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IL-10 Receptor Signaling is essential for T_R1 cell function *in vivo*

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Abstract

Interleukin-10 (IL-10) is essential to maintain intestinal homeostasis. $CD4^+$ T regulatory type 1 (T_R1) cells produce large amounts of this cytokine and being therefore currently examined in clinical trials as T-cell therapy in patients with inflammatory bowel disease (IBD). However, factors and molecular signals sustaining T_R1 cell regulatory activity still need to be identified in order to optimize the efficiency and to ensure the safety of these trials. We investigated the role of IL-10 signaling in mature T_R1 cells *in vivo*.

Double IL-10^{eGFP} $Foxp3^{mRFP}$ reporter mice and transgenic mice with impairment in IL-10 receptor signaling were used to test the activity of T_R1 cells in a murine IBD model, a model that

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resembles the trials performed in humans. The molecular signaling was elucidated *in vitro*. Finally, we used human T_R1 cells, currently employed for cell therapy, to confirm our results.

We found that murine T_R1 cells expressed functional IL-10 receptor α. T_R1 cells with impaired IL-10 receptor signaling lost their regulatory activity *in vivo*. T_R1 cells required IL-10 receptor signaling in order to activate p38 MAP kinase, thereby sustaining IL-10 production, which ultimately mediated their suppressive activity. Finally, we confirmed these data using human T_R1 cells.

In conclusion T_R1 cell regulatory activity is dependent on IL-10 receptor signaling. These data suggest that in order to optimize T_R1 cell-based therapy, IL-10 receptor expression has to be taken into consideration.

Keywords

IBD; T cell therapy; IL-10; immune regulation

Introduction

Interleukin-10 (IL-10) plays an essential role in controlling inflammation. IL-10-deficient mice spontaneously develop inflammatory disease and, patients with mutations in either *IL-10* or *IL-10R* suffer from severe early onset colitis (1, 2). Thus IL-10 plays a fundamental role in maintaining intestinal immune homeostasis (3, 4). Resetting the physiological concentration of IL-10 in the intestine is considered the ‘Holy Grail’ for all therapies, which aim to cure inflammatory mediated diseases, especially in the intestine.

$CD4^+$ T regulatory type 1 cells (T_R1) secrete high levels of IL-10 and are known to play a major role in maintaining immune tolerance through their strong immune-regulatory activity (5–10). Due to the strong immune-regulatory potential of T_R1 cells, which has been proved in several pre-clinical mouse models (6–10), they are a major target of new approaches in the field of T cell-based therapy (11–13).

T_R1 cells are characterized by co-expression of CD49b and LAG-3, high secretion of IL-10 and the lack of Foxp3 expression (14, 15). The expression of Granzyme B and TGF-β1 also contributes to the suppressive capacity of T_R1 cells and the expression of CTLA-4 allows a cell-cell contact dependent suppression of T cells by T_R1 cells (16, 17). Chronic stimulation of $CD4^+$ T-helper cells with IL-10 is sufficient to induce *in vitro* functional mouse and human T_R1 cells. However IL-10 is dispensable to induce mouse T_R1 cells *in vivo* (18). Indeed, interleukin-27 (IL-27) was found to promote the differentiation of T_R1 cells *in vitro* and *in vivo*. Consistent with this observation, mice deficient in IL-27R ($WSX1^{-/-}$) have a strong reduction of T_R1 cells upon infection (19). IL-27 induces the differentiation of mouse T_R1 like cells by activating STAT1 and STAT3, which promotes LAG-3 expression and IL-10 production through the transcriptional factors Egr-2 and Blimp1 (encoded by *Prdm1*) (20). Other factors, such as Ahr, c-Maf, Nfil3 and ROR-α play a key role in the differentiation of T_R1 cells (20–22). Although several transcriptional factors have been shown to drive the differentiation of T_R1 cells, whether this requires one “master regulator” factor or a finely tuned network comprising several factors, still remains unknown.

On the basis of global transcriptomic analysis, it has been shown that T_R1 cells are distinct, not unlike how the other T-helper cell subsets are from each other (23, 24). Recently, their peculiar metabolic activity and their specific activity during the night/day cycle have further highlighted the distinction of T_R1 cells from T_H17 and Foxp3$^+$ Treg cells (25). As a consequence of all these differences, the protective and anti-inflammatory function of T_R1 cells is what ultimately distinguishes them from other subsets such as T_H1, T_H2 and T_H17 cells (17, 26, 27).

Several pre-clinical studies and mouse models of immune mediated disease have sustained the translation of T_R1 cell-based therapy into clinic (8, 9, 28, 29). Human T_R1 cells are being tested in clinical trials to treat autoimmune diseases, such as inflammatory bowel disease (IBD) or to limit donor vs. host-reactivity (GvHD) after hematopoietic stem cell transplantation (HSCT) (11, 13). Treatment of Crohn's patients with antigen-specific T_R1 cells showed good tolerability and high potential. Also the proof-of-concept trial with HSCT patients showed a positive outcome for patients who were treated with IL-10-anergized T cells containing T_R1 cells (11, 13, 30). A new clinical trial for kidney transplanted patients is planned (31).

Although T_R1 cell-based therapies are ongoing, it is unknown which molecular signal maintains T_R1 cell regulatory activity: a basic biological aspect which is fundamental to design successful therapies. IL-10, the signature cytokine produced by T_R1 cells, has been shown to regulate Foxp3$^+$ Treg cells (Tregs) by maintaining their regulatory function (32). However, it is still unclear whether T_R1 cells also express IL-10R and whether IL-10 signaling is responsible for maintaining the functional stability of these cells.

The best-studied IL-10 signaling pathway is the activation of JAK1 and STAT3(33). PI3 kinases as well as p38 MAP kinase pathways can also act downstream of IL-10 by binding to the activated IL-10 receptor complex (34, 35). Whether mature T_R1 cells respond to IL-10, and if so, through which signaling pathway, is currently unknown.

The aim of our study was to analyze the role of IL-10 signaling for the anti-inflammatory activity of mature mouse and human T_R1 cells. Our data show that IL-10 plays a key role in sustaining the function of murine and human T_R1 cells. Mouse T_R1 cells with impaired IL-10 signaling are unstable and lose their regulatory activity *in vivo* in a T cell transfer mediated IBD model (13). Finally, *in vitro* induced human T_R1 cells, which can potentially be employed in a cell therapy approach, do also require IL-10 receptor signaling to maintain IL-10 production.

Materials and Methods

Mice

C57BL/6, C57BL/6 *Rag1*$^{-/-}$, and C57BL/6 *Rag1*$^{-/-}$ CD45.1$^+$ were obtained from the Jackson Laboratory. CD4-DNIL-10R transgenic mice, Foxp3^{RFP}, IL-17A^{eGFP}, and IL-10^{eGFP} reporter mice are described elsewhere (26, 36–39). Age and sex matched littermates between 8–16 weeks of age were used.

Flow cytometry

Anti-CD4, anti-CD62L, anti-CD44, anti-CD45.1, anti-CD45.2, anti-CD45RB, anti-TCR-β, anti-IL-10Rα (clone: 1B1.3a, PE) and isotype control (rat IgG1,K, PE) were purchased from BioLegend. Anti-STAT3 (pY705) and anti-pp38 MAPK were purchased from BD Biosciences. To identify dead cells, 7-AAD (Biolegend) staining was performed.

Anti-human anti-CD4, anti-CD45RA and anti-CD49b (clone: P1E6-C5) were purchased from BioLegend. Anti-LAG-3 was purchased from eBioscience (clone: 3DS223H). The staining for LAG-3 and CD49b was performed at 37°C for 30 min.

For intracellular pSTAT3 and pp38 MAPK staining, cells were fixed with PhosFlow Lyse/Fix Buffer (BD Bioscience) for 10 min at 37°C and permeabilized with Perm Buffer III (BD Bioscience) for 30 min on ice. The cells were stained for pSTAT3 or pp38 MAPK and extracellular markers for 1 hour at room temperature before they were acquired on a LSRII flow cytometer (BD Bioscience).

In vitro T_R1 cells and T_H17 cells differentiation

$CD4^+$ T cells were enriched from splenocytes of IL-10^{eGFP} $Foxp3^{RFP}$ double reporter mice with CD4-microbeads using MACS (Miltenyi Biotec). For naïve T cell enrichment, $CD44^+$ and $CD25^+$ T cells were depleted using biotinylated antibodies and Streptavidin beads (Miltenyi Biotec). T_R1 cell differentiation: naive T cells were cultured for 5 days at a density of 10^6 cells/ml with plate-bound anti-CD3 (2 μg/ml) and soluble anti-CD28 (2 μg/ml) in medium (Click's medium supplemented with 10% FCS, l-glutamine, penicillin, streptomycin and β-Mercaptoethanol) under T_R1-inducing conditions (0.5 ng/ml TGF-β1, 30 ng/ml IL-27). IL-10 (eGFP) and Foxp3 (mRFP) expression were determined by flow cytometry. T_H17 cell differentiation: naïve T cells were cultured for 5 days at a density of 10^6 cells/ml with soluble anti-CD3 (3 μg/ml) and soluble anti-CD28 (1 μg/ml) in the presence of irradiated APCs (ratio 1:4) in medium (Click's medium supplemented with 10% FCS, l-glutamine, penicillin, streptomycin and β-Mercaptoethanol) under T_H17 polarizing conditions (0.5 ng/ml TGF-β1, 10 ng/ml IL-6, 20 ng/ml IL-23, 10 ng/ml IL-1β). IL-17A (eGFP) expression was determined by flow cytometry.

