa2	Col17a1	Gorasp1	Lrrc23	Prrc2a	Spns1	Vps37c
Akap8l	Cpd	Gpat2	Lrrc71	Prrt1	Spry1	Vps54
Anks1a	Cry2	Gpat4	Lrtm2	Psap	Srrm2	Vwa7
Anxa3	Csnk1g2	Gsdma	Luc7l3	Ptpv	Ssbp4	Vwf
Apoe	Dap3	Gtpbp6	Ly49s7	Pwwp3a	Sstr3	Wwc2
Arhgap21	Dcaf6	Hap1	Lyst	Pxdc1	St3gal5	Ypel3
Arhgef40	Dclre1c	Herc2	Mab2113	Pygo2	Stk38	Zbtb17
Armc5	Ddx17	Hgs	Macf1	Rab12	Stox2	Zc3h7a
Arrdc2	Ddx39b	Hmox1	Map2k5	Rab4b	Stra6	Zcchc7
Arvcf	Def8	Hpgds	Map4k4	Ralgds	Stx1a	Zfp347
Ash1l	Dennd1c	Hps5	Mier2	Ralgps2	Supt5h	Zfp641
Atad3a	Dgka	Hsd11b2	Mink1	Rapgef2	Syne4	Zfpml
Atp13a1	Dgkz	Hspbp1	Mir155hg	Rapgef5	Tdp2	Zswim8
Atpaf1	Dhx30	Hus1	Mnt	Rarg	Tfb2m	
Aurkc	Dhx37	Ift43	Mon2	Rarres1	Tfrc	
Bcl2l1	Dkc1	Ikbke	Mrap2	Rbfox2	Timp1	
Bco2	Dlx4	Il2	Mrc1	Rbpjl	Tincr	
Begain	Dnase1l3	Il6r	Mtmr12	Rere	Tmem156	
Blnk	Dpp4	Il6st	Mzb1	Rexo1	Tmem161a	
Brd8	Dse	Il9r	Ncf1	Rftn1	Tmem176a	
Brf2	Dtx2	Inpp4a	Nemf	Rfx1	Tmem176b	
C1qa	Dus3l	Inpp5b	Neur13	RGD1307100	Tnfrsf11b	
C1qb	Dusp5	Ins13	Nr4a1	RGD1564149	Tnfrsf25	
C1qc	Edc4	Irf4	Nsf	Ripor1	Tnk2	
Cacnb1	Edrf1	Itga6	Nt5dc2	Rn45s	Tnks	
Cadm4	Egln2	Itgb5	Olig3	Rrp9	Tnnc2	
Camta2	Emid1	Itm2c	Pced1b	Rspo2	Tnnt3	
Capn5	Erc1	Itpr2	Pdzd4	Rsrp1	Tns3	
Cbx7	Ercc2	Jag1	Phf1	Runde3a	Tpcn2	
Cc2d1b	Ercc6	Jak3	Phyhd1	Runx1	Traf3ip2	
Ccdc134	Faap20	Jmjd1c	Pik3c2a	Sat1	Trappc10	
Ccl1	Fam117a	Kbtbd11	Pim3	Sbf1	Trim34	
Ccnd1	Fam193b	Kcnn4	Pip4p1	Sbsn	Trip6	
Ccnk	Fam214a	Kdm4a	Pitpnm2	Sdc3	Trir	
Cd163	Fam43a	Kdr	Pkib	Sdc4	Trpm2	
Cd5l	Fam98c	Kiaa0408L	Pkn1	Setd4	Ttc22	
Cd83	Fastkd1	Kif13a	Pla2g2d	Sf3a2	Ube2h	
Cdh1	Fcho1	Krit1	Plcd1	Slc17a9	Ubt1	
Cdk16	Fcna	Laptm4b	Plod1	Slc35e4	Ubxn6	
Celf5	Foxi1	Lats1	Plpp3	Slc40a1	Ulk1	
Cep95	Fyttd1	Ldb1	Pms2	Slc4a11	Usf2	
Cfd	Fzd8	Leng8	Pnn	Smpd2	Usp30	
Cfp	Gabbr1	Lingo4	Pou2f2	Smurf2	Utrn	

