Efficient IL-2R signaling differentially affects the stability, function, and composition of the regulatory T-cell pool

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Signaling via interleukin-2 receptor (IL-2R) is a requisite for regulatory T (Treg) cell identity and function. However, it is not completely understood to what degree IL-2R signaling is required for Treg cell homeostasis, lineage stability and function in both resting and inflammatory conditions. Here, we characterized a spontaneous mutant mouse strain endowed with a hypomorphic Tyr129His variant of CD25, the α-chain of IL-2R, which resulted in diminished receptor expression and reduced IL-2R signaling. Under noninflammatory conditions, Cd25Y129H mice harbored substantially lower numbers of peripheral Treg cells with stable Foxp3 expression that prevented the development of spontaneous autoimmune disease. In contrast, Cd25Y129H Treg cells failed to efficiently induce immune suppression and lost lineage commitment in a T-cell transfer colitis model, indicating that unimpaired IL-2R signaling is critical for Treg cell function in inflammatory environments. Moreover, single-cell RNA sequencing of Treg cells revealed that impaired IL-2R signaling profoundly affected the balance of central and effector Treg cell subsets. Thus, partial loss of IL-2R signaling differentially interferes with the maintenance, heterogeneity, and suppressive function of the Treg cell pool.

Keywords: Regulatory T cells; IL-2R signaling; scRNA sequencing; Treg heterogeneity

Cellular & Molecular Immunology (2021) 18:398–414; https://doi.org/10.1038/s41423-020-00599-z

INTRODUCTION
Regulatory T cells (Treg cells), which are potent immunosuppressors, are characterized by the expression of the lineage-specification factor Foxp3.⁴⁵ Efficient interleukin-2 receptor (IL-2R) signaling is essential for the suppressive activity of Treg cells and their homeostasis. These findings have been inferred from germline knockout animal models,⁶,⁷ neutralizing antibody,⁵,⁶ adoptive cell transfer,⁵,⁷ and in vitro studies.⁹,¹⁰ However, efforts to evaluate the requirements for IL-2R signaling in Treg cell development, peripheral homeostasis, and suppressive function have been hampered by the emergence of massive lymphocyte proliferation and lethal autoimmunity in mice carrying germline or cell-type-specific mutations affecting IL-2R signaling components.⁴¹¹–¹³ Furthermore, some of the initial studies addressing Treg cell biology primarily relied on CD25 as a Treg cell marker, which made it difficult to isolate or detect these cells in IL-2R-deficient mice.

IL-2R consists of three components known as IL-2Rα (CD25), IL-2Rβ (CD122), and the common γc chain (CD132). IL-2 binds weakly to either monomers of IL-2Rα or heterodimers of IL-2Rβ/γc, but its affinity increases ~1000-fold when all three subunits form a heterotrimer. Only the heterotrimeric high-affinity receptor seems to be physiologically relevant, since IL-2- and IL-2Rα-deficient mice show similar phenotypes.¹²,¹⁴ IL-2Rβ and γc also serve as subunits of IL-15R that contributes to Treg cell differentiation.¹⁵ Although it is well established that IL-2 maintains peripheral Treg cells, it is not fully understood at which stage and to what degree IL-2R signaling contributes to the induction of Foxp3 expression and Treg cell lineage stability or whether this requirement differs in resting versus inflammatory conditions. For example, antibody-mediated IL-2 neutralization was found to reduce Foxp3 expression in Treg cells.⁶,¹⁶ However, IL-2-specific neutralizing antibodies act only for a short period of time, cause an immediate reduction in Treg cell numbers⁶,¹⁷ and have opposite effects on other T-cell populations,¹⁸ thus preventing the assessment of Treg cell stability in the context of defective IL-2R signaling. Alternatively, several reports have also shown diminished Foxp3 expression in IL-2−/−, IL-2Rα−/−, and IL-2Rβ-deficient mice.³,⁴ Since IL-2R signaling is also essential during thymic Treg cell development,⁶,¹⁹ it is difficult to ascribe the early disease symptoms found in germline knockout mice to either defective thymic Treg cell development or to the malfunctioning of extrathymic Treg cells.

In the present study, we identified mice harboring a novel spontaneous mutation within the CD25 locus (Cd25Y129H) that markedly abrogated the surface and intracellular expression of CD25 in T cells. The reduced levels of CD25 significantly affected responsiveness to IL-2, the frequency of Treg cells and their in vitro suppressive capacity.Surprisingly, despite the defects in Treg cell maintenance, low IL-2R signaling was sufficient to preserve the lineage stability and suppressor function of Treg cells under homeostatic conditions. The unexpected lack of spontaneous autoimmune disease in Cd25Y129H mice offered the possibility to further address the role of CD25 for stability and function of the Treg cell pool in the context of lifelong decreased IL-2R signaling.

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Received: 21 April 2020 Accepted: 14 November 2020
Published online: 6 January 2021
We provide evidence that—in contrast to the findings in steady-state situations—efficient IL-2R signaling is indispensable for Treg cell lineage stability and suppressor function under inflammatory conditions. Moreover, single-cell (sc) RNA sequencing revealed that continuously suboptimal IL-2R signaling profoundly affected the balance of Treg cell subsets, which were strongly shifted toward an effector phenotype in Cd25 Y129H mice.

**MATERIALS AND METHODS**

**Animals**

The following mouse strains were used: C57BL/6 (B6), B6(Cg)-Rag2tm1.1Cgn/J (Rag2−/−), B6.129S4-Ccr2tm1Ifc/J,20 and B6.129P2-Ccr2tm1Mae,21 which were kindly provided by Natalio Garbi (Bonn, Germany), and B6.Cg-Foxp3tm1McJ (Foxp3GF.P),22 which was kindly provided by Bernard Malissen (Marseille, France). Ccr2tm1Mae mice were obtained from The Jackson Laboratory in 2008 and bred under specific pathogen-free conditions at the animal facilities of the University of Bonn, Germany.

**Cd25 Y129H mice** were derived from Ccr2tm1Mae mice and were purchased from Taconic in 2008 and bred under specific-pathogen-free conditions at the animal facilities of the University of Bonn, Germany. Cd25 Y129H mice were derived from Ccr2tm1Mae mice for at least three further generations with C57BL/6 mice and carried the WT alleles of Ccr2 and the Cd25 Y129H point mutation. Cd25 Y129H mice were intercrossed with Foxp3GF.P mice to generate Cd25 Y129H/Foxp3GF.P mice. The homozygous Cd25 Y129H mice and cohoused littermate wt/Cd25 Y129H mice were obtained from The Jackson Laboratory in 2008 and bred under specific-pathogen-free conditions at the animal facilities of the University of Bonn, Germany. Cd25 Y129H mice were intercrossed with Foxp3GF.P mice to generate Cd25 Y129H/Foxp3GF.P mice. The homozygous Cd25 Y129H mice and cohoused littermate WT controls (Cd25 WT) used in all experiments were obtained by intercrossing heterozygous Cd25 Y129H/Cd25 Y129H mice. For mouse genotyping, genomic DNA was extracted from tail biopsies by lysis with 200 mM NaCl, 100 mM Tris-HCl pH 8.5, 5 mM EDTA, 0.2% SDS, and 100 μg/ml Proteinase K followed by isopropanol precipitation. PCR was performed using DreamTag Green PCR Master Mix (Thermo Scientific), and PCR products were purified using a QiAquick PCR Purification Kit (Qiagen) following the manufacturer’s recommendations. Sanger DNA sequencing was performed by GATC (Konstanz, Germany). Mice were bred under specific pathogen-free conditions at the Central Animal Facility at Hannover Medical School. Both males and females were used equally throughout the experiments. All experiments were conducted in accordance with the local animal welfare regulations reviewed by the institutional review board and the Niedersächsisches Landesamt für Verbraucherschutz und Lebensmittelsicherheit (LAVES).