In vitro suppression assay

Responder T cells were isolated from C57Bl/6 mice and labelled with 5 μM violet dye. The cells were activated in the presence of irradiated APCs and 1.5 μg/ml anti-CD3 antibody and cultured either alone or in the presence of IL-$10R\alpha^{WT}$ or IL-$10R\alpha^{Impaired}$ T_R1 cells at a 1:2 (T_R1:Responder) ratio. After 72 hours the proliferation of the responder T cells was measured via flow cytometry.

In vitro kinase inhibition

SB203580, PD98059, JNK inhibitor II or STAT3 inhibitor VI in DMSO were added to the culture medium in the indicated concentrations every 24 hours (Calbiochem, Darmstadt, Germany). DMSO was added to control cultures at equivalent concentrations.

In vitro IL-10 receptor blocking

In vitro differentiated wild type T_R1 cells were re-stimulated (CD3/CD28 antibodies) in the presence of 50 μg/ml IL-10R antibody (clone: 1B1) or isotype control antibody (rat IgG1,K).

In vivo T cell stimulation and Intestinal lymphocyte isolation

Mice were injected with anti-CD3 (clone 2C11, 15μg,) i.p. two times every other day, and sacrificed 4 hours or 48 hours after the second injection. As controls, mice were injected with isotype-matched antibody or PBS. We collected intraepithelial lymphocytes (IEL) after the dissection of the peyer's patches by incubating the small intestine in the presence of 5mM EDTA at 37°C for 30 min. Lamina propria lymphocytes (LPL) were collected by digesting gut tissue with collagenase IV (100 U, Sigma) at 37°C for 45 min. The cells were further separated with a Percoll gradient (GE Healthcare).

Adoptive Transfer models

Splenocytes were collected from 8 to 12 week old IL-17A^{eGFP} reporter mice (CD45.1/2) and $CD4^+$ T cells were enriched by MACS system (Miltenyi Biotec). The $CD4^+$ T cells were further sorted to collect CD45RB^{hi} Foxp3^{RFP-} cells using FACS Aria II. 1.5×10^5 CD45RB^{hi} cells were i.p. injected either alone or together with *in vitro* differentiated 1.5×10^5 wild type or IL-10R^{Impaired} T_R1 cells into *Rag1*$^{-/-}$ mice. For generating highly pathogenic T_H17 cells 4×10^5 CD45RB^{hi} cells were injected i.p. into *Rag1*$^{-/-}$ mice (CD45.1). The mice were weighed once a week to monitor IBD development. After establishment of colitis as determined by endoscopy, mice were sacrificed and lymphocytes were isolated from the colon and mesenteric lymph nodes. The cells were further FACS-sorted to purify IL-17A^{eGFP+} T cells (eT_H17 cells). (e)T_H17 cells (3×10^4) were transferred, and isolated WT or Tg T_R1 cells (3×10^4) from the small intestine of anti-CD3 treated IL-10^{eGFP} reporter mice were transferred either alone or with (e)T_H17 cells into CD45.1 *Rag1*$^{-/-}$ mice. Colitis development was monitored using endoscopy.

Endoscopic procedure

We performed colonoscopy in a blinded fashion for colitis scoring using the Coloview system (Karl Storz, Germany) (40). Colitis scoring was based on the granularity of the mucosal surface, stool consistency, vascular pattern, translucency of the colon and number of fibrin visible (0–3 points for each).

Histopathology procedure

Colons were fixed in Bouin's fixative solution or 4% PFA in PBS and embedded in paraffin. HE stained sections were evaluated by a semi quantitative criterion-based method (score 0–5) as described before (41).

Cytokine assay

We stimulated 4×10^4 mouse T cells/ml for 60 hours with plate-bound CD3 antibodies (10 μg/ml) and soluble CD28 antibodies (10 μg/ml) in medium (Click's medium supplemented with 10% FCS, l-glutamine, penicillin, streptomycin and β-Mercaptoethanol). Cytokines

were quantified by Cytometric Bead Array (mouse $T_H1/T_H2/T_H17$ Cytokine Kit, BD Bioscience) following the manufacturer's instructions.

We stimulated 3×10^4 human T cells/200 μl for 96 hours with CD3/CD28 beads (Dynabeads Human T-Activator CD3/CD28 for T Cell Expansion and Activation, Life technologies) in a 1:1 ratio in medium (Click's medium supplemented with 10% FCS, l-glutamine, penicillin, streptomycin and β-Mercaptoethanol). As indicated, 50 μg/ml human IL-10 Rα Antibody (R&D Systems) were added to the cell culture. Cytokines were quantified by Legendplex Assay (Human T helper Cytokine Panel, BioLegend).

Relative gene expression analysis

RNA from cells was isolated with TRIzol LS reagent (Life Technologies) in accordance with the manufacturer's protocol. RNA was subjected to reverse transcription with Superscript II (Invitrogen) with oligo (dT) primer in accordance with the manufacturer's protocol. cDNA was semi-quantified using commercially available primer/probe sets (Applied Biosystems) and analyzed with the ΔCt (change in cycle threshold) method. All results were normalized to *Hprt* quantified in parallel amplification reactions during each PCR quantification.

Western Blot

To analyze STAT3 activation, total cell lysates of indicated cell populations were separated in a 10% Sodium Dodecyl Sulphate Polyacrylamide Gel electrophoresis (SDS-PAGE) assay, transferred to PVDF membranes (Merck Millipore), probed with anti-phospho-Stat3 (Tyr705) or STAT3 antibody (Cell signaling), followed by incubation with the appropriate HRP-conjugated secondary antibodies (Dako) and were visualized with the enhanced chemiluminescence substrate (Merck Millipore).

Immunofluorescence

In brief, cells were fixed for 10 min in 4% PFA at RT. Cells were washed with PBS and incubated in PBS-Triton 0.3% for 5 min. After washing they were incubated for 60 min in blocking buffer. Samples were stained overnight with biotinylated IL-10Rα antibody (primary rat anti-mouse antibody, 1:100, clone: 1B1.3a, BioLegend) at 4 °C. After washing secondary antibody (Alexa Fluor 568 goat anti-rat IgG, Invitrogen) staining was performed (1 hour, RT) followed by 5 min staining with Hoechst 33258 (1:5000). For isotype control, the primary antibody was omitted.

Isolation of circulating human T_R1 cells

PBMC were isolated from buffy coats of healthy donors using the Biocoll Separating Solution (Biochrome AG). $CD4^+$ T cells were enriched with the Human $CD4^+$ T Cell Enrichment Kit in accordance with the manufacture's protocol (Stemcell Technologies). The $CD4^+$ T cells were further sorted to collect $CD4^+$ $CD45RA^{low}$ LAG-3^+ CD49b^+ T_R1 cells using FACS Aria II.

Generation of human T_R1 cells *in vitro*

T_R1 cells were generated *in vitro* as previously described (42). Briefly, naïve $CD4^+$ cells were cultured for 10 days with tolerogenic DC-10 in an allogeneic setting, in the presence of

exogenous rhIL-10 to produce a T_R1-enriched product (T_R1 cells) or with mDC in the absence of exogenous rhIL-10 as control (non-T_R1 cells).

ELISPOT

Dual IFNγ/IL-10 ELISPOT (Diaclone, Besancon, France) was performed according to manufacturer's instructions, with slight modifications. Briefly, the filter-bottom plate was wetted then coated with both anti-IFNγ and anti-IL-10 capture. T_R1 and non-T_R1 cells were plated after thawing and overnight resting in serum-free X-VIVO15 (tissue-culture grade, Lonza). Stimulation was performed with aCD3 (2μg/ml)/TPA (20ng/ml) or with allogeneic mDC in the presence of rat anti-human IL-10R blocking antibody (50μg/ml) or its isotype control (BD Biosciences). After a 48-hour incubation, detection antibodies were added then visualization was performed with AEC and Vector Blue (Vector Labs). The plate was acquired on an A.EL.VIS 4-Plate ELISPOT Reader (A.EL.VIS GmbH). Analysis was performed using ImageJ (version 1.48) to quantify IFNγ-producing cells (red spots) and IL-10-producing cells (blue spots).

Microarray analysis

Gene expression analysis—Affymetrix Mouse Gene 1.0ST Array data were analyzed using R and the Bioconductor package "affy". Samples were normalized by RMA with standard parameters. To define genes functionally related to mouse T_R1 signature genes, genes interacting with their human orthologs with an interaction probability of at least 80% were extracted from the Immunet database (43) downloaded on May 11, 2016. Mouse orthologs were then found for these functionally related genes. Data are available at NCBI GEO (https://www.ncbi.nlm.nih.gov/geo/ Accession number: GSE89080).

Gene Ontology analysis—Gene Ontology (GO) term analysis was performed using GOrilla (44). GO terms enriched for T_R1 signature genes (p-Value threshold 0.001) were exported. Any genes >2-fold differentially expressed also mapping to these terms were retrieved using Biomart (http:\\www.biomart.org). Enrichment of these genes in the GO term category was calculated as described in (44). Log2-values of enrichment were calculated to better visualize the spread of the data.

Statistics

The Mann–Whitney U test, paired t test or one-way ANOVA (post-test Tukey) were used to calculate statistical significance. A p-value < 0.05 was considered significant. Statistical calculations were performed using Prism program 5.0 (GraphPad Software, Inc.)

Study approval

All experiments involving animals were carried out in accordance with the Institutional Animal Care and Use Committee of Yale University or in accordance with the Institutional Review Board 'Behörde für Soziales, Familie, Gesundheit und Verbraucherschutz' (Hamburg, Germany).

