# Technical University of Denmark

**DTU**

# IL-18R-deficient CD4+T cells induce intestinal inflammation in the CD45RBhitransfer model of colitis despite impaired innate responsiveness

**Holmkvist, Petra; Pool, Lieneke; Hägerbrand, Karin; Agace, William Winston; Rivollier, Aymeric Marie Christian**

## DTU Library
Technical Information Center of Denmark

# IL-18Rα-deficient CD4+ T cells induce intestinal inflammation in the CD45RBhi transfer model of colitis despite impaired innate responsiveness

*Petra Holmkvist[1], Lieneke Pool[2], Karin Hägerbrand[1], William W. Agace[1,2] and Aymeric Rivollier[2]*

[1] Immunology Section, Lund University, Lund, Sweden
[2] Section of Immunology and Vaccinology, National Veterinary Institute, Technical University of Denmark, Frederiksberg, Denmark

IL-18 has been implicated in inflammatory bowel disease (IBD), however its role in the regulation of intestinal CD4+ T-cell function remains unclear. Here we show that murine intestinal CD4+ T cells express high levels of IL-18Rα and provide evidence that IL-18Rα expression is induced on these cells subsequent to their entry into the intestinal mucosa. Using the CD45RBhi T-cell transfer colitis model, we show that IL-18Rα is expressed on IFN-γ+, IL-17+, and IL-17+IFN-γ+ effector CD4+ T cells in the inflamed colonic lamina propria (cLP) and mesenteric lymph node (MLN) and is required for the optimal generation and/or maintenance of IFN-γ-producing cells in the cLP. In the steady state and during colitis, TCR-independent cytokine-induced IFN-γ and IL-17 production by intestinal CD4+ T cells was largely IL-18Rα−dependent. Despite these findings however, IL-18Rα−deficient CD4+ T cells induced comparable intestinal pathology to WT CD4+ T cells. These findings suggest that IL-18-dependent cytokine induced activation of CD4+ T cells is not critical for the development of T-cell-mediated colitis.

Additional supporting information may be found in the online version of this article at the publisher's web-site

## Introduction

IL-18 is a member of the IL-1 cytokine superfamily produced primarily by macrophages, dendritic cells, and intestinal epithelial cells under homeostatic conditions [1, 2]. It is produced as an inactive 24 kDa precursor (pro-IL-18) and processing to its 18 kDa bioactive form requires inflammasome activation and the proteolytic activity of caspase-1 [3], or alternative processing pathways, notably involving proteinase-3 [4]. The IL-18 receptor (IL-18R) is composed of two subunits, IL-18 receptor alpha (IL-18Rα)

and IL-18Rβ (also known as IL-18R accessory protein IL-18Rap), both of which consist of three extracellular immunoglobulin-like domains and one intracellular Toll/IL-1 receptor (TIR) domain. Ligand binding triggers receptor heterodimerization [5] and initiates downstream signaling events via the two TIR domains [6], which recruit MyD88 and signal through IL-1R-associated kinases (IRAK) to initiate p38 MAPK and NF-κB-mediated responses [3, 7].

IL-18 expression is upregulated in a range of inflammatory and autoimmune diseases (for recent reviews see [3, 8]), including inflammatory bowel disease (IBD), the two major forms of which are Crohn's disease (CD) and ulcerative colitis (UC) [9, 10]. IL-18 expression is notably increased in mucosal biopsies of IBD patients compared with those of control patients, but also in inflamed versus noninflamed intestinal tissues of IBD patients [9, 10]. The

*Correspondence:* Dr. Aymeric Rivollier
e-mail: ayri@vet.dtu.dk

predominant source of IL-18 in IBD is thought to be intestinal epithelial cells, however in CD patients during the late and most severe phase of the disease, lamina propria (LP) macrophages and dendritic cells become an additional source of this cytokine [9]. Further we have recently demonstrated high levels of IL-18 expression in lymphoid follicles in the LP of CD patients in close association with CD4^{+} T cells [11].

IL-18 expression is also increased in animal models of IBD [12–14] and, depending on the model, has been reported to have either pro or anti-inflammatory activities [15, 16]. A possible explanation for these results is that IL-18 may have distinct functions depending on the stage of the disease, the cellular sources of IL-18 and the presence of specific IL-18R-expressing effector cells [9, 17–21]. Subsets of CD4^{+} T helper cells in both humans and mice express IL-18Rα [11, 22, 23] and we recently demonstrated that most CD4^{+} T cells in the healthy and inflamed human intestine express IL-18Rα and thus represent a potentially important IL-18-responsive population in IBD [11]. Regarding direct effects on CD4^{+} T cells, IL-18 promotes both Th1 and Th2 development [24] and synergizes with IL-12 to drive the proliferation of and IFN-γ production by Th1 cells in the context of T cell receptor (TCR) stimulation [7]. Interestingly in human effector/memory CD4^{+} T cells, IL-18 in combination with IL-12 can induce IFN-γ production and can synergize with IL-23 to induce potent IL-17 production from Th17-polarized effectors, independently of TCR stimulation, a property often referred to as "innate responsiveness" [7, 25–27]. In this regard, we recently demonstrated that IL-15 or the TNF-like ligand 1a (TL1a), together with IL-12/IL-18, induce the production of a wide range of cytokines in human intestinal CD4^{+} T cells, in a TCR-independent but IL-18-dependent manner [11]. The role of IL-18-dependent innate CD4^{+} T-cell responses in the setting of intestinal inflammation is however unclear.

In the current study we assess the role of IL-18Rα expression by murine CD4^{+} T cells in cytokine-induced cytokine production and its contribution to driving inflammation in vivo during CD4^{+} T-cell-dependent colitis.

