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Original research

24-Nor-ursodeoxycholic acid improves intestinal inflammation by targeting T_H17 pathogenicity and transdifferentiation

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ABSTRACT

Background 24-Nor-ursodeoxycholic acid (NorUDCA) is a novel therapeutic bile acid for treating immune-mediated cholestatic liver diseases, such as primary sclerosing cholangitis (PSC).

Objective Since PSC strongly associates with T helper-type-like 17 (T_H17)-mediated intestinal inflammation, we explored NorUDCA's immunomodulatory potential on T_H17 cells.

Design NorUDCA's impact on T_H17 differentiation was assessed using a $CD4^+T_{Naive}$ adoptive transfer mouse model, and on intraepithelial T_H17 pathogenicity and transdifferentiation using an αCD3 stimulation model combined with interleukin-17A-fate-mapping. Mechanistic studies used molecular and multiomics approaches, flow cytometry and metabolic assays with pathogenic (p) T_H17. Pathogenicity of pT_H17 exposed to NorUDCA *in vitro* was evaluated following adoptive transfer in intestinal tissues or the central nervous system (CNS). Key findings were validated in an αCD3-stimulated humanised NSG mouse model reconstituted with peripheral blood mononuclear cells from patients with PSC.

Results NorUDCA suppressed T_H17 effector function and enriched regulatory T cell (Treg) abundance upon $CD4^+T_{Naive}$ cell transfer. NorUDCA mitigated intraepithelial T_H17 pathogenicity and decreased the generation of proinflammatory 'T_H1-like-T_H17' cells, and enhanced T_H17 transdifferentiation into Treg and Tr1 (regulatory type 1) cells in the αCD3-model. *In vivo* ablation revealed that Treg induction is crucial for NorUDCA's anti-inflammatory effect on T_H17 pathogenicity. Mechanistically, NorUDCA restrained pT_H17 effector function and simultaneously promoted functional Treg formation *in vitro*, by attenuating a glutamine-mTORC1-glycolysis signalling axis. Exposure of pT_H17 to NorUDCA dampened their pathogenicity and expansion in the intestine or CNS upon transfer. NorUDCA's impact on T_H17 inflammation was corroborated in the humanised NSG mouse model.

Conclusion NorUDCA restricts T_H17 inflammation in multiple mouse models, potentiating future clinical applications for treating T_H17-mediated intestinal diseases and beyond.

WHAT IS ALREADY KNOWN ON THIS TOPIC

⇒ Primary sclerosing cholangitis (PSC) is an immune-mediated cholestatic liver disease highly associated with T helper-type-like 17 (T_H17)-driven intestinal inflammation. Novel therapeutics targeting both liver and intestinal disease in PSC are of high clinical relevance.

⇒ Independent of anti-cholestatic effects, 24-Nor-ursodeoxycholic acid (NorUDCA) directly modulates $CD8^+$T cell metabolism, lymphoblastogenesis and expansion by targeting mTORC1 signalling.

⇒ mTORC1 metabolically regulates T_H17 differentiation, therapeutic inhibition on mTORC1 may therefore counteract T_H17 differentiation and associated inflammation during intestinal inflammation.

WHAT THIS STUDY ADDS

⇒ Beyond its therapeutic efficacy against hepatobiliary inflammation and injury, NorUDCA targets intraepithelial T_H17 pathogenicity and transdifferentiation *in vivo* to reduce intestinal inflammation.

⇒ NorUDCA curtails T_H17 pathogenicity and expansion by metabolically conditioning the induction of anti-inflammatory regulatory T cells.

⇒ NorUDCA limits T_H17 inflammation in a humanised NSG model reconstituted with peripheral blood mononuclear cells from patients with PSC.

INTRODUCTION

T helper-type-like 17 (T_H17) lineage is characterised by interleukin-17A (IL-17A) secretion, and

HOW THIS STUDY MIGHT AFFECT RESEARCH, PRACTICE OR POLICY

⇒ This study provides a scientific basis for future investigations into the impact of therapeutic or naturally occurring bile acids (BAs) within intestine and liver on $CD4^+T_{Helper}$ cell transdifferentiation, under homeostatic and pathological conditions. Such knowledge of the pleiotropic functions of BAs may lead to novel therapeutic strategies targeting tissue autoimmunity.

⇒ Independent of anti-cholestatic effects, NorUDCA directly modulates $CD8^+$T cell metabolism, lymphoblastogenesis and expansion by targeting mTORC1 signalling.

expression of C-C-chemokine receptor 6 (CCR6) and transcription factor retinoic acid orphan receptor-gamma (RORγt).[1] T_H17 may undergo 'transdifferentiation', a process enabled by their inherent 'instability', whereby IL-17A expression is discontinued, and 'plasticity', where they may secrete cytokines typical of other lineages, such as T helper-type-like 1 (T_H1),[2] regulatory type 1 (Tr1) or regulatory T (Treg) cells.[2–4] Moreover, T_H17 can retain IL-17A expression while inducing cytokine expression of other lineages, such as interferon (IFN)-γ, resulting in IL-17A$^+$IFN-γ$^+$ proinflammatory 'T_H1-like-T_H17' cells.[5] During homeostasis, T_H17 develops in the intestine and is pivotal to host defence against microbial pathogens, as T_H17-produced IL-17A maintains intestinal mucosal barrier integrity.[6] However, dysregulation of T_H17 immune responses and transdifferentiation can lead to tissue inflammation and autoimmunity.[7] Hence, T_H17 represents a highly attractive target for therapeutic intervention.[8]

24-Nor-ursodeoxycholic acid (NorUDCA) (recently termed 'Norucholic acid'[9] is a novel therapeutic modified bile acid (BA) that has demonstrated promising results in phase II clinical trials for primary sclerosing cholangitis (PSC),[10] a progressive, poorly treatable immune-mediated hepatobiliary disease.[11] NorUDCA is currently under evaluation for its long-term effect on PSC in a phase III trial (NCT03872921). Previously, we reported that NorUDCA modulates $CD8^+$T cell metabolism and immune responses through blunting mTORC1 activity during hepatic immunopathology, profoundly distinct from its parent compound UDCA.[12] Thus, the therapeutic potential of NorUDCA against T cell-mediated liver diseases is promising. PSC is highly associated with intestinal inflammation driven by dysregulated T_H17 immune responses often linked with mTORC1 hyperactivation.[13] We hypothesised, that NorUDCA might modulate T_H17 immunity during intestinal inflammation, beyond its well-established therapeutic effects against hepatobiliary inflammation and injury.

Currently, no experimental model exists that replicates both hepatic and intestinal inflammatory manifestations of PSC.[14] PSC-liver disease models such as the *Mdr2*$^{-/-}$ model and DDC-feeding model do not show pronounced T_H17-driven intestinal inflammation as T_H17 cells were barely detectable in the gut. Therefore, we used various models of T_H17-associated intestinal immunopathology to explore NorUDCA's efficacy on T_H17-mediated intestinal inflammation. Here, we report that NorUDCA targets intraepithelial T_H17 pathogenicity and transdifferentiation, and that NorUDCA curtails T_H17 autoimmunity by inducing a distinct metabolic reprogramming facilitating generation of suppressive Tregs. Moreover, we confirmed that NorUDCA limits T_H17 inflammation in a hαCD3-stimulated humanised NSG model reconstituted with peripheral blood mononuclear cells (PBMCs) from patients with PSC. These findings propose NorUDCA as a potential therapeutic for treating T_H17 cell-mediated autoimmune disorders of the intestine and beyond.

METHODS

Mice

All mice were housed under specific pathogen free (SPF) conditions at the Medical University of Vienna. Details of the mouse strains used are provided in the online supplemental materials and methods. For most experiments, littermate mice were used, matched for both age (8 and 12 weeks) and sex (including both male and female mice). An exception was the C57BL/6N *Rag2*$^{-/-}$ line, where age-matched female mice from the same genetic background were used specifically for adoptive pathogenic T_H17 cell transfer models. All animal experiments were approved by the Federal Ministry for Education, Science and Art and under national laws in agreement with guidelines of the Federation of European Laboratory Animal Science Associations and the National Centre for the Replacement, Refinement and Reduction of Animals in Research (ARRIVE).

$CD4^+T_{Naive}$ adoptive cell transfer model

$CD4^+T_{Naive}$ cells were flow-sorted from C57BL/6 mice and 0.5×10^6 cells transferred (intraperitoneal (*i.p.*)) into *Rag2*$^{-/-}$ recipients which received standard chow or 0.5% (weight/weight (wt/wt)) NorUDCA-supplemented diet. The diet was produced by a commercial vendor (SAFE-lab). The standard chow diet (SAFE A04) and the NorUDCA-supplemented diet are matched in formulation, differing only by the presence of NorUDCA (0.5% w/w). Leucocytes were analysed 8 weeks later (see online supplemental material).

αCD3 stimulation-induced intestinal inflammation model

IL-17A fate-mapping (IL-17A^{Cre}R26R^{eYFP}) mice[2] (IMSR_JAX: 016879) were injected with αCD3 (20 µg, clone 145–2 C11 *i.p.*) twice every other day and fed with a standard chow or NorUDCA-supplemented diet for 100 hours post the first injection.[2–4] Intraepithelial lymphocytes (IEL) and lamina propria lymphocytes (LPL) were isolated ex vivo (see online supplemental material).

Treg *in vivo* ablation in αCD3-stimulated DEREG mice

αCD3-stimulated DEREG (Foxp3-DTR^{eGFP})[15] mice receiving either standard chow or 0.5% (wt/wt) NorUDCA-supplemented diet were injected with diphtheria toxin (DT, Merck) (1 µg/mouse *i.p.*) daily. Intestinal IEL and LPL were analysed 100 hours after αCD3 injection.

Adoptive cell transfer models of intestinal inflammation and experimental autoimmune encephalomyelitis

C57BL/6 or TCR^{2D2}CD4$^+T_{Naïve}$ cells were polarised towards pathogenic T_H17 (pT_H17) and treated±NorUDCA for 4 hours *ex vivo*, then transferred into *Rag2*$^{-/-}$ recipients as described in online supplemental materials and methods.

Flow cytometry and cell sorting

Flow cytometry and cell sorting were performed as described in online supplemental table 1 and online supplemental material.