Results

Intestinal T_R1 cells express IL-10Rα and can respond to IL-10

We used Foxp3^{RFP} IL-10^{eGFP} double reporter mice and a model of anti-CD3-specific antibody mediated transient intestinal inflammation to test whether a pure population of intestinal T_R1 cells can respond to IL-10 (36, 37). This murine model has already been largely validated by others and by us as being capable of inducing CD4$^+$ Foxp3^{RFP-} IL-10^{eGFP+} CD49b$^+$ LAG-3$^+$ *bona fide* T_R1 cells in the small intestine (26). First, we found that *ex vivo* isolated T_R1 cells express IL-10Rα at a comparable level to intestinal Foxp3$^+$ pTreg cells and levels about 6 times higher than splenic naïve T cells (Fig. 1A and 1B).

We then wanted to test whether IL-10Rα was functional on T_R1 cells. We therefore checked the level of phosphorylated STAT3 (45), the main downstream molecule of the IL-10R signaling, in intestinal T_R1 cells upon *in vitro* stimulation with IL-10. (45).

By testing STAT3 phosphorylation at different times and in response to different doses of IL-10 we found that intestinal T_R1 cells respond to IL-10 (Fig.1C and 1D). As controls, splenic naïve T cells, in which IL-10R was very low, did not show an elevated level of pSTAT3 after stimulation with IL-10 (Fig. 1C), whereas Foxp3$^+$ Treg cells responded to IL-10 with a distinct increase of pSTAT3 level (Fig. 1C). To further strengthen our data, we used CD4-DN-IL10R transgenic (IL-10R^{Impaired}) T_R1 cells, which overexpress a dominant-negative IL10Rα chain and therefore have largely impaired IL-10 signaling (37). We detected that these IL-10R^{Impaired} T_R1 cells only slightly up-regulated pSTAT3 upon IL-10 stimulation (Fig.1C and 1D). STAT3 can also be phosphorylated by pro-inflammatory cytokine IL-6. As a control, to confirm that STAT3 activation in CD4-DN-IL10R transgenic T_R1 cells is not impaired *per se*, we stimulated wild type (WT) and IL-10R^{Impaired} T_R1 cells with IL-6. In contrast to what we observed with IL-10 stimulation, the activation of pSTAT3 by IL-6 was normal in IL-10R^{Impaired} T_R1 cells (Fig. 1D).

Finally, we wanted to test whether the capacity of T_R1 cells to respond to IL-10 is restricted to the mouse model used or if it is a more general feature of T_R1 cells. We therefore used the standard protocol (i.e. IL-27+TGF-β1) (21, 46) to induce *bona fide* T_R1 cells *in vitro*. Initially, we confirmed IL-10Rα expression on *in vitro* differentiated wild type and IL-10R^{Impaired} T_R1 cells using Immunofluorescence (Fig. S1A). Next, we FACS-sorted a pure population of differentiated T_R1 cells and stimulated them at different times and with different doses of IL-10 and measured STAT3 phosphorylation via FACS and western blot analysis. As for the *in vivo* induced intestinal T_R1 cells, we observed that also mature T_R1 cells induced *in vitro* respond to IL-10 by activating STAT3 (Fig. S1B–S1E).

Altogether these data show that *in vivo* and *in vitro* differentiated murine T_R1 cells express functional IL-10Rα and are, in principle, able to respond to IL-10 stimulation.

No phenotypically and early functional defect among wild type and DN-IL10R transgenic T_R1 cells

The above results show that T_R1 cells express functional IL-10R. However, what exactly the role of this receptor is in T_R1 cell biology remained elusive. To investigate this, wild type

(WT) and CD4-DN-IL10R transgenic (IL-10R^{Impaired}) mice were treated with anti-CD3 mAb. Shortly after the second injection, the induction of intestinal T_R1 cells was evaluated without any *in vitro* manipulation due to the use of the Foxp3^{RFP} IL-10^{eGFP} double reporters. Surprisingly, we did not observe any changes in the frequency and number of intestinal T_R1 cells between WT and CD4-DN-IL10R transgenic mice upon treatment (Fig. 2A).

We further analyzed the surface markers, which typically characterize T_R1 cells. The FACS analysis on WT and IL-10R^{Impaired} T_R1 cells showed similar levels of CD49b and LAG-3 expression (Fig. 2A). To further broaden our data, we performed a microarray analysis of IL-10R^{WT} T_R1 and IL-10R^{Impaired} T_R1 cells. The gene expression profiles between IL-10R^{WT} T_R1 and IL-10R^{Impaired} T_R1 cells were largely similar (Pearson correlation coefficient r=0.989). For all transcriptional factors, extracellular receptor and cytokines known to be crucial for T_R1 cell differentiation and function (T_R1 signature genes) such as *Prdm1*, *Maf* and *Ahr*, as well as *Gzmb*, *Tgfb1* and *Ctla4* mRNA expression was unaffected in WT T_R1 cells compared to IL-10R^{Impaired} T_R1 cells (Fig. 2B). The similar expression of some of these genes was also confirmed by conventional mRNA analysis (Fig. 2C). We further expanded our analysis to those genes that were found to be functionally related to the T_R1 signature genes (related genes) based on the ImmuNet Data Base (43): none of those genes were more than two fold differentially expressed among the two genotypes. Although most genes were similarly expressed, we found 300 genes higher than two fold differentially expressed among IL-10R^{Impaired} T_R1 cells compared to wild type T_R1 cells (NCBI GEO https://www.ncbi.nlm.nih.gov/geo/ Accession number: GSE89080). We therefore asked whether any of these genes are functionally related to the genes known to be involved in T_R1 cell differentiation. A Gene Ontology (GO) analysis revealed 379 GO terms enriched for T_R1 signature genes. Within these GO categories, enrichment of T_R1 signature genes was consistently higher than enrichment of the genes more than two fold differentially expressed between IL-10R^{Impaired} T_R1 cells and wild type T_R1 cells (Fig. S2). Based on the current knowledge about T_R1 cells, we could not reveal any significant defect of IL-10R^{Impaired} T_R1 cells. However we cannot exclude that among the differentially expressed genes are some genes, which could affect T_R1 cells. Thus we tested the functional activity of WT and IL-10R^{Impaired} T_R1 cells isolated shortly after the *in vivo* induction using anti-CD3 antibody treatment to further exclude a defect of IL-10R^{Impaired} T_R1 cells. The *in vitro* suppressive assay showed that both WT and IL-10R^{Impaired} T_R1 cells have similar suppressive capacities (Fig. 2D). These data indicate that shortly after T_R1 cell induction, IL-10R^{Impaired} T_R1 cells do not show any obvious defect compared to WT T_R1 cells, suggesting that IL-10 signaling is not essential for T_R1 cell differentiation.

IL-10 signaling in T_R1 cells maintains their suppressive function in vivo in a murine IBD model

Although IL-10R signaling is dispensable for acquiring a T_R1 like phenotype and *in vitro* suppressive function, we wondered whether IL-10R signaling influences the long-term functional stability of T_R1 cells, as is the case with Foxp3$^+$ Treg cells (32, 47). We tested the functionality of IL-10R^{Impaired} T_R1 cells and wild type T_R1 in the CD45RB^{hi} T cells transfer colitis model. Transfer of CD4$^+$Foxp3$^-$CD45RB^{hi} cells caused severe disease that could be

prevented by the co-transfer of *in vitro* differentiated wild type T_R1 cells. IL-10R^{Impaired} T_R1 cells showed a significantly reduced regulatory activity compared to wild type T_R1 cells. Interestingly, we observed that IL-10R^{Impaired} T_R1 cells still have the ability to reduce the colitis caused by the transfer of CD4$^+$Foxp3$^-$CD45RB^{hi} T cells into *Rag1*$^{-/-}$ by trend (Fig. S3). Nevertheless, in patients suffering from Crohn's disease a possible T_R1-based cell therapy is required to not only suppress the development of naïve T cells into pathogenic effector T cells, but to control already differentiated cells. Thus, we wanted to test IL-10R^{Impaired} T_R1 cells and wild type T_R1 in the more challenging model of T_H17 cell induced colitis (26). CD4$^+$ IL-17A^{eGFP+} effector (e)T_H17 cells were generated using the CD45RB^{hi} transfer colitis model and isolated from the intestine and mesenteric lymph nodes of diseased mice. The (e)T_H17 cells were then co-transferred into *Rag1*$^{-/-}$ recipients together with wild type T_R1 cells or IL-10R^{Impaired} T_R1 cells. Transfer of (e)T_H17 cells caused severe disease, characterized by histological and endoscopic findings of colitis and weight loss. Transfer of wild type T_R1 cells prevented the development of colitis mediated by (e)T_H17 cells. In contrast, IL-10R^{Impaired} T_R1 cells failed to block colitis mediated by (e)T_H17 cells (Fig. 3A). These recipient mice showed similar weight loss, endoscopic and histological colitis score to the experimental group of mice that received (e)T_H17 cells alone (Fig. 3B and 3C). Adoptive transfer of IL-10R^{Impaired} T_R1 cells alone did not cause colitis, nor did the transfer of wild type T_R1 cells alone. The recipient mice showed no signs of weight loss (Fig. 3A) or colitis as evaluated by endoscopic and histological colitis score (Fig. 3B and 3C).

In summary, wild type, but not T_R1 cells with impaired IL-10 signaling, could suppress colitis development. However, 10R^{Impaired} T_R1 did not aggravate disease caused by (e)T_H17. Furthermore, transfer of T_R1 cells with impaired IL-10 signaling alone did not cause disease, backing up the argument for the safety of T_R1 cells in human trials.

IL-10 receptor signaling in T_R1 cells maintains IL-10 production

Although IL-10 receptor is dispensable for the development of T_R1 cells, this receptor becomes fundamental to maintain the functional activity of T_R1 cells *in vivo*.

However, the reason why T_R1 cells require IL-10 receptor signaling to maintain their functional activity remained unresolved. Knowing that other CD4$^+$ T cells, such as T_H17, T_H2 and Foxp3$^+$ Treg cells are required to respond to their own cytokines to maintain their functional activity, we wondered if T_R1 cells are also required to respond to IL-10 to maintain the production of IL-10, which mediates their suppressive activity.