Common genes (49) between CD4+EGFP+ vs. CD4+EGFP- cells and CD8+EGFP+ vs. CD8+EGFP- cells

Abcc5	Wdr25
Abhd17c	Zc3h12d
Adora2a	
Aff3	
Arl5c	
Art2b	
Atp6v0a1	
Bcl3	
Bcl9l	
Bex1	
C1qtnf6	
Cipc	
Cyp2s1	
Dst	
Eno2	
Foxp3	
Il21r	
Il4r	
Inpp5a	
Irak3	
Ly6e	
Manba	
Nfkb1a	
Nsg1	
Paqr5	
Patj	
Pcbp3	
Pde2a	
Phlda1	
Pik3ip1	
Ppfbp1	
RGD1565410	
Rhoh	
Rilpl2	
Scarb2	
Serpini1	
Sesn1	
Sez6	
Slc12a7	
Slc41a3	
Snx20	
Thrb	
Tiam1	
Tnfrsf26	
Tox	
Ttyh3	
Vipr1	

Unique genes (256) between CD4+EGFP+ vs. CD8+EGFP+ Treg

Abca7	Cers2	Gpx4	Ndufa9	Rnf125	Trappc3
Abhd4	Chid1	Gstm1	Ndufb11	Rpain	Trappc4
Acadv1	Chmp3	Gtf2a2	Ndufb4	Rpe	Trat1
Acp1	Cklf	Hdhd2	Nfyb	Rps19l2	Trmt112
Actl6a	Cldnd1	Hist1h2bq	Nmi	Rsu1	Tsnax
Adi1	Clec4a3	Hmgn3	Nucb1	RT1-Da	Tspo
Adprm	Clic1	Hsd17b11	Oas1i	Rtp4	Txndc17
Aga	Clk4	Hspa13	Ormdl2	Sar1b	Ubb
Agk	Cnih4	Ifi47	Ost4	Scp2	Ubl4a
Agtrap	Coa3	Ifitm2	Papss1	Scpep1	Ubxn8
Alkbh6	Coa4	Ift172	Pcnp	Selenok	Utp11
Alox5ap	Cops2	Igtp	Pdcd10	Selplg	Vamp8
Amelx	Cr1l	Il2rg	Pde4b	Sfr1	Vdac3
Anapc13	Crcp	Irf7	Pex16	Skap2	Vps29
Anp32a	Cript	Isca2	Pex19	Smco4	Ythdf2
Anxa1	Cryl1	Isg15	Pkm	Smyd3	Zfp566
Anxa4	Cst3	Jpt1	Pnkd	Snx3	
Anxa5	Cstb	Krtcap2	Pnrc2	Spcs1	
Ap2m1	Ctsb	Leng1	Pdpf	Spib	
Aprt	Ctss	LOC100125368	Ppil3	Spsb2	
Arf4	Cyba	LOC100302372	Ppp1r11	Ssr3	
Arhgap15	Daam1	LOC100910973	Ppp2r5c	Ssr4	
Arhgdib	Dctn6	LOC688090	Prdx2	Stom	
Arpp19	Ddost	Lrpap1	Prdx4	Strada	
Atp5f1c	Dguok	Lsm10	Prr13	Stt3a	
Atp5if1	Dnajc19	Ly86	Prrt3	Stub1	
Atp5pb	Dusp6	Lyc2	Psenen	Stx18	
Atp5pf	Ech1	Mal	Psmb7	Sugt1	
Atp6v0e1	Eef1akmt1	Mapre1	Psmd1	Sumo1	
Avpi1	Eef1e1	Med10	Psmd10	Sv2a	
Bcap31	Emd	Med19	Psmd11	Szrd1	
Bccip	Erg28	Mien1	Psmd12	Taf9b	
Bpgm	Erp44	Mpc1	Psmd14	Tapbp	
Bud31	Evi2a	Mpc2	Rab11b	Tceal1	
Calm1	Exoc6	Mrpl18	Rab2a	Tceal8	
Calm2	Fcnb	mrpl24	Rab8b	Tcf4	
Camk4	Fermt3	Mrpl34	Rab9a	Tgoln2	
Capg	Fkbp3	Mrpl50	Rabac1	Tmbim4	
Ccl6	Frg1	Mrps12	Rbks	Tmbim6	
Ccr6	Fubp1	Mrps16	Rdh8	Tmco1	
Cd19	Gabarap	Ms4a1	RGD1306063	Tmed10	
Cd2	Galm	Mthfs	RGD1359127	Tmem128	
Cd300c2	Gda	Naa38	RGD1359508	Tmem179b	
Cd4	Gemin7	Naa60	RGD1559482	Tmem205	
Cd48	Gimp	Nagk	RGD1563941	Tmem50a	
Cd53	Gm2a	Napsa	Rhno1	Tmem59	
Cd74	Gmfg	Ncoa4	Rida	Tnfaip8l2	
Cdc123	Gpx1	Ndufa13	Rnasek	Trappc1	