Murine pathogenic T_H17 and Treg differentiation *in vitro*

Murine pathogenic (p)T_H17 and Treg were differentiated *in vitro* (see online supplemental material).

Intestinal lymphocyte isolation
Intestinal lymphocytes were isolated as described in online supplemental material.

In vitro suppression assay
In vitro suppression assay was performed as described in online supplemental material.

Tissue H&E and periodic acid-Schiff staining
H&E and periodic acid Schiff staining of tissue sections were performed as described.[16] Histological intestinal immunopathology was assessed by a board-certified pathologist blinded to the experimental assignments (see online supplemental material).

Metabolic assays
Glucose uptake assay, Seahorse assay and αketoglutarate assay were performed as described in online supplemental material.

Glucose uptake assay
Murine pT_H17 cells differentiated±NorUDCA were incubated with 10 µM 2-(N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino)−2-deoxy-D-glucose, a fluorescent analogue of D-glucose, for 30 min at 37°C prior to flow cytometric analysis.

Targeted metabolomics and bulk RNA sequencing
Multiomic analysis were performed as described in online supplemental material.

In vitro human T_H17 cell differentiation assay
In vitro human T_H17 cell differentiation assay was performed as described in online supplemental material.

Humanised NSG mouse model reconstituted with PBMCs from patients with PSC
PBMCs were collected from patients with PSC with approval from the regional Committees for Medical and Health Research Ethics of South East Norway (2012–286 and 2016–1540) (online supplemental table 2). Biobanking of PBMCs and associated functional analyses were approved by the Regional Ethics Committees in South-Eastern Norway (15 368 and 18 221). All patient samples were collected, stored and used with written, informed consent. Frozen PBMCs were thawed and adoptively transferred into NOD/SCID/IL-2rγ$^{-/-}$ (NSG) mice (IMSR_JAX: 005557) (4−5×10^6 PSC-PBMCs/mouse *i.p.*). 2 weeks after transfer, mice received 10 µg human (h)αCD3 antibody (teplizumab, clone hOKT3γ1, *i.p.*) and were fed with either chow or NorUDCA-supplemented diet for 2 weeks. Details for experimental design are shown in online supplemental material . Animals exhibited no symptoms of graft-versus-host disease.

Serum biochemistry
Serum biochemistry was performed as described previously.[17]

Patient and public involvement statement
Patients were not involved.

Quantification and statistical analysis
P values were determined using GraphPad Prism V.10 software and details of statistical testing can be found in the figure legends. Comparisons for two groups were calculated using unpaired or paired two-tailed Student's *t*-tests (for two groups meeting the normal distribution criteria, according to the Shapiro-Wilk normality test). Comparisons of more than two groups or grouped data were analysed using one-way or two-way analysis of variance, followed by post hoc tests, such as Dunnett's test for comparisons to a reference group, or other corrections (i.e., Tukey's honestly significant difference (HSD) or Bonferroni's test) for multiple comparisons, to control for type I error. Differences were considered statistically significant when $p \leq 0.05$ ($^*p \leq 0.05$, $^{**}p \leq 0.01$, $^{***}p \leq 0.001$, $^{****}p \leq 0.0001$). Data are shown as mean and SEM.

Data availability
All data for the study is available on request. Bulk RNA sequencing data sets with accession number GSE285363 were deposited in gene expression omnibus (GEO) database.

RESULTS
NorUDCA reduces T_H17 and enriches Tregs within intestine upon CD4$^+$T$_{Naive}$ adoptive transfer *in vivo*
To explore whether NorUDCA modulates T_H17 differentiation *in vivo*, CD4$^+$T$_{Naive}$ cells were transferred into *Rag2*$^{-/-}$ recipients who received a chow or NorUDCA-supplemented diet (figure 1A). Following interaction with intestinal antigen-presenting cells (APCs), the majority of transferred CD4$^+$T-$_{Naive}$ cells differentiate into T_H17 cells,[18] disrupting the balance with Tregs, resulting in progressive intestinal inflammation.[19] Increased plasma BA levels confirmed NorUDCA systemic enrichment *in vivo* (online supplemental figure 1A). In contrast to controls, NorUDCA treatment reduced the size of mesenteric lymph nodes (mLNs), spleen and protected mice against colon shortening reflecting the level of tissue inflammation (figure 1B). Decreased leucocyte infiltration in tissues of NorUDCA-treated mice indicated reduced inflammation (figure 1C). Reduced immune cell infiltration, goblet cell loss, and preserved intestinal architecture and mucus barrier integrity all support NorUDCA-induced mitigation of immunopathology (Figure 1D,E).

Similarly to the small intestine and colon of NorUDCA-treated mice, LPL contained reduced frequencies of T_H17 accompanied by increased frequencies of Tregs (figure 1F, online supplemental figure 1B). The proportion of T_H17 and Tregs was similarly impacted in secondary lymphoid organs such as mLNs and spleen (figure 1G). Cell numbers of Tregs across various tissues were not changed by NorUDCA (online supplemental figure 1C). Additionally, NorUDCA did not alter tissue-infiltrating T_H17 expression of RORγt or CCR6, which control T_H17 differentiation and their migration into intestinal tissues, respectively (figure 1H,I). Despite the reduction in T_H17 frequencies by NorUDCA, IL-17A expression (based on geometric mean-fluorescence intensity; gMFI) was comparable to controls (online supplemental figure 1D), indicating that NorUDCA might affect T_H17 effector functions rather than differentiation.

IELs expressing CD8αα homodimers or CD8αβ heterodimers that have prominent roles in mediating intestinal inflammation[20] can differentiate from CD4$^+$T$_{Naive}$ cells,[20] and Tregs,[21] but were unaffected by NorUDCA (online supplemental figure 2).

The frequency, maturation or expression of various receptors[22 23] and cytokines[24] known to impact T_H17 and Treg differentiation on lamina propria APC were unaffected by NorUDCA, so unlikely to influence T_H17 or Treg immunity (online supplemental figure 3).

NorUDCA targets T_H17 pathogenicity and transdifferentiation during intestinal inflammation in an αCD3 model
An αCD3 model of intestinal inflammation was used to corroborate our observation that NorUDCA modulates T_H17 effector