## Results

### Intestinal IFN-γ^{+}, IL-17^{+}, and Foxp3^{+} CD4^{+} T cells express IL-18Rα

To determine which subsets of murine CD4^{+} T cells express IL-18Rα and are thus potentially responsive to IL-18, cells were isolated from murine MLN, spleen, small intestinal lamina propria (siLP) and cLP and IL-18Rα expression assessed by FACS (Fig. 1). IL-18Rα was expressed on a small proportion of total splenic and MLN CD4^{+} T cells (Fig. 1A) and further sub-setting of these cells revealed high IL-18Rα expression on 20–40% of central memory and effector memory, but not naïve, CD4^{+} T cells (Fig. 1B and C, data not shown). Specificity of the IL-18Rα staining was confirmed by the absence of staining on IL-18Rα^{−/-} cells (Supporting Information Fig. 1A). In contrast to MLN and spleen, a large fraction of the steady-state cLP and siLP CD4^{+} T cells expressed high levels

of IL-18Rα (Fig. 1D). The majority of the intestinal CD4^{+} T cells displayed an effector/memory phenotype and this was unaltered in IL-18Rα^{−/−} mice (Supporting Information Fig. 1B and C). Further, all cLP CD4^{+} IL-18Rα^{+} cells exhibited an effector/memory phenotype (Supporting Information Fig. 1D–F). The proportions of IL-18Rα^{+} cells varied among cLP CD4^{+} T cell subsets, with IL-18Rα expressed almost ubiquitously on IL-17^{+} and IFN-γ^{+} IL-17^{+} cells (>90%), on the majority of IFN-γ+ cells (70-80%) and on approximately 60% of FoxP3^{+} T cells (Fig. 1E–G). Collectively these results demonstrate that IL-18Rα^{hi} CD4^{+} T cells are enriched in the intestinal mucosa compared with MLN and spleen, and that IL-18Rα expression is not restricted to a specific functional subset of intestinal CD4^{+} T cells.

### IL-18Rα is induced preferentially on activated OT-II CD4^{+} T cells localizing to the intestinal LP

To gain further insights into the regulation of IL-18Rα expression on CD4^{+} T cells, naïve OVA-specific OT-II cells were labeled with violet cell tracer (VCT) and injected i.v. into congenic recipients. One day later recipient mice were immunized with OVA + R848 (oral gavage) or OVA + LPS + antiCD40 (i.p.). (Fig. 2A). IL-18Rα expression was then assessed on responding OT-II cells 4, 7, and 12 days after immunization. While injected OT-II cells did not express IL-18Rα (Fig. 2A), OT-II cells expressing high levels of IL-18Rα were readily detected in the siLP but not MLN or spleen at day 7 and 12 after oral immunization (Fig. 2B–D). Similar results were obtained after i.p. immunization of animals with OVA + anti-CD40 + LPS and confirmed that OT-II cells start to express IL-18Rα relatively late after activation and primarily upon localization to the intestine (Fig. 2E and F). IL-18Rα, was also detected on a minor subset of OT-II cells in the MLN, PP, spleen suggesting some degree of IL-18Rα upregulation in these tissues or that these cells represent OT-II cells that have first circulated through intestinal sites. Finally a significant proportion of OT-II cells also started expressing high levels of IL-18Rα in the lungs after i.p. immunization, but to a lesser extent compared to siLP OT-II cells (Fig. 2E and F).Together these results suggest that IL-18Rα is induced on CD4^{+} T cells subsequent to their entry into the intestinal mucosa.