**Whole-genome sequencing**

Genomic DNA was prepared using a DNeasy Kit (Qiagen) according to the manufacturer’s instructions and subjected to whole-genome sequencing by Macrogen (Macrogen Korea, Seoul, South Korea). Briefly, a 100 bp paired-end read library was prepared and sequenced on an Illumina HiSeq4000 (Illumina, San Diego, USA), which generated raw images utilizing HCS (HiSeq Control Software v3.3) for system control and base calling through the integrated primary analysis software RTA (Real Time Analysis v2.5.2). The BCL binary files were converted into FASTQ using the illumina package bcl2fastq2 (v2.16.0.10). Reads were aligned to a mouse reference genome (GRCm38.p4) with Burrows-Wheeler Aligner (http://bio-bwa.sourceforge.net/), duplicates were identified and removed using Picard software (http://broadinstitute.github.io/picard/), and variant information was found using SAMTools (http://samtools.sourceforge.net/). Deep sequencing yielded 514.93 million mapped reads corresponding to 27.1-fold coverage (see Supplementary Table 1 for a complete list of variants).

**Preparation of lymphocytes from tissues**

Secondary lymphoid organs (spleen, lymph nodes, or thymus) were homogenized through a 70 μm filter and washed with FACS buffer (PBS containing 3% fetal bovine serum [FBS]). Erythrocytes were removed by resuspension in ACK lysis buffer (0.15 M NH4Cl, 1 mM KHCO3, and 0.1 mM EDTA) for 5 min, followed by washing in FACS buffer. Euthanized mice underwent cardiac perfusion with 20 ml ice-cold PBS with heparin (100 U/ml). Lung tissue was cut into <1 mm fragments and then digested with 1 mg/ml collagenase D (Roche) at 37 °C for 60 min. The cells were then filtered through a 70 μm filter. The liver was minced and homogenized through a 70 μm filter. Lymphocytes were obtained from homogenates by histopaque (Sigma) density separation.

**Antibodies, cell staining, and flow cytometry**

Staining for cell-surface markers was performed in FACS buffer for 20 min at 4 °C using the following antibodies: APC-Cy7-conjugated anti-CD8 (53-6.7), BV785-conjugated anti-CD4 (GK1.5), PerCP-Cy5.5-conjugated anti-CD4 (RM4-5), Pacific Blue-conjugated anti-CD3 (17A2), APC-Cy7-conjugated anti-CD3 (145-2C11), APC-conjugated anti-CD103 (2E7), BV421-conjugated anti-CD127 (IL-7Rα) (A7R34), APC-conjugated anti-CTLA-4 (UC10-489), APC-Cy7- or BV605-conjugated anti-CD62L (Mel-14), PE-conjugated anti-IFN-γ (XM1G1.2), AF647-conjugated anti-IL-4 (11B11), and streptavidin-FITC (all from BioLegend); PE-conjugated anti-CD25 (PC61.5), Pacific Blue- or FITC-conjugated anti-CD25 (PC61.5), APC-conjugated anti-GITR (DTA-1), PE-conjugated anti-ICOS (7E.17G9), PE-Cy7-conjugated anti-PD-1 (543), FITC-, PE- or APC-conjugated anti-Foxp3 (FJK-16S), APC- or eFluor450-conjugated anti-CD44 (IM7), APC-conjugated anti-CCR7 (4B12), APC-conjugated anti-ICXCR3 (CXCR3-173), biotinylated anti-CD29 (eBioHMb1-1), anti-CD18 (M18/2), anti-β7 (FIB504), anti-αv (RM7-1), and PE-Cy5-conjugated anti-IL-17A (eBio17B17) (all from Thermo Fisher Scientific); PE-conjugated anti-Thy1.1 (CD90.1) (OX-7) and AF647-conjugated anti-pStat5 (pY694) (clone 47) (from BD Biosciences); and Cy5-conjugated anti-Thy1.2 (CD90.2) (M1T; made in-house). For intracellular staining, fixation and permeabilization were performed according to manufacturer recommendations. Cytoselects were stained with PE-conjugated anti-CD45.2 (104), Cy5-conjugated anti-CD3 (17A2, made in-house), Cy5-conjugated anti-CD220 (TB146, made in-house), APC-conjugated anti-TCRβ (H57-597, BioLegend), GFP Booster (ATTO488, Chromotek) and DAPI. For intracellular cytokine staining, cells were treated with 50 ng/ml ionomycin, and 10 μg/ml brefeldin A for 3 h and then fixed and permeabilized with the Intracellular Fixation & Permeabilization Buffer Set (ebiScience). To assess STAT5 phosphorylation, sorted GFP+ Treg cells were cultured in complete RPMI medium for 2 h at 37 °C and then stimulated with different concentrations of recombinant hIL-2 (Sigma) for 20 min, followed by staining using Phosflow Lyse/Fix Buffer and Phosflow Perm Buffer III (BD Biosciences). Simultaneous staining of surface and intracellular Cd25 was performed using the same antibody labeled with different fluorochromes (clone PC61.5) together with an anti-Foxp3 antibody (FJK-16S) and the Foxp3/Transcription Factor Staining Buffer Set (ebiScience). Flow cytometry was performed on an LSR II, and data were analyzed using FlowJo software, while cell sorting was performed on a FACSAria Ii Ll or a FACSAria Fusion (all BD Biosciences).

**T-cell expansion and an in vitro suppression assay**

GFP− Treg cells were sorted from pooled LNs and spleens isolated from Foxp3GF.P mice and cultured in RPMI 1640 medium ( Gibco) supplemented with 10% heat-inactivated FBS (Gibco Healthcare Life Sciences), 2 mM l-glutamine, 1% penicillin-streptomycin (both from Gibco), and 50 μM β-mercaptoethanol (Sigma-Aldrich) at 37 °C with 5% CO2. For in vitro expansion, Treg cells were cultured in 96-well plates coated with 1 μg/ml anti-CD3 antibody (clone 17A2, prepared in-house) and 1 μg/ml anti-CD28 antibody (clone 37.51, ebioscience) in RPMI medium supplemented with 300 μU/ml hIL-2 (Sigma) for 14 days. The cells were reseed with fresh medium every second day. Total CD4+ T cells were isolated using the MACS
Conventional CD4+ T cells were stimulated with Dynabeads® Mouse T-Activator CD3/CD28 (Thermo Fisher) in the presence of WT or Cd25Y129H Treg cells for 72 h.

In vitro IL-2 capture assay
Sorted GFP+ Treg cells were cultured in 96-well U-bottom plates (5 x 10^4/well) in 50 μl complete RPMI medium supplemented with recombinant hIL-2 (1.6 U/ml) for 3 h at 37°C. The remaining IL-2 in the supernatant was detected using a cytometric bead array and cDNA from WT and CD25Y129H mice was amplified by using the following primers: forward 5′-CATCATGATCATGCTGCTTCT-3′ and reverse 5′-CATCATGATCATGCTGCTTCTCCT-3′, which inserted BamHl and EcoRI restriction sites into the 5′ and 3′ ends, respectively. The PCR product was digested with BamHl and EcoRI restriction enzymes and subcloned into pcdNA3-mRFP (Addgene plasmid #13032). A total of 5 x 10^6 293T cells per 10-cm dish were seeded and transfected the next day using a calcium phosphate transfection kit (Sigma) with 10 μg plasmid. Transfected cells were cultured for 24 h in complete DMEM supplemented with 20 mM HEPES (Sigma-Aldrich) at 37 °C with 5% CO₂ before FACS analysis.

Induction of oral tolerance and assessment of delayed-type hypersensitivity (DTH) responses
To induce oral tolerance, mice were fed 25 mg ovalbumin (OVA) (grade III; Sigma-Aldrich) dissolved in 200 μl PBS or only PBS as a control by oral gavage on days 0 and 2. On day 7, the mice were immunized by subcutaneous injection of 300 μg OVA (grade VI; Sigma-Aldrich) in 200 μl PBS/CFA emulsion (containing 100 μg Mycobacterium tuberculosis; Sigma-Aldrich). On day 21, the mice were challenged by intradermal injection of 50 μg OVA (grade VI; Sigma-Aldrich) in 10 μl PBS into the right ear pinna, while 10 μl PBS without OVA was injected into the left ear pinna as a control. Ear swelling was then measured in a blinded fashion before and 48 h after injection by using a micrometer. OVA-specific ear swelling was calculated as follows: (right ear thickness – left ear thickness)_inh – (right ear thickness – left ear thickness)_oh.

Adoptive transfer colitis model
Colitis was induced in Rag2-/- mice by intraperitoneal injection of 5 x 10^6 FACs-sorted naive CD4+ CD62L+CD44+ CD25- cells isolated from pooled spleens and lymph nodes from B6 (Thyl.2+) mice with or without 5 x 10^5 sorted CD4+ CD25+ T cells isolated from pooled spleens and lymph nodes from WT or Cd25Y129H Foxp3+GFP mice. Mice were monitored for weight loss and analyzed 8 weeks after cell transfer or immediately upon loss of more than or equal to 20% of their initial body weight.

