The Charcot-Leyden Crystal protein Galectin-10 is not a major determinant of human regulatory T cell function

To the Editor,

Recent studies have highlighted an immunogenic role for Galectin-10 (Gal10) in type 2 inflammation[1,2]. Activated eosinophils release Gal10, which can auto-crystallize to form Charcot-Leyden Crystals (CLCs)[2,3]. Importantly, the dissolution of CLCs by crystal-dissolving antibodies may provide a novel therapeutic target for the treatment of severe asthma and eosinophil-mediated inflammation[1,2]. Concomitantly, FOXP3$^+$ regulatory T cells (Tregs) play a crucial role in controlling allergic diseases[4]. Interestingly, Gal10 has been reported as being significantly overexpressed in Tregs and actively involved in their function[5]. Given the potential therapeutic application of CLC-dissolving antibodies in eosinophilic disorders and the essential immunoregulatory function of Tregs, we have investigated the role of Gal10 and the effects of Gal10 crystals and an anti-Gal10 antibody capable of CLC dissolution in human T cell subsets in detail.

A previous study reported almost exclusive Gal10 expression in CD25$^+$-enriched Tregs compared to CD4$^+$CD25$^-$ conventional T cells (Tconvs)[5]. Although no evidence of Gal10 secretion or crystal formation was observed, Gal10 downregulation in Tregs enhanced IFNγ and TNF-α secretion and inhibited their suppressive capacity[5]. We have re-examined Gal10 expression in highly pure FACS-sorted CD4$^+$CD25$^-$CD127$^+$Tconvs and CD4$^+$CD25$^+$CD127$^-$ Tregs (FigureS1A). However, we observed only low *Gal10* mRNA expression in both T cell subsets using two sets of previously described primers[5,6] (Figure1A). Following *in vitro* stimulation, *Gal10* expression only increased in Tconvs but not Tregs (Figure1A). To independently confirm our data, we reanalyzed published transcriptomic datasets of Tregs and observed again in contrast to a previous study[5] lower *Gal10* than *FOXP3* mRNA, and comparable low *Gal10* expression between different Tconv and Treg subsets (FigureS1B-F). Moreover, epigenetic analysis of the *Gal10* locus in Tconvs and Tregs predicted quiescent/weak expression of this gene, while expectedly weak or strong *FOXP3* expression was predicted in Tconvs or Tregs respectively (FigureS1G-H).

Gal10 protein expression was analyzed using two antibodies which specificity was verified in detail (FigureS2A-C). We observed low Gal10 expression in a minor population of Tconvs and Tregs (Figure1B-D), which was quantified to represent less than 2.5% of the total Tconvs and Tregs (Figure1D). Similar Gal10 protein expression levels were observed in 4 day-stimulated Tconvs and Tregs (Figure1E and FigureS2D). The discrepancies to a previous report are possibly due to insufficient purity of cell isolates[5].

To determine whether external Gal10 may affect Treg function, Tconvs and Tregs were stimulated *in vitro* with allogeneic DCs or anti-CD2/CD3/CD28-coated-beads in the presence or absence of recombinant Gal10 crystals[2]. Gal10 crystals did not affect Tconv proliferation (Figure2A and FigureS2E) and we only observed subtle changes in cell viability at high Gal10 concentrations (Figure2B and FigureS2F). Similarly, Gal10 crystals had no effect on viability, FOXP3 expression or suppressive potency and cytokine expression of Tregs (Figure2C-E and FigureS2G-J).

Next, we silenced the minimal activity of endogenous Gal10 in Tregs. In contrast to previous data[5], this had no effect on Treg survival, FOXP3, *IFNG* expression or suppressive activity (Figure2F and FigureS2K-N). Moreover, 1D11 anti-Gal10 antibody[2] had no effect on proliferation or viability of CD4$^+$Tconvs (Figure2G-H and FigureS2O-P). Similarly, Treg phenotype and activity were not significantly altered by 1D11, although a tendency towards increased suppressive potency was observed (Figure2I-K and FigureS2Q-S).

In summary, by using multiple primer sets and transcriptomic datasets, as well as several antibodies and methods of protein detection, we did not find significant Gal10 expression in

human Tregs. The inhibition of Gal10, either through siRNA or antibody treatment, had no significant effect on Treg function, nor could we see enhanced Treg activity by Gal10 crystals, suggesting no potential Treg-based side effects of targeting Gal10 crystallization. Overall, our data indicate that Gal10 likely plays a neglectable role in the context of Tregs and eosinophilic inflammation.

References

1. Aegerter H, Smole U, Heyndrickx I, et al. Charcot-Leyden crystals and other protein crystals driving type 2 immunity and allergy. *Current opinion in immunology.* 2021;72:72-78.
2. Persson EK, Verstraete K, Heyndrickx I, et al. Protein crystallization promotes type 2 immunity and is reversible by antibody treatment. *Science.* 2019;364(6442).
3. Ueki S, Tokunaga T, Melo RCN, et al. Charcot-Leyden crystal formation is closely associated with eosinophil extracellular trap cell death. *Blood.* 2018;132(20):2183-2187.
4. Noval Rivas M, Chatila TA. Regulatory T cells in allergic diseases. *The Journal of allergy and clinical immunology.* 2016;138(3):639-652.
5. Kubach J, Lutter P, Bopp T, et al. Human CD4+CD25+ regulatory T cells: proteome analysis identifies galectin-10 as a novel marker essential for their anergy and suppressive function. *Blood.* 2007;110(5):1550-1558.
6. Andersson J, Cromvik J, Ingelsten M, et al. Eosinophils from hematopoietic stem cell recipients suppress allogeneic T cell proliferation. *Biol Blood Marrow Transplant.* 2014;20(12):1891-1898.

Author list and affiliations

Rebeca Arroyo-Hornero[1, 2], Helena Aegerter[3, 4], Ibrahim Hamad[1, 2], Beatriz Corte-Real[1, 2], Katrien Staes[5, 6], Bas van der Woning[7], Kenneth Verstraete[8, 9], Savvas N. Savvides[8, 9], Bart N. Lambrecht[3,4,10,#], Markus Kleinewietfeld[1, 2,#, *]

[1]VIB Laboratory of Translational Immunomodulation, VIB Center for Inflammation Research (IRC), Hasselt University, Diepenbeek, Belgium.

[2]Department of Immunology, Biomedical Research Institute, Hasselt University, Diepenbeek, Belgium.

[3]Immunoregulation Unit, VIB Center for Inflammation Research, Ghent, Belgium.

[4]Department of Internal Medicine and Pediatrics, Ghent University, Ghent, Belgium.

[5]Transgenic Core Facility, VIB Center for Inflammation Research, Ghent, Belgium

[6]Department of Biomedical Molecular Biology, Ghent University, Ghent, Belgium.

[7]argenx BV, 9052, Zwijnaarde, Belgium.

[8]Unit for Structural Biology, VIB Center for Inflammation Research, Ghent, Belgium.

[9]Department of Biochemistry and Microbiology, Ghent University, Ghent, Belgium.

[10]Department of Pulmonary Medicine, ErasmusMC, Rotterdam, Netherlands.

[#]These authors jointly supervised this work

***Correspondence:** markus.kleinewietfeld@uhasselt.vib.be (M.K.)

Acknowledgments

We thank Anneleen Geuzens and Dries Swinnen for technical assistance. We further thank the VIB-IRC Flow Cytometry and the VIB-IRC Microscopy Core Facilities for assistance.

Funding statement

H.A. was supported by a Vlaio grant (Vlaio-Baekeland HBC.2019.2632). This project has received further funding from an Excellence of Science UHEAD consortium grant (B.N.L.), a Concerted Research Action grant to the University of Ghent (B.N.L.), the European Research Council (ERC) under the European Union's Horizon 2020 research and innovation programme (789384, B.N.L.; 640116, M.K.), by a GOA grant from Ghent University (B.N.L and S.N.S.), by a SALK-grant from the government of Flanders (M.K.) and by an Odysseus-grant of the Research Foundation Flanders (FWO), Belgium (M.K.).