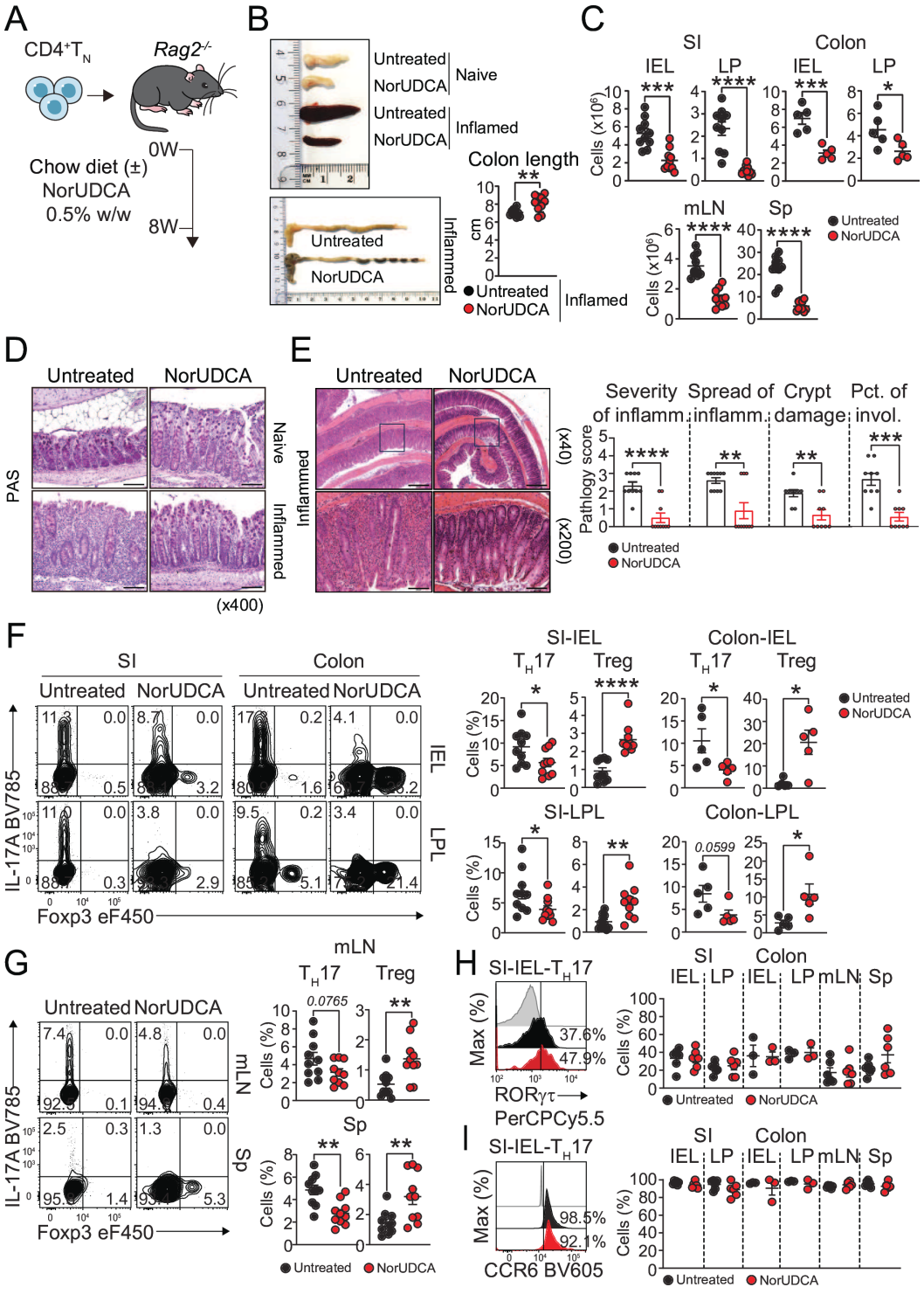

Figure 1 NorUDCA decreases T_H17 frequency and enriches Tregs in multiple tissues upon $CD4^+T_{Naive}$ adoptive transfer *in vivo*. NorUDCA decreases T_H17 frequency and enriches Tregs in multiple tissues upon $CD4^+T_{Naive}$ adoptive transfer *in vivo*. (A) Experimental design. (B) Photographs of mesenteric lymph nodes and spleens from indicated groups are depicted. Quantitative analysis of colon length of indicated groups. (C) Quantitative analysis of numbers of leucocytes extracted from indicated tissues. (D, E) PAS and H&E staining of colon sections (magnifications as indicated, scale bar 200 µm). (E) Histopathological scores are shown alongside. (F, G) Representative flow cytometric plots and quantitative analysis of T_H17 and Treg cells extracted from indicated tissues. (H, I) Representative histogram plots presenting RORγt and CCR6 expression on small intestine-IEL-infiltrating T_H17 cells. Summary of the frequency of T_H17 cells expressing RORγt (H) or CCR6 (I) within indicated tissues. Data summarise three independent experiments. At least three mice were used per group for each experiment. Each point represents one mouse. Two samples from colonic fractions (IEL or LPL) were pooled to achieve sufficient cell numbers for flow cytometric analysis. Mean and SEM are presented. *P* values were calculated using the unpaired Student's *t*-test (two-tailed). *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$, ****$p \leq 0.0001$. $CD4^+T_N$, $CD4^+T_{Naive}$: CCR6, C-C-chemokine receptor 6; IEL, intraepithelial lymphocytes; IL, interleukin; Inflamm, inflammation; invol, involvement; LPL, lamina propria lymphocytes; mLN, mesenteric lymph nodes; NorUDCA, 24-Nor-ursodeoxycholic acid; PAS, periodic Acid Schiff; Pct, percentage; RORγt, retinoic acid orphan receptor-gamma; SI, small intestinal; Sp, spleen; T_H17, T helper-type-like 17; Treg, regulatory T cell.

function in a $CD4^+T_{Naive}$ cell adoptive transfer model. αCD3 stimulation *in vivo* leads to activation-induced cell death of T cells.[25] Systemic upregulation of IL-6 and transforming growth factor-β1 (TGF-β1) follows phagocyte engulfment of apoptotic T cells, resulting in a 'cytokine storm' favouring T_H17 differentiation and robust T_H17 immune response causing local inflammation mainly in the small intestine.[4] Within an inflamed small intestine, T_H17 exhibits plasticity in effector programmes, either retaining IL-17A expression while inducing expression of cytokines of T_H1 lineage (i.e., IFN-γ) to become IL-17A-double-producing T_H1-like-T_H17 cells[18] or losing expression of IL-17A and converting to T_H1, Tr1[26] or Treg.[3 4] Using the αCD3 model with the IL-17A-fate-mapping system[2] (i.e., $IL\text{-}17A^{Cre}R26R^{eYFP}$ mice, in which IL-17A-producing cells permanently express yellow fluorescent protein (eYFP)), we investigated whether NorUDCA influences T_H17 fate decision within the inflamed small intestine (figure 2A). Severe oedema, inflammation and lymphocyte infiltration induced by αCD3 in vivo were attenuated by NorUDCA (figure 2B,C). NorUDCA decreased frequencies of 'effector T_H17' ($IL\text{-}17A^+eYFP^+$) cells and total $eYFP^+$ cells (figure 2D,E). αCD3-mediated inflammation critically depends on CCR6-mediated T_H17 migration to the small intestine.[4] Of note, CCR6 expression on $eYFP^+$ cells from NorUDCA-treated and untreated-mice were comparable (figure 2F), thus intestinal T_H17 frequencies were not reduced due to altered CCR6 expression.

On αCD3-stimulation, T_H17 in untreated mice maintained a high level of 'plasticity' towards 'T_H1-like T_H17' cells, which was impeded in NorUDCA-treated mice (figure 2G), implying T_H17 plasticity towards reportedly pathogenic 'T_H1-like T_H17' cells[18] is restrained. T_H17 transdifferentiation into 'T_H1-like T_H17' is driven by mTORC1 signalling.[27] Accordingly, $eYFP^+$ cells from NorUDCA-treated mice demonstrated blunted mTORC1 activity revealed as reduced phosphorylation of mTORC1 downstream target RPS6 at Ser235/236 (figure 2H). Additionally, the frequencies of 'T_H1-like T_H17' cells and T_H1 cells derived from 'exT_H17' cells were reduced in NorUDCA-treated mice (figure 2G).

We assessed whether NorUDCA potentiates 'exT_H17' cells towards an anti-inflammatory fate. NorUDCA-treated mice exhibited a higher frequency of Tr1 cells co-expressing CD49b and Lag3, with similar IL-10 levels compared with control mice (online supplemental figure 4A-C). Consistent with findings from the $CD4^+T_{Naive}$ adoptive transfer model, $Foxp3^+$Tregs were enriched in NorUDCA-treated mice, although their IL-10 expression remained unchanged (figure 2I, online supplemental figure 4D). Interestingly, $eYFP^+$cells in NorUDCA-treated mice displayed increased IL-10 expression (online supplemental figure 4D). Using the IL-17A-fate-mapping system, we observed that NorUDCA-induced Tregs originated from both 'exT_H17' cells and $CD4^+$T cells that had never expressed IL-17A (figure 2J,K). These results suggest that NorUDCA not only facilitates T_H17 transdifferentiation into Tregs, but also promotes Tregs development from non-T_H17 $CD4^+$T cells (figure 2J,K).

Treg induction is essential for NorUDCA's restriction on T_H17 pathogenicity during intestinal inflammation *in vivo*

Tr1 and Treg cells can limit T_H17 pathogenicity and are both induced by NorUDCA *in vivo* (figure 2I, online supplemental figure 4B).[28 29] However whether induction of Tr1 or Treg, or both is essential for NorUDCA's effect on restricting T_H17 pathogenicity during intestinal inflammation is unclear. Although NorUDCA promotes Tr1 differentiation *in vitro* (online supplemental figure 5), the role of Tr1 *in vivo* could not be assessed as transgenic models for Tr1 *in vivo* ablation are unavailable. Therefore, we examined the role of Tregs in DEREG mice, which express the DT receptor-eGFP fusion gene under the control of the Foxp3 regulatory elements, stimulated with αCD3 *in vivo (figure 3A)*. $Foxp3^+$Treg can be selectively depleted by the administration of DT during an ongoing immune response. $Foxp3^+$Tregs were successfully ablated from IELs in DEREG mice receiving DT. This was accompanied by an exacerbated T_H17 immune response and body weight loss, compared with littermate controls without DT (figure 3C,D, online supplemental figure 6), suggesting that Tregs play an important role in controlling the extent of T_H17 pathogenicity in the αCD3 model. Notably, DT-induced ablation of Tregs not only attenuated the suppressive efficacy of NorUDCA on T_H17 inflammation, but also abolished NorUDCA's protective effect on body weight loss (figure 3B-D). Thus, induction of Tregs is pivotal for NorUDCA to exert its anti-inflammatory action in T_H17-driven intestinal inflammation.

NorUDCA induces functional Tregs during pT_H17 *in vitro* differentiation, which inhibits the expansion of pT_H17 cells

To investigate how NorUDCA affects T_H17 effector function, murine $CD4^+T_{Naive}$ cells were polarised *in vitro* towards pT_H17 cells (figure 4A, online supplemental figure 7). The addition of NorUDCA after activation decreased the frequency of proliferating $IL\text{-}17A^+$ cells, while IL-17A expression (as assessed by gMFI) remained unchanged (figure 4B). Interestingly, a 4-hour NorUDCA treatment was sufficient to reduce the frequency of differentiated pT_H17 cells that produce IL-17A (figure 4B). RORγt expression on NorUDCA-treated T_H17 cells was unaffected (figure 4C). Consistent with our *in vivo* data, NorUDCA-treated pT_H17 cells display reduced expansion but intact differentiation.

mTORC1 regulates pT_H17 differentiation and expansion.[13] Consistent with *in vivo* data (figure 2F), mTORC1 activity was downregulated in long- or short-term NorUDCA-treated pT_H17 similarly to mTORC1 inhibitor rapamycin treatment, indicating that mTORC1 activity was inhibited by NorUDCA during pT_H17 differentiation (figure 4D).

A population of $Foxp3^+IL\text{-}17A^-$ cells was induced simultaneously within the pT_H17 culture by NorUDCA (figure 4E). Short-term NorUDCA treatment did not result in a significant increase in the percentage of Foxp3-expressing cells, although Foxp3 expression per cell was enhanced (figure 4E). Notably, $Foxp3^+IL\text{-}17A^-$ cells induced by NorUDCA displayed high levels of CD25, a marker of effector Tregs,[30] compared with untreated cells (figure 4E).

Next, we investigated whether $Foxp3^+IL\text{-}17A^-$ cells induced by NorUDCA are functional Tregs that have the ability to suppress IL-17A-producing T_H17 cells. For *in vitro* suppression assays, we polarised $CD4^+T_{Naive}$ cells isolated from $IL\text{-}17A^{eGFP}\times Foxp3^{hCD2/CD52}$ dual reporter mice into pT_H17 cells in the presence or absence of NorUDCA. The dual reporter system enabled us to accurately flow-sort $IL\text{-}17A^{eGFP+}Foxp3^{hCD2/CD52-}$ and $Foxp3^{hCD2/CD52+}IL\text{-}17A^-$ cells from pT_H17 cultures. $IL\text{-}17A^{eGFP+}Foxp3^{hCD2/CD52-}$ cells sorted from untreated cultures were labelled with proliferation dye (figure 4G) and co-cultured with $Foxp3^{hCD2/CD52+}IL\text{-}17A^{eGFP-}$ cells sorted from untreated-NorUDCA or NorUDCA-treated cultures. IL-17A expression was impaired and proliferation was reduced in $IL\text{-}17A^{eGFP+}Foxp3^{hCD2/CD52-}$ cells when co-cultured with $Foxp3^{hCD2/CD52+}IL\text{-}17A^{eGFP-}$ cells sorted from NorUDCA-treated cultures, to an