Histopathological analysis of colon samples
Colon samples were removed, fixed in neutral buffered 4% PFA, dehydrated (Shandon Hypercenter, XPL), and subsequently embedded in paraffin (TES, Medite). Sections (2–3 μm thick; Reichert-Jung 2030 microtome) were deparaffinized in xylene and H&E stained according to standard protocols. Histological scoring was performed in a blinded manner as described previously.

RNA expression analysis
Total RNA was isolated from cells using a RNeasy kit (Qiagen) or from PFA-fixed, paraffin-embedded colon tissue sections (6 μm sections; four sections per sample) using an RNeasy FFP kit (Qiagen) according to the manufacturer’s instructions. Equivalent quantities of total RNA were reverse transcribed with SuperScript III reverse transcriptase (Invitrogen) and random hexamers according to the manufacturer’s protocol. Gene expression levels were determined by quantitative real-time PCR using SYBR Green PCR Master Mix (Takara) on an ABI 5700 sequence detector system (Applied Biosystems) using the following primers specifically designed to span intron/exon boundaries: CD25, 5′-GGGGGGACTC-3′ and 5′-CTGGTGGTGTATGAGGCAG-3′; IL-17, 5′-ACTACCTCAACGGTCCAGC-3′ and 5′-GAAGGTCCATCAACAGAGA-3′; IFN-γ, 5′-AGGCAAGGAAAGGAATGC-3′ and 5′-TTCTAGGACCTCCTCCCTGCG-3′; TF-β, 5′-CTGTCGACACCCCTGAGTCAG-3′ and 5′-AGCAGTTTGCCGTTGCTCTTT-3′; TNF-α, 5′-TTCATGACCCGACAGA-3′ and 5′-GGAACGGCCAGAACGAG-3′.

Gene expression levels were normalized to the housekeeping gene Hprt.

Immunohistology
Sacrificed mice were perfused with 20 ml ice-cold PBS with heparin (100 U/ml). The salivary gland, lacrimal gland, and pancreas were removed, embedded in optimum cutting temperature (OCT) compound, and frozen on dry ice. Eight-micrometer sections were prepared with a cryostat (CM3050 Leica) and fixed for 10 min in ice-cold acetone. Imaging of tissue sections was performed using an automated slide scanner (AxioScan Z1; plan-apochromat objective: 10x/0.45 M27) equipped with an AxioCam 506 mono camera and ZenBlue analysis software (all from Zeiss). Organs were collected and fixed in 2% PFA/30% sucrose overnight (thymus and spleen) or for 1.5 h (LN) before they were embedded in OCT compound and snap-frozen. Cryosections (7 μm in thickness) were blocked with 4% BSA, permeabilized with 0.5% Triton X-100 for 20 min, and stained with conjugated antibodies and DAPI (Sigma). Slides were mounted with MOWIOL (home-made), and images were acquired using a Zeiss Axiosvert 200M microscope with a 20x objective. Cryostats of stained cells were performed by centrifugation of 100 μl cell suspension (5 x 10^6 cells/ml PBS) at 450 rpm for 5 min in a Shandon Cytospin 4 (Thermo Scientific). Images were acquired with a Leica SP2 confocal microscope with a 63x Plan-Apochromat objective and analyzed using Imaris scientific software (Bitplane).

DNA methylation analysis
Genomic DNA was isolated from sorted GFP+ Treg cells and GFP- T cells derived from WT or Cd25Y129H Foxp3+GFP mice using a DNaseasy kit (Qiagen) and concentrated using the DNA Clean & Concentrator Kit (Zymo Research), both following the manufacturer’s instructions. Methylation analysis of Treg cell-specific demethylated regions (TSDRs) was performed using bisulfite sequencing as described previously. We used the primers mTSR-for (5′-bio-TAAGGGGGTGTATGAGGCAG-3′), mTSR-rev (5′-TCTAATACGACTCACTATAGGG-3′), mTSR-seq1 (5′-ACCAAAATATAAATATAAAT-3′), mTSR-seq2 (5′-ACCAAAATATAAATATAAAT-3′), and mTSR-seq3 (5′-GAAGGTCCATCAACAGAGA-3′) for standard amplification and pyrosequencing, whereas...
we used the primers mTSDR-sen-for (5′-AGGTGTGTTTTTGGGATAT AGAATATG-3′), mTSDR-sen-rev (5′-ACCTAAAAAAATATCTACCC CTTTC-3′), and mTSDR-sen-seq1 (5′-GTTGTTAATTGGAATTTGT TAG-3′) for low-input samples. Exclusively, cells from male mice were used for the methylation analysis. For all samples analyzed, cells from two mice per condition were pooled to collect a sufficient amount of DNA for methylation analysis.

Single-cell sequencing of cell-hashed Treg cells and data preprocessing

CD4+ GFP+ Treg cells were FACS sorted from the spleen of WT or CD25Y129H Foxp3GFP mice and labeled with TotalSeq cell hashing antibodies (BioLegend) according to the cell hashing protocol (https://cite-seq.com/protocol/; PMID: 30567574). After labeling, the cells from three mice per genotype were pooled and used to generate single-cell gel beads in emulsions using the Chromium Single Cell 3′ Library & Gel Bead Kit v3 (10x Genomics) according to the manufacturer’s protocol (https://support.10xgenomics.com/single-cell-gene-expression/library-prep/doc/user-guide-chromium-single-cell-3-reagent-kits-user-guide-v3-chemistry). During reverse transcription, 1 μl HTO PCR additive primer (0.2 μM) was added to the cDNA amplification mixture to increase the yield of HTO products. After cDNA amplification, HTO-derived cDNAs were separated from mRNA-derived cDNAs using SPRI selection and further purified, amplified, and fragmented according to the cell hashing protocol or Chromium Single Cell 3′ Reagent Kit v3 user guide (10x Genomics), respectively. For sequencing, sample indexes were added during index PCR (12 cycles), and purified libraries were sequenced on a NextSeq 550 (Illumina) with 26 cycles of read 1, 8 cycles of 17 index, and 56 cycles of read 2. For cDNA libraries, Cell Ranger v3.0.2 was used for alignment to a mouse genome (mm10), filtering, barcode counting, and unique molecular identifier counting. This setup allowed us to confidently map 82% of 270,993,014 or 311,497,445 reads from WT or CD25Y129H CD4+ GFP+ Treg cells, respectively, to the genome. Downstream analysis was performed using the Seurat R package (version 3.0, Satija Lab; PMID: 31178118), which enables the integrated processing of multimodal (RNA and HTO) single-cell datasets (PMID: 29608179). Initially, raw cDNA library datasets were loaded, and genes detected in less than three HTO single-cell datasets (PMID: 29608179). Initially, raw cDNA library datasets were loaded, and genes detected in less than three HTO-derived cDNAs were further filtered using Seurat’s standard preprocessing workflow. Cells with low-quality transcriptomes (<900 counts per cell) were removed independently by Sanger sequencing of the Ccr2 gene (Supplementary Fig. 2a). The presence of the Y129H mutation was confirmed using RT-qPCR and Sanger sequencing of the Ccr2 gene from WT and CD25Y129H Foxp3GFP mice.

RESULTS

Characterization of Treg cells with a hypomorphic mutation in the Ccr2 gene

During routine flow cytometric analysis, we observed reduced CD25 expression in Treg cells and defined thymocyte subpopulations, as well as in in vitro activated CD4+ T cells from one Ccr2-deficient (Ccr2tm1Jfi) strain but not from another Ccr2-deficient (Ccr2tm1Mar) strain (Fig. 1a and Supplementary Fig. 1). We performed whole-genome DNA sequencing to determine whether a spontaneous mutation in Ccr2tm1Mar mice could be responsible for the low CD25 expression. From the sequence variants found, we identified one affecting the CD25 gene (Supplementary Table 1). The identified variant carried a point mutation (T→C) at position Chr2:11680290 in exon 4 that resulted in the replacement of tyrosine (Y) with histidine (H) at codon 129 (Y129H) (Fig. 1b).