Common genes (48) between CD4+EGFP+ vs. CD8+EGFP+ cells and CD4+EGFP+ vs. CD4+EGFP- cells

Acp5
Ak3
Apobec2
Bnip3l
Cd9
Cdkn1b
Cdkn2c
Ctsc
Ctse
Cyb5r3
Ethel
Gata3
Gimap7
Glrx
Grb14
Hps6
Icos
Ifngr1
Ipcef1
Lbh
Lrrc8c
MGC105567
Mgst1
Ms4a6b1
Mxd4
Ncr3
Nmnat1
Nmur1
Nrp1
Phlda2
Pja1
Prxl2b
Ptafr
Ptp4a2
Pts
Pvr
Rab25
Sec11c
Serpib6a
Shisa5
Smagp
Snx2
Sri
Tank
Tmem140
Traf3ip3
Wls
Zfp148

Common genes (5) between CD4+EGFP+ vs. CD8+EGFP+ and CD8+EGFP+ vs. CD8+EGFP- cells

Cd79b
Cyb561a3
Lgmn
Lta
Ramp3

Common genes (13) between CD4+EGFP+ vs. CD8+EGFP+ and CD8+EGFP+ vs. CD8+EGFP- and CD4+EGFP+ vs. CD4+EGFP- cells

Apbb1
Ctsl
Fcmm
Il2ra
Lrrc32
Maf
Prtfde1
Samhd1
Selenop
Smpd13a
Tnfrsf18
Tnfrsf4
Tnfrsf9

Supplementary table 6. Genes that are downregulated in Venn diagrams.