### Cytokine-induced cytokine production by intestinal CD4^{+} T cells is diminished in IL-18Rα^{−/−} mice

To determine whether IL-18Rα expression impacted on the number and proportion of colonic CD4^{+} T-cell subsets, we next compared the composition of cLP CD4^{+} T cells in IL-18Rα^{−/−} and WT littermate controls (Fig. 3A). The absolute numbers of CD4^{+} T cells in the cLP of IL-18Rα^{−/−} mice did not differ from those of littermate controls (Fig. 3B). Similarly, no significant differences in the proportions of IFN-γ[+], IL-17^{+}, IFN-γ[+]IL-17^{+}, or FoxP3^{+} cells were observed between these mice (Fig. 3B). Consistent with our findings in the human intestine [11], murine intestinal IL-18Rα^{+}CD4^{+}
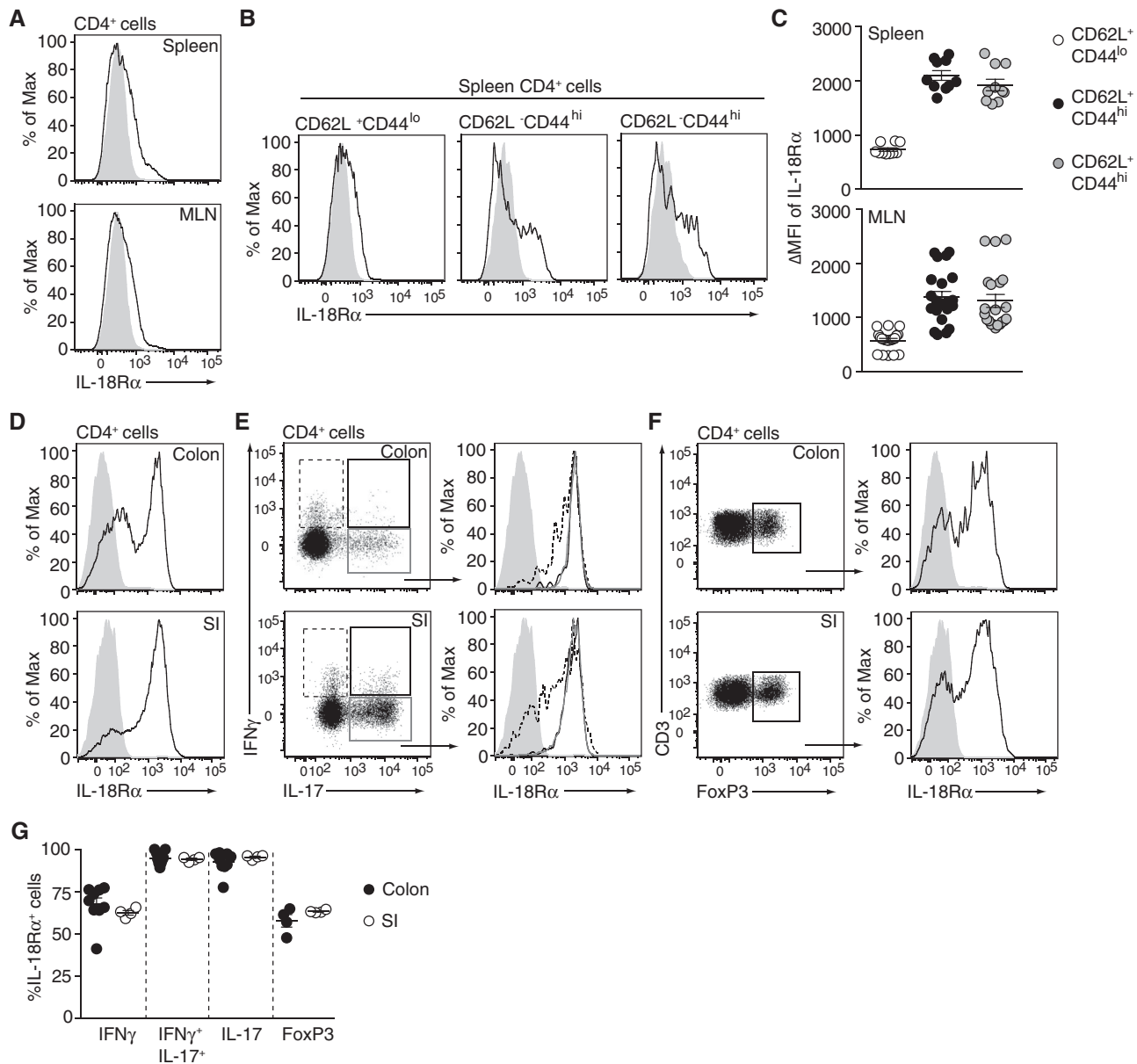
**Figure 1.** IL-18Rα on a broad range of functionally distinct intestinal lamina propria CD4$^+$ T cells. (A, B) IL-18Rα expression on (A) CD4$^+$ cells from spleen and MLN and (B) CD4$^+$TCRβ$^+$ T-cell subsets in the spleen was measured by flow cytometry. (C) The pooled MFI of IL-18Rα expression on the indicated CD4$^+$TCRβ$^+$ T-cell subsets in the spleen and MLN of steady state WT mice is also shown. Each symbol represents an individual mouse and results are pooled from three to four independent experiments. (A and B) IL-18Rα staining (black line), isotype control (filled histogram). (C) Results are expressed as (ΔMFI) = (MFI of IL-18Rα expression on a given T-cell subset) - (MFI of the relevant isotype control on the same T-cell subset). Of note, CD4$^+$TCRβ$^+$ and CD4$^+$CD3$^+$ T cells are completely overlapping populations in the MLN, spleen, siLP, and cLP. Therefore both gating strategies have been used interchangeably in this work. (D) IL-18Rα expression on total live CD4$^+$CD3$^+$ intestinal LP T cells. IL-18Rα staining (black line), isotype control (filled histogram). (E–G) Surface IL-18Rα expression on IFN-γ$^+$, IFN-γ$^+$IL-17A$^+$, IL-17A$^+$, and Foxp3$^+$ CD4$^+$CD3$^+$ T cells from SI and cLP of steady state WT mice, assessed by flow cytometry. (E) Representative gating for IFN-γ$^+$ (dashed line), IFN-γ$^+$IL-17A$^+$ (black line), IL-17A$^+$ (grey line) cells (left). Histogram overlay