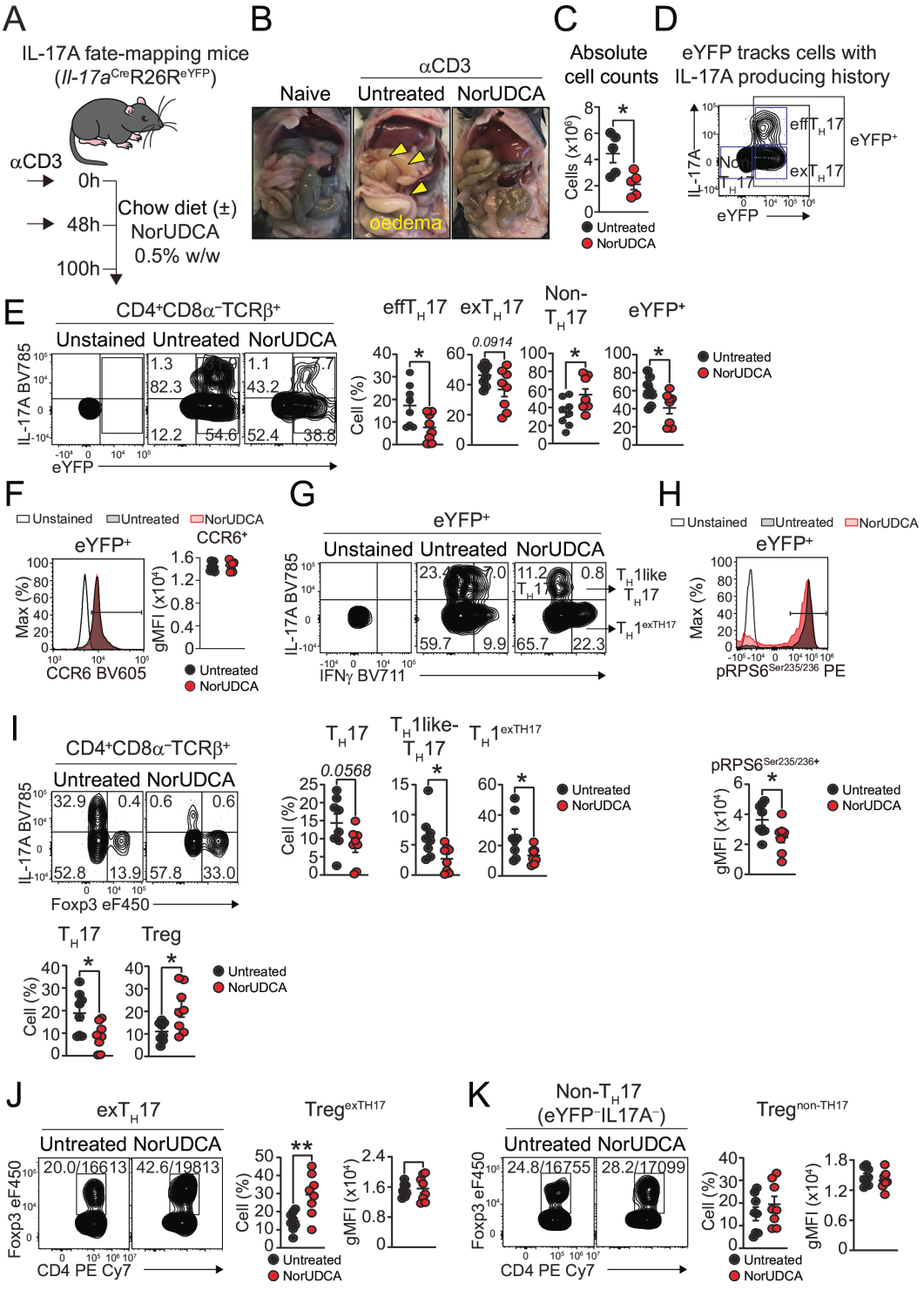

Figure 2 NorUDCA targets T_H17 pathogenicity and transdifferentiation during intestinal inflammation *in vivo* in an αCD3 model. (A) Experimental design. (B) Photographs of intestines from IL-17A-fate-mapping mice±*i.p.* αCD3 fed either standard chow or NorUDCA enriched diet. (C) Absolute cell counts from leucocytes extracted from small intestines. (D) Gating strategy for small intraepithelial effector(eff)T_H17, exT_H17 and $CD4^+$ lymphocytes with IL-17A producing history (YFP^+). (E) Representative and summary of frequencies of effT_H17 cells, exT_H17 cells, YFP^+ cells and non-T_H17 cells of indicated groups. (F) Representative expression of CCR6 on YFP^+ cells and corresponding quantitative analysis. (G) Gating strategy for T_H17, T_H1^{exTH17} and T_H1 cells transdifferentiated from exT_H17 cells (T_H1-likeT_H17). Representative flow cytometric plots and analysis of indicated cell types. (H) Representative expression of phosphorylated RPS6 (serine 235/236) on YFP^+cells and corresponding quantitative analysis. (I, J, K) Representative flow cytometric plots and analysis of Tregs derived from exT_H17 cells and non-T_H17 cells. Data presented throughout this figure were analysed from *ex vivo* isolated small intestinal intraepithelial lymphocytes . Data are cumulative of two independent experiments, with at least three mice per group for each experiment. Each point represents one mouse. Mean and SEM are shown. *P* values were calculated using the unpaired Student's *t*-test (two-tailed). *$p\leq0.05$, **$p\leq0.01$, ***$p\leq0.001$, ****$p\leq0.0001$. *i.p.*, intraperitoneal; eYFP, express yellow fluorescent protein; gMFI, geometric mean-fluorescence intensity; IFN, interferon; IL, interleukin; NorUDCA, 24-Nor-ursodeoxycholic acid; RPS6, ribosomal protein S6; T_H17, T helper-type-like 17; Treg, regulatory T cell.

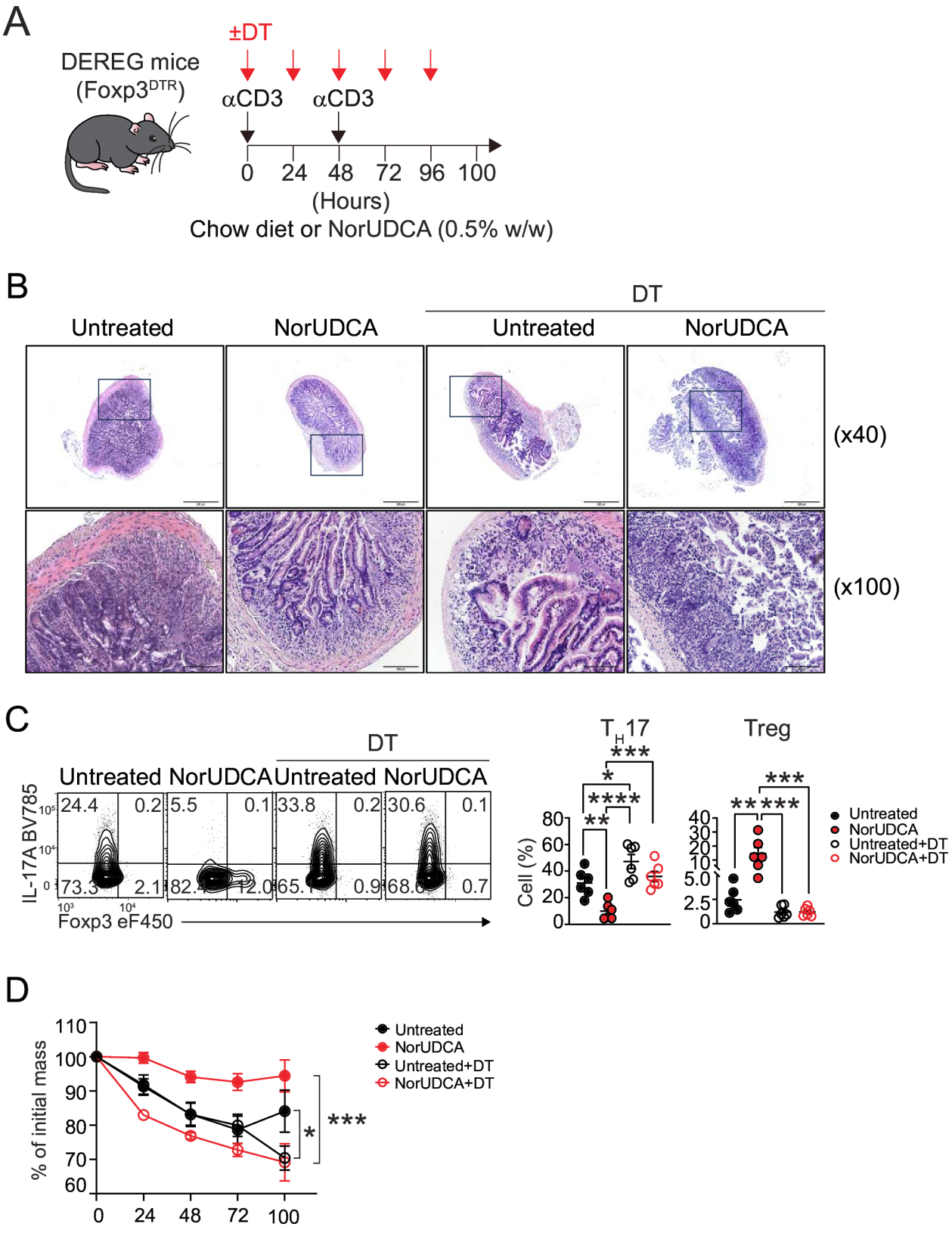

Figure 3 Treg induction is essential for NorUDCA's restriction on T_H17 pathogenicity during intestinal inflammation *in vivo*. (A) Experimental design. (B) H&E staining of representative histological sections (scale bar 200 µm) of indicated groups. (C) Representative flow cytometric plots and frequency of T_H17 and Treg in αCD3 challenged untreated or NorUDCA-treated DEREG mice±Treg depletion by DT administration. Data are analysed from ex vivo isolated small intestinal intraepithelial lymphocytes . Data summarises two independent experiments, with at least three mice per group per experiment. Each point represents one mouse. (D) Body weight loss data is presented, derived from one experiment with five mice per group. Results (C, D) are expressed as mean and SEM are indicated. *P* values were calculated using the one-way ANOVA analysis (C) or two-way ANOVA with Tukey's multiple comparisons test (D). *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$, ****$p \leq 0.0001$. ANOVA, analysis of variance; DT, diphtheria toxin; IL, interleukin; NorUDCA, 24-Nor-ursodeoxycholic acid; Treg, regulatory T cell.