Unique genes (239) in CD4+EGFP+ vs. CD4+EGFP- cells

Adh6	Espn	Lingo4	Pgam1	Shisa8
Akr1e2	Etv6	Lmf2	Pip5k1b	Slc11a1
Ankrd13d	Exog	Lmna	Plaur	Slc22a4
Ankrd39	Exosc2	LOC100910945	Plk3	Slc37a3
Apoo	F8	LOC286960	Poc1a	Smc5
Arntl	Fabp5	LOC312273	Pola2	Smim3
Atad2	Fam111a	LOC361914	Pomt1	Snrpa
Atf6	Fam136a	LOC365238	Ppa1	Sorcs3
Atp10a	Fbxo41	LOC498265	Ppp1r14b	Sox17
Atp2b4	Fhad1	Lsm2	Ppp1r9b	Specc1
Baiap211	Fkbp11	Map3k6	Pprc1	Sphk1
Bik	Foxm1	Mboat1	Prdm8	St3gal5
Bpifb4	Fut7	Mgat4a	Prmt5	ST7
Capn10	Fyco1	Mir155hg	Prpf19	Sv2b
Ccl3	Gar1	Mpp4	Prr51	Sytl1
Ccr6	Gch1	Mrpl12	Psat1	Tbl3
Cd226	Gcsh	Mrpl38	Ptn	Tfb1m
Cd40lg	Gem	Mrto4	Pus7	Tfdp1
Cd99	Ggt1	Mtfr2	Pxdc1	Tgm3
Cdca7	Glod5	Mvd	Qtrt1	Thg11
Cenpn	Gna14	Myadml2	Rab38	Thop1
Cfap45	Gpatch4	Myom3	Rad23a	Tigar
Chd7	Gpr146	N5	Rad51	Timd2
Ciapi1	Gsdma	Naglt1	Rap1gap2	Timm9
Ckb	Herpud1	Nat10	Rapgef2	Tma16
Clcn4	Hspd1	Nck2	Rbm28	Tmem97
Cldn10	Hyou1	Ncl	Rbpj	Tnf
Clspn	Ier3	Ncln	Rcc11	Tnfrsf21
Cluh	Ifitm1	Nfasc	Resp18	Tnfrsf25
Cnr2	Ifrd2	Nkx6-2	Rfc3	Tpi1
Cntd1	Igflr1	Nme2	Rflna	Trim2
Col14a1	Igsf9	Nmral1	Rflnb	Tsga10ip
Col4a1	Il17f	Noc4l	RGD1308065	Tufm
Cpm	Il17rc	Nolc1	RGD1309350	Txlng
Ctps1	Il17re	Nop9	RGD1310852	Ube2s
Cyp4f17	Il3ra	Nr1d1	RGD1560289	Uck2
Dclre1b	Kcng4	Nthl1	RGD1564149	Usp36
Dctpp1	Kcnk1	Nup93	RGD1564664	Usp44
Dear	Kif5c	Olah	Rhbdd3	Vars1
Depdc1	Klhl8	Olfm1	Rhbdf2	Vegfb
Dnajb7	Klrc1	Pa2g4	Rpf2	Vwa7
Dnajc11	Knop1	Pak3	Rps6ka2	Xcl1
Dync2i2	Kntc1	Pcdhb2	S100a4	Ybey
Ecm1	Lama3	Pde11a	S100a5	Ydjc
Ehhadh	Lap3	Pde7a	Scg5	Zbtb8a
Elmod2	Large1	Pelp1	Sdf2l1	Zfp707l1
Emb	Lat	Pex1	Sh3bp5	Zfp9
Enpp3	Lgals3	Pex11b	Shisa3	