Based on the known three-dimensional structure of human CD25,28 Y129 is located in the extracellular part of the protein and probably forms a hydrogen bond with P8 that may help to stabilize a region of the CD25 protein that is in direct contact with IL-2 (Supplementary Fig. 2A). The presence of the Y129H mutation was confirmed independently by Sanger sequencing of the genomic DNA fragment in Ccr2tm1Mar mice (data not shown). Moreover, to avoid confounding effects, the mutated Ccr2 alleles were replaced by backcrossing to B6 mice for at least three generations, leading to the generation of CD25wt/Y129H mice.

Subpopulation detection in single-cell sequencing data and differential expression analysis

Unsupervised clustering and uniform manifold approximation and projection (UMAP) plots were generated using a resolution of 0.5 and the first 30 principal component analysis (PCA) dimensions by applying the SCTransform function of the Seurat package. After inspection, we detected the presence of a few contaminating B cells (Ms4a1+Cd3ε+) that were excluded from further analyses. The remaining events were reclustered with a resolution of 0.5 and the first 30 PCA dimensions using the SCTransform function of the Seurat package, resulting in six clusters. Data were log normalized and scaled using the NormalizeData and ScaleData functions of Seurat, and differential expression testing was performed using the FindAllMarkers or FindMarkers function of Seurat to generate a list of DEGs. All neighboring clusters with fewer than 20 DEGs (adjusted p value <0.05) were merged, resulting in a final UMAP plot with four clusters. Heatmaps and Venn diagrams for the DEGs were generated in R using the ggplot2 package. To identify enriched Gene Ontology (GO) biological processes, data were exported from R and analyzed with PANTHER classification system software (v.14.1) using Fisher’s exact test with a false discovery rate (FDR) calculated to be <0.05 and a significance level adjusted to 0.05 (http://pantherdb.org; PMID: 23193289). To identify subset-specific markers, we manually compared the DEGs from each cluster, determined by a threshold for the FDR <0.001, to markers previously reported in canonical pathways and Treg cell subsets using predefined25 and hallmark gene sets (https://www.gsea-msigdb.org/gsea/msigdb). The full set of signature genes is provided in Supplementary Table 2. The log2 fold change was calculated as log2(Δ) – log2(Δ), where B and A are the proportions of Treg cells in each cluster in Cd25Y129H and WT mice, respectively.

Pseudotime analysis of Treg cell differentiation

For trajectory analysis, we used the open-source R package Slingshot (available from the GitHub repository https://github.com/kstreel13/slingshot).26 Cells were ordered in pseudotime using PCA and Seurat cluster assignments. With cluster 2 (central Treg (cTreg) cells) chosen as the starting point, two main trajectories were found: one including cells from clusters 2, 3, and 4 and the other including cells from clusters 2, 3, and 1.

Statistics

Statistical analysis was performed with Prism 8 (GraphPad Software). When comparing two groups, statistical significance was determined using a two-tailed unpaired t test. To compare multiple groups, one-way or two-way ANOVA was used. Statistical details, including the statistical test used and the number of mice analyzed (n), can be found in the legend of each figure. p values <0.05 were considered statistically significant; data with p >0.05 were considered to be not significant (ns). The following annotations were used: *p < 0.05, **p < 0.01, ***p < 0.001, and ****p < 0.0001. All data are presented as the mean ± SD.
together with their WT littermates as controls. Homozygous Cd25<sup>Y129H</sup> mice were fertile and survived until adulthood with no obvious developmental defects (data not shown). To better understand the molecular mechanism by which the Cd25<sup>-Y129H</sup> mutation results in decreased CD25 expression, we analyzed the protein and mRNA levels of CD25 by Foxp3<sup>+</sup> Treg cells from WT and Cd25<sup>Y129H</sup> mice. The mean fluorescence intensity (MFI) of both cell surface and intracellular CD25 was strongly diminished in Cd25<sup>Y129H</sup> mice (Fig. 1c). In addition, we found similar results in in vitro activated CD4<sup>+</sup> T cells from WT and Cd25<sup>Y129H</sup> mice and in 293T cells transiently transfected with plasmids encoding the CD25-WT or CD25-Y129H gene (Supplementary Fig. 3a, b). This
result was confirmed by confocal immunofluorescence microscopy, which showed that CD25 downregulation was especially prominent at the cell-surface level, whereas the intracellular CD25 expression levels were better sustained (Fig. 1d). Moreover, quantification of CD25 transcript levels by real-time PCR revealed that WT and Cd25<sup>Y129H</sup> Treg cells expressed comparable amounts of Cd25 mRNA, rendering the possibility that the Cd25<sup>Y129H</sup> mutation affects miRNA transcription or stability unlikely (Fig. 1c). However, following in vitro stimulation of CD4<sup>+</sup> T cells with anti-CD3 and anti-CD28 mAbs, we found reduced levels of the Cd25 transcript in Cd25<sup>Y129H</sup> cells compared to WT cells, suggesting that transcriptional activity might be affected in mutant Cd25<sup>Y129H</sup> T cells subsequent to activation via the TCR (Supplementary Fig. 3c). Alternatively, reduced CD25 expression could restrict the IL-2/CD25 positive feedback loop, resulting in decreased mRNA synthesis. Moreover, we also showed that the CD25 protein does not accumulate intracellularly, excluding a hypothetical defect in protein transport to the cell membrane in Cd25<sup>Y129H</sup> cells. Irrespective of the underlying cause, our data indicated that the Cd25<sup>Y129H</sup> mutation led to reduced levels of CD25.

To demonstrate a functional consequence of the diminished expression of CD25 in Cd25<sup>Y129H</sup> Treg cells, we assessed IL-2-induced phosphorylation of signal transducer and activator of transcription-5 (pSTAT5), which reflects a main outcome of IL-2R signaling. WT and Cd25<sup>Y129H</sup> Treg cells displayed different dose responses to recombinant hIL-2 (Fig. 1f). At a low IL-2 concentration (0.32 U/ml), the magnitude of the response (as measured as % of pSTAT5<sup>+</sup> cells) was significantly higher (fourfold) in WT Treg cells than in Cd25<sup>Y129H</sup> Treg cells, whereas at a high IL-2 concentration (8 U/ml), the fraction of pSTAT5<sup>+</sup> cells was comparable between WT and Cd25<sup>Y129H</sup> Treg cells. These results show that the reactivity of Cd25<sup>Y129H</sup> Treg cells to physiological amounts of IL-2, which usually do not exceed 0.05 U/ml, is diminished. We next asked whether Cd25<sup>Y129H</sup> Treg cells can take up and deplete exogenous IL-2, which has been suggested to be an essential mechanism of Treg cell-mediated suppression. Using an in vitro assay, we found that IL-2 consumption by Cd25<sup>Y129H</sup> Treg cells was significantly reduced compared to that by WT Treg cells (Fig. 1g). This finding suggests that in vivo, low CD25 expression by Cd25<sup>Y129H</sup> Treg cells may decrease their capacity to compete for IL-2 produced by activated T cells, which in turn could potentially lead to a diminished capacity to suppress the proliferation of conventional T cells. Moreover, by comparing the in vitro suppressive activity of WT and Cd25<sup>Y129H</sup> Treg cells by evaluating their ability to inhibit CD4<sup>+</sup> T-cell proliferation at different cell ratios, we found that the suppressive capacity of Cd25<sup>Y129H</sup> Treg cells was strongly reduced (Fig. 1h). Altogether, these results demonstrate that reduced expression of CD25 in Cd25<sup>Y129H</sup> Treg cells significantly impairs their responsiveness to IL-2 and their immunosuppressive capacity.