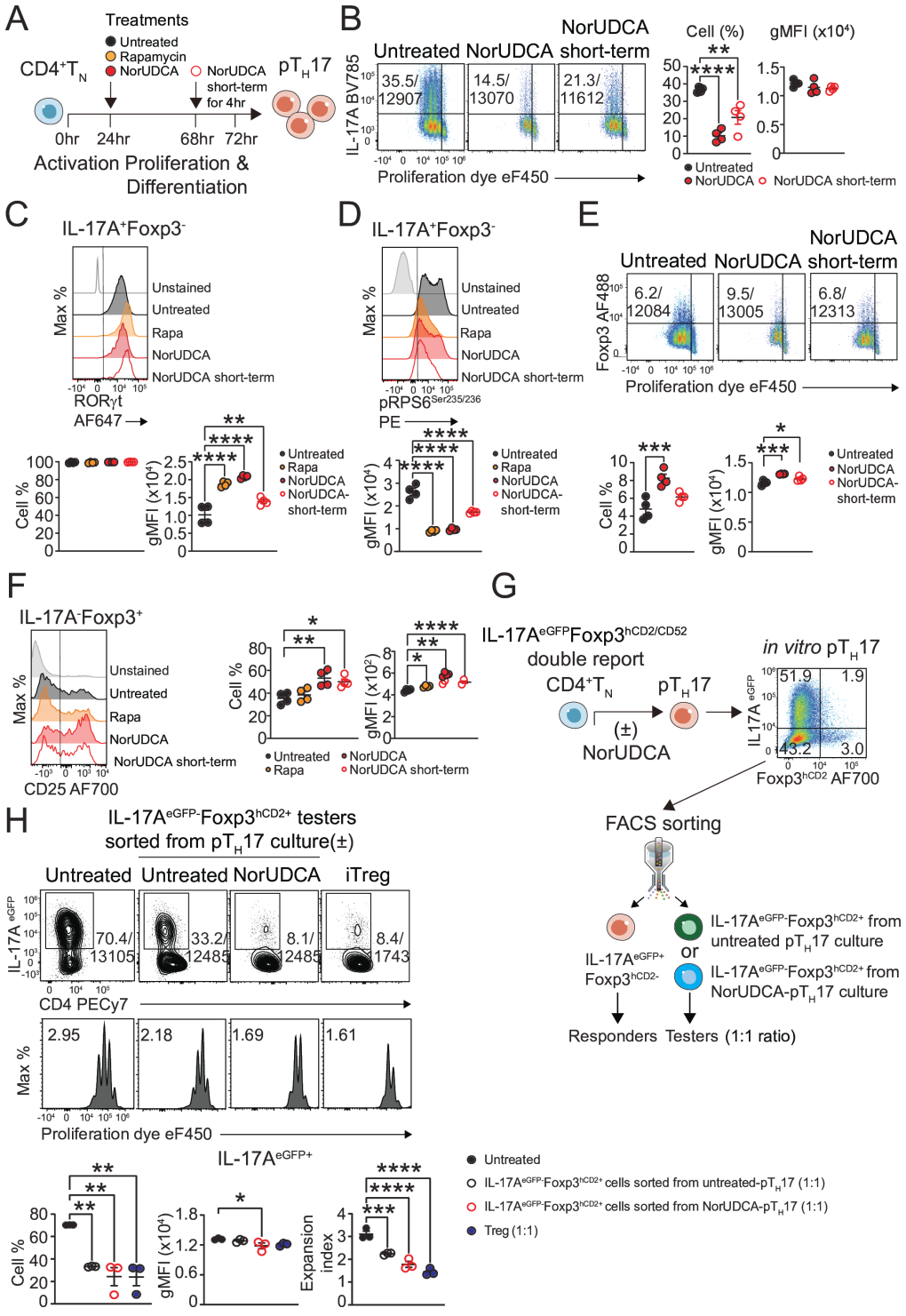

Figure 4 NorUDCA restrains pathogenic T_H17 cell differentiation and promotes functional suppressive Treg development *in vitro*. (A) Experimental design. (B) IL-17A production and proliferation of pathogenic (p)T_H17 cells. gMFI of IL-17A are shown. (C) Expression of RORγt and (D) phosphorylated RPS6 (serine 235/236) in IL17A$^+$Foxp3$^-$cells from pT_H17 culture. Rapamycin (mTORC1 inhibitor) was used for comparison (C,D). (E) Foxp3 expression and proliferation of pT_H17 cells. gMFI of Foxp3 are shown. (F) CD25 expression in IL-17A$^-$Foxp3$^+$ cells from pT_H17 culture. (B–F) are data cumulative of two independent experiments (n=4 biologically independent samples per group). (G) Experimental design of the *in vitro* suppression assay. IL-17A^{eGFP+}Foxp3$^{hCD2/CD52-}$cells and Foxp3$^{hCD2/CD52+}$IL-17A^{eGFP-}cells were sorted from pT_H17 cultures, IL-17A^{eGFP+}Foxp3$^{hCD2/CD52-}$cells (responders) labelled with proliferation dye eF450 were mixed with Foxp3$^{hCD2/CD52+}$IL-17A^{eGFP-}cells (testers) or *in vitro* differentiated induced (i)Tregs at 1:1 ratio. (H) IL-17A^{eGFP} expression and dilution of proliferation dye gated on IL-17A^{eGFP+}Foxp3$^{hCD2/CD52-}$cells (number depicts expansion index) (n=3 biologically independent samples per group). Frequency (upper) and gMFI (lower) are shown in the representative flow cytometric plots of (B–F, H). Mean and SEM are indicated. *P* values were calculated using a one-way analysis of variance corrected for multiple comparisons with the Dunnett's post hoc test. *$p\leq0.05$, **$p\leq0.01$, ***$p\leq0.001$, ****$p\leq0.0001$. gMFI, geometric mean-fluorescence intensity; IL, interleukin; NorUDCA, 24-Nor-ursodeoxycholic acid; pT_H17, pathogenic T_H17; Rapa, rapamycin; RORγt, retinoic acid orphan receptor-gamma; RPS6, ribosomal protein S6; T_H17, T helper-type-like 17; Treg, regulatory T cell.

extent comparable to IL-17A^{eGFP+}Foxp3$^{hCD2/CD52-}$ cells co-cultured with conventional cultured Tregs (figure 4H). Therefore, Foxp3$^{hCD2/CD52+}$IL-17A^{eGFP-} cells induced by NorUDCA are functional Tregs that have suppressive activity.

Additionally, NorUDCA enhances Foxp3 expression among IL-17A^{eGFP+}Foxp3$^{hCD2/CD52-}$ cells sorted from untreated-pT$_H$17 cultures on restimulation, further supporting our hypothesis that NorUDCA drives the transdifferentiation of pT$_H$17 cells into Tregs (online supplemental figure 8).

NorUDCA did not affect CD4$^+$T$_{Naive}$ cells differentiating into Tregs under Treg polarising conditions with various concentrations of TGF-β (online supplemental figure 9). Intriguingly, Treg induction by NorUDCA under T$_H$0 condition suggests that NorUDCA's induction of Tregs might be selective for certain T$_{Helper}$ lineages (online supplemental figure 9).

NorUDCA induces a distinct metabolic programme that drives Treg generation among differentiating pT$_H$17 cells

pT$_H$17 differentiation, plasticity and effector function is regulated by metabolic processes.[31] mTORC1 is pivotal in orchestrating pT$_H$17 cellular metabolic pathways including glycolysis.[32] Considering NorUDCA's ability to inhibit mTORC1 during pT$_H$17 immune response, we hypothesised that NorUDCA may reprogramme pT$_H$17 metabolism, thereby influencing pT$_H$17 effector function.

Bulk RNA sequencing revealed that brief exposure to NorUDCA reshaped the transcriptomic landscape of murine pT$_H$17 cells, with enriched pathways including mTORC1 signalling and associated metabolic processes (online supplemental figure 10A-D). Additionally, NorUDCA altered the expression of genes involved in glutamine metabolism (online supplemental figure 10E). Targeted metabolomics corroborated these findings, showing that NorUDCA short-term treatment reduced glutamine-to-glutamate conversion and decreased levels of tricarboxylic acid (TCA) cycle metabolites (online supplemental figure 11A).

Long-term treatment with NorUDCA further disrupted glycolytic signalling by downregulating transcription factors Hif1α[33] and c-Myc[34] (figure 5A,B). This was accompanied by reduced expression of glucose transporter 1 (GLUT1), impaired glucose uptake (figure 5C,D) and lower extracellular acidification rates under both baseline and mitochondrial stress conditions during pT$_H$17 differentiation (figure 5E), as measured by Seahorse analysis.

Integrated with glycolysis, pT$_H$17 expedites glutamine uptake and glutaminolysis to meet the increasing bioenergetic demands for rapid expansion and acquisition of effector functions.[35] Hydrolysis of glutamine by the enzyme glutaminase2 (*Gls2*) produces glutamate, which is metabolised to α-ketoglutarate to fuel the TCA cycle. Glutamine deprivation hampers pT$_H$17 development and promotes Foxp3 expression in a process linked to mTORC1 signalling.[35] The major glutamine transporter ASCT2 (also known as *Slc1A5*) expression (figure 5F) was unaffected by NorUDCA, suggesting that glutamine uptake may remain unaltered. NorUDCA transcriptionally downregulated *Gls2*, the rate-limiting enzyme in glutaminolysis (figure 5G), and decreased α-ketoglutarate in pT$_H$17 (figure 5H).

Cell-permeable α-ketoglutarate supplementation of pT$_H$17 cultures, abrogated mTORC1 inhibition by NorUDCA under glutamine deprivation and reverted NorUDCA's effect on Foxp3 and IL-17A expression (figure 5I). Furthermore, the addition of α-ketoglutarate restored mTORC1 activity and GLUT1 expression under glutamine-deprived conditions in the presence of NorUDCA (figure 5J,K), indicating NorUDCA targets a glutaminolysis-mTORC1-glycolysis feedback loop (figure 5L). Taken together, our data reveal that NorUDCA reshapes a distinct metabolic programme favouring Treg generation among differentiating pT$_H$17.

