Journal of Autoimmunity 98 (2019) 86–94

Contents lists available at ScienceDirect

Journal of Autoimmunity

journal homepage: www.elsevier.com/locate/jautimm

Multi-faceted inhibition of dendritic cell function by CD4$^+$Foxp3$^+$ regulatory T cells

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ARTICLE INFO

Keywords:
Regulatory T cells
Dendritic cells
Costimulation
CTLA-4
PD-L2

ABSTRACT

CTLA-4 is required for CD4$^+$Foxp3$^+$ regulatory T (Treg) cell function, but its mode of action remains incompletely defined. Herein we generated *Ctla-4$^{ex2fl/fl}$Foxp3-Cre* mice with Treg cells exclusively expressing a naturally occurring, ligand-independent isoform of CTLA-4 (liCTLA-4) that cannot interact with the costimulatory molecules CD80 and CD86. The mice did not exhibit any signs of effector T cell activation early in life, however, at 6 months of age they exhibited excessive T cell activation and inflammation in lungs. In contrast, mice with Treg cells completely lacking CTLA-4 developed lymphoproliferative disease characterized by multi-organ inflammation early in life. *In vitro*, Treg cells exclusively expressing liCTLA-4 inhibited CD80 and CD86 expression on dendritic cells (DC). Conversely, Treg cells required the extra-cellular part of CTLA-4 to up-regulate expression of the co-inhibitory molecule PD-L2 on DCs. Transcriptomic analysis of suppressed DCs revealed that Treg cells induced a specific immunosuppressive program in DCs.

1. Introduction

CD4$^+$Foxp3$^+$ regulatory T (Treg) cells comprise a subset of T cells that suppress immune responses and constitute a therapeutic target in autoimmune diseases, infectious diseases, and cancer [1]. Numerous mechanisms and cellular targets of Treg cell-mediated suppression have been suggested but their relative importance remain controversial [2]. *In vitro* studies suggest that Treg cells have the capacity to directly suppress a large number of cell types, including T cells and dendritic cells (DCs) [3,4]. Recent studies aiming to elucidate how Treg cells function have focused on their ability to suppress DCs, as it is clear that Treg cells interact with DCs *in vivo* [5]. It has been demonstrated by *in vivo* imaging studies that Treg cells can decrease the time of contact between effector T cells and DCs prior to effector T cell activation [6]. Also, Treg cells can either actively down-regulate or prevent the up-regulation of the co-stimulatory molecules CD80 and CD86 during DC maturation [4,7–9]. Finally, Treg cells can modulate cytokine production by DCs, for example by inhibiting the production of IL-6 while promoting the production of IL-10 [10]. In several of these studies, CTLA-4 has been implicated as the effector mechanism by which Treg cells suppress DC function.

The co-inhibitory molecule CTLA-4 is a key negative regulator of immune responses [11]. CTLA-4 acts in part by outcompeting the co-stimulatory molecule CD28 for their shared ligands, CD80 and CD86, which are primarily expressed by antigen presenting cells. A complete loss of CTLA-4 in mice leads to severe immune dysregulation and autoimmunity [12,13] and polymorphisms in the *CTLA4* gene are associated with several human inflammatory diseases including systemic lupus erythematosus, Graves' disease, and type I diabetes [14,15]. Also, individuals with *CTLA4* haploinsufficiency present an immune dysregulation syndrome characterized by hypogammaglobulinemia, recurrent infections and autoimmunity [16,17]. Finally, CTLA-4 has recently attracted substantial interest as an anti-cancer target and now represents the archetypal example of immune checkpoint blockade therapy [18]. An increasing number of clinical studies show that blocking CTLA-4 enhances anti-tumor immunity and produces durable clinical responses. However, anti-CTLA-4 therapeutics also frequently result in immune-mediated adverse events such as skin lesions and

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https://doi.org/10.1016/j.jaut.2018.12.002
Received 10 August 2018; Received in revised form 12 December 2018; Accepted 13 December 2018
Available online 05 January 2019
0896-8411/

colitis [19].

Although all T cells express CTLA-4 following TCR stimulation, constitutive CTLA-4 expression on Treg cells is especially vital for immune homeostasis. Mice lacking CTLA-4 specifically in Treg cells develop lethal lymphoproliferative disease early in life [20]. Meanwhile, CTLA-4-intact Treg cells are sufficient to suppress autoimmunity mediated by CTLA-4-deficient effector T cells [21,22]. Qureshi et al. demonstrated that T cells, and Treg cells in particular, can down-regulate both CD80 and CD86 expression on DCs by physically removing these ligands and degrading them by trans-endocytosis [23], indicating a cell-extrinsic function of CTLA-4. Contrariwise, protein kinase C (PKC)-η associates with CTLA-4 in the immunological synapse in Treg cells and this CTLA-4-PKC-η signaling axis is required for Treg cells ability to properly function [24]. Herein we aim to elucidate the relative importance of the cell-intrinsic versus cell-extrinsic actions of CTLA-4 in Treg cells.

2. Material and methods

2.1. Vector design and generation of *Ctla-4$^{ex2fl/fl}$Foxp3-Cre* mice

An 8.9 kb region used to construct the targeting vector was first subcloned from a positively identified C57BL/6 bacterial artificial chromosome (BAC) clone (RP23:388N14) using a homologous recombination-based technique. The region was designed such that the long homology arm (LA) extends 6.01 kb 5′ to the single LoxP site. The short homology arm (SA) extends 2.02 kb 3′ to the LoxP/Flp recombinase target (FRT)-flanked Neo cassette. The single LoxP site is inserted 249 bp upstream of exon 2 of CTLA-4 gene in intron 1–2, and the LoxP/FRT-flanked Neo cassette is inserted 271 bp downstream of exon 2 in intron 2–3. The BAC was subcloned into a ~2.45 kb pSP72 (Promega) backbone vector containing an ampicillin selection cassette for retransformation of the construct prior to electroporation. A pGK-gb2 LoxP/FRT-flanked neomycin cassette was inserted into the gene. The targeting construct was linearized using *Not*I prior to electroporation into embryonic stem (ES) cells. ES cells were then microinjected into Balb/c blastocysts. Resulting chimeras were mated to C57BL/6 Flp mice to remove the Neo cassette and to obtain *Ctla-4$^{ex2fl/fl}$* mice. The *Ctla-4$^{ex2fl/wt}$* mice were then bred with *Foxp3-Cre* mice, all on a C57BL6/J background, to yield *Ctla-4$^{ex2fl/fl}$Foxp3-Cre* mice, in which only the Treg cell population expresses liCTLA-4. The C57BL6/J *Ctla-4$^{ex2-3fl/fl}$Foxp3-Cre* mice have been previously described [20]. Throughout the study *Foxp3-Cre^{+}* littermates were used as controls. The study was performed according to local ethical guidelines.

2.2. PCR

Highly purified (> 95%) Treg cell populations (CD4^{+}CD25^{hi}) were obtained from wild type or *CTLA-4$^{ex2fl/fl}$Foxp3-Cre* mice splenocytes using fluorescence-activated cell sorting (FACS) after labeling with anti-CD4, anti-CD25, on a FACSJazz (BD Biosciences). Total RNA was isolated using the RNeasy Micro Kit and treatment with the RNase-free DNase set (both from Qiagen) according to the manufacturer's instructions. cDNA was generated by using random hexamers and Superscript II reverse transcriptase (Life Technologies). Amplification was performed with FirePol kit (Solis Biodyne) using forward primer (targeting exon 1) 5′ AAC TGC AGC TGC CTT CTA GG 3′ and reverse primer (targeting exon 4; described in Oaks et al. Cell Immunol 2000) 5′ TCA CAT TCT GGC TCT GTT GG 3’.