To test this, we adoptively transferred *in vivo* generated wild type and IL-10R^{Impaired} T_R1 cells, which had been isolated from the small intestine of Foxp3^{RFP} IL-10^{eGFP} double reporter mice, into congenic *Rag1*$^{-/-}$ mice. Five weeks later, transferred cells were isolated from different organs and IL-10^{eGFP} expression was analyzed. Approximately 45% of the previously transferred wild type T_R1 cells isolated from the colon or mesenteric lymph nodes still expressed IL-10. However, only 10% of the previously transferred CD4-DN-IL10R transgenic T_R1 cells still demonstrated IL-10 production (Fig. 4A). To balance out the bias resulting from the transfer of T_R1 cells into immune deficient mice, we continued testing this aspect. However, this time we used an *in vitro* reductionist approach to

investigate whether intestinal T_R1 cells require IL-10 receptor signaling to sustain the expression of IL-10 upon *in vitro* reactivation. In line with the *in vivo* experiments, *in vitro* re-stimulated IL-10R^{Impaired} T_R1 cells produce significantly lower amounts of IL-10 as compared to wild type T_R1 cells. As expected based on our previous results which show that IL-10 suppresses IL-17A but not IFN-γ production by T cells *in vivo* (26), we observed *in vitro* that the production of IFN-γ was similar, while IL-10R^{impaired} T cells produced more IL-17A (Fig. 4B). Notably, the mRNA level of *Tgfb1*, *Ctla4* and *Gzmb*, which are involved in the suppressive function of T_R1 cells, were not altered in IL-10R^{Impaired} T_R1 cells compared to WT T_R1 cells (Fig. 4C). Altogether, these data suggest that IL-10 is fundamental to sustain IL-10 production in T_R1 cells over time.

When cells are not synchronized *in vivo*, it is difficult to clearly dissect early IL-10 receptor related biological effects occurring during the cell differentiation phase from later effects happening when cells have already acquired their phenotype. We had to find a solution when faced with this technical limitation, so once more we took advantage of a better controllable *in vitro* experimental setting. We therefore differentiated T_R1 cells starting with naïve wild type and IL-10R^{Impaired} T cells in the presence of IL-27 and TGF-β1. In line with the *in vivo* experiment, we did not observe any difference after the first 5 days of T_R1 differentiation, even when IL-10 was added to the culture (Fig. 5A). Then, we FACS-sorted the T_R1 cells and monitored IL-10 expression over time after *in vitro* poly-clonal TCR stimulation. To exclude that the observed defect in IL-10 production by IL-10R^{Impaired} T_R1 cells was caused by side effects due to the over expression of a dominant negative IL-10Rα, we included an additional control. We re-stimulated wild type T_R1 cells either in the presence of a blocking IL-10R antibody or isotype control. Thus, we were able to assess the influence of IL-10 signaling on the IL-10 production of mature T_R1 cells starting from the same pool of cells. The resulting data confirmed our *in vivo* findings that IL-10R^{Impaired} T_R1 cells showed a faster decrease of IL-10 expression over time compared to wild type T_R1 cells (Fig. 5B). Likewise, T_R1 cells re-stimulated in the presence of blocking IL-10R antibody demonstrated a strong decrease of IL-10 expression over time compared to T_R1 cells in the presence of an isotype antibody (Fig. 5B).

IL-10 maintains IL-10 production in T_R1 cells via activation of p38 MAP kinase

It has previously been shown that IL-10 production by Foxp3$^+$ Treg cells is controlled in a STAT3 dependent manner following IL-10R signaling. Furthermore, induction of IL-10 during *in vitro* differentiation of T_R1 cells with IL-27 also depends on STAT3 activation (48, 49). However, p38 MAP kinase signaling has also been linked to IL-10 production in cell types other than T cells, such as human monocytes and macrophages, and it has also been linked to the regulatory function of iTreg cells (50–53). To address the role of STAT3 and p38 MAP kinase in maintaining IL-10 production in T_R1 cells, we compared the phosphorylation status of STAT3 and p38 MAP kinase in a pure population of *in vitro* induced WT and IL-10R^{Impaired} T_R1 cells over time upon re-stimulation.

We could not observe a reduction of pSTAT3 in IL-10R^{Impaired} T_R1 cells compared to WT T_R1 cells over time (Fig. 6A). In contrast, phosphorylation of p38 MAP kinase was reduced by trend in IL-10R^{Impaired} T_R1 cells compared to WT T_R1 cells (Fig. 6A). These results

suggested a possible correlation between IL-10 production and the activation level of p38 MAP kinase in T_R1 cells. To further test the functional role of STAT3 and p38 MAP kinase signaling in T_R1 cells, kinase inhibitors were used. As a control, we also tested inhibitors of other major MAPK pathways, ERK1/2 and JNK, since these kinases have also been linked to IL-10 expression in other immune cell types, such as T_H1 and T_H2 cells or human monocytes and macrophages (54–56). Wild type T_R1 cells were differentiated *in vitro* from $CD4^+$ T cells, isolated from $Foxp3^{RFP}$ $IL10^{eGFP}$ double reporter mice, and re-activated for 48 hours. To test the role of p38 MAP kinase signaling in the stability of T_R1 cells, a specific p38 inhibitor (SB203580), which has previously been shown by others and us to selectively block p38 MAP kinase signaling in T cells (57, 58), was added during the re-activation (Fig. 6B). The frequency of $IL\text{-}10^{eGFP+}$ cells was tested. Both STAT3 and JNK inhibitors slightly reduced the frequency of $IL\text{-}10^{eGFP+}$. However, high concentrations of STAT3 or JNK inhibitor also decrease the viability of T_R1 cells (data not shown). Inhibition of ERK1/2 activation did not have any effect on the IL-10 production of T_R1 cells. Strikingly, inhibition of p38 MAP kinase resulted in a significant and strong reduction in the frequency of $IL\text{-}10^{eGFP+}$ in a dose dependent manner (Fig. 6B). p38 MAP kinase inhibitor neither compromised viability of the cells nor resulted in overgrowth of $IL\text{-}10^{eGFP-}$ cells in culture (Fig. S4A). Moreover, in line with our previous *in vivo* data, *in vitro* differentiated wild type T_R1 cells, re-activated in the presence of p38 MAP kinase inhibitor, did not show significant changes in the expression of *Gzmb*, *Tgfb1* and *Ctla4* genes (Fig. 6C).

STAT3 has been previously reported to be important during the differentiation of T_R1 cells in the presence of IL-27 (59, 60). We therefore tested the effect of STAT3 on the differentiation of T_R1 cells from naïve T cells. As expected, the addition of STAT3 inhibitor blocked T_R1 cell differentiation demonstrating the activity of this compound (Fig S4B). We also tested p38 MAP kinase inhibitors and we observed that this compound also blocks the differentiation of T_R1 cells almost completely, whereas inhibition of ERK1/2 or JNK did not affect the differentiation of T_R1 cells *in vitro*. (Figure S4B).

In summary, our findings suggest that IL-27 initiates the differentiation of naïve $CD4^+$ T cells into T_R1 cells through STAT3 and p38 MAP kinase activation. In addition, differentiated T_R1 cells, which express functional IL-10R, are required to respond to IL-10 in order to maintain p38 MAP kinase activation and in turn, sustain IL-10 production.

IL-10 maintains IL-10 production in human T_R1 cells

Human T_R1 cells are of great interest for regulatory T cell-based immunotherapy, because of their strong potential to induce immune homeostasis (14, 61, 62). Thus, we aimed to investigate the role of IL-10 signaling in mature human T_R1 cells. Using CD49b and LAG-3 as markers, we isolated circulating human T_R1 cells from PBMCs of healthy donors (Fig. 7A). We re-stimulated the isolated T_R1 cells *in vitro* together with IL-10Rα blocking antibody (or isotype) and measured cytokine release. In line with the data obtained with mouse T_R1 cells, we did not observe differences in the production of IFN-γ, IL-4 or IL-6 between T_R1 cells upon blocking IL10R compared to control. IL-10 production of human T_R1 cells was however decreased by more than 30% in the presence of IL-10Rα blocking antibody, highlighting the importance of IL-10 signaling to maintain the production of IL-10

(Fig. 7B and data not shown). In line with our murine data, we saw no significant difference in the mRNA expression of *GZMB*, *TGFB1* and *CTLA4* between re-stimulated T_R1 cells in the presence or absence of IL-10Rα blocking antibody (Fig. 7C).

Next, we used IL-10-anergized T cells containing T_R1 cells that were generated *in vitro* with tolerogenic dendritic cells (DC-10) (42). This is the most efficient protocol to induce human T_R1 cells which are going to be used in the upcoming clinical trials (63). We observed that differentiated T_R1 cells re-activated in the presence of IL-10Rα blocking antibody showed reduced numbers of IL-10 producer cells compared to isotype treated cells. Of note, the effect we observed was independent of the activation used: both polyclonal and allogeneic mDC stimulation in the presence of IL-10Rα blocking antibody led to a reduction of IL-10-producing cells (Fig. 7D), confirming our results obtained with circulating human T_R1 cells regarding the IL-10 production. Interestingly, blockade of IL-10 signaling led to increased IFN-γ production in the pool of IL-10-anergized T cells. This difference might be due to the presence of non-T_R1 cells in the IL-10-anergized T cell pool, which might be affected differently by the blocking of IL-10 signaling. $CD4^+$ T cells generated *in vitro* in the absence of IL-10 (non-T_R1 cells) were also tested and they did not show an increased IFN-γ production and only a slight reduction in IL-10 production upon blockage of IL-10 signaling (Fig. 7D).