Unique genes (333) in CD8+EGFP+ vs. CD8+EGFP- Treg

Abrac1	Cd81	Fcnb	Larp7	Nudt1	Rdh8	Stt3a
Acat1	Cdc123	Fen1	Lck	Nudt21	Rfc4	Stx11
Acp1	Cdc20	Fxyd5	Ldhb	Nusap1	RGD1359127	Sub1
Actg1	Cdc25b	Fyn	LOC102554317	Nxt2	RGD1359508	Suclg1
Actr3	Cdca8	Gale	LOC688090	Oprd1	Rpa3	Susd2
Agk	Cdhr1	Galm	Lsm10	Ost4	Rpain	Svbp
Agpat4	Cdk4	Gas7	Lsm12	Ostf1	Rpe	Szrd1
Aifm1	Cdkn3	Gbp2	Ly49s3	Ostm1	Rps19l2	Tagln2
Ak2	Cenpm	Gemin7	Ly49s6	Oxr1	Rps27l	Tapbp
Ak8	Cers2	Gimap4	Lyc2	Pabpc1	Rrm1	Tcea1
Anapc13	Cfl1	Gins1	Mad2l1	Papss1	Rrm2	Tcf19
Anxa2	Chchd5	Glr2	Magohb	Pcnp	Rsu1	Tent5a
Anxa6	Cisd3	Gmfg	Mapre1	Pdcd1	RT1-Da	Tes
Arpc1b	Clic1	Gnai3	Mapre2	Pde4b	RT1-Db1	Tgln2
Arpc5	Cnrip1	Gpr68	Mbd2	Pgk1	RT1-DMa	Timm17b
Asf1b	Cops2	Gyg1	Mcm4	Phf11	RT1-DMb	Timm23
Atp5f1a	Cox5b	Gzma	Mdh1	Phf11b	Rtp4	Tk1
Atp5f1b	Cox6b1	Gzmk	Med19	Pik3cg	S100a10	Tmbim6
Atp5f1c	Crep	H2ax	MGC105649	Pkm	S100a9	Tmed2
Atp5pb	Cst7	H2az1	Mkrn1	Plaatz	S1pr5	Tmem126a
Atp5pf	Cuedc2	H2bc12	Mpc1	Plek	Samd3	Tmsb15b2
Aurka	Cx3cr1	Haus4	Mpst	Plk4	Sar1b	Tnfsf9
Aurkb	Cxcr3	Hcst	Mrip	Pnrc2	Sema4a	Tpgs2
B4galt5	Cxcr6	Hdgf	Mrpl18	Pole	Serbp1	Tprg11
Banf1	Cyria	Hikeshi	Mrpl34	Pop4	Serpinb9	Tpst2
Bcap29	Dapk2	Hltf	Mthfd1	Ppib	Sh2d1a	Traf1
Becn1	Dars2	Hmgb1	My112a	Ppil3	Sh2d2a	Trappc1
Birc5	Dbi	Hmgb3	My1pf	Ppp2r5c	Sh3bgr	Tubb4b
Borcs8	Def6	Hmgcs1	Naa38	Prdx4	Sh3bgrl3	Txn1
Calm1	Dhcr7	Hopx	Naa60	Prim2	Siva1	Ube2a
Calm2	Dhfr	Hsd11b1	Nans	Prkcb	Skap1	Ubl4a
Cap1	Dmrct2	Hsd17b11	Ncald	Prmt2	Slamf7	Uchl3
Casp3	Dnajc19	Hspa13	Ncr1	Prrt3	Slbp	Uchl5
Ccl5	Dstn	Idh2	Ndufa10	Psma5	Slc25a5	Uqcrfsl
Ccl6	Dynl1	Idh3a	Ndufa2	Psmb8	Slc39a8	Uxt
Ccnb1	E2f1	Isca2	Ndufa9	Psmc1	Slc9a3r1	Vdac3
Ccnb2	Eef1akmt1	Isg15	Ndufb11	Psmc11	Slirp	Vim
Ccnd3	Eef2	Itgb2	Ndufb4	Psmc12	Spes2	Xpo1
Ceng1	Eif4e2	Itm2b	Ndufb6	Psmc14	Spink2	Xrcc5
Ccr5	Eipr1	Jpt1	Ndufb9	Psmf1	Spint1	Ybx3
Cd164	Emd	Kif20a	Ndufs1	Pxn	Spn	Yif1b
Cd2	Emp1	Kif22	Ndufs3	Pycard	Spr	Ythdf2
Cd38	Emp3	Klra22	Ngp	Rab11a	Sptssa	Ywhae
Cd3e	Erp44	Klrb1a	Nicn1	Rab27a	Ssr2	Ywhaq
Cd3g	Esd	Klre1	Nmi	Rab2a	Ssr3	Zbtb32
Cd53	Etfb	Knstrn	Nptn	Rab3d	Stgal6	
Cd59	Evi2b	Lage3	Nt5c	Rab9a	Stmn1	
Cd74	Fam89b	Lamp2	Nucb1	Rbms1	Strada	

Common genes (24) between CD4+EGFP+ vs. CD4+EGFP- cells and CD8+EGFP+ vs. CD8+EGFP- cells

Ahnak
Amt
Cdc42ep3
Chchd6
Chek1
Ctla2a
Faslg
Gapdh
Gzmb
Gzmc
Id2
Klrk1
Lgals1
Lgals3bp
Mcm2
Myadm
Myo1f
Nkg7
Pcp4
Pwp1
Ran
S100a6
Tmem107
Tmem204