2.3. FACS

Single-cell suspensions were prepared from thymuses, spleens, lymph nodes and lung. Bronchoalveolar lavage were performed as described previously (http://www.bio-protocol.org/e1875). Dead cells were stained using the Live/Dead Fixable Dead Cell Staining Kit (Life Technologies). Intracellular staining was performed using eBioscience's Foxp3 staining kit according to the manufacturer's instructions. Samples were acquired on an LSR Fortessa flow cytometer (BD Biosciences) and analyzed using FlowJo software (Version 10.1r1 for Mac, TreeStar). The following antibodies were used for flow cytometry: CD3 (clone 17-A2, Biolegend), CD4 (clone RM4-5, Biolegend), CD8 (clone 53–6.7, BD Biosciences), CD11c (clone N418, Biolegend), CD25 (clone PC61, BD Biosciences), CD28 (37.51, Biolegend), CD44 (clone IM7, Biolegend), CD45.2 (clone 104, Biolegend), CD45R (clone RA3-6B2, Biolegend), CD62L (clone MEL-14, Biolegend), CD69 (clone H1.2F3, BD Biosciences), CD80 (clone 16-10A1, Biolegend), CD86 (Clone PO3, Biolegend), CD90 (clone 30-H12 Biolegend), CTLA-4 (clone UC10-4B9, Biolegend), CD103 (clone 2E7, eBioscience), Foxp3 (clone FJK-16s, eBioscience), Foxp3 (clone MF23, BD Biosciences), GATA3 (clone TWAJ, eBioscience), GATA3 (clone L50-823, BD Biosciences), Ki67 (clone B56), Ly6C (clone AL-21, BD Biosciences), MHC II (clone M5/114.15.2, BD Biosciences), PD-1 (RMP1-30, Biolegend), PD-L1 (clone 10F.9G2, Biolegend), PD-L2 (clone TY25, Biolegend), RORγt (clone Q31-378, BD Biosciences), RORγt (clone B2D, eBioscience), Siglec F (clone ESO-2440, BD Bioscience), TCR-beta (clone H57-597, BD Biosciences), T-bet (4B10, Biolegend) and T-bet (REA102, Miltenyi).

2.4. Cytokine bead array

Serum cytokines were measured with Mouse Th1/Th2/Th17 Cytometric Bead Array kit (CBA; BD Biosciences) according to the manufacturer's instructions. Data acquisition was performed on a FACS Calibur (BD Biosciences) and analyzed with FCAP Array software v3.0 (BD Biosciences).

2.5. Bone marrow chimeras

For generation of mixed bone marrow chimeras, 1×10^7 total bone marrow cells from *Ctla-4$^{ex2fl/fl}$Foxp3-Cre* mice (expressing CD45.2) or as a control wild type bone marrow cells (expressing CD45.2) and WT bone marrow cells (expressing CD45.1) at a 1:1 ratio were transplanted via intravenous injection into lethally irradiated (13 Gy) WT C57BL/6 (expressing CD45.2) recipient animals.

2.6. Cell culture

High-purity CD4^{+}CD25^{+} Treg cells (> 98%) were sorted from peripheral lymph nodes using FACS Jazz (BD Biosciences). Sorted CD4^{+}CD25^{+} T cells were pre-activated for 3 days with 5 μg/ml plate-bound mouse anti-CD3 (BD Biosciences) and 500 U/ml recombinant murine IL-2 (Peprotech). CD4^{+}CD25^{+} T cells were then washed and rested for 24 hours with 500 U/ml mouse recombinant IL-2 (Peprotech). High-purity CD11c^{+} DCs were separated from mouse spleens using manual column separation (Miltenyi). In brief, DCs were enriched by depletion of T cells, NK cells, and B cells followed by positive selection of CD11c^{+} DCs using mouse CD11c microbeads. Purified CD11c^{+} DCs were co-cultured with pre-activated CD4^{+}CD25^{+} Treg cells at a 1:1 ratio in the presence of 100 ng/ml LPS. After 24 hours, CD11c^{+} DCs were either analyzed by FACS or re-sorted from the co-culture using FACS Jazz (BD Biosciences). The re-sorted DCs were either used for FACS analysis, RNA-sequencing or co-cultured with CD4^{+}CD25^{-} effector T cells at a 1:10 ratio (DC:Teff cells) with 0.25 μg/ml soluble mouse anti-CD3 for 3 days. Proliferation of CD4^{+}CD25^{-} T cells was measured by 3H-thymidine incorporation.

2.7. RNA-sequencing and data analysis

RNA extraction and RNA sequencing were performed by PrimBio Research Institute, Philadelphia, USA. The final cDNA libraries were loaded onto a chip and run on an Ion Proton system. The mm10

reference genome was used for alignment using the Ion Torrent alignment plugins. Post-alignment QC were determined using StrandNGS bioinformatic software and total reads were filtered based on read metrics (alignment score > 90, Match count, vendors QC). Read counts were further analyzed in R. Between-sample distribution was adjusted using quantile normalization on log-transformed counts. Samples were originated from two independent experiments and, thus, differential expression was carried out using TMM-normalized GLM method in EdgeR package [25] accounting for known batch effects on the experimental design. Differences were considered significant when FDR < 0.05 and fold change > 2. Heatmaps were plotted using the CRAN pheatmap package. Differentially expressed genes were also applied to gene set enrichment analysis (GSEA) [26] using the LPS-activated DCs gene set (GSE22886) [27].

3. Results

3.1. Generation of *Ctla-4$^{ex2fl/fl}$Foxp3-Cre* mice that lacks the extracellular part of CTLA-4 in Treg cells

CTLA-4 is essential for Treg cells' ability to function and it acts either by (1) receiving and transmitting signals into the Treg cells (intrinsic), (2) transmitting signals from the Treg cell to surrounding cells (extrinsic), or (3) down regulating CD80 and CD86 expression on antigen-presenting cells through transendocytosis (extrinsic). To define the relative contribution of these modes of action in Treg cell mediated suppression of DCs we generated conditional knockout mice with LoxP sites flanking exon 2 of the *Ctla-4* gene (*Ctla-4$^{ex2fl/fl}$Foxp3-Cre*). Exon 2 of CTLA-4 encodes for the whole extracellular ligand-binding domain of CTLA-4 (Fig. 1A) and *Foxp3-Cre*-mediated deletion resulted in the exclusive expression of a naturally occurring isoform of CTLA-4, known as ligand-independent CTLA-4 (liCTLA-4) in Treg cells. liCTLA-4 cannot interact with extracellular factors but still inhibit T cell activation, albeit not as efficiently as full-length CTLA-4 [28–30]. We reasoned that if *Ctla-4$^{ex2fl/fl}$Foxp3-Cre* mice would appear healthy, then CTLA-4 mediated transendocytosis and extracellular signaling would be largely dispensable for Treg cells' ability to maintain immune homeostasis during steady state.