Disclosure of Conflicts of Interest

Bart Lambrecht has received consultancy fees from Astra-Zeneca, Sanofi, GSK, Novartis, Oncoarendi and argenx BV. Bas van der Woning is a full-time employee of argenx BV. These disclosures do not affect content of this article.

Authorship contributions

R.A.H., I.H., B.C.R. and H.A. designed and performed research and analyzed and interpreted data; K.S., B.W., K.V. and S.N.S. contributed vital reagents; B.W. and S.N.S gave conceptual input; B.N.L. and M.K. conceived the project, designed and supervised research and interpreted data. R.A.H. and M.K. wrote the manuscript with key editing by H.A. and B.N.L. and further input from all authors.

Figure legends

FIGURE 1. Gal10 expression in human Tregs. A, Relative *Gal10* mRNA expression measured in Tconvs and Tregs isolated as shown in Figure S1 and stimulated *in vitro* with anti-CD3/CD28 mAbs and IL-2. *Gal10* mRNA expression studied using Taqman probe Hs00171342_m1[6] and normalized according to *B2M* mRNA expression (left graph), or using *Gal10* primers previously described by Kubach *et al*.[5] and normalized according to *B2M* (middle graph) or *EF1A* (right graph) mRNA expression. Mean ± SD, n=3-12 independent donors. **B,** Western blot assay using anti-Gal10 Ab AF5447. n=11 independent donors isolated as shown in Figure S1. Gal10 protein expression normalized to beta-actin expression. HEK293T cells, untransfected or transfected with a *Gal10*-expressing plasmid, were used as negative or positive control respectively. **C,** $CD4^+CD25^-$Tconvs and $CD4^+CD25^+$Tregs isolated by magnetic bead enrichment, stained with anti-Gal10 Ab AF5447 and analyzed by fluorescence microscopy. Images obtained with a Carl Zeiss LSM780 confocal microscope (25x objective). The scale shows 20 µm. **D,** Representative FACS analysis of $CD4^+$T cells stained for viability, FOXP3 and Gal10 expression (anti-Gal10 mAb B-F42, see FigureS2C) **(**left**)** and summary of Gal10 expression within $FOXP3^-$Tconvs or $FOXP3^+$Tregs in 5 independent donors. **E,** Western blot assay performed in 4 day-stimulated Tconvs and Tregs using anti-Gal10 Ab AF5447 in 6 independent donors. Gal10 protein expression was normalized to beta-actin expression. Recombinant His-Tag Gal10 was used as a positive control. Normal distribution was assessed by Shapiro-Wilk normality test with a significance

level of 0.05. Statistical significance was studied by (A) Kruskal-Wallis test with Dunn's post-test for multiple comparisons or (B, D, E) two-tailed unpaired *t* test for normally distributed data.

FIGURE 2. Assessment of Gal10 activity on human T cell function. A-E, $CD4^+CD25^-$ Tconvs or Tregs were stimulated *in vitro* by co-culturing with monocyte derived-DCs in the presence of soluble anti-CD3 mAb and 10µg/ml Gal10 crystals and IL-2 for 4 days. **A,** Cell proliferation and **B-C,** cell viability studied by flow cytometry by CFSE labelling or by staining with a live/dead fixable dead cell stain kit respectively. **D,** FOXP3 expression studied in Treg cultures. **E,** Treg suppressive activity assessed *in vitro* by co-culturing Tregs with autologous CFSE-labelled $CD4^+CD25^-$Tconvs and monocyte derived-DCs for 4 days in the presence of anti-CD3 mAb and 10µg/ml Gal10 crystals. $CD4^+$Tconv cell proliferation was studied by FACS. FOXP3 expression and cell proliferation were normalized to control condition without Gal10 crystals. **F,** FACS-sorted $CD4^+CD25^+CD127^-$Tregs, cultured *in vitro* for 6-7 days, were nucleofected with 100nM control siRNA (CTRL) or siRNA targeted to *Gal10* (*Gal10* siRNA). Suppressive activity of Tregs was assessed *in vitro* by co-culturing Tregs with autologous CFSE labelled-PBMCs (hereafter named as Tresp) and anti-CD2/CD3/CD28 mAb coated beads. Histogram plots show CFSE expression of $CD4^+$ and $CD8^+$Tresp. Tregs were excluded from Tresp by gating out $CFSE^-CD4^+$T cells. **G-J,** $CD4^+CD25^-$Tconvs or Tregs were stimulated *in vitro* by co-culturing with monocyte derived-DCs in the presence of soluble anti-CD3 mAb and 10µg/ml 1D11 (Gal10 crystal-dissolving Ab) and IL-2 for 4 days. **G,** Cell proliferation and **H, I,** cell viability studied by FACS by CFSE labelling or by staining with a live/dead fixable dead cell stain kit respectively. **J,** FOXP3 expression studied in Treg cultures. **K,** Treg suppressive activity was assessed *in vitro* by co-culturing Tregs with autologous CFSE-labelled $CD4^+CD25^-$Tconvs and monocyte derived-DCs for 4 days in the presence of anti-CD3 mAb and 10µg/ml 1D11 Ab. $CD4^+$Tconv cell proliferation studied by FACS is represented. FOXP3 expression and cell proliferation were normalized to control condition without 1D11 Ab. Mean ± SD, n=4-6 independent cell donors. Normal distribution was assessed by Shapiro-Wilk normality test with a significance level of 0.05. Statistical significance was studied by two-tailed paired *t* test for normal distributed data.