of IL-18Rα expression on the subsets defined in the left panels (right). An isotype control staining on total CD4$^+$CD3$^+$ T cells (filled histogram) was also included in the histograms overlay. (F) Representative FoxP3 intracellular staining on live CD4$^+$CD3$^+$ T cells from the SI and cLP of steady state WT mice (left). IL-18Rα expression on the defined FoxP3$^+$ T cells (black line) and respective isotype control (filled histogram, right). (G) Quantification of IL-18Rα$^+$ cells within SI (gray circles) and colon LP (black circles) IFN-γ$^+$, IL-17A$^+$, IL-17A$^+$IFN-γ$^+$, and FoxP3$^+$ cells defined in (E) and (F). Results are shown as mean ± (SEM) from two independent experiments; each symbol represents results from a single mouse.
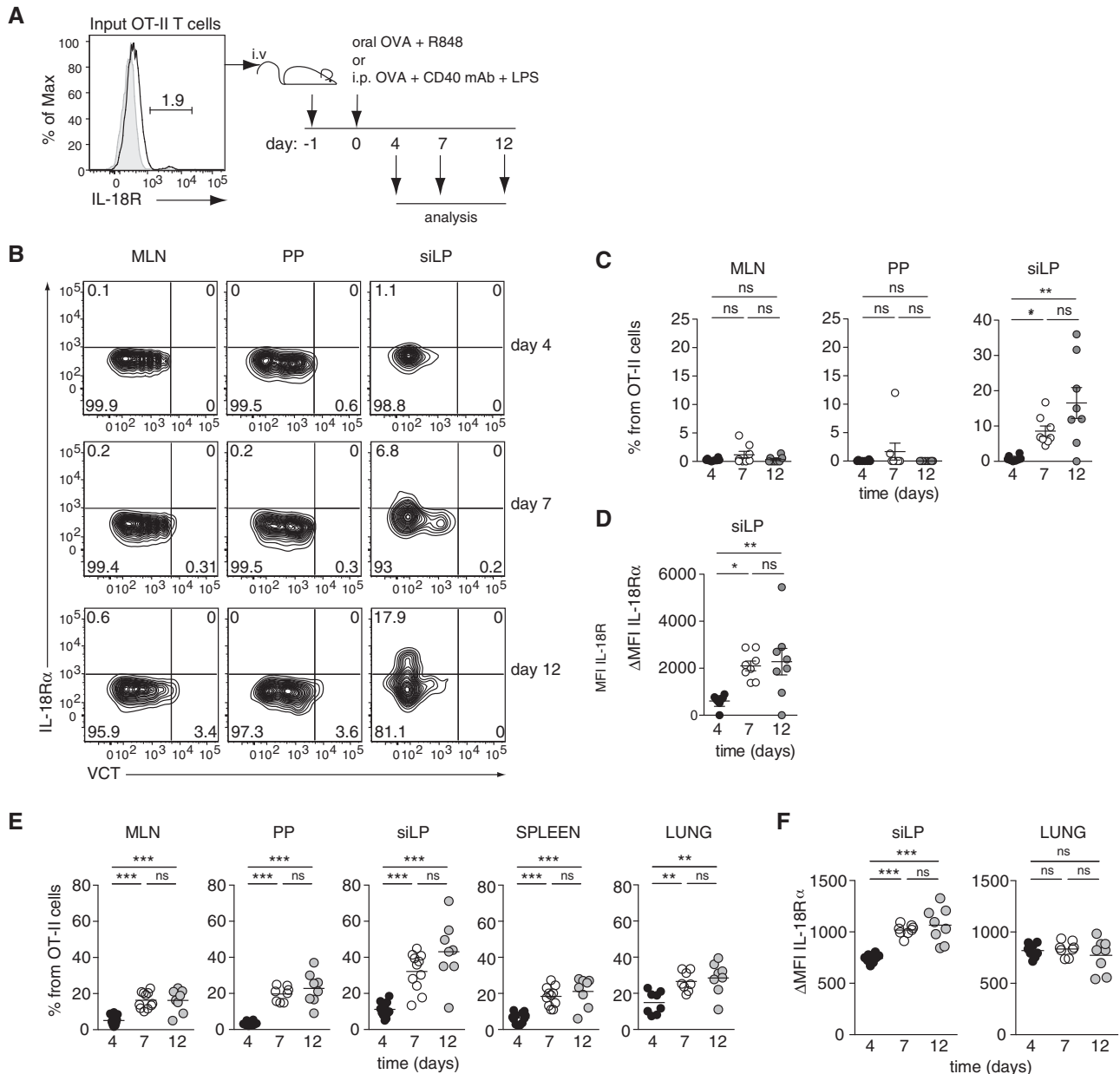
**Figure 2.** IL-18Rα is induced preferentially on activated OT-II CD4$^+$ T cells localizing to the intestinal LP. VCT-labeled naïve OT-II cells were injected i.v. into WT recipient mice subsequently immunized with OVA + R848 (oral gavage) or OVA + LPS + antiCD40 (i.p.). (A) IL-18Rα expression on input OT-II cells and experimental workflow. Isotype control (filled histogram). (B–D) IL-18Rα expression on OT-II cells in the indicated organs at day 4, 7, and 12 after oral immunization was evaluated by flow cytometry and shown as (B) representative plots, (C) cellular percentages, and (D) IL-18Rα MFI. (D) ΔMFI = (MFI of IL-18Rα on IL-18Rα$^+$ cells) - (MFI of the relevant isotype control on total donor-derived OT-II cells). (C and D). Results are shown as mean ± SEM of data pooled from two experiments; each symbol represents a single mouse. (E-F) IL-18Rα expression by OT-II cells in the indicated organs at day 4, 7, and 12 after i.p. immunization is shown as (E) cellular percentages and (F) MFI. Results are shown as mean ± SEM of data pooled from two independent experiments; each symbol represents a single mouse. (D and F) ΔMFI = (MFI of IL-18Rα on IL-18Rα$^+$ cells) - (MFI of the relevant isotype control on total donor-derived OT-II cells). Statistical analysis was performed using a nonparametric Student's *t*-test (*$p<0.05$, **$p<0.01$, ***$p<0.001$, n.s. nonsignificant).