Homozygous *Ctla-4$^{ex2fl/fl}$Foxp3-Cre* mice were born at the expected Mendelian frequency, appeared healthy at birth and displayed normal body weight gain. To ensure that exon 2 of CTLA-4 was efficiently deleted in Treg cells, we first used PCR to determine the relative proportion of CTLA-4 isoforms. As expected, Treg cells from *Ctla-4$^{wt/wt}$Foxp3-Cre* control mice predominantly expressed full-length CTLA-4 and low amounts of soluble CTLA-4 and liCTLA-4, whereas Treg cells from *Ctla-4$^{ex2fl/fl}$Foxp3-Cre* mice exclusively expressed liCTLA-4 (Fig. 1B). Additionally, flow cytometry was used to assess the presence of the extracellular domain of CTLA-4 in CD4$^+$ effector T cells and Treg cells. We found that Treg cells from *Ctla-4$^{ex2fl/fl}$Foxp3-Cre* mice, but not *Ctla-4$^{wt/wt}$Foxp3-Cre* control mice, completely lacked expression of the extracellular portion of CTLA-4, which contains the epitope of the α-CTLA-4 antibody used herein (Fig. 1C). The proportion of CD4$^+$Foxp3$^-$ effector T cells expressing CTLA-4 was comparable between *Ctla-4$^{wt/wt}$Foxp3-Cre* control mice and *Ctla-4$^{ex2fl/fl}$Foxp3-Cre* mice (3.31% ± 1.3 versus 4.93% ± 0.62, mean ± SD, n = 5, P = 0.37).

3.2. *Ctla-4$^{ex2fl/fl}$Foxp3-Cre* mice are healthy early in life

To assess if *Ctla-4$^{ex2fl/fl}$Foxp3-Cre* mice displayed any signs of breakdown in immune tolerance we first used flow cytometry to characterize the phenotype of T cells in spleen. At 12 weeks of age we found no differences in the absolute numbers of activated memory-like CD44$^+$CD62L$^-$ CD4$^+$ T cells in the spleen when comparing *Ctla-4$^{wt/wt}$Foxp3-Cre* and *Ctla-4$^{ex2fl/fl}$Foxp3*-Cre mice (Fig. 1D). In contrast, mice that completely lacked CTLA-4 in Treg cells (*Ctla-4$^{ex2-3fl/fl}$Foxp3-Cre* hereafter denoted CKO mice) displayed a significant increase of CD44$^+$CD62L$^-$ CD4$^+$ effector T cells (Fig. S2A), which is in line with a previous report [20]. While *Ctla-4$^{ex2fl/fl}$Foxp3-Cre* mice had normal numbers of effector T cells they exhibited significantly increased numbers of Treg cells in the spleen (Fig. 1D). Phenotypic analysis of the peripheral Treg cell compartment showed that a significantly larger proportion of the CD4$^+$Foxp3$^+$ Treg cells mice were negative for CD25 expression in *Ctla-4$^{ex2fl/fl}$Foxp3-Cre* mice compared to control mice (Fig. 1D). Analysis of serum cytokines using a cytometric bead array assay mirrored these findings as *Ctla-4$^{fl/fl}$Foxp3-Cre* displayed similar levels of effector cytokines to those of *Ctla-4$^{wt/wt}$Foxp3-Cre* control mice but an increase of the immunosuppressive cytokine IL-10 that can be produced by Treg cells (Fig. 1E).

3.3. *Ctla-4$^{ex2fl/fl}$Foxp3-Cre* mice display increased numbers of Treg cells

We went on to elucidate why *Ctla-4$^{ex2fl/fl}$Foxp3-Cre* mice had such strikingly increased numbers of Treg cells. Flow cytometric analysis of Foxp3 expression in thymocytes demonstrated a 2-fold increased proportion of Treg cells in both CD4$^+$CD8$^+$ and CD4$^+$CD8$^-$ thymocytes (Fig. 2A and B). We next analyzed the proliferation of splenic Treg cells by flow cytometric staining for Ki67 and found that Treg cells from *Ctla-4$^{ex2fl/fl}$Foxp3-Cre* mice proliferated at a 2-fold higher rate than Treg cells from control mice (Fig. 2C). The increased thymic output and peripheral proliferation of Treg cells resulted in an increase of both resting central Treg cells and activated effector Treg cells (Fig. S1) [31]. Additionally, the Treg cells from *Ctla-4$^{ex2fl/fl}$Foxp3-Cre* demonstrated loss of CD62L expression and increased CD44 expression (Fig. 2D). No difference was seen in the MFI of PD-1 (127 ± 28 versus 84 ± 7, mean ± SD, n = 5, P = 0.119) and CD28 (346 ± 146 versus 309 ± 53, mean ± SD, n = 5, P = 0.648) when comparing *Ctla-4$^{ex2fl/fl}$Foxp3-Cre* and control mice. These phenotypic changes correspond with an activated Treg cell phenotype and raised the question whether young *Ctla-4$^{ex2fl/fl}$Foxp3-Cre* mice harbor a low-grade inflammatory response that has yet to result in appreciable effector T cell expansion. We created bone marrow chimeras to directly address if such a low-grade inflammatory response was necessary for the dramatic increase of Treg cells. Wild type CD45.2 hosts were irradiated and reconstituted with CD45.1 wild type and CD45.2 *Ctla-4$^{ex2fl/fl}$Foxp3-Cre* bone marrow or as a control CD45.1 wild type and CD45.2 *Ctla-4$^{ex2wt/wt}$Foxp3-Cre* bone marrow. The bone marrow chimeras did not display increased amounts of CD45.2 *Ctla-4$^{ex2fl/fl}$Foxp3-Cre* Treg cells demonstrating that the increase of Treg cells in *Ctla-4$^{ex2fl/fl}$Foxp3-Cre* require cell-extrinsic factors (Fig. 2E).

3.4. Old *Ctla-4$^{ex2fl/fl}$Foxp3-Cre* mice develop inflammation in the lungs

We next examined if the immunological tolerance in *Ctla-4$^{ex2fl/fl}$Foxp3-Cre* breaks down later in life. At 24 weeks of age *Ctla-4$^{ex2fl/fl}$Foxp3-Cre* mice exhibited significantly increased amounts of activated memory-like CD44$^+$CD62L$^-$ CD4$^+$ effector T cells in the spleen even though 60–80% of all CD4$^+$ T cells exhibited a Treg cell phenotype (Fig. 3A). Histological examination revealed no inflammatory lesions in the heart, liver, pancreas, kidney, or colon of *Ctla-4$^{ex2fl/fl}$Foxp3-Cre* mice. However, a subset of mice developed inflammatory lesions in the lungs (Fig. 3B and C). In contrast, CKO mice developed multi-organ inflammation with involvement of liver, pancreas, kidney, lung, and stomach, which is also in line with previous studies using these mice (Fig. S2). To define the lung infiltrates we analyzed both bronchoalveolar lavage (BAL) fluid and dissociated lung tissue using flow cytometry. *Ctla-4$^{ex2fl/fl}$Foxp3-Cre* mice exhibited increased numbers of infiltrating leukocytes, and in particular CD4$^+$ T cells, in both BAL fluid and lung tissue (Fig. 3D–F). As in other tissues *Ctla-4$^{ex2fl/fl}$Foxp3-Cre* mice had a significantly increased number of Treg cells (Fig. 3E). Among the infiltrating cells in the lungs, there was a marked increase in GATA-3$^+$ Th2 cells and eosinophils (Fig. 3G and H). In summary, our data demonstrates that Treg cells exclusively expressing liCTLA-4 are