Fig. 1

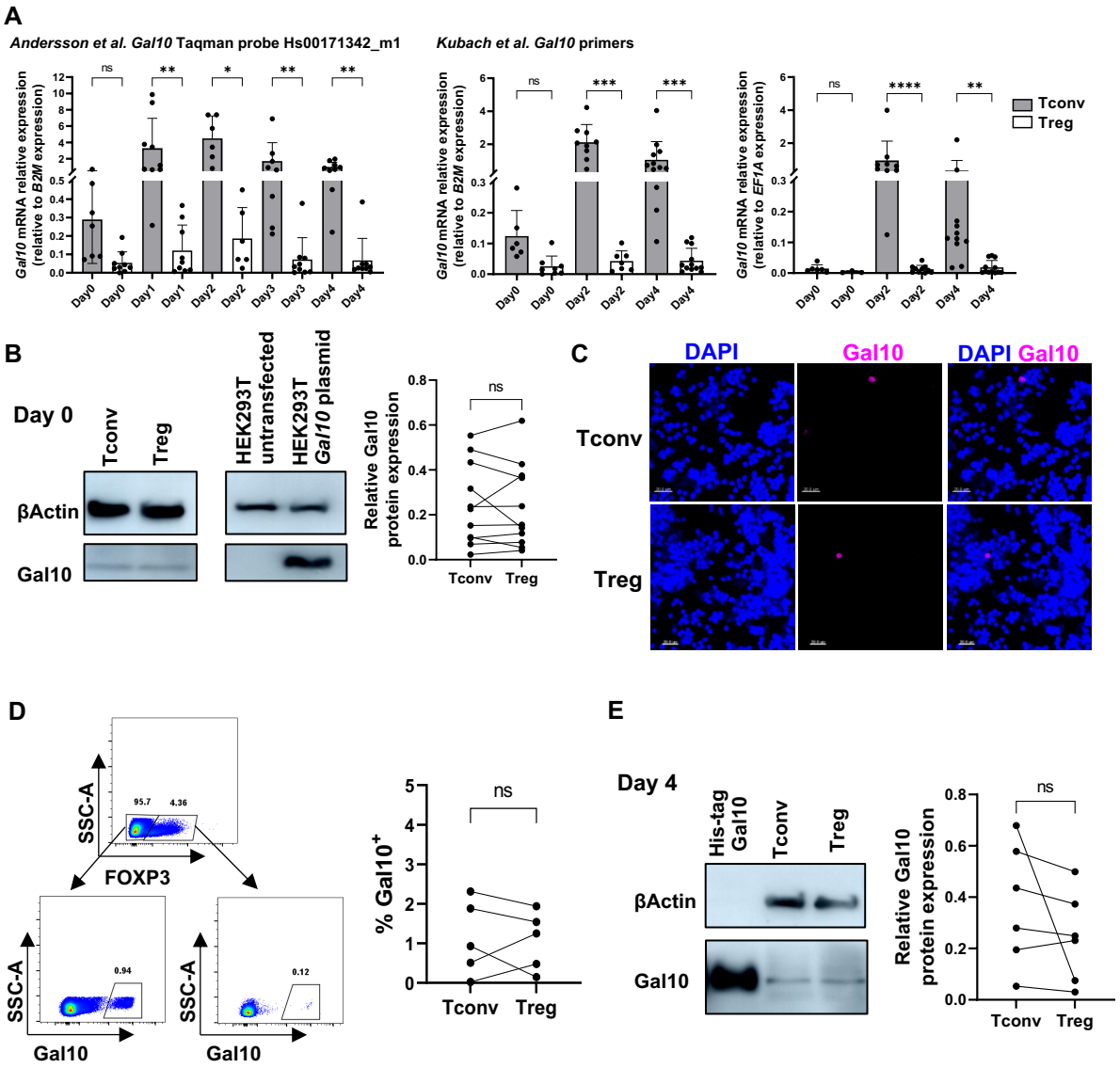
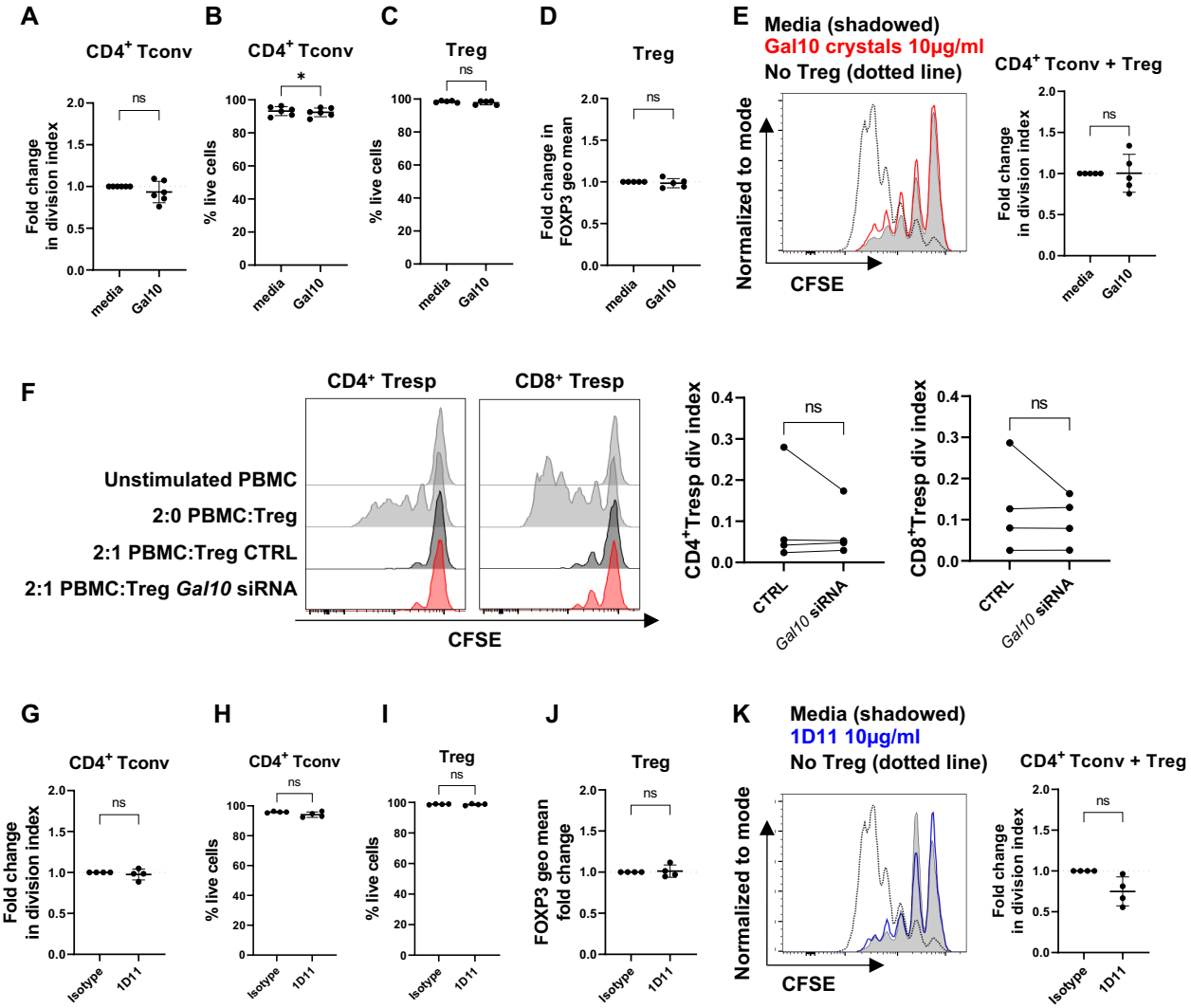

A
Andersson et al. Gal10 Taqman probe Hs00171342_m1
Kubach et al. Gal10 primers
Gal10 mRNA relative expression (relative to B2M expression)
Gal10 mRNA relative expression (relative to EF1A expression)
Tconv
Treg
Day0
Day1
Day2
Day3
Day4
B
Day 0
Tconv
Treg
HEK293T untransfected
HEK293T Gal10 plasmid
βActin
Gal10
Relative Gal10 protein expression
C
DAPI
Gal10
DAPI Gal10
Tconv
Treg
D
SSC-A
FOXP3
95.7
4.36
0.94
0.12
Gal10
% Gal10+
E
Day 4
His-tag Gal10
Tconv
Treg
βActin
Gal10
Relative Gal10 protein expression
ns

Fig. 2


A
CD4+ Tconv
Fold change in division index
ns
media
Gal10
B
CD4+ Tconv
% live cells
*
C
Treg
% live cells
ns
D
Treg
Fold change in FOXP3 geo mean
ns
E
Media (shadowed)
Gal10 crystals 10µg/ml
No Treg (dotted line)
Normalized to mode
CFSE
CD4+ Tconv + Treg
Fold change in division index
F
CD4+ Tresp
CD8+ Tresp
Unstimulated PBMC
2:0 PBMC:Treg
2:1 PBMC:Treg CTRL
2:1 PBMC:Treg Gal10 siRNA
CFSE
CD4+Tresp div index
CD8+Tresp div index
CTRL
Gal10 siRNA
G
CD4+ Tconv
Fold change in division index
Isotype
1D11
H
CD4+ Tconv
% live cells
I
Treg
% live cells
J
Treg
FOXP3 geo mean fold change
K
Media (shadowed)
1D11 10µg/ml
No Treg (dotted line)
Normalized to mode
CFSE
CD4+ Tconv + Treg
Fold change in division index

The Charcot-Leyden Crystal protein Galectin-10 is not a major determinant of human regulatory T cell function.