In summary, IL-10 signaling is also essential to maintain IL-10 production by freshly isolated and *in vitro* induced human T_R1 cells.

Discussion

Chronic TCR stimulation in the presence of IL-10 has been shown to be sufficient to induce highly regulatory mouse and human T_R1 cells (29, 64). However, findings by Maynard *et al.* (2007) demonstrated that in a totally IL-10 deficient mouse, T_R1 cells were still present in the intestine (18). Our study allowed us to test the direct effect of IL-10 on intestinal T_R1 cells and therefore exclude possible extrinsic effects and side effects related to the use of a totally IL-10 deficient mouse model.

We found that IL-10 signaling is not essential to induce T_R1 cells *in vivo*. Of note, the over expression of a dominant negative IL-10Rα suppressed IL-10 mediated STAT3 activation. Our data suggest that the differentiation of T_R1 cells is depended on STAT3 activation, but independent of IL-10 signaling. However, the activation of STAT3 by other factors, such as IL-27 and IL-6 is not affected in transgenic mice with impaired IL-10 signaling, which explains this finding. Furthermore, it is widely accepted that IL-27 is sufficient to induce T_R1 cells *in vivo* (46, 48, 59, 60). Our data further support this as they show that T_R1 cells with a strongly impaired IL-10 signaling do not show any obvious defects shortly after induction. However, we showed that mature T_R1 cells can respond to IL-10. More importantly IL-10 signaling is crucial for their function: By responding to IL-10, T_R1 cells maintain the production of IL-10. This allows them to preserve their regulatory activity. When mouse intestinal T_R1 cells do not respond to IL-10, they lose IL-10 production and therefore their suppressive function. Accordingly, IL-10 signaling in T_R1 cells was crucial to maintain their high level of potential to prevent $CD4^+Foxp3^-CD45RB^{hi}$ T cell and T_H17 cell

mediated colitis. Interestingly, we observed that IL-10R^{Impaired} T_R1 cells still have the ability to reduce the colitis caused by CD45RB^{hi} T cells by trend. In contrast IL-10R^{Impaired} T_R1 cells were not able to improve colitis induced by the transfer of T_H17 cells. This difference could be due to the different disease severity in the two colitis models or the different T helper cell composition, since the CD45RB^{hi} colitis model is dominated by T_H1 and T_H17 cells. Thus, it would be possible that IL-10 signaling in T_R1 cells is more important to control T_H17 cells than T_H1 cells like it has been proposed for Foxp3 Treg (32). Taken together these studies with our current findings suggests that Foxp3$^+$ Tregs and T_R1 cells need to respond to IL-10 to sustain IL-10 production, which is then essential to directly control T_H17 cells. Nevertheless, we cannot exclude that other T_R1 associated suppressive mechanisms, such as the secretion of Granzyme B or TGF-β, are more important to suppress other cell types.

T_R1 cells can exert their regulatory function through several mechanisms (16, 17, 65). Among these, we found that T_R1 cells that do not respond to IL-10 lose their IL-10 production and their capacity to suppress T_H17 mediated colitis, but still express normal levels of, Granzyme B and CTLA-4. All of these molecules have been considered as being involved in alternative regulatory mechanisms of T_R1 cells (29, 66, 67). Nevertheless IL-10 seems to be essential to control T_H17 cells (26, 32). The possibility remains that in the absence of IL-10 secretion, other regulatory mechanisms of T_R1 cells would be directed to other pro-inflammatory cells. For example, Granzyme B secreted by T_R1 cells could kill antigen presenting cells (16).

Several signaling molecules have been identified to induce and maintain IL-10 expression in a variety of cells, for example STAT3, p38 MAP kinase and ERK1 and ERK2 (ERK1/2) (50–53, 68). Furthermore, it has been previously shown that IL-10 signaling sustains the production of IL-10 via activation of STAT3 in Foxp3$^+$ Treg cells. T_R1 cells also depend on STAT3 signaling for differentiation (32, 48), but surprisingly we found that it is dispensable for the maintenance of IL-10 secretion in mature T_R1 cells, whereas p38 MAP kinase plays a crucial role in this process. However, since addition of JNK and STAT3 inhibitors affected cell viability at high concentrations, we cannot completely exclude that JNK or STAT3 signaling in addition to p38 MAP kinase signaling might affect IL-10 production. But STAT3 requires Foxp3 and histone acetyl transterase-1 to epigenetically modify the *Il10* promoter region to allow for gene regulation via STAT3 in Foxp3$^+$ Treg cells (69). The absence of Foxp3 in mature T_R1 cells could mitigate the role of STAT3 in this process and explain why p38 MAP kinase, which does not require Foxp3 mediated stabilization, sustains IL-10 expression in T_R1 cells.

Considering the literature, we propose IL-27/Erg-2/Blimp1 in conjunction with STAT3, Ahr and c-Maf as the driving force for T_R1 cell differentiation. However, IL-10 signaling, through p38 MAP kinase, is required for the stabilization and function of intestinal T_R1 cells. Whether and how IL-10 and IL-21 (another cytokine which has been proposed to sustain the T_R1 cell phenotype) synergistically sustain IL-10 production, remains to be further investigated (21, 46, 59).

Human T_R1 cells display a high level of potential to maintain and re-establish immune homeostasis. They therefore receive major focus in current immunological and clinical research into the design of T_R1 cell-based therapies to treat human diseases such as IBD (61, 62). In such trials the success of a T_R1-based therapy is strongly linked to their high IL-10 production (67). The functional stability of T_R1 cells could be critical for the efficiency and safety of these cells as therapeutics.

We extended our key findings to human biology. We show that IL-10 is also essential for human T_R1 cells to maintain their IL-10 production. The capacity of T_R1 cells to sustain themselves by IL-10 is therefore key to determine the long-term success of the therapy. Currently, it has been proposed to select *in vitro* induced T_R1 cells based on the expression of CD49b and LAG-3 (67). Further enriching T_R1 cells by high expression of IL-10 receptor could optimize the efficiency and ensure the safety of T_R1 cell-based trials. Furthermore, measuring the expression of IL-10 receptor could serve as a marker to determine the efficacy of a T_R1 cell therapy.

Collectively, we show that T_R1 cells require the presence of their immunoregulatory cytokine IL-10 to maintain the cell function. Accordingly, T_R1 cell stability is dependent on IL-10 signaling in T_R1 cells themselves. Overall, our data indicate the crucial role of IL-10 signaling in T_R1 cells and suggest that T_R1-based T cell therapy is safe and efficient for the treatment of IBD.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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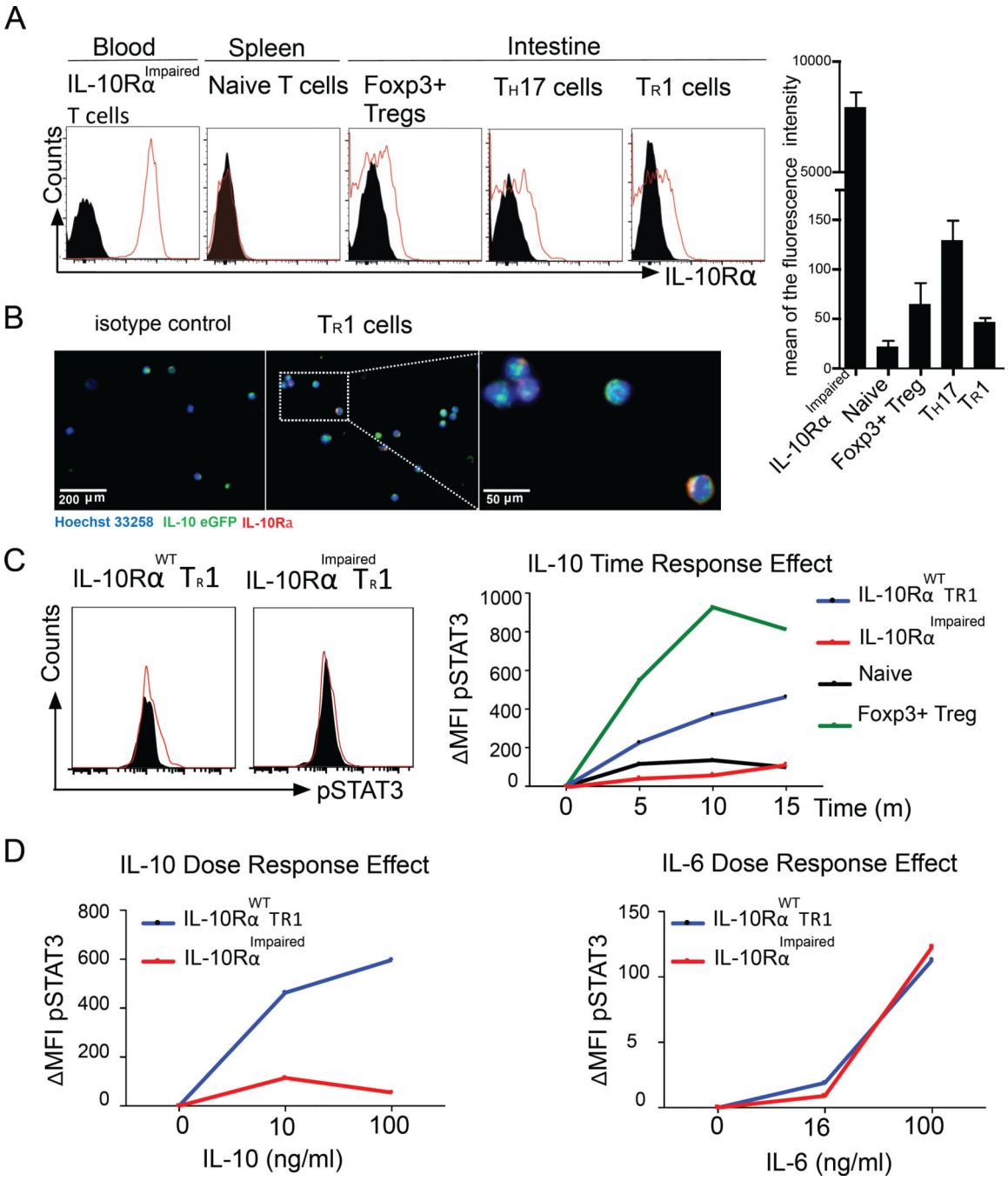