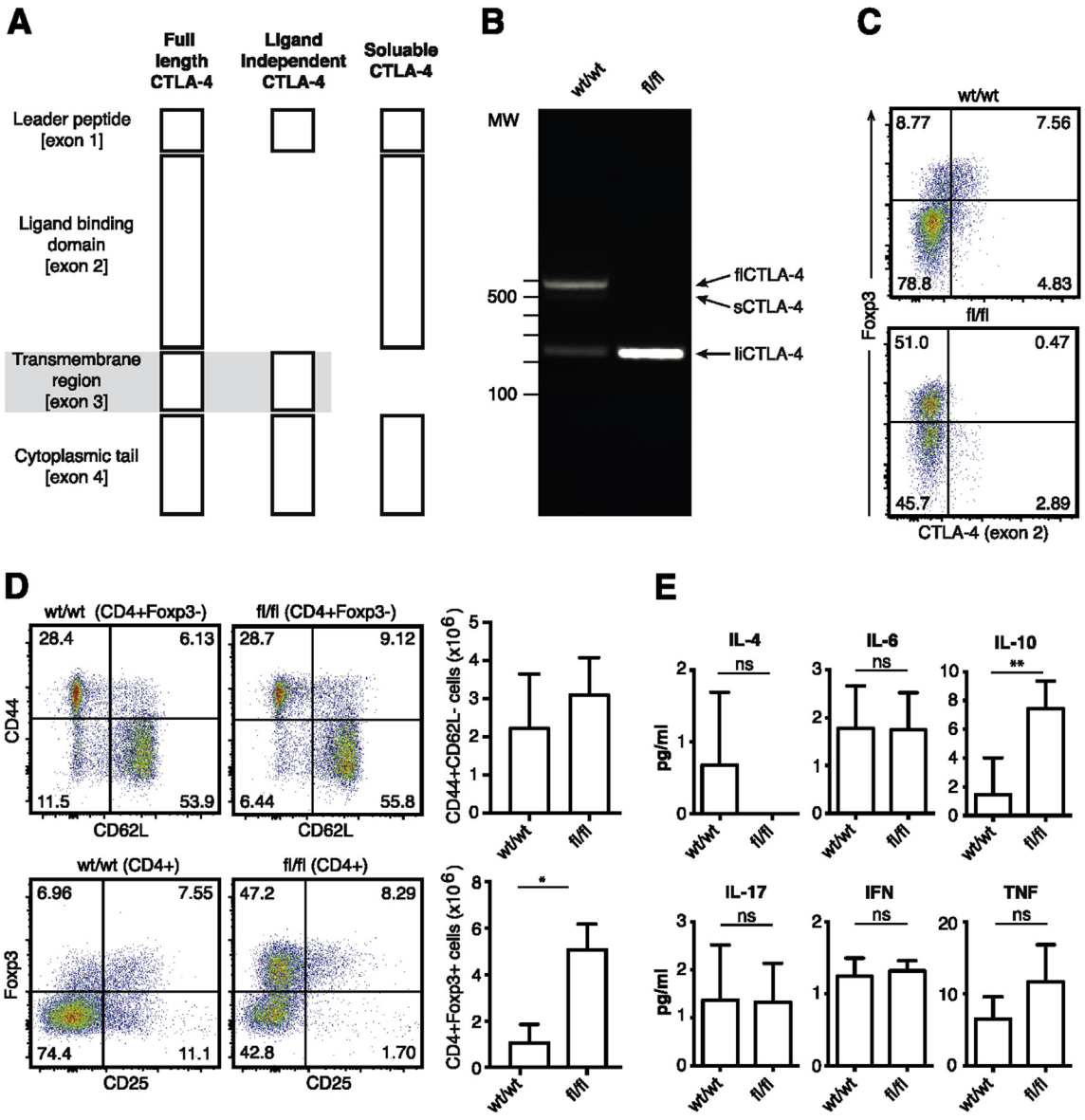

Fig. 1. *Ctla-4$^{ex2fl/fl}$Foxp3-Cre* mice have increased numbers of Treg cells but not effector T cells early in life. Overview of the different CTLA-4 isoforms, displaying the signal peptide, ligand-binding domain, trans-membrane region and cytoplasmic tail **(A)**. Reverse transcription PCR analysis of CTLA-4 isoform expression in *Ctla-4$^{wt/wt}$Foxp3-Cre* wild type mice (denoted wt/wt) and *Ctla-4$^{ex2fl/fl}$Foxp3-Cre* mice (denoted fl/fl) **(B)**. Flow cytometric analysis of splenocytes isolated from 12 to 16 weeks-old wt/wt and fl/fl mice **(C, D)**. Cytometric bead array analysis of serum cytokines in wt/wt and fl/fl mice **(E)**. Data are representative of 2–4 experiments with 4–5 mice per group and are presented as the mean ± standard deviation (SD). $^{*}P < 0.05$, $^{**}P < 0.005$ was considered significant (two-tailed unpaired Student's *t*-test).

unable to control Th2 cell responses in the lung.

3.5. Treg cells lacking the extracellular portion of CTLA-4 can suppress CD80 and CD86 expression on DCs

The striking increase of Treg cells in *Ctla-4$^{ex2fl/fl}$Foxp3-Cre* mice raised the possibility that the increased Treg cell numbers might compensate for any potential dysfunction in their ability to suppress DCs. To address this possibility, we performed an *in vitro* suppression assay to determine if Treg cells from *Ctla-4$^{ex2fl/fl}$Foxp3-Cre* mice could down-regulate the expression of CD80 and CD86 on DCs. Splenic CD11c^{+} DCs from wild type C57BL/6 mice were co-cultured with Treg cells from either *Ctla-4$^{ex2fl/fl}$Foxp3-Cre* or wild type control mice at a 1:1 ratio for 24 hours with lipopolysaccharide (LPS) stimulation. Flow cytometric analysis demonstrated that Treg cells from *Ctla-4$^{ex2fl/fl}$Foxp3-Cre* mice and control mice were equally potent in down-regulating CD80 and CD86 expression (Fig. 4A and B). Moreover, the suppressed DCs from the DC-Treg co-cultures had a decreased ability to support CD4^{+}CD25^{-} effector T cell proliferation (Fig. 4C). Similar results we seen in co-cultures with without exogenous ligands, with CpG or with poly I:C (data not shown). This data shows that Treg cells do not require the extracellular functions of CTLA-4 to suppress DCs *in vitro*.