Rebeca Arroyo-Hornero, Helena Aegerter, Ibrahim Hamad, Beatriz Corte-Real, Katrien Staes, Bas van der Woning, Kenneth Verstraete, Savvas N. Savvides, Bart N. Lambrecht, Markus Kleinewietfeld

Methods

Cell isolation and culture

The study was conducted according to the Declaration of Helsinki principles and was approved by the institutional ethics committee of Hasselt University (Comité voor Medische Ethiek UHasselt). Peripheral blood (collected in Lithium Heparin coated tubes, 455084, Greiner bio-one) or buffy coats (purchased from the Belgium Red Cross) were obtained from healthy donors, after providing written informed consent. Human peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll-Paque PLUS (GE17-1440-02, Sigma-Aldrich) gradient centrifugation. In some cases, $CD4^+$ T cells were isolated from whole blood using RosetteSep Human $CD4^+$ T Cell Enrichment Cocktail (15062, Stemcell Technologies) according to manufacturer's protocol. PBMCs or $CD4^+$ T cells were then incubated with CD25 Microbeads II (130-097-044, Miltenyi Biotec) and separated using LS columns (130-042-401, Miltenyi Biotec). Tregs were isolated from $CD25^+$ enriched cells, while Tconvs were isolated from CD25-depleted cells. For further FACS sorting, cells were labelled with Propidium Iodide (PI) (556463, BD Biosciences), anti-CD4 APC-Cy7 (557851, BD Biosciences), CD25 PE-Cy7 (557741, BD Biosciences) and CD127 PerCP-Cy5.5 (351322, Biolegend) antibodies prior being sorted as $PI^-CD4^+CD25^+CD127^-$ Tregs or as $PI^-CD4^+CD25^-CD127^+$ Tconvs using a BD FACSAria II cell sorter as described before[1,2]. FACS-sorted Tconvs and Tregs were cultured in X-VIVO15 media (BE02-060F, Lonza) supplemented with 5% heat inactivated Fetal Bovine Serum (S1400, BioWest). Cells were stimulated with 1 or 10 μg/ml plate bound anti-CD3 mAb (555329, BD Biosciences) and 1μg/ml soluble anti-CD28 mAb (555725, BD Biosciences). Where indicated, media was supplemented with IL-2 (Proleukin, Novartis or 11147528001, Sigma-Aldrich).

Quantitative polymerase chain reaction with reverse transcription (qRT-PCR)

Cells were lysed in RLT buffer (74034, Qiagen) containing 2-mercaptoethanol according to the manufacturer's protocol, and stored at -80°C until RNA was extracted. RNA was isolated using the RNeasy plus Micro Kit (74034, Qiagen) and further converted to cDNA using qScriptTM cDNA SuperMix kit (95048, QuantaBio) following manufacturer's instructions. Real Time PCR was performed in duplicates on a Step ONE Plus RT-PCR machine (Applied Biosciences). The following primers were used for PCR with TaqMan Fast Universal PCR Master Mix (2X) (4352042, Thermofisher Scientific): *Gal10/CLC*- Hs0017342_m1 (ThermoFisher Scientific[3]), *IFNG*- Hs00989291_m1, and *B2M*- 4326319E-1402015 from Applied Biosystems. Additionally, the following primers were used for PCR with PowerUp SYBR Green Master Mix (A25742, ThermoFisher Scientific): *Gal10/CLC* forward- 5'-TAC CCG TGC CAT ACA CAG AGG CTG-3', *Gal10/CLC* reverse- 5'-CTT ATC TGG CAG CAC TGA GAT GCT C-3' (described by[4]), *B2M* forward- 5'-TTC TGG CCT GGA GGC TAT-3', *B2M* reverse- 5'-TCA GGA AAT TTG ACT TTC CAT TC-3' (described by[5]), *EF1A* forward- 5'-GAT TAC AGG GAC ATC TCA GGC TG-3', *EF1A* reverse- 5'-TAT CTC TTC TGG CTG TAG GGT GG -3' (described by[4]). Fold-changes in expression were calculated using the $\Delta\Delta$CT method based on human *B2M* or *EF1A* as endogenous controls for mRNA expression as described before[6].

Reanalysis of published transcriptomic datasets

EGAS00001004470, GSE90600, GSE76598 and GSE148267 were downloaded from the European Genome-phenome Archive (EGA) or gene expression omnibus (GEO) database. GSE76598 was analyzed with GEO2R. For reanalysis of RNA sequencing in EGAS00001004470, GSE90600 and GSE148267, quality of raw sequence reads was checked using FastQC version 0.11.8, and nucleotide calls with a quality score of 28 or higher were considered high quality. Adapters were removed using cutadapt v.2.4. Reads were aligned to the hg19 genome reference, using STAR (2.5.0e) and a maximum of five mismatches were allowed. Gene counts were retrieved using htseq-count using the "union" option.

Reanalysis of published epigenetic datasets

Histone epigenetic marks and chromHMM data from the Roadmap Epigenomics Project were downloaded from WashU EpiGenome Browser (v52.1.0)[7]. Data was extracted from human $CD4^+CD25^-$ Th and $CD4^+CD25^+CD127^-$ Treg primary cells isolated from blood of healthy donors[1,8].

Western Blot

FACS-sorted $CD4^+CD25^-CD127^+$ Tconvs or $CD4^+CD25^+CD127^-$ Tregs were lysed in RIPA buffer (150mM sodium chloride, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulphate and 50mM Tris pH 8.0) containing protease inhibitor (05 892 970 001, Roche). Alternatively, FACS-sorted Tconvs and Tregs were *in vitro* stimulated for 4 days with 1μg/ml plate bound anti-CD3 mAb (555329, BD Biosciences), 1μg/ml soluble anti-CD28 mAb (555725, BD Biosciences) and 125IU/ml Proleukin prior lysis in RIPA buffer. Protein was quantified by BCA assay (23227, ThermoFisher Scientific). His-tag recombinant galectin-10 (generated in house by B. Lambrecht) or HEK293T cells

transfected with a *Gal10*-expressing plasmid were used as a positive control. The lysates were electrophoresed in a 10% SDS gel and transferred onto a PDVF membrane. The membrane was blocked with 5% BSA for 1 hour at room temperature and sequentially reacted with anti-Gal10 Ab (AF5447, R&D Systems) or anti-β-Actin (3700, Cell Signalling) overnight at 4ºC and a peroxidase-conjugated secondary antibody (P0260 or P0449, Agilent Technologies) for 1.5 hours at room temperature. Membranes were revealed with Pierce ECL reagent (32134, ThermoFisher Scientific) in an Amersham Imager 680. Protein quantification was performed using ImageJ.

Fluorescence microscopy

Galectin-10 expression was studied in $CD4^+CD25^-$ Tconvs and $CD4^+CD25^+$ Tregs isolated from PBMCs using RosetteSep Human $CD4^+$ T Cell Enrichment Cocktail (15062, Stemcell Technologies) and CD25 Microbeads II (130-097-044, Miltenyi Biotec). Galectin-10 crystals (generated in house by S. Savvides) and the human eosinophilic cell line AML14.3D10 were used as a positive control. A *Gal10*-knockout of the AML14.3D10 cell line was used as a negative control. The AML14.3D10 cell line was a kind gift of C. Paul and M. Baumann (Wright State University, Dayton, OH) and was cultured at 37°C and 5% CO2 in RPMI 1640 (LifeTechnologies) containing 10% FCS (Life Technologies), 50mM 2-ME (Sigma-Aldrich), 0.1 mM nonessential aminoacids (Life Technologies), 1 mM sodium pyruvate (Sigma-Aldrich), with a culture density of below $1x10^6$/ml. For Galectin-10 staining, cells were fixed in 2% paraformadehyde for 15 minutes at room temperature, washed with PBS and permeabilized with 0.025% Triton-X (Sigma-Aldrich) for 10 minutes at room temperature. Cells were then blocked in 2% Bovine-Serum Albumin (Sigma-Aldrich) + 3% Donkey serum for 1 hour at room temperature. Cells were incubated overnight with anti-Galectin-10 (AF5447, R&D Systems) or isotype (AB-108-C, R&D) in PBS. The following day, cells were washed three times in PBS and incubated with a donkey polyclonal-goat IgG (A-21447, ThermoFisher) conjugated to Alexa-Fluor647 for 1 hour at room temperature in the dark, followed by DAPI (D3571, Life Technologies) and washed in PBS prior to being mounted on Poly-L-Lysine (Sigma-Aldrich) coated slides with Polyvinyl alcohol (Sigma-Aldrich). Images were acquired with a Zeiss LSM 780 confocal microscope (Carl Zeiss) with a 10x or 25x objective and analyzed using iMaris v7.6.4 software.