T cells co-expressed the TL1a receptor, death receptor-3 (DR3) (Fig. 3C). Since IL-12, TL1a, IL-15 have been shown to synergistically induce TCR-independent IL-18-dependent cytokine production in human siLP IL-18Rα$^+$DR3$^+$ CD4$^+$ T cells [11], we next assessed the role of IL-18Rα in cytokine-induced cytokine production by murine intestinal CD4$^+$ T cells. SiLP and cLP CD4$^+$ T from

IL-18Rα$^{-/-}$ mice or WT littermate controls were stimulated in vitro with IL-12, IL-18, IL-15, and TL1a and their expression of IFN-γ and IL-17 assessed after 48 h by FACS (Fig. 3D and E). IL-12/IL-18/TL1a/IL-15 induced IFN-γ and IL-17 production in siLP and cLP CD4$^+$ T cells from WT mice, while little cytokine production was observed from IL-18Rα deficient CD4$^+$ T cells (Fig. 3D and
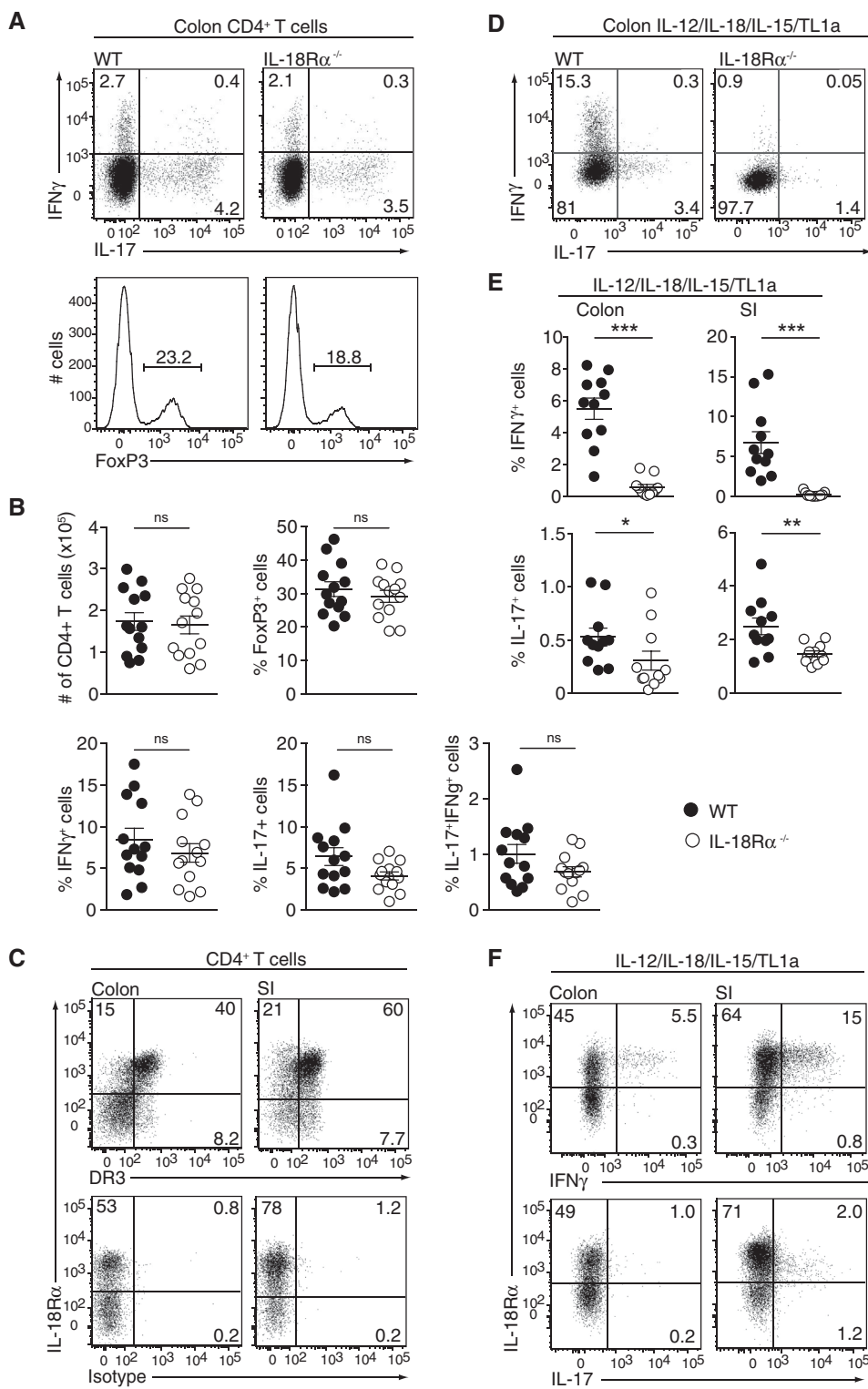
**Figure 3.** IL-18Rα is important for TCR-independent cytokine-induced cytokine production by colonic intestinal CD4+ T cells. (A) Representative flow cytometry plots and (B) quantification of the absolute numbers of CD4+TCRβ+ cLP T cells and of the percentages of IFN-γ+, IL-17+, IFN-γ+IL-17+, and FoxP3+ subsets within these cells in IL-18Rα−/− and WT littermate control mice. (B) Mean ± SEM of three independent experiments, each symbol represents a single mouse. (C) Representative plots of IL-18Rα and death receptor 3 (DR3) expression on colonic and SI LP CD4+CD3+ T cells. Plots are representative of nine biological replicates pooled from three experiments. (D–F) Intestinal LPMCs from IL-18Rα−/− mice and littermate controls were cultured with IL-12, IL-18, IL-15, and TL1a for 48 h. (D) Representative cLP flow cytometry plots and (E) quantification of the percentages of IFN-γ+ and IL-17+ subsets within CD4+TCRβ+ T cells in the cLP and the siLP. (E) Mean ± SEM of three independent experiments, each circle represents cells from an individual mouse. Statistical analysis was performed using a nonparametric Student's t-test (*p<0.05, **p<0.01, ***p<0.001, n.s. nonsignificant).

E). Consistent with these findings cytokine production was largely restricted to IL-18Rα expressing CD4+ T cells (Fig. 3F). Further, stimulation of total cLP and siLP cells with IL-12/IL-18/TL1a/IL-15, confirmed that IL-18Rα expression was required for optimal secretion of IFN-γ, IL-17, GM-CSF, IL-13 but not IL-10 or IL-6 (Supporting Information Fig. 2A). To assess the individual con-

tribution of each cytokine in driving the TCR-independent IFN-γ production by CD4+ T cells, purified cLP CD4+ T cells were incubated with each cytokine alone or in combination (Supporting Information Fig. 2B). While each cytokine alone induced little IFN-γ production, addition of only IL-12 and IL-18 resulted in a dramatic increase in IFN-γ production (Supporting Information

Fig. 2B). In contrast to what we have previously observed with human intestinal CD4$^+$ LP T cells, the addition of TL1a, IL-15, or TL1a and IL-15, to IL-12 and IL-18 did not further increase the production of IFN-γ by these cells (Supporting Information Fig. 2B). Previous reports have suggested that the synergy between IL-12 and IL-18 in driving IFN-γ production may be related to the capacity of IL-12 to upregulate IL-18Rα in mouse Th1 cells [28, 29]. While cLP CD4$^+$ T cells, stimulated with IL-12+IL-18 ± IL-15+TL1a showed a trend toward enhanced IL-18Rα expression compared with medium alone, this did not reach statistical significance (Supporting Information Fig. 2C and D). In comparison, expression of IL-15Rα was not regulated by addition of any of the cytokines (Supporting Information Fig. 2C and D). Collectively these results suggest an important role for IL-18Rα in cytokine-induced cytokine production by intestinal LPMC and cytokine-induced IFN-γ and IL-17 production by intestinal LP CD4$^+$ T cells.

### IL-18Rα is expressed on most intestinal CD4$^+$T cells during experimental colitis

To assess whether IL-18Rα is expressed on intestinal CD4$^+$ T cells during experimental colitis, WT CD45RB$^{hi}$CD4$^+$ T cells were injected into RAG$^{-/-}$ recipients and IL-18Rα expression on donor CD4$^+$ T cells was assessed in the MLN and cLP after 6–7 weeks (Fig. 4A), a time point where mice had developed active colitis. IL-18Rα was expressed at high levels on the majority of CD4$^+$ T cells in the cLP and MLN (Fig. 4A). Furthermore IL-18Rα was expressed on almost all effector CD4$^+$ T cells, irrespective of whether they produced IFN-γ, IL-17, or both cytokines (Fig. 4B and C). Similarly to the steady-state condition, 50–60% of cLP and MLN FoxP3$^+$ Treg cells expressed IL-18Rα during colitis. Of note, the majority of MLN, cLP and spleen CD4$^+$ T cells also expressed IL-18Rα in RAG $^{-/-}$ recipients of naïve CD45RB$^{hi}$ T cells and WT CD45RB$^{lo}$ T cells (which do not develop inflammation) (data not shown), indicating that the high proportions of IL-18Rα-expressing T cells was a result of the RAG$^{-/-}$ lymphopenic and not of the chronic inflammatory environment.