3.6. Treg cells do not cause general inhibition of DC maturation

Notably, although CD80 and CD86 were down-regulated on DCs co-cultured with Treg cells in the presence of LPS, the levels of CD80 and CD86 on these suppressed DCs were still substantially higher than that of freshly isolated, unstimulated DCs (data not shown). Consequently, we reasoned that Treg cells might inhibit the maturation of DCs using CTLA-4-independent effector mechanisms leading to reduced levels of CD80 and CD86 and an impaired ability to support T cell proliferation. To address this possibility, we used RNA-sequencing to determine the global gene expression of unstimulated DCs, LPS stimulated DCs

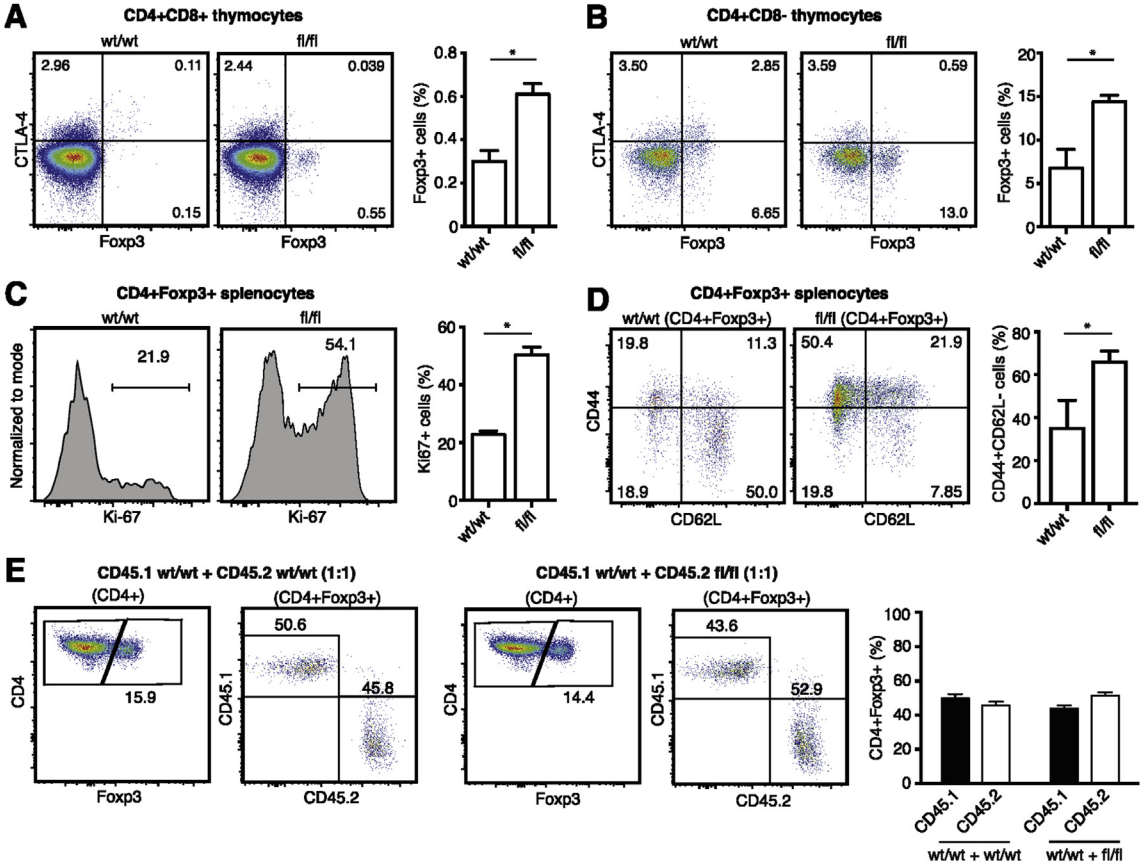

Fig. 2. Full-length CTLA-4 regulates the pool size of Treg cells. Flow cytometric analysis of CTLA-4 and Foxp3 expression in $CD4^+CD8^+$ and $CD4^+CD8^-$ thymocytes, respectively, from 6-week-old wt/wt and fl/fl mice (**A, B**). Flow cytometric analysis of splenic $CD4^+$ T cells from 8-week-old *Ctla-4$^{wt/wt}$Foxp3-Cre* (wt/wt) and *Ctla-4$^{ex2fl/fl}$Foxp3-Cre* (fl/fl) mice (**C-D**). Lethally irradiated $CD45.2^+$ mice were reconstituted with either a 1:1 mixture of $CD45.1^+$ wild type and $CD45.2^+$ *Ctla-4$^{ex2fl/fl}$Foxp3-Cre* bone marrow or as a control $CD45.1^+$ wild type and $CD45.2^+$ wild type bone marrow. T cell composition in the spleen was analyzed 7 weeks post reconstitution (**E**). Data are representative of at least 2 experiments with 5 mice per group and are presented as the mean ± SD. *$P < 0.05$ was considered significant (two-tailed unpaired Student's *t*-test).

cultured alone or with Treg cells and then resorted using flow cytometry. While LPS induced a dramatic change in gene expression when compared to unstimulated DCs, suppression mediated by Treg cells influenced the expression of far fewer genes (Fig. 4D and E). Principal component analysis on indicated that although LPS-stimulated DCs and Treg suppressed DCs are not separated in the first principal component (PC1), they were distant in the second component (PC2), suggesting that minor transcriptional changes still might exist between these conditions (Fig. 4F). In addition, we performed gene-set–enrichment analysis (GSEA) [26] to detect transcriptome-wide changes in expression and we identified a robust enrichment for genes with high expression in LPS-activated DCs [27] in the suppressed DCs population (Fig. 4G). Thus, contrary to our initial hypothesis, Treg cells appear to induce a specific immunosuppressive program in DCs, rather than generally inhibiting the activation of DCs.

3.7. Treg cells use CTLA-4 to induce PD-L2 expression in DCs

The observed Treg cell-mediated changes in DC transcription encompass genes regulating cell signaling (*GPR157, DGKA, GPR4, Ttc32*), cellular metabolism (*HFE, FADS1, SLC40A1, COQ4, Slc25a29, Slc14a1*), transcription (*TRIM34b, EAR, BHLHA15*), endocytosis (*HIP1R, RAB3A, RAB19*) and immune function (e.g. *PD1, CD300C IL-12 p40, Slamf1, LIF, ITGB8, GPR4, Fcgr2b*) (Fig. 5A). While several of these molecules merit further investigation, we chose to interrogate the involvement of the PD1/PD-L1/PD-L2 pathway in the suppression of DCs as these molecules relate directly to antigen presentation. In addition to reduced expression of CD80 and CD86, DCs suppressed by *in vitro* co-culture with wild type C57BL/6 Treg cells expressed significantly increased levels of PD-L2, but not PD1 or PD-L1. This increased expression of PD-L2 was particularly evident in the $CD8^+$ DC subset (Fig. 5B). Interestingly, we did not observe any increased expression of PD-L2 on DCs co-cultured with *Ctla-4$^{ex2fl/fl}$Foxp3-Cre* Treg cells (Fig. 5C), demonstrating that cell-extrinsic CTLA-4 signaling by Treg cells is responsible for the increased expression of PD-L2 under these conditions.

3.8. There is a strong association between FOXP3, CTLA-4 and PD-L2 expression in human disease

The co-inhibitory molecule PD-L2 has, just as CTLA-4, attracted substantial interest as a checkpoint inhibitor of anti-tumor immune responses. To further investigate the relation between CTLA-4 and PD-L2 we used the "R2: Genomics Analysis and Visualization Platform" (http://r2.amc.nl), which contains genome-wide gene expression data from human diseases. We found strong associations between *FOXP3* and *PD-L2* mRNA expression as well as *CTLA-4* and *PD-L2* mRNA expression in biopsies from treatment-naïve inflammatory bowel disease patients and in biopsies from patients with lung adenocarcinoma and breast cancer (Fig. 5D–I). These results indicate that Treg cells may use extrinsic CTLA-4 signaling to induce PD-L2 expression and promote immunosuppression via this pathway in a variety of human diseases.