Short-term exposure of pT$_H$17 cells to NorUDCA *ex vivo* dampens pathogenic potential and expansion on transfer *in vivo*

To study whether NorUDCA affects the T$_H$17 pathogenic potential in a T cell-intrinsic manner, we combined *ex vivo* culture with two adoptive cell transfer models. First, we adoptively transferred pT$_H$17, either short-term exposed to NorUDCA or not (online supplemental figure 12), into *Rag2*$^{-/-}$ recipients to induce intestinal inflammation. Mice were cohoused to normalise the macrobiotic environment (figure 6A). 4 weeks post transfer, *Rag2*$^{-/-}$ recipients that received non-treated pT$_H$17 developed pronounced oedema, while in mice reconstituted with pT$_H$17 briefly exposed to NorUDCA *ex vivo*, oedema was much milder (figure 6B). Accordingly, in mice transferred with NorUDCA-treated pT$_H$17, the frequency of intraepithelial T$_H$17 was decreased, whereas the frequency of Treg among both IEL and LPL from small intestines was increased (figure 6C,D). CCR6 expression was similar, suggesting *ex vivo* NorUDCA-treatment of T$_H$17 might not affect *in vivo* migration mediated by CCR6 (figure 6E). However, IL-17A-expressing T$_H$17 from IEL or LPL isolated from mice transferred with NorUDCA-treated T$_H$17 exhibited disrupted mTORC1 kinase activity and reduced proliferation as evidenced by lower phosphorylation level of RPS6 and expression of Ki67, respectively, compared with those of mice transferred with untreated-cells (figure 6G,F).

To test whether NorUDCA alleviates T$_H$17-mediated inflammation outside intestine, we used an antigen-specific T-cell transfer-induced experimental autoimmune encephalomyelitis (EAE) model (figure 6H). CD4$^+$T$_{Naive}$ cells derived from 2D2-transgenic mice (expressing myelin oligodendrocyte glycoprotein antigen (MOG$_{33-35}$)-specific T-cell receptors) were differentiated into pT$_H$17±short-term exposure to NorUDCA *ex vivo* (figure 6H, online supplemental figure 13). Subsequently, cells were adoptively transferred into *Rag2*$^{-/-}$ recipients which were immunised with MOG peptide and received pertussis toxin to induce EAE (figure 6H). On immunisation, mice reconstituted with untreated-TCR^{2D2}pT$_H$17 exhibited progressive clinical signs of EAE (figure 6I,J). In contrast, in mice receiving NorUDCA-treated TCR^{2D2}pT$_H$17, disease onset was significantly delayed (figure 6I,J). Similar to the pT$_H$17 adoptive transfer-induced intestinal inflammation model, decreased frequencies of IL-17A-expressing T$_H$17 cells and increased frequencies of Tregs among central nervous system-infiltrating CD4$^+$ T cells were observed in mice transferred with NorUDCA-treated TCR^{2D2}pT$_H$17 cells, without alteration of migratory markers, such as CCR6 and CXCR3, but with reduced Ki67 expression (figure 6L,M). Although our data suggest a promising role for NorUDCA in modulating pT$_H$17 inflammation in a T cell-intrinsic manner, further studies are required to confirm whether oral administration of NorUDCA in EAE mice yields the same anti-inflammatory effects as observed *ex vivo*.

Together, we found that NorUDCA *ex vivo* treatment can diminish the pathogenic potential of T$_H$17 and drive Foxp3$^+$Treg formation post-transfer *in vivo*. Importantly, this clearly indicates that NorUDCA modulates T$_H$17-mediated inflammation in a T cell-intrinsic fashion.

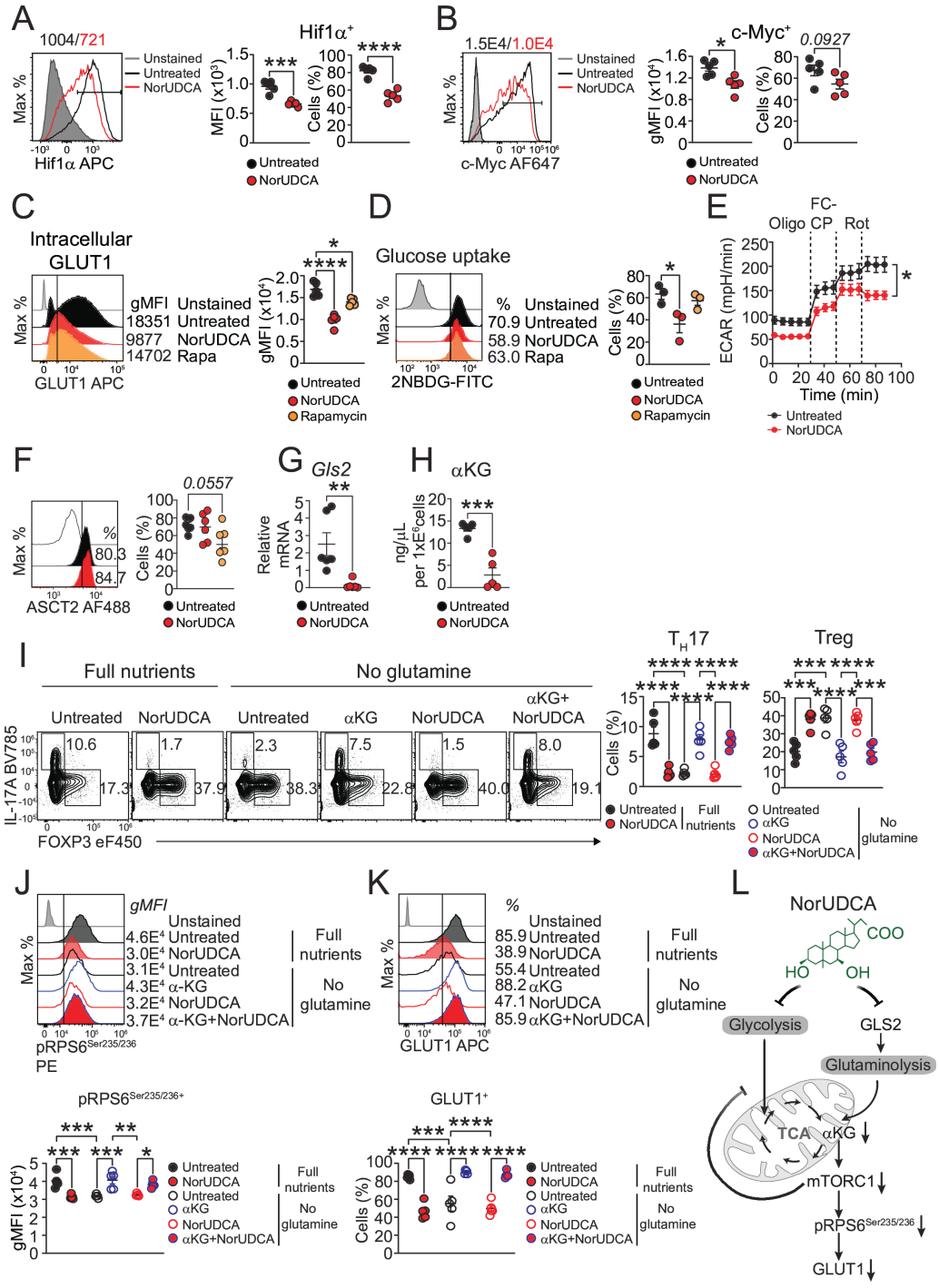

Figure 5 NorUDCA restricts glutamine metabolism that licenses mTORC1 activation and glycolysis in differentiating pT$_H$17 cells. (A, B) Expression of Hif1α (A) and c-Myc (B) on in vitro differentiated pathogenic (p)T$_H$17 cells (gated on IL-17A$^+$Foxp3$^-$fraction). Numbers depict gMFI (A,B). (C) Expression of intracellular GLUT1 expression on differentiating pT$_H$17 cells. (D) Glucose uptake by differentiating pT$_H$17 cells. (E) Seahorse ECAR analysis in real time of activated CD4$^+$T cells treated±NorUDCA. (F) Expression of ASCT2 on *in vitro* differentiated pT$_H$17 cells (gated on IL-17A$^+$Foxp3$^-$ cells). (G) Real-time PCR analysis of *Gls2* expression (normalised to house-keeping *Hprt*) in pT$_H$17 cells. (H) Intracellular αKG level in pT$_H$17 cells. (I) Flow cytometric analysis of T$_H$17 and Treg within pT$_H$17 culture under indicated conditions. (J, K) Expression of phosphorylated RPS6 (serine 235/236) and GLUT1 on pT$_H$17 cells (gated on IL-17A$^+$Foxp3$^-$fraction) under indicated conditions. (L) A model showing NorUDCA's impact on glutaminolysis-mTORC1-glycolysis signalling in differentiating pT$_H$17 cells. Data summaries three independent experiments. Data shown in (A, B, C, G, H, I, J, K) n=5, (D) n=3, (E) n=10 biologically independent samples per group. Mean and SEM are shown. *P* values in (A, B, G, H) were calculated using the unpaired Student's *t*-test (two-tailed), in (E) were calculated using a two-way ANOVA followed by Bonferroni's multiple comparison post hoc tests and in (C, D, F, I, J, K) were calculated using a one-way ANOVA corrected for multiple comparisons with Dunnett's post hoc test. *$p\leq$ 0.05, **$p\leq$0.01, ***$p\leq$0.001, ****$p\leq$0.0001. ANOVA, analysis of variance; ASCT2, alanine serine cysteine transporter 2; ECAR, extracellular acidification rate; FCCP, carbonyl cyanide-4 (trifluoromethoxy) phenylhydrazone; GLUT1, glucose transporter 1; gMFI, geometric mean fluorescence intensity; IL, interleukin; mRNA, messenger RNA: NorUDCA, 24-Nor-ursodeoxycholic acid; Oligo, oligomycin; Rapa, rapamycin; Rot, rotenone; RPS6, ribosomal protein S6; T$_H$17, T helper-type-like 17; Treg, regulatory T cell; 2-NBDG, 2-deoxy-2-((7-nitro-2,1,3-benzoxadiazol-4-yl)amino)-D-glucose; αKG, alpha-ketoglutarate.