Y129H Treg cells may decrease their capacity to compete for IL-2. Treg cell homeostasis and phenotypic diversity maintenance. To demonstrate a functional consequence of the diminished expression of CD25 in Cd25<sup>Y129H</sup> Treg cells, we assessed IL-2-induced phosphorylation of signal transducer and activator of transcription-5 (pSTAT5), which reflects a main outcome of IL-2R signaling. WT and Cd25<sup>Y129H</sup> Treg cells displayed different dose responses to recombinant hIL-2 (Fig. 1f). At a low IL-2 concentration (0.32 U/ml), the magnitude of the response (as measured as % of pSTAT5<sup>+</sup> cells) was significantly higher (fourfold) in WT Treg cells than in Cd25<sup>Y129H</sup> Treg cells, whereas at a high IL-2 concentration (8 U/ml), the fraction of pSTAT5<sup>+</sup> cells was comparable between WT and Cd25<sup>Y129H</sup> Treg cells. These results show that the reactivity of Cd25<sup>Y129H</sup> Treg cells to physiological amounts of IL-2, which usually do not exceed 0.05 U/ml, is diminished. We next asked whether Cd25<sup>Y129H</sup> Treg cells can take up and deplete exogenous IL-2, which has been suggested to be an essential mechanism of Treg cell-mediated suppression. Using an in vitro assay, we found that IL-2 consumption by Cd25<sup>Y129H</sup> Treg cells was significantly reduced compared to that by WT Treg cells (Fig. 1g). This finding suggests that in vivo, low CD25 expression by Cd25<sup>Y129H</sup> Treg cells may decrease their capacity to compete for IL-2 produced by activated T cells, which in turn could potentially lead to a diminished capacity to suppress the proliferation of conventional T cells. Moreover, by comparing the in vitro suppressive activity of WT and Cd25<sup>Y129H</sup> Treg cells by evaluating their ability to inhibit CD4<sup>+</sup> T-cell proliferation at different cell ratios, we found that the suppressive capacity of Cd25<sup>Y129H</sup> Treg cells was strongly reduced (Fig. 1h). Altogether, these results demonstrate that reduced expression of CD25 in Cd25<sup>Y129H</sup> Treg cells significantly impairs their responsiveness to IL-2 and their immunosuppressive capacity.
The hypomorphic Cd25<sup>Y129H</sup> mutation leads to reduced CD25 expression and loss of Foxp3<sup>+</sup> Treg cells. Representative FACS plots showing the expression of Foxp3 and CD25 by CD4<sup>+</sup> T cells in the peripheral lymph nodes (pLNs), spleen, thymus, mesenteric lymph nodes (mLN), liver, and lungs of 8–12-week-old WT and Cd25<sup>Y129H</sup> mice. Percentages and absolute cell numbers of Foxp3<sup>+</sup> Treg cells in different organs of WT (filled circles) and Cd25<sup>Y129H</sup> (open circles) mice. Mean fluorescence intensities (MFIs) of CD25 (d) and Foxp3 (e) on Treg cells from WT and Cd25<sup>Y129H</sup> mice. Data were derived from at least three experiments with 5–8 mice per genotype and are represented as the mean ± SD. ns not significant; *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001; two-tailed unpaired Student’s t test.
levels of key Treg cell signature molecules in WT and Cd25Y129H mice using flow cytometry. Cd25Y129H Treg cells expressed higher levels of markers associated with cell activation, including CD103 (Fig. 3a), GITR (Fig. 3c), and ICOS (Fig. 3d), while the expression of markers associated with Treg cell suppressive function, including CTLA-4 (Fig. 3b) and PD-1 (Fig. 3e), was not significantly different from that of WT Treg cells. Furthermore, reduced expression of CD25 in Cd25Y129H Treg cells was accompanied by increased expression of CD127 (IL-7Ra) (Fig. 3f). Similar results were found in both CD62L+CD44− Treg cells and CD62L−CD44+ Treg cells (data not shown). However, Cd25Y129H mice had reduced percentages and absolute cell numbers of CD62L+CD44− Treg cells in lymphoid tissues and NLTs (Fig. 3g). Conversely, the absolute cell numbers of CD62L−CD44+ Treg cells were comparable between WT and Cd25Y129H mice, although increased percentages of CD62L−CD44+ Treg cells were observed in Cd25Y129H mice (Fig. 3h). Altogether, these results indicated that reduced expression of CD25 specifically impaired the maintenance of CD62L+CD44− Treg cells, regardless of whether they resided in lymphoid tissues or NLTs, a finding that is consistent with previous reports showing that IL-2R signaling is specifically required for cTreg cell homeostasis.31,32

Fig. 3 Activated/effector-like phenotype of Foxp3+ Treg cells in Cd25Y129H mice. a-f Representative histograms (upper panels) and quantitative analysis (lower panels) of the MFIs of the indicated Treg surface markers on spleen CD4+Foxp3+ Treg cells from WT (filled circles) and Cd25Y129H (open circles) mice. Data were derived from at least three independent experiments with 5 mice per genotype. Percentages (left) and absolute cell numbers (right) of CD62L+ (g) and CD44+ (h) Treg cell populations in different organs of WT and Cd25Y129H mice. Data were derived from at least three independent experiments with 6–8 mice per genotype and are represented as the mean ± SD; ns not significant; *p < 0.05, **p < 0.01, ***p < 0.001; two-tailed unpaired Student’s t test

Cellular & Molecular Immunology (2021) 18:398–414
Lack of spontaneous autoimmunity in $\text{Cd}25^{\text{Y129H}}$ mice

We next investigated whether $\text{Cd}25^{\text{Y129H}}$ mice exhibited signs of autoimmunity, as previously reported for mice lacking expression of various IL-2R signaling components.\textsuperscript{4,11–14} Compared with WT mice, $\text{Cd}25^{\text{Y129H}}$ mice showed a similar gain in body weight with age (Fig. 4a), had no substantial expansion of CD4$^+$ or CD8$^+$ T cells, and showed comparable percentages and absolute numbers of cells displaying a CD44$^+$ memory phenotype (Fig. 4b).

In addition, histopathological analysis of aged mice (35–40 weeks old) of both strains revealed comparable lymphocyte infiltration in multiple organs, such as the salivary gland, lacrimal glands, and pancreas (Fig. 4c, d). Thus, the absence of autoimmunity in $\text{Cd}25^{\text{Y129H}}$ mice likely indicates that Treg cells developing under this condition of impaired IL-2R signaling are still functional and maintain their suppressive capacity during aging. Alternatively, the lack of autoimmunity in $\text{Cd}25^{\text{Y129H}}$ mice could also reflect a loss of IL-2R function in autoreactive CD4$^+$ and CD8$^+$ T cells. To explore this possibility, conventional CD4$^+$ T cells from these mice were stimulated with anti-CD3/CD28 antibodies and subjected to T helper cell differentiation in vitro. Compared to WT cells, T cells from $\text{Cd}25^{\text{Y129H}}$ mice exhibited significantly lower intracellular Th1 (IFN-$\gamma$) cytokine expression, similar Th2 (IL-4) cytokine expression, and modestly elevated Th17 (IL-17) cytokine expression (Supplementary Fig. 6). These data suggest that IL-2R signaling is required for the maximal generation of IFN-$\gamma$-producing cells during a type 1 immune response. Moreover, the abnormal responsiveness of potentially autoreactive T cells may partially contribute to the lack of spontaneous autoimmunity in $\text{Cd}25^{\text{Y129H}}$ mice.