Fig. 1. *In vivo* differentiated T_R1 cells can respond to IL-10

(**A**) IL-10Rα expression and MFI of indicated cell populations. Black area represents the isotype control. Four independent experiments were performed. (**B**) Immunofluorescence staining (two independent experiments) of IL-10Rα on wild type T_R1 cells. (**C–D**) ΔMFI (compared to unstimulated cells) of pSTAT3 levels. (**C**) Naïve T cells, $Foxp3^+$ T cells and $IL\text{-}10R\alpha^{WT}$ or $IL\text{-}10R\alpha^{Impaired}$ T_R1 cells were stimulated with IL-10 (100 ng/ml) for indicated time points. Black area represents the unstimulated control. (**D**) $IL\text{-}10R\alpha^{WT}$ or

IL-10Rα^{Impaired} T_R1 cells were stimulated for 20 min with the indicated concentrations of IL-10 or IL-6. Data are representative of three independent experiments.

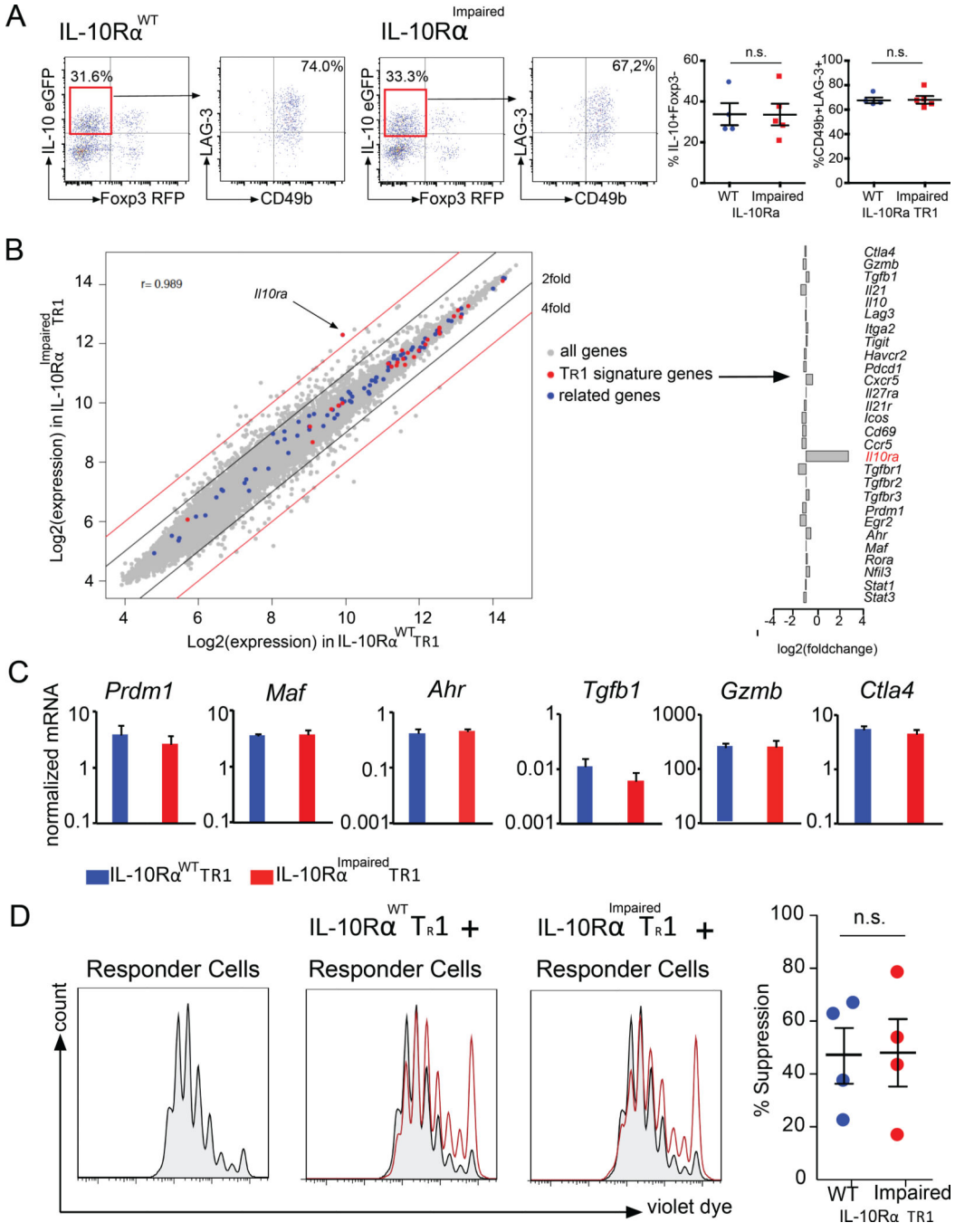

Fig. 2. IL-10 signaling in T cells is not essential for the differentiation of T_R1 cells

IL-10Rα^{WT} or IL-10Rα^{Impaired} Foxp3^{RFP} IL-10^{eGFP} double reporter mice were treated with anti-CD3. (**A**) Frequency of T_R1 cells in the small intestine and CD49b and LAG-3 expression by T_R1 cells are shown (IL-10Rα^{WT} n=4; IL-10Rα^{Impaired} n=5) (**B**) Gene expression analysis, comparing IL-10Rα^{WT} T_R1 cells and IL-10Rα^{Impaired} T_R1 cells. Fold change expression of T_R1 cell signature genes of IL-10Rα^{Impaired} T_R1 cells compared to IL-10Rα^{WT} T_R1 cells. (**C**) *Maf*, *Ahr*, *Prdm1*, *Tgfb1*, *Ctla4* and *Gzmb* mRNA expression

normalized to *Hprt* (data pooled of three independent experiments). (**D**) T_R1-mediated *in vitro* suppression. Data are representative of five independent experiments.

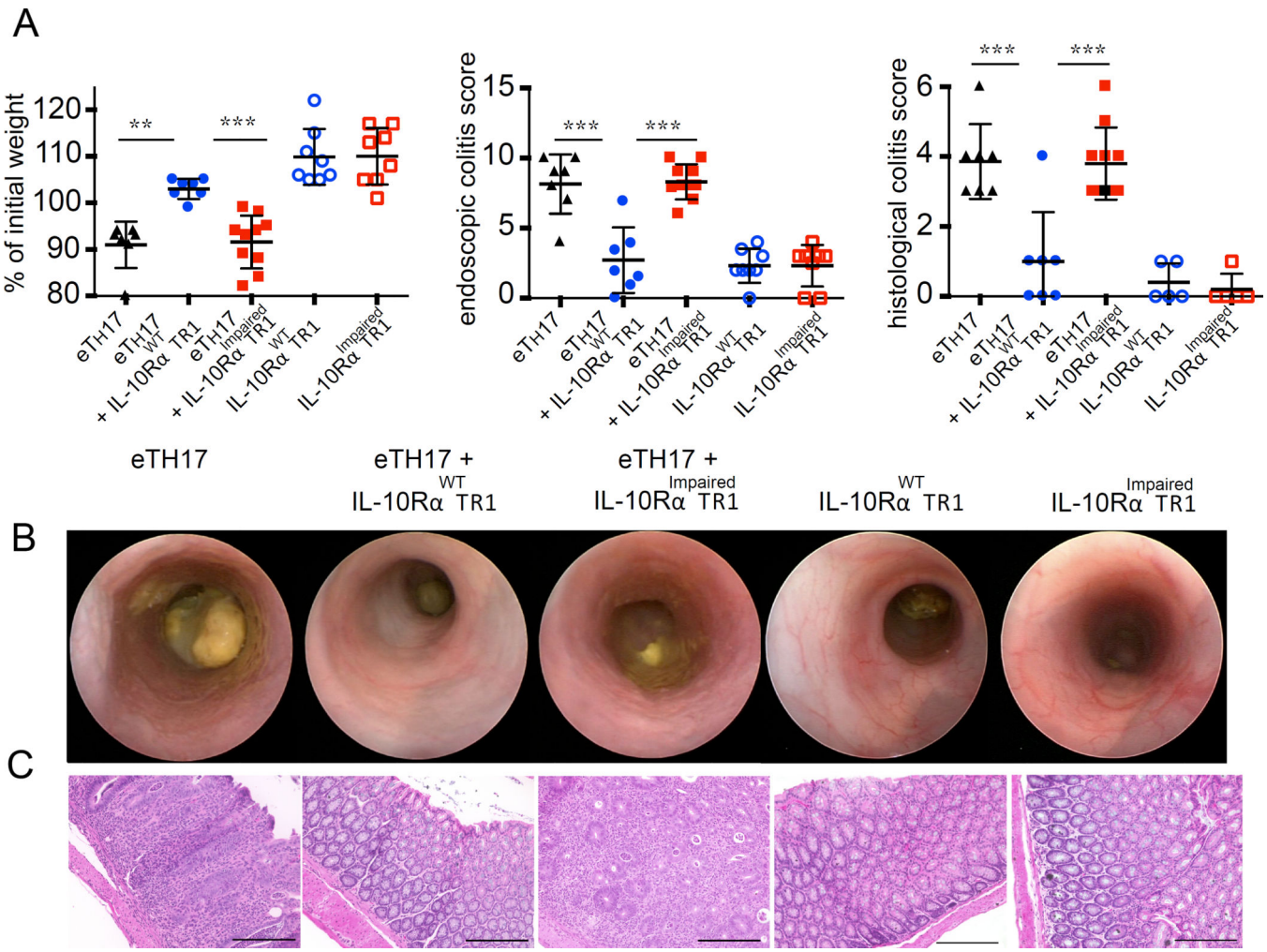

Fig. 3. IL-10 signaling in T_R1 cells is essential for their *in vivo* function

In vivo induced IL-10Rα^{WT} or IL-10Rα^{Impaired} T_R1 were injected alone or together with *in vivo* differentiated effector (e)T_H17 cells. (**A**) Mass loss, endoscopic and histological colitis score 5 weeks upon transfer (eT_H17 n=7; eT_H17+ IL-10Rα^{WT} T_R1 n=7; eT_H17+ IL-10Rα^{Impaired} T_R1 n=10; IL-10Rα^{WT} T_R1 n=8; IL-10Rα^{Impaired} T_R1 n=8; lines indicate mean ± SEM). Representative endoscopic pictures (**B**) and histology (scale bars, 200 μm) (**C**) are shown. Results are cumulative of two independent experiments. One-way ANOVA (post-test Tukey) was used to calculate significance (** $p < 0.01$; *** $p<0.001$).