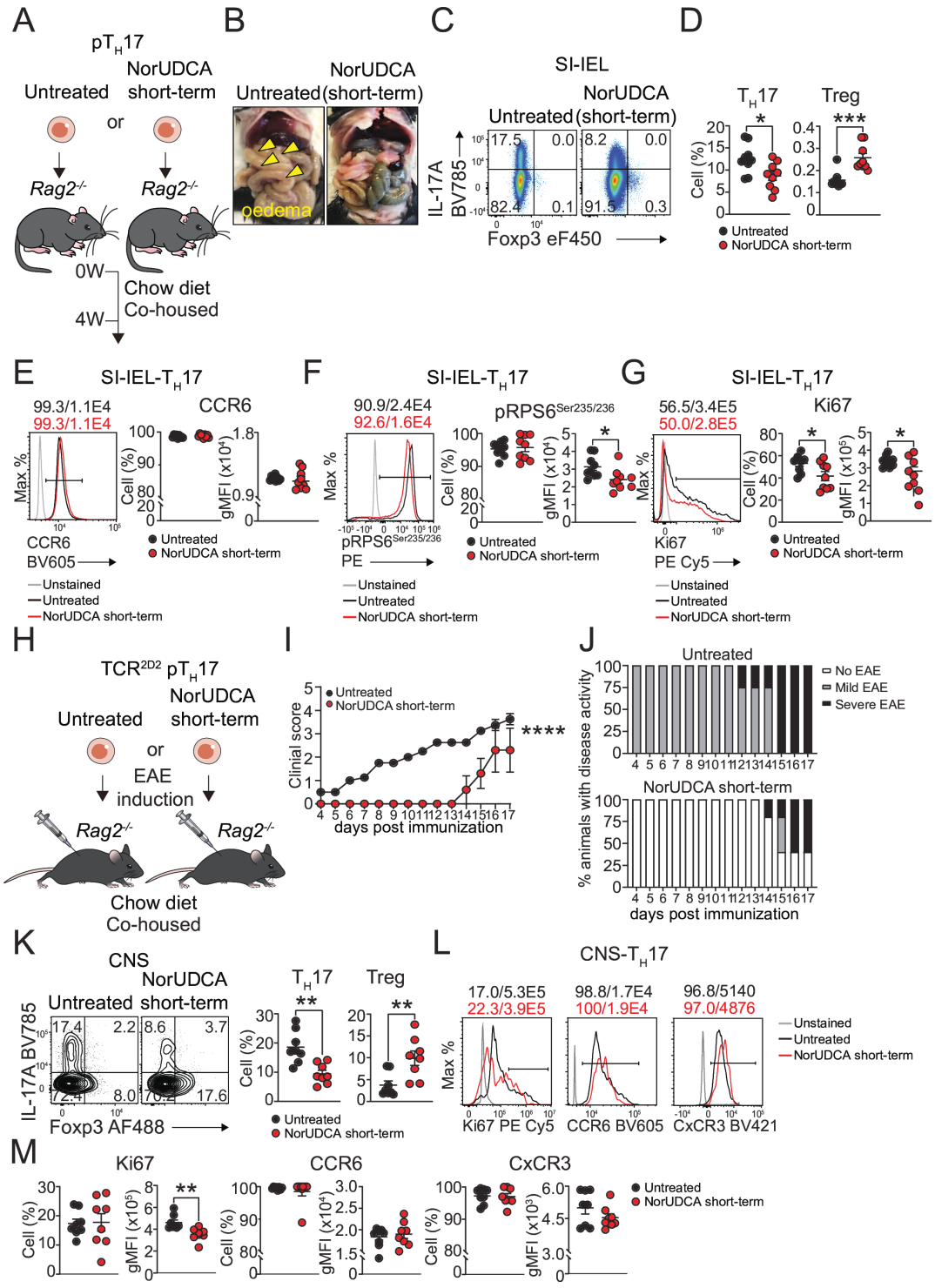

Figure 6 pT_H17 cells briefly exposed to NorUDCA *ex vivo* show dampened pathogenic potential and expansion upon transfer *in vivo*. (A) Experimental design for adoptive cell transfer (ACT)-induced intestine inflammation model. (B) Intestines from *Rag2*$^{-/-}$ mice reconstituted with pT_H17 cells briefly exposed±NorUDCA. (C, D) Frequency of T_H17 and Treg among small intestinal IELs *ex vivo* isolated from ACT-induced intestine inflammation mice. (E–G) Expression of CCR6, phosphorylated RPS6 (serine 235/236) or Ki67 on small intestinal intraepithelial T_H17 cells from ACT-induced intestine inflammation mice. Data presented in (C–G) are cumulative of two independent experiments (n=8 biologically independent samples per group). (H) Experimental design for ACT-induced experimental autoimmune encephalomyelitis model (ACT-EAE). (I) Clinical scoring. (J) Distribution of disease severity. On a scale of 1–5, No EAE=score <0.5; mild EAE=score range 0.5–2.5; severe EAE=score >3. (K) Frequency of T_H17 and Treg cells found in the CNS. (L, M) Expression of Ki67, CCR6 and CXCR3 on CNS-infiltrating T_H17 cells of ACT-EAE mice. Data presented in (I, J) is representative of two independent experiments and in (K–M) are cumulative of two independent experiments (n=8 biologically independent samples per group). Numbers depict frequencies and gMFI (E–G, L). Mean and SEM are shown. *P* values in (C–G, K–M) were calculated using the unpaired Student's *t*-test (two-tailed) and in (I) were calculated using a two-way analysis of variance followed by Bonferroni's multiple comparison tests. *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$, ****$p \leq 0.0001$. CNS, central nervous system; gMFI, geometric mean-fluorescence intensity; IEL, intraepithelial lymphocytes; IL, interleukin; NorUDCA, 24-Nor-ursodeoxycholic acid; pT_H17, pathogenic T_H17; SI, small intestine; T_H17, T helper-type-like 17; Treg, regulatory T cell.

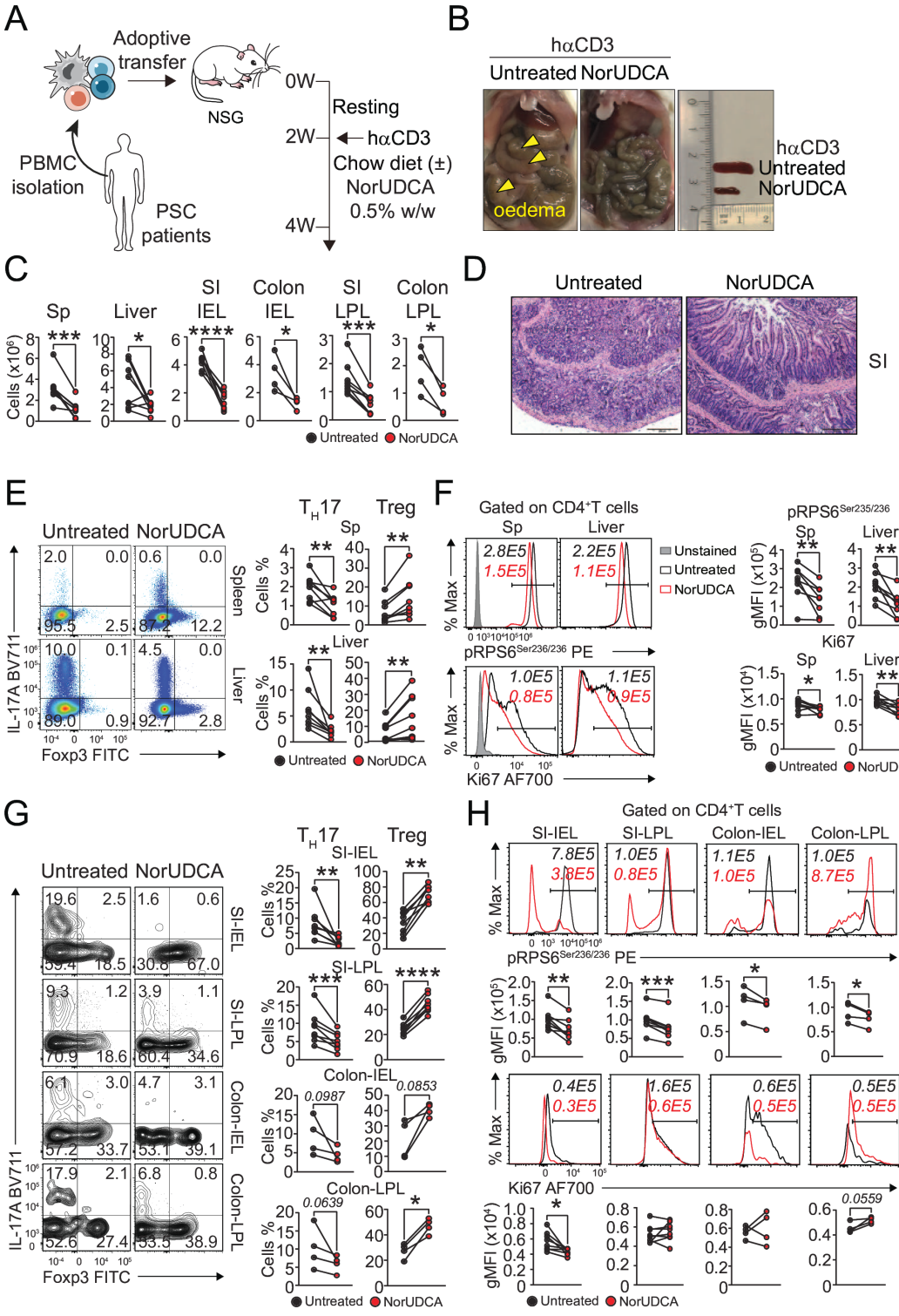

Figure 7 NorUDCA represses T_H17 inflammation in humanised NSG model reconstituted with PBMCs from patients with PSC. NorUDCA represses T_H17 inflammation in humanised NSG model reconstituted with PBMCs from patients with PSC. (A) Experimental design. (B) Intestines of NSG mice reconstituted with patient with PSC-derived PBMCs±*i.p.* hαCD3 fed either standard chow or NorUDCA-supplementary diet. (C) Absolute cell counts of ex vivo isolated leucocyte fractions. (D) H&E staining of small intestine (scale bar 200 µm). (E) Flow cytometric analysis of splenic and hepatic T_H17 and Treg cells. (F) Expression of phosphorylated RPS6 (serine 235/236) or Ki67 on splenic and hepatic T_H17 cells. (G) Flow cytometric analysis of T_H17 and Treg cells within intraepithelial or lamina propria fractions of small intestine or colon. (H) Expression of phosphorylated RPS6 (serine 235/236) or Ki67 expression on T_H17 cells. (F, H) Depict frequency (upper) and gMFI (lower) of positive cell populations. Data are cumulative of two independent experiments, n=8 biologically independent samples per group. Every two samples from colonic fractions (IEL or LPL) were pooled to achieve sufficient cell numbers for flow cytometric analysis. Mean and SEM are shown. *P* values were calculated using a paired Student's *t*-test (two-tailed). *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$, ****$p \leq 0.0001$. gMFI, geometric mean fluorescence intensity; IEL, intraepithelial lymphocytes; IL, interleukin; *i.p.*, intraperitoneal; LPL, lamina propria lymphocytes; NorUDCA, 24-Nor-ursodeoxycholic acid; PBMC, peripheral blood mononuclear cell; PSC, primary sclerosing cholangitis; h, human; pT_H17, pathogenic T_H17; SI, small intestine; Sp, spleen; T_H17, T helper-type-like 17; Treg, regulatory T cell.