Generation of Gal10-deficient AML14.3D10 knockout cells

Gal10/*CLC*-deficient AML14.3D10 knockout (KO) cells were generated with the CRISPR/Cas9 system. Guide RNAs were designed using CRISPOR software, and inserted in pX458 (plasmid #48138, Addgene). Exon 3 was targeted by gRNA clc2211 with protospacer sequence 5'-CACGACGACCAAAGCACACT-3'. Cells were electroporated (NEPA21, NepaGene) with the indicated plasmid and GFP positive cells were single cell sorted. The targeting region of the resulting clones was sequenced and analysed with ICE (Synthego) and TIDE (NKI). Galectin-10 depletion was further confirmed by WB.

Flow cytometry (FACS)

For FACS, cells were stained with LIVE/DEAD kit (L34976, Invitrogen) for 10 minutes at room temperature to exclude dead cells. For surface staining, cells were labelled with respective antibodies for 20 minutes in MACS buffer (PBS with 0.5% BSA, 2mM EDTA) at 4°C. For intracellular staining, cells were first fixed and made permeable using eBioscience FOXP3/Transcription Factor Staining Buffer Set (00-5523-00, Invitrogen) according to manufacturer's instructions and stained with respective antibodies diluted in Perm buffer for 30 minutes at 4°C, washed and assayed in MACS buffer. For cytokine detection, cells were stimulated with 50ng/ml phorbol12-myristate13-acetate (PMA) (P1585, Sigma) and 250ng/ml Ionomycin (1063, Sigma) in the presence of GolgiPlug (555029, BD) for 5 hours prior intracellular staining. Antibodies used were anti-CD4 APC-Cy7 (557851, BD Biosciences), anti-CD4 PerCP-Cy5.5 (300530, Biolegend), anti-CD3 Pacific Blue (317314, Biolegend), anti-CD3 PE (317308, Biolegend), anti-CD25 PE-Cy7 (557741, BD Biosciences), anti-CD127 PerCP-Cy5.5 (351322, Biolegend), anti-IFNγ FITC (eBioscience 11-7319-81), anti-IL10 APC (BD 554707), anti-FOXP3 PE (320108, Biolegend) and anti-FOXP3 AF700 (56-4776-41, eBioscience). When indicated, cells were stained with the cell trace dyes CFSE or CTV, at a final concentration of 1μM or 2.5μM respectively. Gal10 protein expression was studied by flow cytometry in HEK293T cells or in $CD4^+$ T cells sorted from freshly isolated human PBMCs using EasySep Human $CD4^+$ T cell isolation Kit (17952, StemCell Technologies), before and after 4 days of stimulation (1μg/ml plate bound anti-CD3 mAb, 1μg/ml soluble anti-CD28 mAb and 125IU/ml Proleukin). $CD4^+$ T cells were stained with LIVE/DEAD Kit prior being fixed and permeabilized as described above. Cells were stained with anti-Gal10 mAb (852.963.020, Diaclone) or mouse IgGκ isotype (401408, Biolegend) for 1 hour at 4°C in Perm buffer, washed and incubated with anti-mouse IgG labelled with AF555 (A21425, ThermoFisher Scientific) and anti-FOXP3 AF700 for 30 minutes at 4°C in perm buffer. Data was acquired on a BD LSR Fortessa II and analyzed with FlowJo software (TreeStar).

Galectin-10 overexpression in HEK293T cells

HEK293T cells were transfected using jetOptimus buffer (117-07, Polyplus) with 300ng *Gal10*-expression plasmid (RC219689, Origene) according to manufacturer's protocol and incubated at 37°C for 48 hours before read-out.

siRNA mediated knock-down

FACS-sorted CD4$^+$CD25$^+$CD127$^-$ Tregs were *in vitro* cultured for 6-7 days prior siRNA nucleofection. Cells were stimulated in X-VIVO15 media (BE02-060F, Lonza) supplemented with 5% heat inactivated Fetal Bovine Serum (S1400, BioWest) with 10 μg/ml plate bound anti-CD3 mAb (555329, BD Biosciences), 1μg/ml soluble anti-CD28 mAb (555725, BD Biosciences) and 1500IU/ml Proleukin. Cells were rested 24 hours prior nucleofection in media containing 500IU/ml Proleukin in the absence of TCR stimulation. Control siRNA (ON-TARGETplus non-targeting control pool, D-001810-10-05) and a pool of 4 specific siRNA for *Gal10/CLC* (ON-TARGETplus SMARTpool#1178, L-012397-01-0005) were obtained from Horizon Discovery LTD, Dharmacon. 1x10^6 Tregs were transfected in 20μl P3 Primary Cell 4D-Nucleofector X Kit S (V4XP-3032, Lonza) with 100nM of siRNA by using Nucleofection cuvette strips (4D-Nucleofector X Kit S, Lonza) and the 4D-Nucleofector Core Unit (AAF-1002B, Lonza) and X Unit (AAF-1002X, Lonza) with program EO115. After transfection, Tregs were cultured in X-VIVO15 media supplemented with 5% FBS and 500IU/mL Proleukin and incubated at 37°C for 2 days. Dead cells were removed by staining with propidium iodide (PI) (556463, BD Biosciences) and FACS-sorting negative cells using a BD FACSAria II cell sorter, resulting in a viability greater than 99%. RNA measurements and suppression assays were assessed on sorted live cells.

Generation of monocyte-derived dendritic cells (mo-DCs)

For monocyte-derived DCs, CD14$^+$ monocytes were magnetic bead-isolated from PBMCs (17858, StemCell Technologies) and cultured with 50U/ml IL-4 (11340045, Immunotools) and 50ng/ml GM-CSF (300-03, Peprotech) in X-VIVO15 supplemented with 10% FBS. After 5 days of incubation, DCs were harvested and stored in liquid nitrogen for later use.

Functional in vitro assays

FACS-sorted CD4$^+$CD25$^-$CD127$^+$ Tconvs or CD4$^+$CD25$^+$CD127$^-$ Tregs were labelled with 1μM Cell Trace CFSE (C34554, ThermoFisher Scientific) or 2.5μM Cell Trace Violet CTV (C34557, ThermoFisher Scientific). Cells were cultured in 96-well U-bottom plates at 5x10^4 cells per well in X-VIVO15 media (BE02-060F, Lonza) supplemented with 5% heat inactivated Fetal Bovine Serum (S1400, BioWest). Cells were stimulated with 2x10^4 allogeneic mo-DCs and 0.5μg/ml soluble anti-CD3 (555329, BD Biosciences) or by anti-CD2/CD3/CD28 mAb coated beads (130-092-909, Miltenyi Biotec) at 1 bead: 1 cell ratio, for 4 days before flow cytometry analysis. Media was supplemented with 25U/ml IL-2 (Sigma-Aldrich) for Treg cultures. When stated in the figures, Gal10 crystals or 1D11 Ab (Gal10 crystal-dissolving antibody)[9] were added to the cell cultures at different concentrations as indicated.