### IL-18Rα signaling in CD4$^+$ T cells is dispensable for the induction of T-cell-mediated colitis

Given the fact that IL-18Rα was expressed on the vast majority of IFN-γ- and IL-17-producing CD4$^+$ T cells in the MLN and cLP of colitic mice (Fig. 4B and C) and that these cells are thought to be directly involved in driving intestinal pathology in this model [30], we sought to evaluate the impact of an IL-18Rα deficiency specifically in CD4$^+$ T cells on colitis development. CD45RB$^{hi}$ CD4$^+$ T cells from IL18Rα$^{-/-}$ mice or WT littermate controls were thus transferred into RAG$^{-/-}$ mice. Six to seven weeks after transfer, recipients of IL-18Rα$^{-/-}$ or WT T cells displayed similar severe colonic inflammation, as assessed by histological score (Fig. 4D and E). In addition, the total numbers of CD4$^+$ T cells accumulating in the MLN and cLP of colitic mice were similar between both

groups of recipient mice (Fig. 4F). Quantitative PCR analyses on total colon tissue showed that the absence of IL-18Rα signaling in CD4$^+$ T cells also had no impact on the production of IL-12 and IL-23 mRNA in the cLP (data not shown). FACS analyses revealed that IL-18Rα deficiency had no impact on the Th1/Th17/Treg cell balance in the MLN, 6-7 weeks after transfer (Fig. 4G–J). In contrast, the cLP of IL-18Rα$^{-/-}$ CD4$^+$ T-cell recipients contained significantly decreased numbers of IFN-γ$^+$ and IFN-γ$^+$IL-17$^+$ but not IL-17$^+$ or FoxP3$^+$ cells, compared to recipient of WT CD4$^+$ T cells (Fig. 4 G–J). Collectively these results demonstrate that although IL-18Rα signaling in CD4$^+$ T cells is required for the optimal generation of Th1 cells, it is dispensable for colitogenic function of CD4$^+$ T cells in this model.

### IL-18Rα is required for cytokine-induced cytokine production by colonic intestinal CD4$^+$ T cells during inflammation

Given that RAG$^{-/-}$ recipients of IL-18Rα$^{-/-}$ and WT CD4$^+$ T cells developed similar levels of intestinal inflammation we sought to determine whether intestinal CD4$^+$ T cells maintained their innate-like properties during inflammation and whether this cytokine production remained IL-18Rα dependant. WT CD4$^+$ T cells from colitic mice maintained their ability to produce cytokines after stimulation with IL-12/IL-18/IL-15/TL1a compared with medium alone and these responses remained absent in IL-18Rα$^{-/-}$ CD4$^+$ T cells (Fig. 5). Collectively these results suggest that the innate-like cytokine response of intestinal CD4$^+$ T cells remains IL-18 dependent during colitis, however this property of CD4$^+$ T cells appears dispensable for the development of intestinal inflammation in the T-cell transfer colitis model.