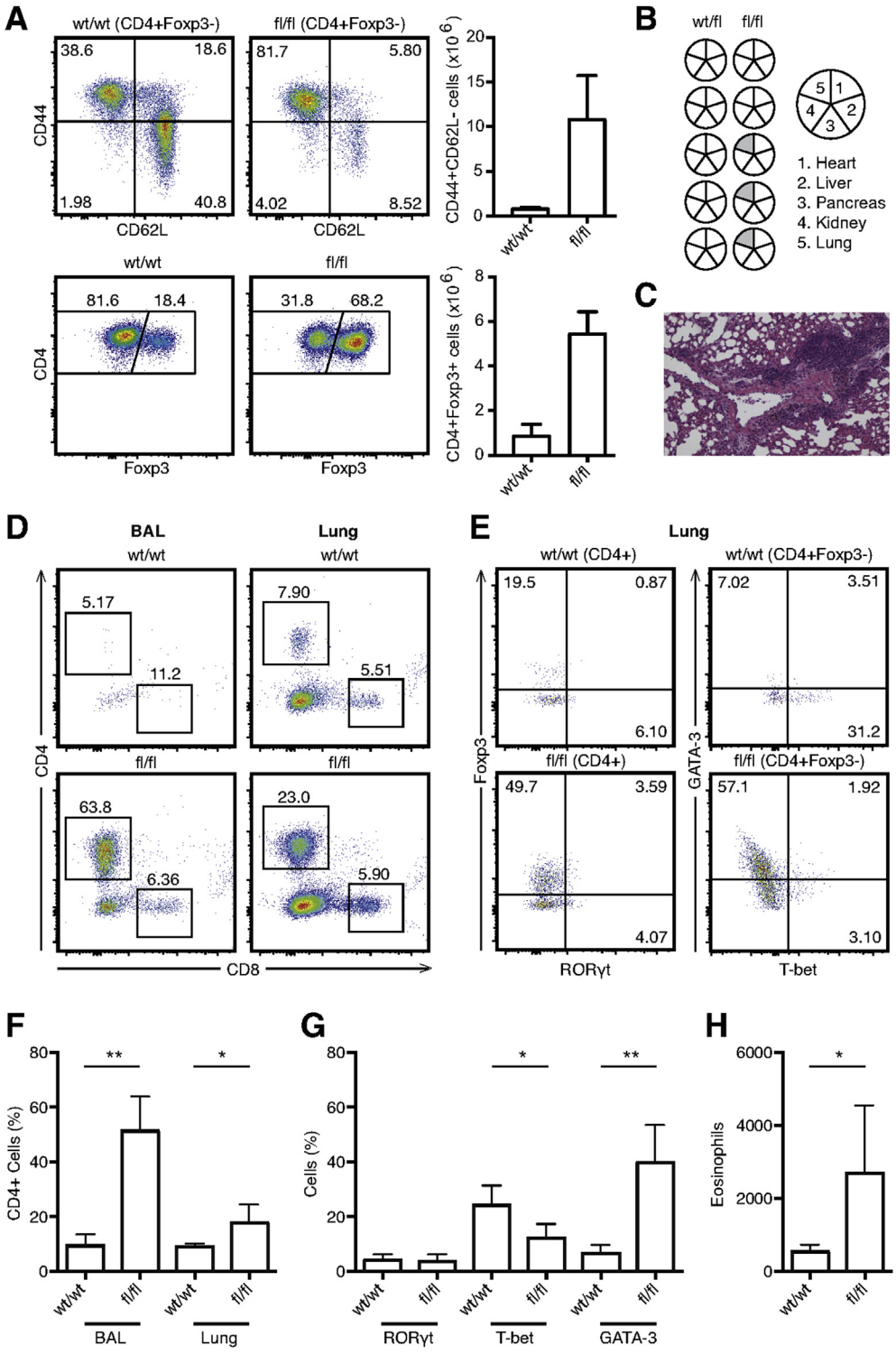

Fig. 3. *Aged Ctla-4$^{ex2fl/fl}$Foxp3-Cre* mice display breakdown of immunological tolerance and Th2 inflammatory responses in lung. (**A**) Flow cytometric analysis and quantification of T cells in spleen of 24-week-old mice. (**B**) Summary of the histologic analysis of wt/fl and fl/fl mice where inflamed organs are indicated in gray. (**C**) A representative section from the lung of fl/fl mice at 24-weeks of age. (**D**) Flow cytometric analysis of CD4$^+$ and CD8$^+$ T cell infiltration in BAL fluid and lungs of 24-week-old *Ctla-4$^{wt/wt}$Foxp3-Cre* (wt/wt) and *Ctla-4$^{ex2fl/fl}$Foxp3-Cre* (fl/fl) mice. (**E**) Flow cytometric analysis of T cell lineage markers in lung tissue of wt/wt mice (top) and fl/fl (bottom). (**F-G**) Quantification of the flow cytometry data. (**H**) Quantification of Siglec F$^+$ eosinophils. Data are representative of 3 experiments with 3–5 mice per group and are presented as the mean ± SD. $*P < 0.05$, $**P < 0.005$ was considered significant (two-tailed unpaired Student's *t*-test).

4. Discussion

The conclusive demonstration that Treg cells require CTLA-4 to function came in 2008 when it became evident that mice specifically deficient of CTLA-4 in Foxp3$^+$ cells displayed impaired Treg cell suppressive function *in vitro* and *in vivo*, and succumbed early in life to lymphoproliferative disease [20]. In this study, we have investigated the requirements of the extracellular portion of CTLA-4 in Treg cells for upholding T cell homeostasis and confer Treg cell function. We found an increased size of the Treg cell pool in *Ctla-4$^{ex2fl/fl}$Foxp3-Cre* mice resulting from both increased thymic output of Treg cells and expansion of Treg cells in the periphery. The increased thymic output of Treg cells contradicts a prior study where exons 2 and 3 of CTLA-4 were flanked by LoxP sites, resulting in complete deletion of CTLA-4 in Treg cells when bred with Foxp3-cre mice. We speculate that this discrepancy is a result of differentially efficient deletion of CTLA-4. Double-positive thymocytes from the *Ctla-4$^{ex2fl/fl}$Foxp3-Cre* mice almost completely lacked expression of the extracellular region of CTLA-4, whereas previous results suggest that both single-positive and double-positive thymocytes from CKO mice only displayed partial deletion of CTLA-4 in