IL-2R signaling balances the landscape of Treg cell subsets

To understand how IL-2R signaling shapes the heterogeneity of different Treg cell subsets at the molecular level, we performed scRNA sequencing of splenic CD4$^+$Foxp$^3^+$ Treg cells from WT and $\text{Cd}25^{\text{Y129H}}$ mice (Fig. 5a). Unsupervised graphical clustering organized the Treg cells into four clusters and identified transcripts that best characterized the different groups (Fig. 5b; Supplementary Table 3). To associate each cluster with known Treg cell subsets, we screened the 60 most significantly upregulated genes of each cluster and overlapped them with previously reported Treg cell markers and canonical signaling pathways (Fig. 5c; Supplementary Table 2). Cluster 1 highly expressed genes, such as Il1f1, Il1f3, Isg15, and Stat1, associated with type I interferon (IFN) responses but also genes that are primarily expressed by naive cells (e.g., Sell and Ly6c1) that most likely reflect an intermediate state of cell activation and/or response to IFN. We named this population Stat-1$^+$ Treg cells, as suggested previously for Treg cells isolated from the brachial LNs.\textsuperscript{13} Cluster 2 included genes expressed in cTreg cells,\textsuperscript{31,32} such as Sell, Ccr7, Bcl2, Ly6c1, and Stat1p. Cluster 1 and cluster 2 Treg cell subsets also showed increased expression of Msi4Aa4b and Msi4Aa6b, which may facilitate the resting state of these Treg cell subpopulations by inhibiting cell cycle progression.\textsuperscript{34} Cluster 3 was enriched for genes that were highly expressed in effector Treg (eTreg) cells.\textsuperscript{35,36} This cluster included key Treg cell signature genes, such as Cita4, Tnfsf9 (4-1BB), and Izumo1r, as well as cell-surface receptors, such as Tnfsf4 (Ox40), Tnfsf18 (GITR), Pdcd1 (PD-1), Icos, and Npr1. Furthermore, the expression of many transcription factors that control the differentiation and function of eTreg cells,\textsuperscript{37} such as Batf, Ikar2 (Helios), Maf, and Gata3, was upregulated in cluster 3, whereas the expression of Klf2, Satb1, Bach2, and Lef1, which antagonize eTreg cell differentiation,\textsuperscript{38,39} was increased in cluster 2 (Fig. 5d). In addition, cluster 3 also overexpressed genes associated with the response to TCR signals, such as Rel, Orai1, and Zap70, which reflects recent engagement of the TCR. Some of the cell-surface markers that are characteristic of activated Treg cells, such as Maf, Npr1, and Icos, were also highly expressed in cluster 4, consistent with the idea that these clusters identify Treg cell subpopulations that represent a continuum of discrete states rather than separate entities.\textsuperscript{33} This cluster also included transcription factors that promote the differentiation or function of tissue-resident Treg cells,\textsuperscript{35} such as Rora, Runx3, Flt1, and Id2, as well as genes that are functionally related to tissue homing and trafficking, including Itgb1, Itgb7, Cxcr3, and Ccr2, indicating that cluster 4 probably corresponds to cells undergoing a later stage of maturation and preadjuvant to their trafficking to NLTs. We named this population NLT-like Treg cells, as previously described.\textsuperscript{53}

Next, we compared the proportion of each Treg cell subset between WT and $\text{Cd}25^{\text{Y129H}}$ mice. Whereas the cTreg cell subset was significantly reduced, the eTreg cell subset was highly enriched in $\text{Cd}25^{\text{Y129H}}$ mice (Fig. 6a, b). The Stat-1$^+$ and NLT-like Treg cell subsets had similar abundances in both WT and $\text{Cd}25^{\text{Y129H}}$ mice. These data were consistent with flow cytometry data showing that $\text{Cd}25^{\text{Y129H}}$ Treg cells contained a higher fraction of CD44$^+$CD62L$^-$ Tregs (Fig. 3h). In addition, differential expression analysis revealed that over 40% of the DEGs in WT and $\text{Cd}25^{\text{Y129H}}$ Treg cells largely overlapped among clusters 2, 3, and 4, indicating that IL-2R signaling is essential in maintaining the transcriptional programs of these different Treg cell subsets (Fig. 6c; Supplementary Table 4). This was in contrast with cluster 1 cells, which displayed very few DEGs between WT and $\text{Cd}25^{\text{Y129H}}$ Treg cells and had reduced overlap with the other clusters, suggesting that the homeostasis of this cluster is largely IL-2R independent (Supplementary Fig. 7a). However, better surface markers must be identified to clarify this point. An overall comparison of the gene expression profiles of splenic WT and $\text{Cd}25^{\text{Y129H}}$ Treg cells showed that 64 genes were upregulated and 29 genes were downregulated in WT Treg cells compared to $\text{Cd}25^{\text{Y129H}}$ Treg cells (Fig. 6d; Supplementary Table 5). WT Treg cells expressed higher levels of genes required for T-cell maintenance, such as the antiapoptotic molecules Mcl-1 and Bclb11 (Supplementary Fig. 7b), consistent with a role of IL-2R signaling in T-cell survival.\textsuperscript{42,43} In contrast, $\text{Cd}25^{\text{Y129H}}$ Treg cells showed higher expression of CD127, indicating that they may take advantage of IL-7 signaling for survival to compensate for reduced IL-2R signaling. In addition, the most significantly upregulated genes in WT Treg cells included many DNA-binding transcription regulators, such as Klf2, Cited2, Pura, Cebzb, JunB, and some trascriptional regulators, including Aes (aka Tie5), Ncor1, and Suds3, confirming that IL-2R signaling regulates the gene transcription of Treg cells.\textsuperscript{53} Next, GO-based enrichment analysis found that biological processes associated with metabolism, biosynthesis, and gene expression were significantly overrepresented in WT Treg cells (Supplementary Fig. 7c). In contrast, the genes upregulated in $\text{Cd}25^{\text{Y129H}}$ Treg cells were associated with eTreg cell development and function, including Ikar2, Maf, Itgb1, Gata3, S100a6, S100a10, and S100a11, as previously suggested (Fig. 6d). Interestingly, this list of genes strongly overlapped with the genes associated with cluster 4 (Supplementary Table 3), reinforcing the idea that IL-2R signaling balances Treg cell heterogeneity, and that decreased IL-2R signaling shifts the balance toward an effector phenotype. To further explore this hypothesis, we evaluated the Treg cell clusters with regard to their expression using the Slingshot software package.\textsuperscript{38} We found that the inferred spectrum of differentiation partially recapitulated cell clustering, ordering cTregs and eTregs followed by NLT-like Tregs on a linear developmental trajectory (Fig. 6e). Using the same approach, we also observed that $\text{Cd}25^{\text{Y129H}}$ cTreg and eTreg cells had a developmental pseudotime tilted toward a more effector phenotype, indicating faster acquisition of effector properties under impaired IL-2R signaling (Fig. 6f). In accordance with recent studies, our scRNA-seq analysis identified the presence of different subpopulations of Treg cells in steady-state lymphoid tissues. In addition, by comparing the single-cell transcriptomes of Treg cells receiving optimal or low IL-2R signaling, we identified gene programs associated with Treg cells that depend on full IL-2
Fig. 4  Lack of autoimmune symptoms in aged Cd25^{Y129H} mice.  

**a** Weekly body weight monitoring of female (left) and male (right) WT (black line) and Cd25^{Y129H} (red line) mice. Data were derived from 6 to 12 mice per genotype and sex. 

**b** Percentages and absolute cell numbers of CD44^{hi} cells among CD4^+ and CD8^+ T cells from the pLN (left) and spleen (right) of WT (filled circles) and Cd25^{Y129H} (open circles) mice. Data were derived from at least three independent experiments with 9–13 mice per genotype. 

**c** Representative immunofluorescence microscopy of the salivary glands, lacrimal glands, and pancreas of WT (top) and Cd25^{Y129H} mice (bottom) stained with the indicated antibodies. Scale bar, 500 μm. 

**d** Individual size (left) and number (right) of lymphoid infiltrates per section in the salivary glands, lacrimal glands, and pancreas of WT (filled circles) and Cd25^{Y129H} (open circles) mice. Data were derived from at least three independent experiments with 9–13 mice per genotype. 

Organs were isolated from 35- to 40-week-old mice. Data are shown as the mean ± SD. ns not significant; *p < 0.05 (two-tailed unpaired Student’s t test)
signaling. Altogether, these data demonstrate that impaired IL-2R signaling does not allow maintenance of the heterogeneity of Treg cells.

Diminished oral tolerance in \textit{Cd25}\textsuperscript{Y129H} mice

Local expansion of Treg cells in the intestinal lamina propria has been shown to be essential for the establishment of tolerance to food antigens.\textsuperscript{46} Thus, we used \textit{Cd25}\textsuperscript{Y129H} mice to examine the role of IL-2R signaling in the induction of oral tolerance in a DTH model. To this end, WT and \textit{Cd25}\textsuperscript{Y129H} mice received either two doses of 25 mg OVA or PBS by oral gavage (Fig. 7a). One week later, the mice were sensitized with subcutaneous immunization with OVA/CFA. Two weeks later, induction of tolerance was assessed by measuring DTH reactions to intradermally injected OVA. WT mice fed OVA showed reduced DTH responses compared to mice fed PBS, indicating the induction of oral tolerance (Fig. 7b). In contrast, under the same conditions, \textit{Cd25}\textsuperscript{Y129H} mice failed to develop oral tolerance, as shown by an impaired capacity to suppress DTH responses, suggesting a defective function of Treg cells in \textit{Cd25}\textsuperscript{Y129H} mice.