Treg suppression assay

Treg suppressive capacity was assessed by their ability to suppress autologous T cell proliferation *in vitro*. PBMCs or CD4$^+$CD25$^-$ Tconvs (referred as Tresp) were labelled with CFSE and cultured at 5x10^4 cells per well with autologous Tregs in 96-well U-bottom plates in X-VIVO15 supplemented with 5% FBS. When stated, Tregs were labelled with CTV. Cells were stimulated for 4 days using Treg Suppression Inspector beads (anti-CD2/CD3/CD28 mAb coated beads) (130-092-909, Miltenyi Biotec) at 1 bead: 1 cell ratio, or by co-culturing with allogenic mo-DCs (2x10^4 cells per well) in the presence of 0.5μg/ml soluble anti-CD3 mAb (555329, BD Biosciences). Co-culture of cells were stained to distinguish CD4$^+$ and CD8$^+$ T cells and CFSE dilution was studied by FACS. Cell proliferation of Tresp was calculated using FlowJo software as division index.

Quantification and statistical analysis

Graphs were produced and statistical analyses were performed with GraphPad Prism Version 8. All data were presented as mean ± standard deviation (SD). Each dot displayed in the figures denotes an independent blood donor. Number of donors (n) and statistical tests that were used can be found in figure legends. Paired tests were selected when comparing Gal10 or 1D11 treatment with control condition for each cell subset and blood donor. Unpaired tests were selected when comparing different cell subsets. Normality of the data was tested by Shapiro-Wilk normality test. For more than two groups with one variable only, one-way ANOVA with Sidak's multiple comparisons test (for normal distributed data) of Friedman test with Dunn's multiple comparisons test (for non-normal distributed data) were used. For data with more than two groups and multiple variables, the non-paired Kruskall-Wallis test with Dunn's post-test for multiple comparisons (for non-normal distributed data) was used. Significance was defined as *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001 and ****p ≤ 0. 0001.

Supplementary figure legends

FIG S1. Purity of FACS-sorted Tconv and Tregs and re-analysis of published data sets. A, Phenotype of FACS-sorted CD4$^+$CD25$^-$CD127$^+$ Tconv and CD4$^+$CD25$^+$CD127$^-$ Treg cells from one representative donor. **B-F**, Re-analysis of *Gal10* mRNA expression on freshly isolated CD4$^+$CD25$^-$ Tconv and Tregs. *FOXP3* and *Gal10* mRNA expression (read counts) was studied in published transcriptomic data: (B) EGAS00001004470[10], (C) GSE90600[11], (D, E) GSE76598[12] and (F) GSE148267[13]. **B,** mRNA levels (read counts) in Tregs isolated from peripheral blood of 14 healthy individuals. **C,** RNAseq was performed in different CD4$^+$ T cell subsets sorted from blood of 3 healthy donors based on CD4, CD25

and CD45RA expression. $CD4^+$ T cells were isolated as: $CD45RA^+CD25^-$ naive T cells (nTconv), $CD45RA^-CD25^-$ memory T cells (mTconv), $CD45RA^-CD25^{int}$ cells, $CD45RA^+CD25^+$ naive Tregs (nTreg) and $CD45RA^-$ $CD25^{high}$ effector Tregs (eTreg). **D-E,** mRNA expression was studied in cells sorted from blood of 7 healthy donors as: $CD4^+CD45RA^+CD25^-$ naive T cells (nTconv), $CD4^+CD45RA^-CD25^-$ memory T cells (mTconv), $CD4^+CD45RA^+$ $CD25^+$ naive Tregs (nTreg) and $CD4^+CD45RA^-CD25^+$ memory Tregs (mTreg). RNA expression was studied (D) prior and (E) after 40 hours of stimulation with anti-CD3 and anti-CD28 antibodies. **F**, Transcriptomic data of $CD4^+CD25^{low}CD127^+$ Tconvs or $CD4^+CD25^{high}CD127^-$ Tregs isolated from healthy individuals before (n= 3) or after 6 hours, 24 hours or 7 days of *in vitro* stimulation with anti-CD3/CD28 mAbs and IL-2 (n= 1). Normal distribution was calculated by Shapiro-Wilk normality test with a significance level of 0.05. Significance was calculated by (B) Wilcoxon matched-pairs test or (C-F) one-way ANOVA with Sidak's multiple comparisons test for normally-distributed data or by a Kruskal-Wallis test with Dunn's multiple comparisons test for data non-normally distributed. **G-H,** Epigenetic signature at the *Gal10* (*CLC gene)* and *FOXP3* locus was studied using WashU epigenome browser (v52.1.0)[7]. Data from human $CD4^+CD25^-$ Th and $CD4^+CD25^+CD127^-$ Treg primary cells obtained from blood of healthy donors were downloaded from the Roadmap Epigenomics Project[1,8]. Gene track from gencodeV29 is shown in purple. The chromatin state (chromHMM) and histone marks (H3K4me1, H3K4me3, H3K36me3, H3K27me3 and H3K9me3) at the **G,** *Gal10* (*CLC gene)* or **H,** *FOXP3* loci are shown for Th (black line) and Tregs (red line).

FIG S2. Gal10 protein expression and functional characterization of T cells. A-D, Validation of anti-Gal10 antibodies and Gal10 expression in activated T cells. **A,** Gal10 crystals were generated as previously described by Persson *et al.*[9], stained with anti-Gal10 antibodies B-F42 or AF5447 and studied by fluorescence microscopy (10x objective, scalebar = 100μm). **B,** Wild type and *Gal10*-knock-out AML cell lines were stained intracellularly with goat IgG isotype or anti-Gal10 Ab (AF5447) and DAPI previous fluorescence microscopy analysis (25x objective, scalebar = 20μm). **C**, Representative FACS analysis of HEK293T cells transfected with a *Gal10*-expression plasmid. Cells were stained with anti-Gal10 (B-F42) antibodies or mouse IgG isotype, followed by incubation with anti-mouse IgG AF555 secondary antibody. **D,** Gal10 expression in 4 day-stimulated T cells within $FOXP3^-$Tconvs or $FOXP3^+$Tregs gated as in Fig.1D (n = 4), normal distribution was calculated by Shapiro-Wilk normality test with a significance level of 0.05. Statistical significance was studied by two-tailed paired *t* test. **E-J,** Effect of Gal10 crystals on $CD4^+CD25^-$ Tconv and Treg function**.** Cells were stimulated *in vitro* by co-culturing with monocyte derived-DCs in the presence of soluble anti-CD3 mAb or by anti-CD2/CD3/CD28 mAb coated beads and Gal10 crystals for 4 days. IL-2 was added to Treg cultures. **E,** Cell proliferation and **F-G,** cell viability were studied by CFSE labelling or by staining with a live/dead fixable dead cell stain kit respectively. **H,** FOXP3 expression was studied in Treg cultures. **I,** CFSE-labelled $CD4^+CD25^-$ Tconvs were co-cultured with Tregs and stimulated with allogenic DCs and anti-CD3 mAb for 4 days in the presence of Gal10 crystals at different concentrations. $CD4^+$ Tconv cell proliferation, studied as CFSE dilution is represented. Cell proliferation and FOXP3 expression levels were normalized to control condition without Gal10 crystals. Mean ± SD is represented for 5-6 independent cell donors. Normal distribution was calculated by Shapiro-Wilk normality test with a significance level of 0.05. Significance was studied by (E, F, H, I) one-way ANOVA with Sidak's multiple comparisons test or (G) Friedman test with Dunn's test for multiple comparisons. **J,** Intracellular FACS analysis of IFNγ and IL-10 expression of bead-stimulated Tregs in the presence or absence of 10μg/ml Gal10 crystals and IL-2 after 4 days (n = 4), normal distribution was calculated by Shapiro-Wilk normality test with a significance level of 0.05. Statistical significance was studied by two-tailed paired *t* test. **K-N,** Characterization of *Gal10*-siRNA knockdown (*Gal10* siRNA) versus controls (CTRL). **K,** Cell viability and **L,** FOXP3 expression studied by FACS. **M,** *Gal10* mRNA expression (Hs00171342_m1) and **N,** *IFNG* mRNA expression as assed by RT PCR, normalized according to *B2M* expression (n=4). Normal distribution was calculated by Shapiro-Wilk normality test with a significance level of 0.05. Statistical significance was studied by two-tailed paired *t* test. **O-S,** Effect of Gal10 crystal-dissolving 1D11 antibody in $CD4^+CD25^-$ Tconvs and Tregs. Cells were stimulated for 4 days *in vitro* by co-culturing with monocyte derived-DCs in the presence of soluble anti-CD3 mAb and 1D11 at different concentrations. IL-2 was added to Treg cultures. **O,** Cell proliferation and **P-Q,** cell viability were studied by CFSE or by staining with a live/dead kit respectively. **R,** FOXP3 expression in Treg cultures. **S,** CFSE-labelled $CD4^+CD25^-$ Tconvs were co-cultured with Tregs and stimulated with allogenic DCs and anti-CD3 mAb for 4 days in the presence of 1D11 at different concentrations and $CD4^+$ Tconv cell proliferation is represented. Cell proliferation and FOXP3 expression levels were normalized to control condition without 1D11. Mean ± SD is represented for 4 independent cell donors. Normal distribution was calculated by Shapiro-Wilk normality test with a significance level of 0.05. Significance was studied by one-way ANOVA with Sidak's multiple comparisons test (O, P, R, S) or (Q) Friedman test with Dunn's test for multiple comparisons.