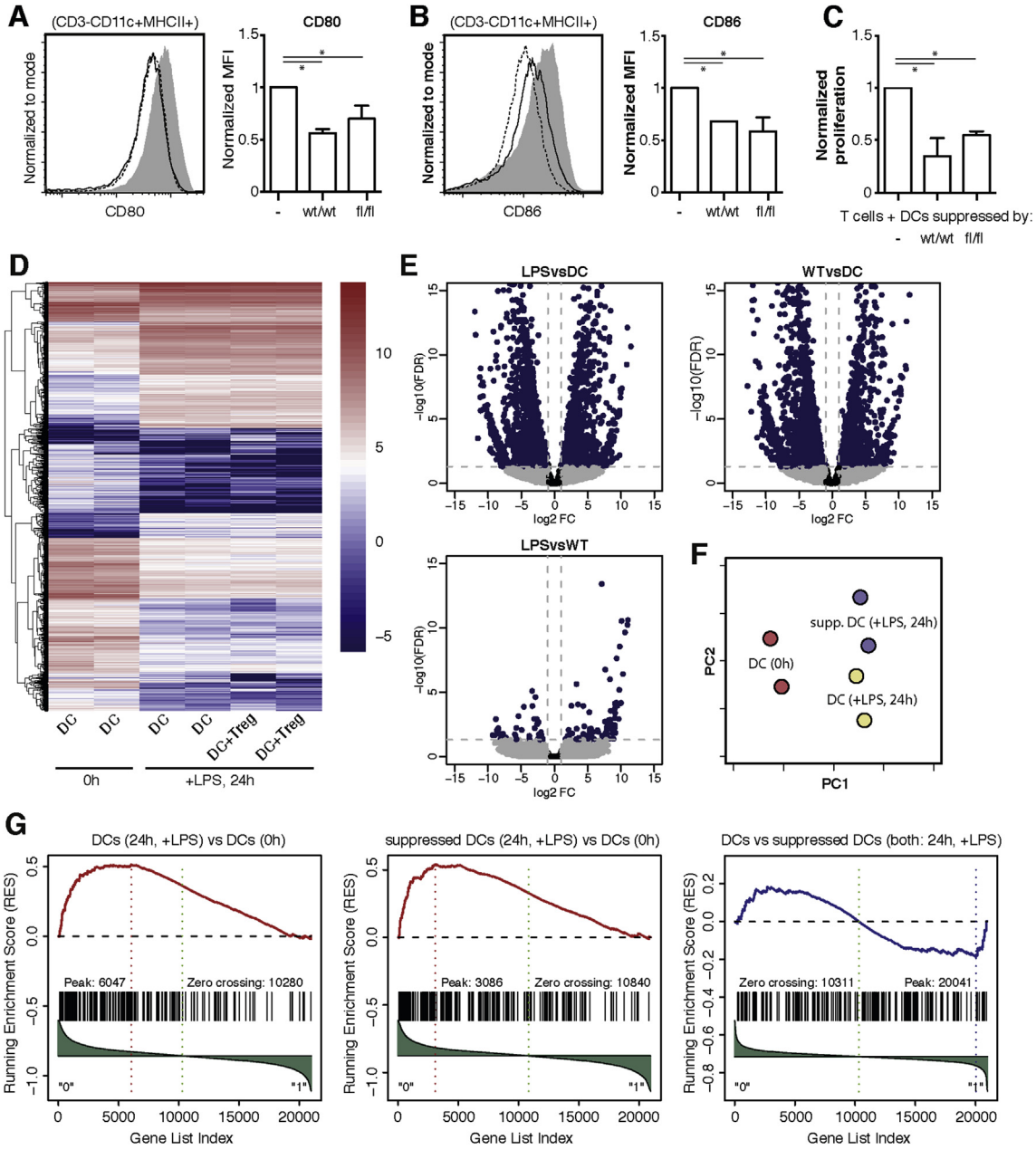

Fig. 4. Treg cells do not require the cell-extrinsic functions of CTLA-4 to inhibit CD80/86 expression on DCs. Flow cytometric analysis of CD80 and CD86 expression on wild type splenic DCs cultured alone (gray histogram), co-cultured with Treg cells at a 1:1 ratio from either *Ctla-4*$^{ex2wt/wt}$*Foxp3-Cre* mice (wt/wt, solid line) or *Ctla-4*$^{ex2fl/fl}$*Foxp3-Cre* mice (fl/fl, dotted line) for 24 h in the presence of 100 ng/ml LPS. The median fluorescence intensity (MFI) of CD80 and CD86 was normalized against the LPS control group (**A, B**). Proliferation of wild-type CD4$^+$CD25$^-$ effector T cells co-cultured with suppressed DCs re-sorted from the co-culture in (A, B) in the presence of 0.25 μg/ml soluble anti-mouse CD3. Proliferation was measured by 3H-thymidine incorporation after 3 days. Data are normalized against the proliferation of the control group (dendritic cells cultured alone for 24 hours with 100 ng/ml LPS) (**C**). Heatmap of differentially expressed genes by RNA-sequencing in freshly sorted DCs, resorted DCs previously cultured alone or with Treg cells for 24 hours in the presence of 100 ng/ml LPS (**D**). Volcano plots comparing the different conditions shown in (D) (**E**) p. Principal component analysis of the DC populations in (D) (**F**). Gene set enrichment analysis between the different groups in (D) using LPS activated activation versus unstimulated DCs gene set (GSE22886) (**G**). (A–C) Data are presented as the mean ± SD of 3 independent experiments. *$P < 0.05$ was considered significant (two-tailed unpaired Student's *t*-test). (D–G) RNA-sequencing was performed in duplicates on cells from 2 independent experiments.

the thymus [20]. Several other studies have addressed the impact of CTLA-4 on thymic development of Treg cells using complete deletion of CTLA-4 in all cells [20,32–34]. A limitation with these studies is that they utilized mice completely devoid of CTLA-4, which affects the thymic microenvironment and thereby indirectly Treg cell development. We also found that Treg cells exclusively expressing liCTLA-4 proliferated in the periphery at a significantly higher rate than Treg cells from control mice. This is in line with previous studies in mice and humans, which have demonstrated that blocking of CTLA-4 results in increased Treg cell proliferation [35–37]. Our experiments using bone marrow chimeras clearly demonstrated that the increase of Treg cells in *Ctla-4*$^{ex2fl/fl}$*Foxp3-Cre* mice require cell-extrinsic factors. However, this does not exclude an additional role for cell-intrinsic restriction of the pool size of Treg cells through CTLA-4. If that would be the case it would imply that liCTLA-4 is unable to restrict Treg cell proliferation as efficient as full-length CTLA-4. We favor this view that CTLA-4 as the