NorUDCA represses T_H17 inflammation in a humanised NSG mouse model reconstituted with PBMCs from patients with PSC

Finally, to translationally validate key findings obtained in mice, we performed *in vitro* T_H17 differentiation assays using human $CD4^+T_{Naive}$ cells and observed that NorUDCA restricts the effector functions and mTORC1 activity of human T_H17 cells (online supplemental figure 14), mirroring the effects seen in murine studies. Moreover, we used a humanised NOD/SCID/IL-2rγ$^{-/-}$ (NSG) mouse model reconstituted with PBMCs from patients with PSC with (70%) and without (30%) inflammatory bowel diseases (IBD) (online supplemental table 2).[4] 2 weeks after PBMC reconstitution, mice received teplizumab (hOKT3γ1), a human (h)αCD3 antibody and fed either chow or NorUDCA-diet for another 2 weeks (figure 7A, online supplemental figure 15). Consistent with a published report,[4] hαCD3 in vivo stimulation led to oedema and inflammation along the intestine, as well as in enlarged spleens which were ameliorated by NorUDCA treatment (figure 7B). Reduced immune cell numbers in the spleen, liver, IEL fractions of small intestine and colon of NorUDCA-treated humanised mice compared with untreated mice confirmed reduced inflammation (figure 7C,D). Consistent with our earlier data, T_H17 frequency was reduced and Treg frequency was increased in multiple tissues, such as spleen, liver, small intestine and colon of the humanised mice receiving NorUDCA, compared with untreated mice (figure 7E,G). Moreover, intraepithelial T_H17 in NorUDCA-treated humanised mice exhibited disrupted mTORC1 activity and Ki67 expression (figure 7F,H), phenocopying key findings observed in the murine models. Taken together, NorUDCA prevents an excessive T_H17 inflammatory response and promotes the expansion of Tregs among T cells from patients with PSC on hαCD3 stimulation.

DISCUSSION

PSC is an immune-mediated cholestatic liver disease highly associated with intestinal inflammation in about 70% of the patients, that currently lacks effective medical treatment.[11 36–39] Although the pathophysiological and clinical implications of this association of PSC and IBD are incompletely understood,[40 41] seeking therapeutic strategies to effectively target both PSC and concomitant intestinal inflammation may be of high clinical interest.[11 37 38 40]

Several studies have highlighted the potential role of the T_H17 inflammatory response in both PSC and IBD pathogenesis.[42] Patients with PSC, whose $CD4^+T_{Naive}$ cells show enhanced T_H17 differentiation potential on *in vitro* cytokine stimulation[43] exhibit increased T_H17 frequency and inflammatory responses.[44 45] Importantly, a pathogenic role of T_H17 has been implicated in IBD development.[46] Therefore, it is tempting to speculate that T_H17 could serve as therapeutic targets for treating PSC-associated intestinal inflammation.

Here, we demonstrate that NorUDCA, a promising therapeutic BA for improving cholestasis in PSC,[10 17] exerts potent anti-inflammatory effects by alleviating intestinal inflammation in multiple *in vivo* models of T_H17-associated immunopathology. The therapeutic role of Tregs in mitigating intestinal inflammation is underscored by a recent pilot study where autologous Tregs were successfully transferred into patients with refractory ulcerative colitis and concomitant PSC.[47] Notably, selective Treg ablation *in vivo* diminished NorUDCA's efficacy in suppressing T_H17-driven inflammatory responses, indicating that Treg induction is integral to NorUDCA's ant-inflammatory mechanism in restricting T_H17 pathogenicity in the αCD3 model. However, it is important to note that Treg depletion did not completely abolish NorUDCA's anti-T_H17 effects, suggesting that other regulatory cell types might also contribute. Given the current lack of transgenic models for *in vivo* Tr1 cell ablation, we cannot exclude the potential involvement of Tr1 cells or other regulatory populations in NorUDCA's anti-inflammatory mechanisms, which needs to be explored by future studies.

Our data, for the first time, demonstrate a yet-unrecognised effect of NorUDCA on T_H17 transdifferentiation during intestinal inflammation. This not only adds cellular insights underlying NorUDCA's anti-inflammatory mechanisms, but also lays the scientific basis for future investigations into the potential impacts of other therapeutic BAs or naturally occurring BAs abundant within intestine and liver, on $CD4^+T_{Helper}$ cell transdifferentiation under homeostatic and pathological conditions.

Since our findings primarily focus on NorUDCA's efficacy in treating intestinal inflammation, the relevance of modulating T_H17 cell pathogenicity and transdifferentiation in hepatobiliary inflammatory conditions including PSC, remains a critical question for future investigations.

Feeding NorUDCA *in vivo* significantly increases systemic BA levels[48] with the potential to influence host homeostasis, innate immunity, tissue structural cells and microbiome constitution, all of which might impact T_H17 immunity. Given the complexity of immune regulation, we cannot exclude that the modulation of T_H17 transdifferentiation induced by NorUDCA might also result from its alteration of the aforementioned factors. However, intriguingly, we revealed that even brief *ex vivo* exposure of NorUDCA can confer long-lasting effects on the pathogenic potential and expansion of T_H17 on transfer *in vivo*. These data clearly imply that NorUDCA directly modulates T_H17 immunity in a T-cell intrinsic manner.

Mechanistically, by focusing on differentiating pT_H17, NorUDCA was found to operate a distinct metabolic programme that conditions Treg generation by restricting the loop of glutaminolysis-mTORC1-glycolysis. Previously, we showed that NorUDCA inhibits mTORC1 by perturbing the Ras-Erk-P90RSK axis,[12] the classic upstream signal transduction network regulating mTORC1.[49 50] Here, NorUDCA remodels glutamine sensing programmes and upstream signalling that license mTORC1 activation, resulting in subsequent alterations in cellular metabolism and effector functions. Our original discoveries about NorUDCA reshaping signal transduction cascades and metabolism at multiple layers are key to understanding cellular and molecular mechanisms underlying NorUDCA's immunomodulatory effects (see visual abstract) (online supplemental file 1). Further metabolic studies on NorUDCA across different T-cell subsets are warranted, that will deepen our mechanistic understanding and potentially extend clinical applications of NorUDCA beyond PSC.

Finally, our translational approach validating the key findings obtained with the murine system demonstrated that NorUDCA rectifies systemic T_H17 inflammation in the αCD3-challenged humanised NSG mouse model reconstituted with PBMCs from patients with PSC. Thus, we envision that NorUDCA may have future therapeutic implications for treating PSC-associated intestinal inflammation, where NorUDCA has already shown promising results in part for liver disease.[10] Further studies are warranted to explore the array of potential therapeutic applications of NorUDCA in T_H17-mediated intestinal and hepatic diseases.

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Contributors CZ conceived the project, designed and performed all experiments, analysed the data and wrote the manuscript. NB and WE contributed to the experimental design, shared reagents and contributed to data interpretation. OA-R, DW, TC and VK provided assistance for in vivo experiments. EH, BKC, LWT and THK performed human peripheral blood mononuclear cell isolation. AO-R, PS and HSt assisted in vitro human T cell experiments. FM and SH assisted in vitro Tr1 differentiation assay. HSc and TS measured serum liver biochemistry. NB, AL and AB assisted for metabolic assays. LK performed histological scoring of intestinal tissue slides. TK performed targeted metabolomic measurement. MS and CB performed bulk RNA sequencing and assisted for analysis. LLC edited the manuscript. WE and MT supervised the project, edited the manuscript, act as guarantors and accept full responsibility for the overall content of the manuscript. All authors approved the manuscript.

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Competing interests MT has served as speaker for Agomab, Albireo, BMS, Chemomab, Falk Foundation, Gilead, Intercept, Ipsen, Madrigal and MSD; he has advised for AbbVie, Albireo, BiomX, Boehringer Ingelheim, Falk Pharma GmbH, Genfit, Gilead, Hightide, Intercept, Ipsen, Jannsen, MSD, Novartis, Phenex, Pliant, Rectify, Regulus, Siemens and Shire. He further received travel grants from AbbVie, Falk, Gilead, Intercept and Jannsen and research grants from Albireo, Almylam, Cymabay, Falk, Gilead, Intercept, MSD, Takeda and Ultragenyx. He is also co-inventor of patents on the medical use of NorUDCA filed by the Medical Universities of Graz and Vienna. THK has served as speaker for Gilead and consulted for Falk Pharma, Albireo, MSD and Boehringer Ingelheim. SH served as speaker and advisor for AbbVie, Janssen, Lilly, Falk, BMS and Ferring outside the submitted work. CB is a cofounder and scientific advisor of Myllia Biotechnology and Neurolentech. All the other authors declare no conflict of interest.

Patient and public involvement Patients and/or the public were not involved in the design, or conduct, or reporting, or dissemination plans of this research.

Patient consent for publication Not applicable.

Ethics approval All animal experiments were performed in accordance with Federal Ministry for Science and Research at Medical University of Vienna (MUV) (BMWFW66.009/0008-WF/V/3b/2015), (BMBWF-66.009/0039-V/3b/2019) and (2023- 0.896.905). Human peripheral T cells were obtained from patients with primary sclerosing cholangitis following the Declaration of Helsinki and approved by the Ethics Committee of the MUV: 747/2011, 2001/2018 and regional Committees of Medical and Health Research Ethics of South East Norway (2012-286 and 2016-1540).

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