Supplementary References

1. Farh KK, Marson A, Zhu J, et al. Genetic and epigenetic fine mapping of causal autoimmune disease variants. *Nature*. 2015;518(7539):337-343.

2. Van Zeebroeck L, Arroyo Hornero R, Côrte-Real BF, Hamad I, Meissner TB, Kleinewietfeld M. Fast and Efficient Genome Editing of Human FOXP3(+) Regulatory T Cells. *Frontiers in immunology*. 2021;12:655122.
3. Andersson J, Cromvik J, Ingelsten M, et al. Eosinophils from hematopoietic stem cell recipients suppress allogeneic T cell proliferation. *Biol Blood Marrow Transplant*. 2014;20(12):1891-1898.
4. Kubach J, Lutter P, Bopp T, et al. Human CD4+CD25+ regulatory T cells: proteome analysis identifies galectin-10 as a novel marker essential for their anergy and suppressive function. *Blood*. 2007;110(5):1550-1558.
5. Beyer M, Thabet Y, Müller RU, et al. Repression of the genome organizer SATB1 in regulatory T cells is required for suppressive function and inhibition of effector differentiation. *Nature immunology*. 2011;12(9):898-907.
6. Kleinewietfeld M, Manzel A, Titze J, et al. Sodium chloride drives autoimmune disease by the induction of pathogenic TH17 cells. *Nature*. 2013;496(7446):518-522.
7. Li D, Hsu S, Purushotham D, Sears RL, Wang T. WashU Epigenome Browser update 2019. *Nucleic Acids Res*. 2019;47(W1):W158-W165.
8. Kundaje A, Meuleman W, Ernst J, et al. Integrative analysis of 111 reference human epigenomes. *Nature*. 2015;518(7539):317-330.
9. Persson EK, Verstraete K, Heyndrickx I, et al. Protein crystallization promotes type 2 immunity and is reversible by antibody treatment. *Science*. 2019;364(6442).
10. Alissafi T, Kalafati L, Lazari M, et al. Mitochondrial Oxidative Damage Underlies Regulatory T Cell Defects in Autoimmunity. *Cell Metab*. 2020;32(4):591-604 e597.
11. Cuadrado E, van den Biggelaar M, de Kivit S, et al. Proteomic Analyses of Human Regulatory T Cells Reveal Adaptations in Signaling Pathways that Protect Cellular Identity. *Immunity*. 2018;48(5):1046-1059 e1046.
12. Pesenacker AM, Wang AY, Singh A, et al. A Regulatory T-Cell Gene Signature Is a Specific and Sensitive Biomarker to Identify Children With New-Onset Type 1 Diabetes. *Diabetes*. 2016;65(4):1031-1039.
13. Bhairavabhotla R, Kim YC, Glass DD, et al. Transcriptome profiling of human FoxP3+ regulatory T cells. *Hum Immunol*. 2016;77(2):201-213.