## Discussion

We recently reported that a large proportion of CD4$^+$ T cells from the healthy and inflamed human intestine expresses IL-18Rα and a key role for IL-18 in TCR-independent cytokine induced pro-inflammatory cytokine production by these cells [11]. The purpose of the current study was thus to evaluate whether this TCR-independent, IL-18-dependent, innate responsiveness of mucosal memory CD4$^+$ T cells contributed to the pathogenesis of IBD. While murine intestinal CD4$^+$ T cells displayed similar TCR-independent IL-18-dependent innate functionality under both homeostatic and inflammatory conditions, we demonstrate that this functionality is dispensable for T-cell-induced colitis development. IL-18-driven TCR-independent IFN-γ production by CD4$^+$ T cells was recently reported to be an important component of the in vivo bactericidal function of Th1 cells during acute Salmonella or Chlamydia infections [31, 32], The T-cell transfer colitis model, in contrast, is a chronic model in which the immune system is continually stimulated by commensal bacteria breaching the intestinal epithelium [33]. It is therefore possible that in this model such chronic antigen exposure compensates for the defect in
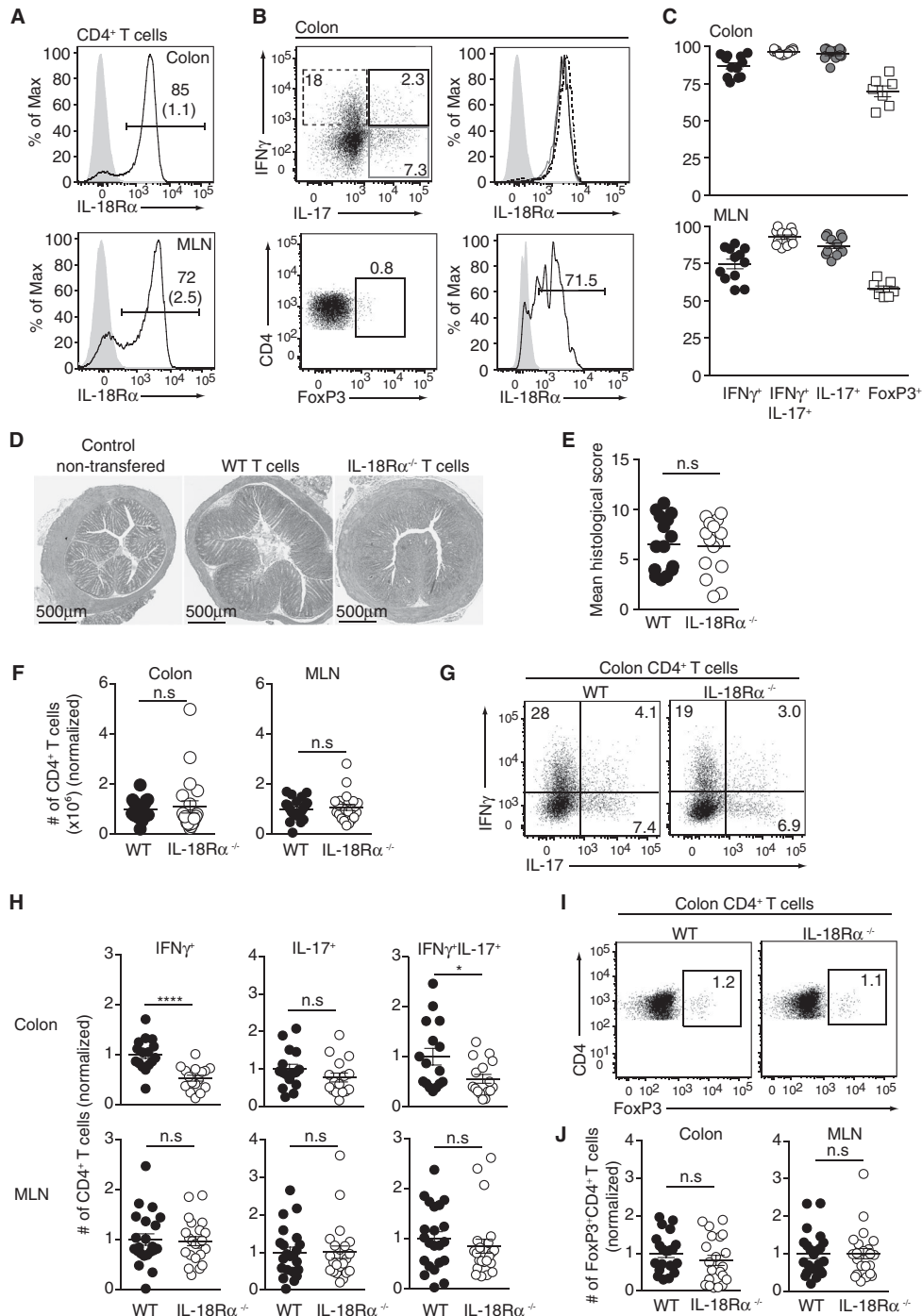
**Figure 4.** IL-18Rα signaling in CD4^{+} T cells is dispensable for induction of T-cell-mediated colitis despite its requirement for optimal generation of IFN-γ-producing Th1 cells. Naïve CD4^{+} T cells from IL-18Rα^{−/−} or control WT littermates were transferred into RAG-1^{−/−} recipients and mice were sacrificed 6–7 weeks after transfer. (A–C) IL-18Rα expression on (A) total cLP and MLN WT donor-derived CD4^{+}TCRβ^{+} T cells and (B and C) IFN-γ^{+}, IL-17^{+}, IFN-γ^{+}IL-17^{+}, and Foxp3^{+} cell subsets was evaluated by flow cytometry. (A) IL-18Rα staining (black line), isotype control (filled histogram). Numbers on the histograms are mean percentages ± (SEM) of IL-18Rα^{+} cells. (B) Representative flow cytometry staining plots and gating strategy for IFN-γ[+], IFN-γ[+]IL-17A^{+}, IL-17A^{+}, and FoxP3^{+} cells (left). Histograms overlay of IL-18Rα expression on the cytokine-producing subsets defined in the left panels (right). An isotype control staining on total CD4^{+} T cells (filled histogram) was also included. (C) Quantification of IL-18Rα expression on the cLP and MLN T-cell subsets defined in (B). Results are shown as mean percentages ± SEM of IL-18Rα^{+} cells from 12 mice pooled from two independent experiments. (D) Representative histological sections and (E) colon inflammation scores of RAG-1^{−/−} mice transferred with IL-18Rα^{−/−} or WT naïve CD4^{+} T cells. (F) Numbers of CD4^{+}TCRβ^{+} T cells recovered from the colon and MLN. (G–J) Representative flow cytometry plots (G and I) and quantification (H and J) of numbers of IFN-γ^{+}, IL-17^{+}, IFN-γ^{+}IL-17^{+}, and FoxP3^{+} CD4^{+}TCRβ^{+} T cells in colon and MLN. Results are pooled from 16 to 22 mice/group in two or three independent experiments. In panels F, H and J, numbers have been normalized to the mean of the WT group in each individual experiment in order to be able to compile separate independent experiments. Statistical analysis was performed using a nonparametric Student's $t$-test (*$p<0.05$, **$p<0.01$, ***$p<0.001$, n.s. nonsignificant).
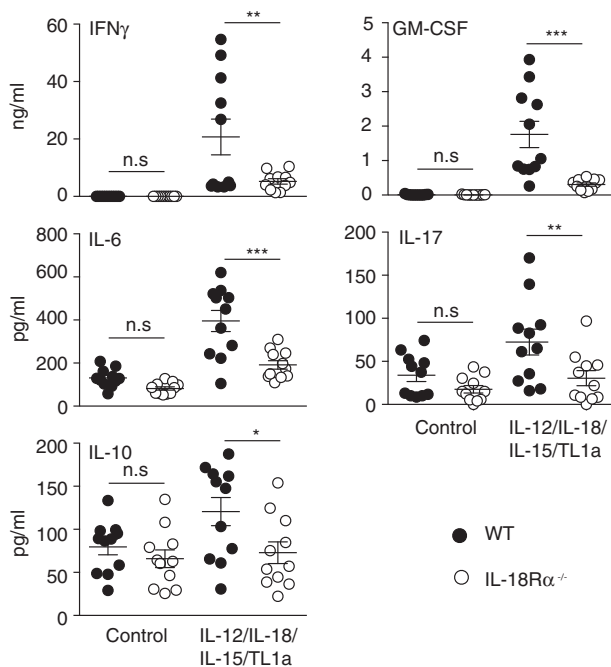
**Figure 5.** IL-18Rα signaling remains required for TCR-independent cytokine-induced cytokine production by colonic intestinal CD4$^+$ T cells under inflammatory conditions. Naïve CD4$^+$ T cells from IL-18Rα$^{-/-}$ mice or WT littermates were transferred into RAG-1$^{-/-}$ recipients. Six weeks after transfer, CD4$^+$ T cells were purified from the cLP and cultured for 48 h in medium alone (control) or in the presence of IL-12, IL-18, IL-15, and TL1a. Levels of the indicated cytokines released in the supernatant were quantified by Bio-Plex technology. Results are shown as mean of 12 mice/group and pooled from two independent experiments. Statistical analysis was performed using a nonparametric Student's t-test ($^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, n.s. nonsignificant).