Unique genes (212) between CD4+EGFP+ vs. CD8+EGFP+ Treg

Acap1	Cstf3	Leng8	Rbm25	Ttc3
Acap3	Cxcl17	LOC100911177	Rbm34	Uap111
Acbd3	Cxxc5	LOC100912312	Rcn1	Ubtf
Acin1	Cyth4	LOC682870	Recql5	Ubxn6
Acrbp	Dcaf6	Lrrc42	Rev1	Uchl1
Adamts10	Defa5	Luc7l2	Rev3l	Unc119b
Adcy6	Dhx30	Luc7l3	RGD1307100	Usp11
Adgrg5	Dhx37	Ly49i4	RGD1560108	Usp38
Agpat3	Dhx9	Ly49i5	Rnf130	Usp40
Akap2	Dis3l2	Ly49s7	Rnf187	Vav3
Akap8	Dnase1l3	Map2k5	Rpsa	Vom2r34
Alms1	Doc2g	Map3k1l	Rsrp1	Vps13a
Ankrd44	Dock5	Mast3	Rundc3a	Vps37c
Ap5z1	Dtx2	Med15	Safb2	Wdr3
Apoe	Dusp2	Mettl3	Senp3	Wdr5
Arf5	Ehbp1l1	Mex3b	Sh2b1	Zbtb17
Arglu1	Elac2	Mink1	Sh2d4a	Zc3h7a
Arhgef2	Emid1	Mms19	Shkbp1	Zfp39
Arhgef7	Eri1	Mnt	Slc16a6	Zfp445
Armc5	Esrra	Mon2	Slc25a38	Zpbp2
Ascc3	Fam13b	Myef2	Slc30a5	
Atf6b	Fam193b	Nat8b	Slc43a1	
Atp13a1	Fam98c	Nemf	Slc4a1l	
Bag1	Fastkd1	Np4	Slc7a6	
Bak1	Fcgbp	Nrros	Slc9a1	
Baz1b	Fcna	Nsun2	Slfn13	
Bin2	Gabpb1	Nt5dc2	Snorc	
Brf1	Glr5	Nufip1	Snrnp48	
C1qa	Gnptg	Pabpc2	Sorl1	
C1qb	Gpsm1	Pabpn1	Sort1	
C1qc	Grk6	Pced1a	Spic	
C2cd2l	Gtpbp10	Pcm1	Spindoc	
Cacnb1	Gtpbp2	Pik3cd	Sppl2b	
Camta2	Hip1r	Plekhg5	Srsf1	
Cc2d1b	Hmox1	Pnn	Srsf2	
Ccdc102a	Hnrnp1	Pnpla7	Ssbp4	
Ccnk	Hps5	Polr1d	Stk38	
Cd8a	Hsd11b2	Ppfia4	Supt5h	
Cdh1	Hsd17b8	Ppp6r1	Syt13	
Cep85	Ins13	Prcc	Tap1	
Cep95	Itpr3	Prr7	Tbcd	
Cfd	Jak3	Ptms	Tfb2m	
Clasrp	Kdm2a	Pwwp3a	Tmem160	
Clcf1	Kdm4a	Pygo2	Tnk2	
Clstn1	Klhdc3	Rab4b	Trafd1	
Cnot1	Klri1	Rabggtb	Trim39	
Cnot10	Lag3	RatNP-3b	Trim41	
Cpsf4	Ldb1	Rbm17	Trir	

Common genes (24) between CD4+EGFP+ vs. CD8+EGFP+ cells and CD4+EGFP+ vs. CD4+EGFP- cel

Abcg312
Agap2
Arrb2
Ccl1
Cd7
Csnk1g2
Dapk3
Dkc1
Fus
Gsto1
Il17ra
Il2
Lmbrd2
Mcoln2
Mical1
Ncbp1
Pcyox11
Pstpip1
Rangap1
Rrp9
Stau2
Tbx21
Tmem273
Usp46