\textit{Cd25}\textsuperscript{Y129H} Treg cells possess a reduced in vivo suppressive capacity

We next investigated the capacity of \textit{Cd25}\textsuperscript{Y129H} Treg cells to suppress intestinal inflammatory responses in vivo. In an adoptive T-cell transfer colitis model, delivery of naïve CD4\textsuperscript{+} T cells into immunodeficient recipients results in severe intestinal inflammation that can be prevented by cotransfer of Treg cells.\textsuperscript{47,48}

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**Fig. 5** Identification of Treg cell subpopulations by single-cell RNA-seq analysis. \textbf{a} Experimental workflow for scRNA-seq analysis including sorting of splenic Foxp3\textsuperscript{GFP}\textsuperscript{*} Treg cells from WT or \textit{Cd25}\textsuperscript{Y129H} mice and use of a Chromium Single Cell 3′ Reagent Kit. \textbf{b} UMAP-based clustering of merged scRNA-seq profiles of WT and \textit{Cd25}\textsuperscript{Y129H} Treg cells. Heatmap showing the expression of selected marker genes (\textbf{c}) and selected transcription factors (\textbf{d}) for each of the four clusters shown in \textbf{b}. A complete list of the genes and expression values is available in Supplementary Table 3.
Fig. 6  Comparison of WT and \(Cd25^{\text{Y129H}}\) Treg cell subpopulations by single-cell RNA-seq. UMAP-based clustering (a) and quantitative analysis (b) of splenic Treg cells from WT and \(Cd25^{\text{Y129H}}\) mice. Data were derived from 3 mice per group and are represented as log2 fold changes (see the “Materials and methods”). c Venn diagram showing the overlap among clusters 2, 3, and 4 for genes differentially expressed between WT and \(Cd25^{\text{Y129H}}\) Treg cells. d Heatmap showing the top 20 differentially expressed genes in WT and \(Cd25^{\text{Y129H}}\) Treg cells. A complete list of the genes and expression values is shown in Supplementary Table 5. e, f Trajectory inference analysis performed with Slingshot software.
Adoptive transfer of effector CD25−CD62L+CD4+ WT T cells (naive CD4+) into Rag2−/− recipients resulted in the development of colitis with extensive infiltration of inflammatory cells and significant induction of inflammatory cytokines within the colonic tissue (Fig. 8a–d). Notably, cotransfer of naive WT CD4+ T cells and Cd25Y129H Treg cells into Rag2−/− recipients did not prevent the development of colitis as efficiently as cotransfer of WT Treg cells. Histological analysis of colon samples from recipient mice coinjected with naive WT CD4+ T cells and Cd25Y129H Treg cells showed significantly increased features of pathological inflammation (Fig. 8a, b) and increased production of inflammatory cytokines compared to recipient mice coinjected with naive WT CD4+ T cells and WT Treg cells (Fig. 8d).

Notably, 30% of the Cd25Y129H Treg cells in the mLNs exhibited reduced Foxp3 expression 8 weeks after colitis induction, while WT Treg cells largely retained Foxp3 expression (Fig. 8e). This observation suggested that impaired IL-2R signaling by Treg cells significantly affected lineage stability and the suppressive capacity during inflammation. Demethylation of the TSDR is required for stable expression of Foxp3 and thus for stable maintenance of their suppressive capacity. Compared to Treg cells isolated ex vivo, Treg cells induced in vitro show an incomplete demethylation phenotype despite displaying high Foxp3 expression, indicating that the DNA demethylation pattern of the TSDRs serves as a marker of long-term Treg cell stability and function.49 To determine whether the instability of adoptively transferred Cd25Y129H Treg cells is due to reduced TSDR demethylation, we compared the methylation status of the TSDR in splenic WT and Cd25Y129H Treg cells. However, pyrosequencing of bisulfite-treated genomic DNA from sorted Foxp3+ and Foxp3− CD4+ T cells revealed a similarly demethylated TSDR in Treg cells from WT and Cd25Y129H mice (Fig. 8f). Given the role of the TSDR in maintaining Foxp3 expression, these results were not unexpected, since Foxp3 protein levels were barely altered in Cd25Y129H Treg cells under steady-state conditions (Fig. 2e). Moreover, this observation confirmed that in Treg cells, a long-term reduction in IL-2R signaling diminishes the size of the Treg cell pool without affecting cell stability. Next, we examined the demethylation of the TSDR in WT and Cd25Y129H Treg cells after transfer into Rag2−/− mice. Consistent with the reduced suppressor function of Cd25Y129H Treg cells, these cells showed a tendency toward a decrease in TSDR demethylation, although this change was not statistically significant (Fig. 8g). Moreover, this reduction correlated with a decrease in Foxp3 levels, indicative of a role for IL-2R signaling in Treg cell stability.

A seemingly unexpected observation was that Cd25Y129H mice did not spontaneously develop autoimmune, while in the adoptive transfer colitis model, Treg cells from these mice could not prevent autoimmune. These findings raised the possibility that Cd25Y129H Treg cells are able to sufficiently deprive only Cd25γ129H effector cells, not WT effector cells, of IL-2, since the latter have higher levels of IL-2R. To test this hypothesis, we performed additional colitis experiments in which Rag2−/− recipients were transferred with naive Cd25γ129H CD4+ T cells alone or in combination with WT or Cd25γ129H Treg cells. In contrast to the above outlined hypothesis, we found that transfer of naive Cd25γ129H CD4+ T cells resulted in disease with a severity similar to that observed in mice transferred with naive WT CD4+ T cells (Fig. 8 and Supplementary Fig. 8). In both situations, Cd25γ129H Treg cells were unable to control inflammation in recipient mice. This finding indicated that the loss of the suppressor function of Cd25γ129H Treg cells in the colitis model might be independent of IL-2 deprivation and points to an intrinsic role for IL-2R signaling in suppressing autoimmunity beyond depriving effector cells of IL-2.

**DISCUSSION**

Previous reports have demonstrated that IL-2R signaling is required for the development, peripheral maintenance, and suppressor function of Treg cells. However, a severe limitation in studying the role of IL-2R in these processes has been the finding that genetically engineered mice deficient in components involved in IL-2R signaling, including IL-2,4,13 IL-2Rα,4,12,50 and IL-2β,11,14 suffer from early systemic autoimmunity and fundamental alterations in Treg cell development. Therefore, it has been difficult to differentiate between thymic and peripheral defects and between resting and inflammatory conditions. Taking advantage of mice bearing a hypomorphic mutation in the Cd25 locus, the present study offers novel insights into the roles of CD25 and IL-2R signaling in the homeostasis and stability of Treg cells under steady-state and inflammatory conditions.

In humans, two patients harboring different mutations in the IL-2Rα gene that markedly abrogate CD25 cell-surface expression have been identified.51,52 One of these mutations leads to an amino acid substitution at codon 166 of the protein (S166F), which is located close to Y129 (Supplementary Fig. 2b), suggesting that mutations in this region may lead to structural rearrangements that affect protein stability. Another reported mutation leads to a substitution of cysteine at codon 51,53 which is in close proximity to the site of interaction with IL-2. Notably, in both patients, CD25 was still detectable at low levels in the cytoplasm, thus resembling the phenotype observed in Cd25γ129H mice. Interestingly, the lack of surface CD25 led to IPEX-like syndrome, which is characterized by early immune dysfunction and systemic autoimmunity, in both patients, similar to other reported cases with mutations within the IL-2Ra gene that completely impaired CD25 expression.53,54 It remains unclear why humans but not Cd25γ129H mice develop spontaneous autoimmunity, even though surface levels of CD25 are reduced to a similar degree. It seems possible that the residual expression of CD25 in Cd25γ129H mice allows sufficient IL-2R-mediated signaling to prevent autoimmunity. Alternatively, this difference in outcome may reflect higher levels of self-antigen challenge in humans than in inbred mice.

We found a substantial reduction in Treg cell numbers in Cd25γ129H mice, indicating that optimal IL-2R signaling is essential for peripheral Treg cell homeostasis, as suggested previously.53,54,55 However, unlike mice deficient in IL-2 or IL-2Rα,51,52,53 Cd25γ129H mice did not exhibit signs of spontaneous autoimmunity. Our study shows that low IL-2R signaling is...
sufficient to maintain basal Treg cell numbers with a sufficient suppressive capacity for maintaining immune system homeostasis under noninflamed conditions. One potential limitation of these observations is that the mutation identified has only been investigated in one genetic background. Thus, the interpretation of our results could be extended by studying the \( \text{Cd}25^{Y129H} \) mutation in mice on other genetic backgrounds, including those known to develop spontaneous autoimmunity.