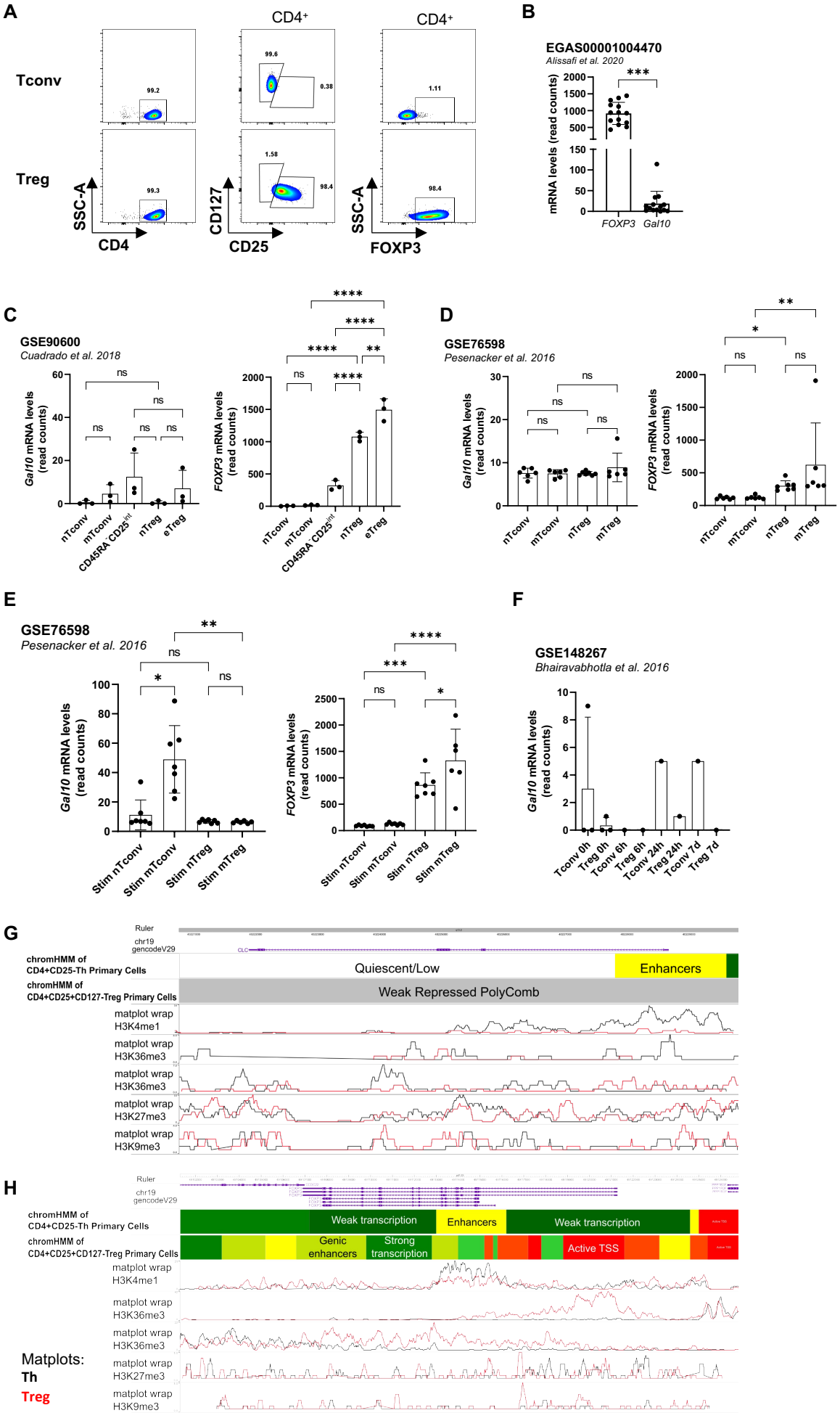

A
CD4+
CD4+
Tconv
Treg
99.2
99.6
0.38
1.11
99.3
1.58
98.4
98.4
SSC-A
CD4
CD127
CD25
SSC-A
FOXP3
B
EGAS00001004470
Alissafi et al. 2020
***
mRNA levels (read counts)
2000
1500
1000
500
150
100
50
0
FOXP3
Gal10
C
GSE90600
Cuadrado et al. 2018
Gal10 mRNA levels (read counts)
FOXP3 mRNA levels (read counts)
ns
****
**
nTconv
mTconv
CD45RA-CD25int
nTreg
eTreg
D
GSE76598
Pesenacker et al. 2016
Gal10 mRNA levels (read counts)
FOXP3 mRNA levels (read counts)
ns
*
**
nTconv
mTconv
nTreg
mTreg
E
GSE76598
Pesenacker et al. 2016
Gal10 mRNA levels (read counts)
FOXP3 mRNA levels (read counts)
Stim nTconv
Stim mTconv
Stim nTreg
Stim mTreg
F
GSE148267
Bhairavabhotla et al. 2016
Gal10 mRNA levels (read counts)
Tconv 0h
Treg 0h
Tconv 6h
Treg 6h
Tconv 24h
Treg 24h
Tconv 7d
Treg 7d
G
Ruler
chr19
gencodeV29
chromHMM of
CD4+CD25-Th Primary Cells
chromHMM of
CD4+CD25+CD127-Treg Primary Cells
Quiescent/Low
Enhancers
Weak Repressed PolyComb
matplot wrap
H3K4me1
matplot wrap
H3K36me3
matplot wrap
H3K36me3
matplot wrap
H3K27me3
matplot wrap
H3K9me3
H
Ruler
chr19
gencodeV29
chromHMM of
CD4+CD25-Th Primary Cells
chromHMM of
CD4+CD25+CD127-Treg Primary Cells
Weak transcription
Enhancers
Weak transcription
Genic enhancers
Strong transcription
Active TSS
matplot wrap
H3K4me1
matplot wrap
H3K36me3
matplot wrap
H3K36me3
Matplots:
Th
Treg
matplot wrap
H3K27me3
matplot wrap
H3K9me3

Fig. S1

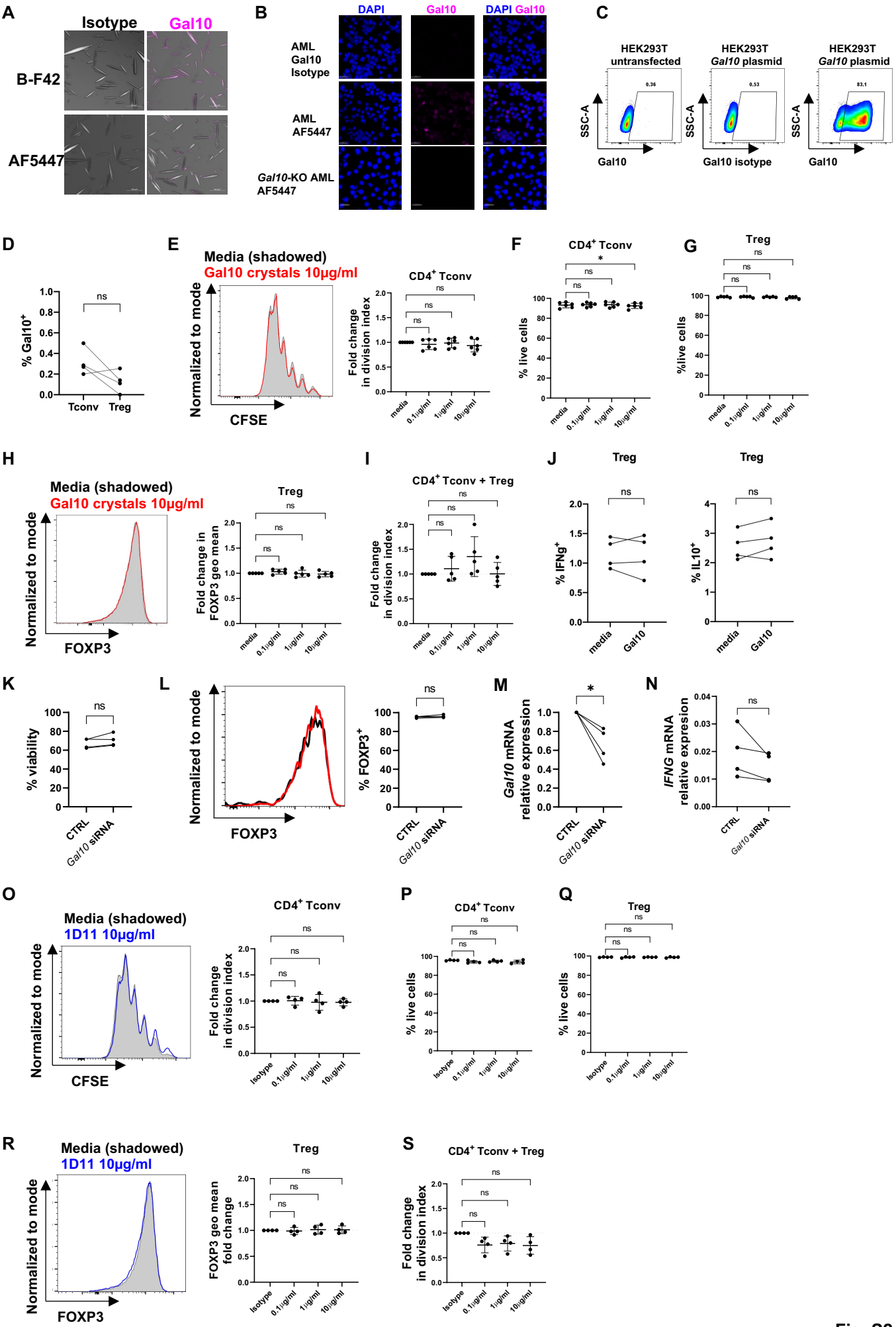

A
Isotype
Gal10
B-F42
AF5447
B
DAPI
Gal10
DAPI Gal10
AML Gal10 Isotype
AML AF5447
Gal10-KO AML AF5447
C
HEK293T untransfected
HEK293T Gal10 plasmid
HEK293T Gal10 plasmid
SSC-A
Gal10
Gal10 isotype
D
% Gal10+
Tconv
Treg
E
Media (shadowed)
Gal10 crystals 10µg/ml
Normalized to mode
CFSE
CD4+ Tconv
Fold change in division index
F
CD4+ Tconv
% live cells
G
Treg
%live cells
H
Media (shadowed)
Gal10 crystals 10µg/ml
Normalized to mode
FOXP3
Treg
Fold change in FOXP3 geo mean
I
CD4+ Tconv + Treg
Fold change in division index
J
Treg
% IFNg+
Treg
% IL10+
media
Gal10
K
% viability
CTRL
Gal10 siRNA
L
Normalized to mode
FOXP3
% FOXP3+
M
Gal10 mRNA relative expression
N
IFNG mRNA relative expresion
O
Media (shadowed)
1D11 10µg/ml
Normalized to mode
CFSE
CD4+ Tconv
Fold change in division index
P
CD4+ Tconv
% live cells
Q
Treg
% live cells
R
Media (shadowed)
1D11 10µg/ml
Normalized to mode
FOXP3
Treg
FOXP3 geo mean fold change
S
CD4+ Tconv + Treg
Fold change in division index
Isotype
0.1µg/ml
1µg/ml
10µg/ml
ns

Fig. S2