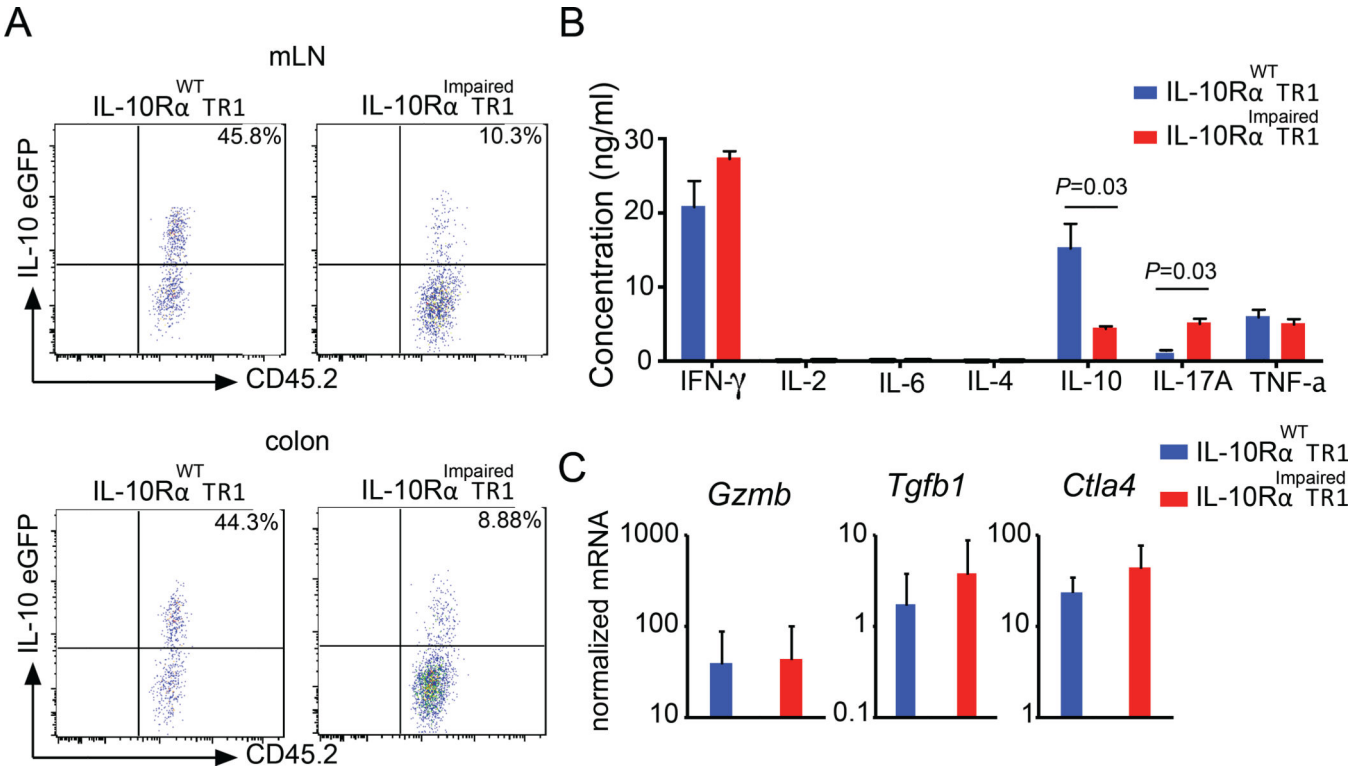

Fig. 4. IL-10 signaling in T_R1 cells sustains IL-10 expression

(**A**) *In vivo* induced IL-10Rα^{WT} or IL-10Rα^{Impaired} T_R1 cells were injected into *Rag1*$^{-/-}$ mice. Cells were isolated 5 weeks after transfer. Representative dot plots of IL-10^{eGFP} expression of 4 pooled mice per group are shown. Data are representative of three independent experiments. (**B**) Cytokine production of T_R1 cells was quantified using Cytometric Bead array. Mean ± SEM from three independent experiments are shown. Mann-Whitney U test was used to calculate significance. (**C**) *Tgfb1*, *Ctla4* and *Gzmb* mRNA expression normalized to *Hprt*. Data are cumulative of three independent experiments.

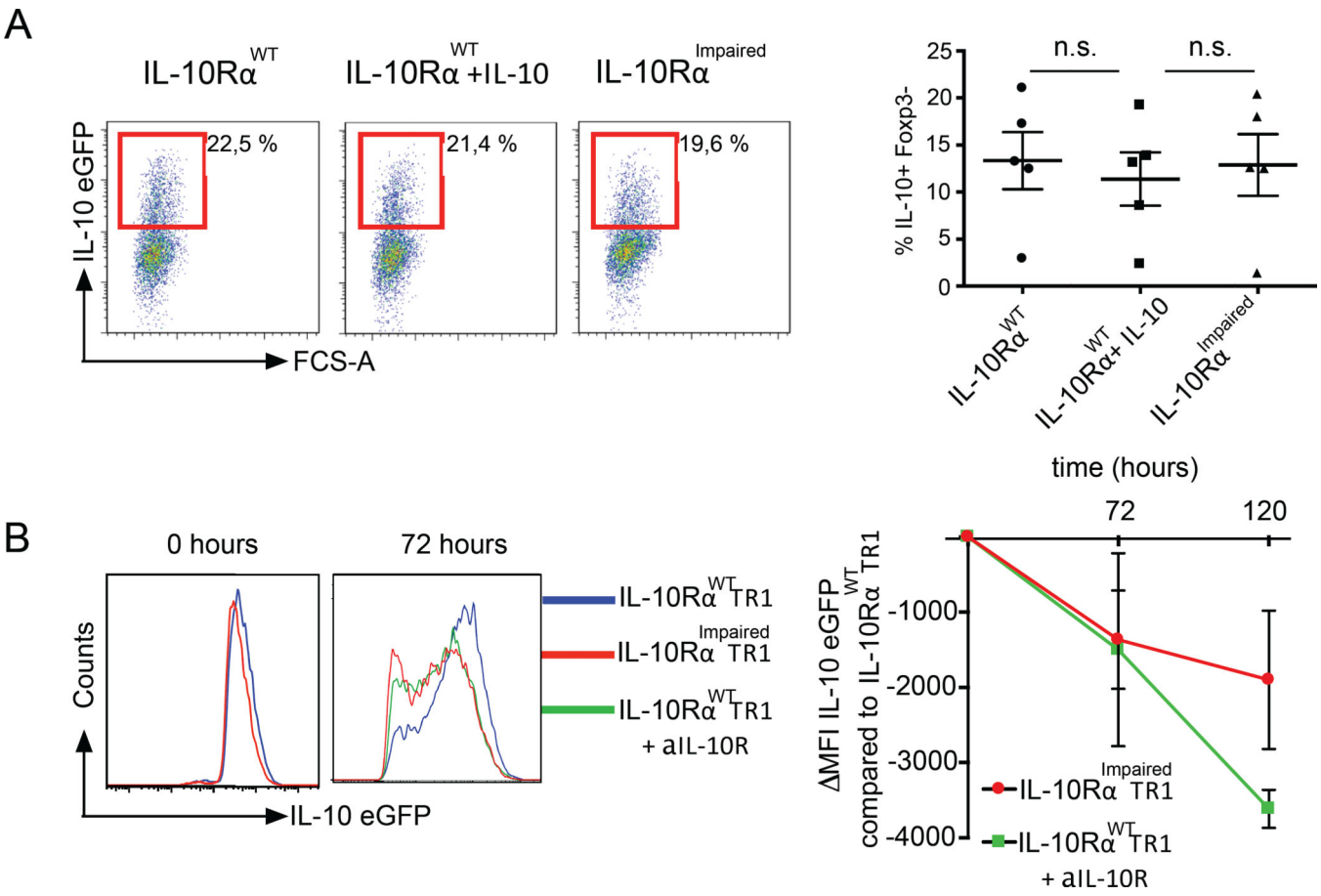

Fig. 5. IL-10 signaling is dispensable for the *in vitro* differentiation of T_R1 cells with IL-27 and IL-10 sustains IL-10 production in *in vitro* differentiated T_R1 cells

(**A**) *In vitro* differentiation of IL-10Rα^{WT} and IL-10Rα^{Impaired} T_R1 cells. Five independent experiments were performed. (**B**) IL-10^{eGFP} ΔMFI of re-stimulated IL-10Rα^{Impaired} T_R1 cells and IL-10Rα^{WT} T_R1 cells + αIL-10R antibody compared to IL-10Rα^{WT} T_R1 cells are shown. Results are cumulative of three independent experiments.