Our findings raise the possibility that mature Treg cells may compensate for low IL-2R signaling by using signals induced by...
other cytokines, such as IL-7 or IL-15, that may sufficiently preserve the function but not the pool of peripheral Treg cells in a steady state. The lack of autoimmunity in Cd25−/− mice could also be explained by an alternative scenario given that activated T cells and Treg cells are known to regulate each other through IL-2 secretion and consumption, respectively.10,11,29,55,56 Therefore, in Cd25−/− mice, impaired IL-2R signaling by autoreactive T cells may lead to decreased IL-2 production, which may be controlled by Treg cells even with low IL-2 activity, thereby maintaining the regulatory loop at a lower functional level. Surprisingly, the lack of spontaneous autoimmunity was not reflected in the in vitro Treg cell suppression assay, which revealed a strong dependency on IL-2R signaling for Treg cell suppressive function in vitro, as previously suggested.9,16 This observation indicates that commonly used in vitro culture suppression assays more likely mimic the requirements for Treg cell function in inflammatory states than in steady-state situations.

Under steady-state conditions, Cd25+ mice maintained Foxp3 expression, although at a moderately lower level, consistent with previous results where sustained Foxp3 expression was observed upon IL-2 deprivation with anti-IL-2 neutralizing antibodies.5,16 Reduced Foxp3 expression was accompanied by the acquisition of an effector phenotype but did not compromise Treg cell identity or lead to the formation of effector Treg cells.47

In contrast, our results from the adoptive transfer collitis model demonstrate that efficient IL-2R signaling is important for Treg cell stability and function during inflammation, implying that IL-2R-independent signals cannot compensate for low IL-2R signaling under such settings. Notably, using Cd25−/− mice allowed us to specifically evaluate the role of IL-2R signaling in Treg cell stability under inflammatory conditions without the intrinsic lymphoproliferative disorder associated with germline depletion of genes involved in the IL-2R signaling machinery, which might explain the different results reported in the past.57–59 Here, we showed that reduced Cd25 expression caused severe defects in Treg cell stability. Approximately 30% of Treg cells lost Foxp3 expression after colitis induction, indicating that efficient IL-2R signaling is required for sustained Foxp3 expression and suppressive function under inflammatory conditions. Loss of Foxp3 expression and subsequent Treg cell stability could be due to exposure to proinflammatory cytokines9,60 or deprivation of IL-2 signals. It has been previously shown that signaling via IL-2R sustains Foxp3 expression by counteracting proinflammatory cytokine-driven Foxp3 silencing mechanisms at the level of the TSDR.61 The present study supports and extends this view. Here, methylation analysis of the TSDR did not reveal any differences between WT and Cd25−/− splenic Treg cells, indicating that the hypomorphic Cd25+ mutation provides sufficient signals for maintaining TSDR demethylation under physiological conditions. However, once Treg cells reside in inflamed tissue, they rely on fully functional IL-2R signaling machinery to maintain Foxp3 expression and the functional phenotype.

The assumption that IL-2R signaling is essential for Treg cell development in the thymus is based on the observation that mice genetically deficient in IL-2, IL-2Ra, or IL-2Rβ exhibit systemic lethal autoimmunity, which has been ascribed primarily to reduced frequencies of thymic Treg cells.5,2,50,62 Alternatively, other studies suggest that thymic Treg cell development may additionally require other cytokines because there is still a substantial proportion of thymic Treg cells in IL-2- or IL-2Ra-deficient mice.6,19 In addition to IL-2, other cytokines, such as primarily IL-7 and IL-15 but also IL-4, IL-9, and IL-21, that signal through the common γ-chain may also contribute to thymic Treg cell development because deficiency in the common γ-chain results in complete absence of thymic Treg cells even though their availability within the thymus may be limiting.63–65 In the present study, decreased Cd25 expression in Cd25−/− mice did not lead to significant reductions in the percentage or absolute number of thymic Treg cells, indicating that unimpaired IL-2R signaling is less important for thymic Treg cell development than for peripheral Treg cell homeostasis. scRNA-seq recently revealed a considerable degree of heterogeneity in the Treg cell compartment in various lymphoid organs and NLTs.33,45,66 In line with these studies, the scRNA-seq data in the present study identified four major Treg cell subsets showing characteristic cell activation phenotypes and gene transcription signatures, as well as different expression patterns for genes regulating migration and adhesion.

Previously, Stat-1+ Treg cells were exclusively identified in the brachial LNs, but not in the spleen or other organs investigated.33 In contrast, the present study identified this Treg cell population in the spleen using unsupervised hierarchical cluster analysis, which might be due to technical details, such as differences in sequencing or sample processing. Nevertheless, the fact that this Treg subpopulation can be found in different lymphoid tissues suggests that their development is governed by external stimuli that are not restricted to a specific environment. Moreover, the fact that Stat-1+ Treg cells showed increased expression of IFN-related molecules suggests that they represent an eTreg cell subset that may arise from the de novo conversion of conventional CD4+ CD25− T cells into Treg cells.67–69 However, this hypothesis still needs to be clarified.

Notably, compared to WT Treg cells, Cd25+ Treg cells were grouped rather differently, highlighting the impact of IL-2R signaling on the organization of the Treg cell compartment. Our data show that efficient IL-2R signaling is particularly required for the maintenance of cTreg cells since we observed a decrease in the absolute number of CD62L+ CD4+ Treg cells but not in that of CD62L+ CD4+ Treg cells. Our findings also suggest that long-lasting impairment in IL-2R signaling may reduce the stability of cTreg cells, which could subsequently transform into eTreg cells. A previous study reported that IL-2R signaling controls chromatin accessibility and the epigenetic landscape of thymic precursor Treg cells in the same mouse model that was used in the present study.70 The results of that study were in agreement with those of earlier reports suggesting that IL-2R regulates gene expression by modulating chromatin conformation at specific transcriptional enhancers.71,72 Along with those findings, our observations indicate that optimal IL-2R signaling is required for Treg cell identity and stability and thus for optimal Treg cell function. Further studies will be needed to determine the precise mechanisms by which IL-2R
signaling promotes Treg cell stability in different environments and whether these mechanisms can be targeted to modulate Treg cell function to dampen inflammatory diseases.

Finally, the scientific community should be reminded that the Ccr2<sup>Y129H</sup> mutation spontaneously occurred in a Ccr2<sup>−/−</sup> mouse strain that has been widely used to study the role of the chemokine receptor CCR2 in the immune system. Since when this mutation occurred is unknown, it might be appropriate to revisit at least those experiments that studied T-cell responses using Ccr2<sup>2m<sup>1/2</sup></sub> mice.

**DATA AVAILABILITY**

The scRNA-seq data that support the findings of this study have been submitted to the National Center for Biotechnology Information/Gene Expression Omnibus database (https://www.ncbi.nlm.nih.gov/geo) under accession number GSE161345. The source data underlying Figs. 1c, e–h, 2b–e, 3a–h, 4a, b, d, 6a, b, 7b, and 8a, c–e, g, and Supplementary Figs. 3a–c, 4a–e, 5a, b, and 8a b are provided as a Source Data file. All other data that support the findings of this study are available from the corresponding author upon reasonable request.

**ACKNOWLEDGEMENTS**

This work was supported by the ERC Advanced Grants 322645, SF8900-B1, and SF8738-BS (all to R.F.) and SF738-C7 (to J.H.). We gratefully acknowledge the assistance of Dr. Matthias Ballmaier of the Cell Sorting Core Facility of Hannover Medical School for superb service. The DNA and MTO libraries used in this publication were sequenced at the Research Core Unit Genomics at Hannover Medical School. We thank Svetlana Piter for providing excellent animal care and Natalio Garbi (IEI Bonn) for providing Ccr2<sup>−/−</sup> mice.

**AUTHOR CONTRIBUTIONS**

M.P. and R.F. designed the study. B.B. and I.O. performed the scRNA-seq analysis. S.G. and H.G. helped with genotyping, cell culture, and microscopy imaging. M.F., L.O.-Q., and H.G. helped with genotyping, cell culture, and IL-2 shape a population of regulatory cells that controls CD4<sup>+</sup> T cell numbers. *J. Immunol.* **169**, 4850–4860 (2002).

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