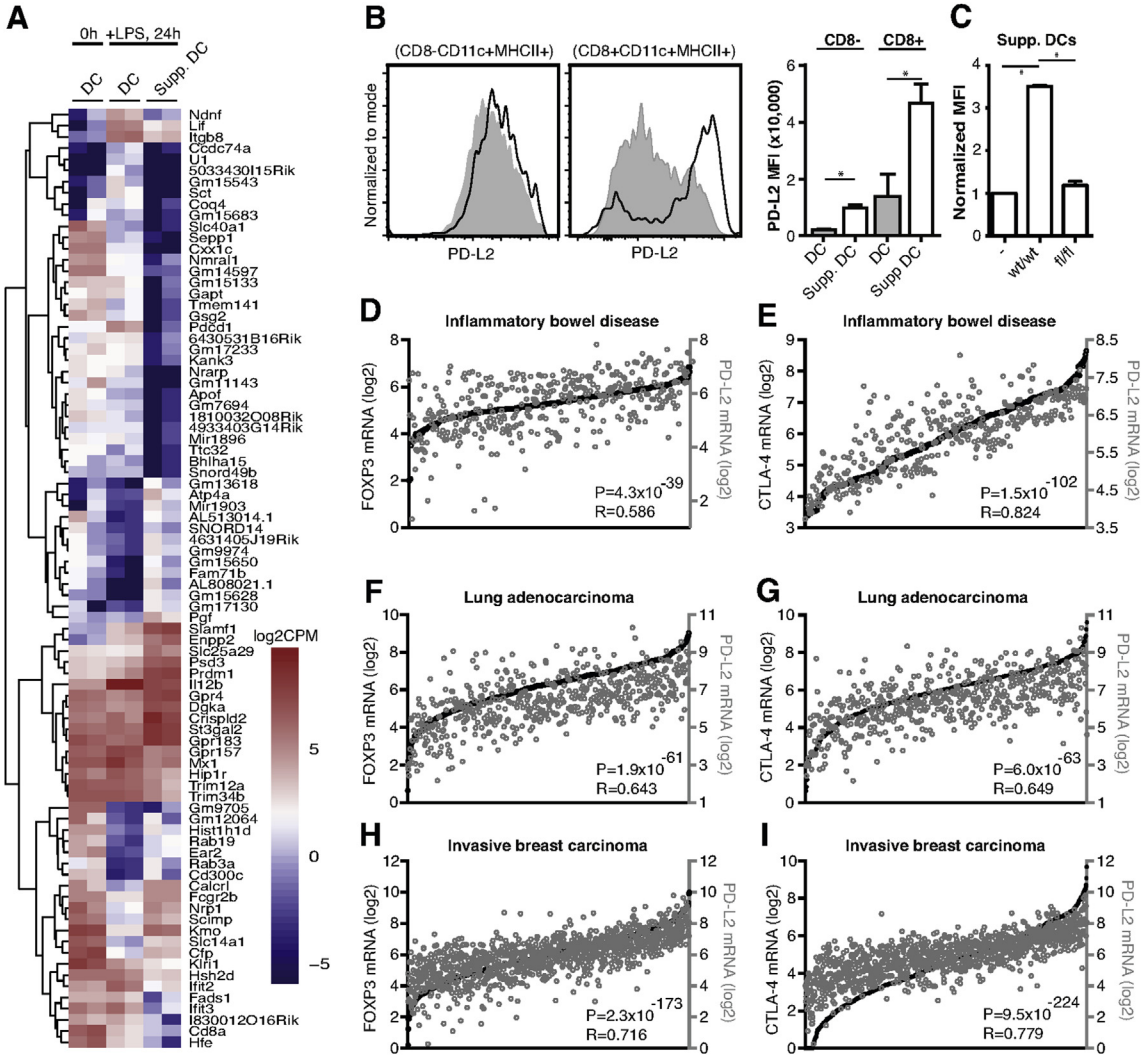

Fig. 5. Treg cells use cell extrinsic CTLA-4 signaling to induce PD-L2 expression on DCs. Heatmap for differentially expressed genes between sorted DCs cultured alone or together with Treg cells for 24 hours, both in the presence of 100 ng/ml LPS. Freshly isolated DCs are included for comparison. RNA-sequencing was performed in duplicates on cells from 2 independent experiments (**A**). Flow cytometric analysis of PD-L2 expression on C57BL/6 wild type splenic $CD8^-$ or $CD8^+$ dendritic cells co-cultured with (solid line) or without (gray) wild type Treg cells for 24 h in the presence of 100 ng/ml LPS (**B**). PD-L2 expression on wild type splenic dendritic cells cultured with 100 ng/ml LPS alone or together with Treg cells from either *Ctla-4$^{ex2wt/wt}$Foxp3-Cre* (wt/wt) or *Ctla-4$^{ex2fl/fl}$Foxp3-Cre* (fl/fl) mice. Data are normalized against the control group (DCs cultured with 100 ng/ml LPS alone) (**C**). Analysis of *FOXP3* (black circles constituting a "solid black line") *versus CTLA-4* (gray circles) mRNA expression (**D, F, H**) and *CTLA-4* (black circles constituting a "solid black line") and *PD-L2* (gray circles) mRNA expression (**E, G, I**) using the "R2: Genomics Analysis and Visualization Platform" in Ileal biopsies from treatment naïve patients with Crohn's disease or ulcerative colitis (n = 322) (D, E), biospices from lung adenocarcinoma (n = 515) (F, G) and tumor biopsies from invasive breast carcinoma (n = 1097) (H, I). (B–C) Data are presented as the mean ± SD of 3 independent experiments. *$P < 0.05$ was considered significant (B: two-tailed unpaired Student's *t*-test, C: ANOVA).

low grade inflammatory response that apparently characterizes *Ctla-4$^{ex2fl/fl}$Foxp3-Cre* mice only facilitates the expansion of T cells expressing liCTLA-4, i.e. Treg cells, and not CTLA-4 sufficient T cells, i.e. the effector T cells. Regardless, CTLA-4 plays an essential role in restraining Treg cell proliferation, and this should be taken into consideration in the context of anti-CTLA-4 blocking antibodies for cancer treatment. It may prove beneficial to combine anti-CTLA-4 antibodies with other treatment modalities that specifically impair or delete Treg cells.

One increasingly controversial aspect of CTLA-4 is how it confers its function. A large number of studies have addressed whether CTLA-4 can signal in cis and several signaling pathways have been implicated. However, to date these studies have yielded different and often conflicting results. More recently, several investigators have suggested that CTLA-4 mainly acts in a cell-extrinsic manner by signaling or transendocytosing CD80 and CD86. These studies demonstrate that transendocytosis of CD80 and CD86 occurs, however, the importance of this process remains unknown [23]. The main point of the current study was to determine where to focus future research efforts in understanding CTLA-4 action in Treg cells. The answer is, perhaps not too surprisingly, that it depends on what function of CTLA-4 we are studying. Our data demonstrate that Treg cells exclusively expressing liCTLA-4 are highly suppressive *in vivo* and that these Treg cells are fully capable of suppressing the expression of CD80 and CD86 on DCs while being unable to induce PD-L2 expression. These findings argue against CTLA-4-mediated transendocytosis of CD80 and CD86 as a major mechanism of Treg cell-mediated suppression. Instead it points towards a cell intrinsic role for CTLA-4 and we favor the view that liCTLA-4 retains some capacity to modulate the suppressive capacity by Treg cells. The presence specific CTLA-4 signaling in Treg cells was recently demonstrated by a study from Kong et al. showing that PKC-η associates with CTLA-4 in the immunological synapse of Treg cells [24,38]. CTLA-4-PKC-η signaling is indeed essential for Treg cell function, as PKC-η-deficient Treg cells displayed defective suppressive ability when co-transferred with effector T cells. PKC-η-deficient mice also developed signs of

lymphoproliferative disease with increased numbers of memory T cells and pro-inflammatory cytokines [39]. Consequently, our data shows that CTLA-4 is not a direct effector mechanism of Treg cells in the context of CD80 and CD86 expression but instead it appears that other, yet to be identified mechanisms, regulate CD80 and CD86 expression. A recent study by Laidlaw et al. demonstrated that the lack of Treg cell-derived IL-10 resulted in enhanced CD80 and CD86 expression in DCs during lymphocytic choriomeningitis virus (LCMV) infection [40]. However, CTLA-4-deficient Treg cells produce more IL-10 than wild type Treg cells and therefore IL-10 may not be the direct effector cytokine [20].

5. Conclusions

In summary, we propose a model whereby the extracellular function of CTLA-4 on Treg cells is not critical for the control of CD80 and CD86 expression of $CD11c^{+}$ DC, but is important for inducing an anti-inflammatory program in DC. This is highlighted by an increase in the co-inhibitory receptor PD-L2. This new mechanism for Treg cells mediated suppression of DCs can help in developing new strategies to modulate immune responses in both cancer and inflammatory diseases.

Funding

This work was supported by the Swedish Cancer Society, the Swedish Childhood Cancer Fund and the Swedish Heart-Lung Foundation.

Appendix A. Supplementary data

Supplementary data to this article can be found online at 5https://doi.org/10.1016/j.jaut.2018.12.002.

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