Common genes (3) between CD4+EGFP+ vs. CD8+EGFP+ and CD8+EGFP+ vs. CD8+EGFP- cells

Cd8b
Clic5
Gzmm

Common genes (3) between CD4+EGFP+ vs. CD8+EGFP+ and CD8+EGFP+ vs. CD8+EGFP- and CD4 vs. CD4+EGFP- cells

Ctsw
Klrd1
Tacc2

Supplementary table 7. Increase in different EGFP+ and EGFP- cell subsets in blood following IL-2 treatment.

			CD3+ CD4+ EGFP+	CD3+CD4+ EGFP-	CD3+CD8+ EGFP+	CD3+CD8+ EGFP-	CD161+ EGFP+	CD161+ EGFP-
Blood	Untreated n=4	% of total cells	5.6 ± 1.4	94.9 ± 0.3	2.2 ± 0.4	96 ± 0.3	0	2 ± 1.4
		N°cellsx10⁵ /ml	0.08 ± 0.06	1.7 ± 0.6	0.016 ± 0.005	0.7 ± 0.3	0	0.06 ± 0.04
	hIL2 treated n=3	% of total cells	17.1 ± 0.6	82.6 ± 0.4	9.8 ± 0.9	89.7 ± 0.9	0	2,7 ± 1.1
		N°cellsx10⁵ /ml	1.5 ± 0.5	7.5 ± 2	0.5 ± 0.1	4.4 ± 1.5	0	0.6 ± 0.2
		Fold induction*	19	4.4	31	6.3	0	10

* Fold induction of absolute numbers of cells in the IL-2-treated vs. untreated animals

Supplementary table 8. Antibodies used in the study.

Marker	Clone	Provider
Viability Dye 506		Invitrogen eBiosciences
Biotin-conjugated mouse anti-rat CD3	G4.18	BD Biosciences
BV421-conjugated mouse anti-rat CD3	1F4	BD Biosciences
PercP-conjugated mouse anti-rat TCR $\alpha\beta$	R7.3	BD Biosciences
PECy7-conjugated mouse anti-rat CD4	OX35	BD Biosciences
BV421-conjugated mouse anti-rat CD25	OX39	BD Biosciences
APC-conjugated mouse anti-rat CD161	3.2.3	Produced in house
APC-conjugated mouse anti-rat CD8a	OX8	Produced in house
PE-conjugated mouse anti rat CD45RC	OX22	BD Biosciences
PE-conjugated anti-Foxp3	FJK-16s	Invitrogen eBiosciences
PE-conjugated mouse anti-Stat5	pY694	BD Biosciences
PE-conjugated mouse anti-rat RT1B	OX6	BD Biosciences
PE-conjugated mouse anti-rat CD28	CD28	BD Pharmingen
PE-conjugated hamster anti-mouse CD27	LG.3A10	BD Biosciences
BV711-conjugated mouse anti-rat CD44	OX49	BD Biosciences
BV421-conjugated hamster anti-rat CD62L	HRL1	BD Biosciences
Purified mouse anti-rat CD71	OX26	Produced in house
Biotin-conjugated mouse anti-rat CD5	OX19	Produced in house
Purified mouse anti-rat CD26	OX61	Produced in house
Purified rabbit anti- rat CX3CR1	TP501	Produced in house
PE-conjugated mouse anti-rat CD106	MR106	BD Biosciences
PE-conjugated mouse anti-rat CD80	3H5	BD Biosciences
Purified mouse anti-rat CD38	14.27	Biolegend
PE-conjugated or BV421-conjugated mouse anti-human CD137	4BB1	BD Biosciences
Streptavidin APC-Cy TM 7		BD Biosciences
AF568 goat anti-mouse IgG2a		Thermofisher
R-Phycoerythrin AffiniPure F(ab') ₂ Fragment Donkey Anti-Rabbit IgG (H+L)		Jackson Immunoresearch