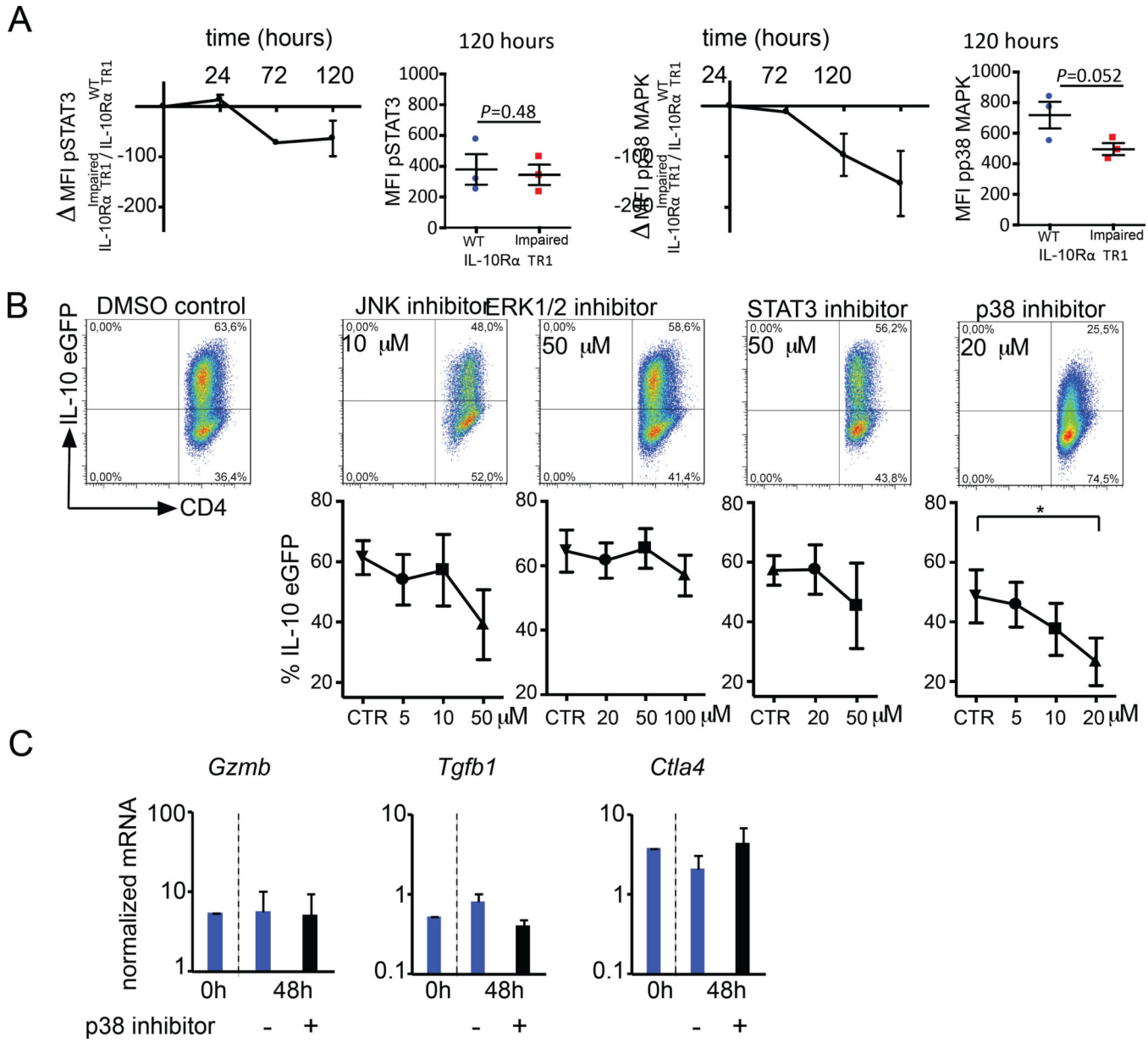

Fig. 6. IL-10 signaling sustains IL-10 production in T_R1 cells via activation of p38 MAPK

(**A**) pSTAT3 and pp38 ΔMFI of re-stimulated IL-10Rα^{Impaired} T_R1 cells compared to IL-10Rα^{WT} T_R1 cells are shown. Results are cumulative of three independent experiments. Paired t test was used to test significance. (**B**) Frequency of IL-10^{eGFP} of re-stimulated T_R1 cells in the presence of indicated inhibitors are shown (mean ± SEM of three independent experiments). One-way ANOVA (post-test Tukey) was used to calculate significance (* P<0,05). (**C**) *Tgfb1*, *Ctla4* and *Gzmb* mRNA expression normalized to *Hprt*. Results are cumulative of three independent experiments.

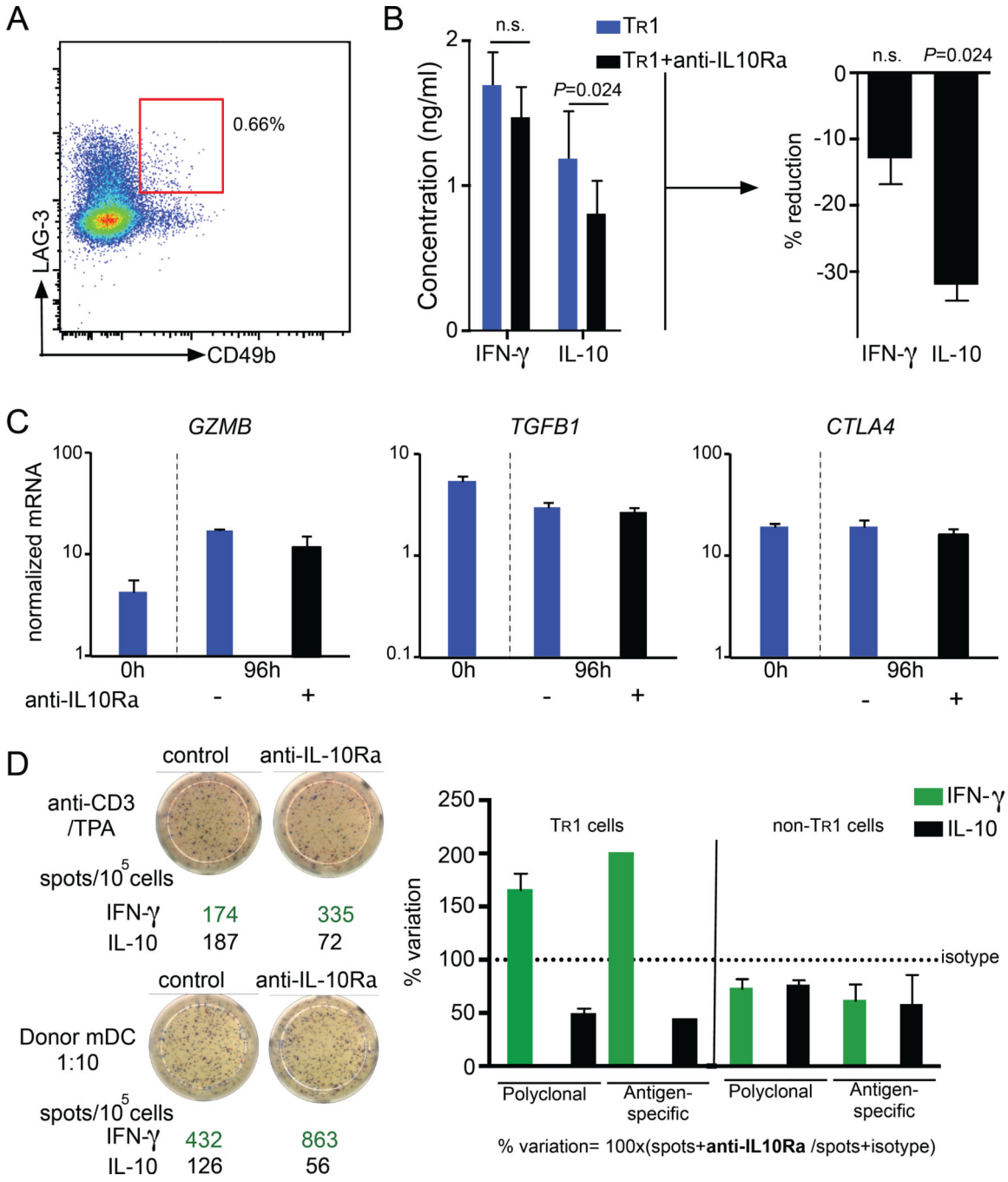

Fig. 7. IL-10 signaling sustains IL-10 production in human T_R1 cell

(**A–C**) Circulating human T_R1 cells ($CD4^+CD45RA^{low}CD49b^+LAG\text{-}3^+$) were FACS-sorted from PBMCs of healthy donors (n=5) (**A**). T_R1 cells were re-stimulated with anti-CD3 and anti-CD28 for 96 hours with either 50 μg/ml human IL-10Rα or isotype control antibody and the indicted cytokines were quantified. A paired t tested was used to calculate significance. (**B**). *TGFB1*, *CTLA4* and *GZMB* mRNA expression normalized to *HPRT* (**C**). (**D**) *In vitro* differentiated T_R1 and non-T_R1 cells were re-stimulated with anti-CD3/TPA or allogeneic mDC for 48 hours with 50 μg/ml human IL-10Rα blocking antibody or isotype

control. A dual IFNγ/IL-10 ELISPOT was performed. Data are cumulative of two independent experiments.