IL-18-dependent innate cytokine response of CD4$^+$ T cells. In this regard it will be of interest in future studies to assess the role of IL-18Rα-dependent innate responses of CD4$^+$ T cells in more acute models of mucosal inflammation and infection.

Similar to our current findings, Harrison et al. demonstrated that IL-18Rα expression by CD4$^+$ T cells is not required for colitis induction [34]. These authors however did not assess alterations in proportions of CD4$^+$ effector cell subsets in the context of IL-18Rα deficiency and colitis. Here we show that cLP CD4$^+$ IL-18Rα$^{-/-}$ T cells exhibit a marked reduction in IFN-γ producing Th1 cells during colitis. One possible mechanism underlying this finding is IL-18s ability to synergize with IL-12 to promote the proliferation and IFN-γ production by Th1 cells in the context of TCR stimulation [7]. In addition, as we show here, IL-18 together with IL-12, in a TCR independent manner, dramatically enhances IFN-γ production by intestinal CD4$^+$ T cells. Thus IL-18 may, in a TCR-independent manner, promote the expansion and/or maintenance of Th1 cells in the context of colitis.

The mechanism by which IL-12 and IL-18 synergize to promote CD4$^+$ T cell functionality has been proposed to involve the reciprocal upregulation of IL-18Rα and IL-12Rβ2 by IL-12 and IL-18 signaling respectively in committed Th1 cells [28, 29, 35], as well as synergistic intracellular signaling pathways directly activating the

IFN-γ promoter [7, 36]. In addition, IL-18 has been shown to stabilize IFN-γ mRNA through the activation of MAPK p38 [37] and to increase Th1 cell proliferation by stimulating their production of IL-2 [38]. Finally Yang et al. recently reported TCR-independent IL-12 and IL-18 synergy to be dependent on IL-18-induced synthesis of GADD45β (MyD118, related member of a family of proteins that activate MAPK kinase kinases), which maintained a prolonged activation of p38 MAPK [39]. The prolonged p38 MAPK activation is a requirement for cytokine-induced but not in TCR-induced prolonged IFN-γ transcription [39]. In the current study, we found that cLP CD4$^+$ T cells incubated with IL-12 and IL-18 or IL-12/IL-18/IL-15 and TL1a lead to a slight increase in IL-18Rα expression compared to medium alone, although changes were not significant. Further our attempts to assess p38 MAPK phosphorylation in cytokine-stimulated cLP CD4$^+$ were highly variable and inconclusive, potentially as a result of the lengthy isolation procedure of these cells. Further studies are thus required to explore the mechanisms underlying the synergistic effects of IL-12 and IL-18 on TCR-dependent and independent cLP CD4$^+$ T-cell functionality in a steady state and during colitis.

The similar degree of colitis development, despite marked reduction in Th1 cell numbers, raises the questions of the role of Th1 cells in the transfer colitis model. It is possible, that the remaining IFN-γ$^+$ cells in RAG$^{-/-}$ recipients of IL-18Rα$^{-/-}$ T cells are sufficient to promote intestinal pathology or that, IL-17-producing CD4$^+$ T cells in this context play a more prominent role. While initial assessment of the role of Th1 cells in this model of colitis have provided conflicting data [40, 41], more recent studies suggest that "classical" Th1 cells are dispensable for the development of intestinal pathology and that Th17 cells were more potent at driving disease, notably by generating colitogenic IFN-γ$^+$ "alternative" Th1cells", via IFN-γ$^+$IL-17$^+$ intermediaries, in a T-bet and STAT-4-dependent manner [42–44]. In our colitis experiments, IL-18R signaling deficiency had no impact on the numbers of IL-17$^+$ cells, but reduced the numbers of both IFN-γ$^+$IL-17$^+$ and IFN-γ$^+$ cells, suggesting that IL-18 could also be implicated in the generation of "alternative Th1cells" in this context, although this did not change the final colitis outcome. Finally Harrison et al. recently demonstrated that IL-18R signaling is required for the optimal suppressive function of FoxP3$^+$ Treg cells and that IL-18R signaling promotes the expression of key FoxP3$^+$ Treg cell effector molecules [34]. Thus, while we found that IL-18R-signaling was not required for the generation of intestinal FoxP3$^+$ Treg cells under steady state or inflammatory conditions, reduced functionality of IL-18Rα$^{-/-}$ FoxP3$^+$ Tregs could partially underlie IL-18Rα$^{-/-}$ CD4$^+$ T cell mediated colitis. The mechanisms underlying IL-18Rα$^{-/-}$CD4$^+$ T cell-mediated colitis and whether they are the same or distinct from that of WT CD4$^+$ T-cell-driven colitis awaits further study.

A surprising finding from the current study was that IL-18Rα$^{-/-}$ and WT littermate control animals exhibited similar numbers and frequencies of intestinal Th1, Th17, and FoxP3$^+$ T cells in the steady state. These results contrast markedly with the report from Harrison et al. suggesting that intestinal epithelial cell-derived IL-18 acted directly on colonic IL-18Rα$^+$CD4$^+$ T cells

to limit their differentiation into Th17 cells under steady state conditions and that IL-1 receptor (IL-1R1) or MyD88 expression by CD4^{+} T cells was required for IL-18-mediated control of Th17 differentiation [34]. Quantitative PCR analyses demonstrated that our IL-18Rα^{−/−} mice and littermate controls contained similar levels of segmented filamentous bacteria (SFB, data not shown). Therefore the lack of differences in the numbers of intestinal IL-17^{+} cells in our IL-18Rα^{−/−} mice compared to control WT mice cannot be explained by an absence of the Th17-inducing SFB in our colony versus the mouse colony analyzed by Harrison et al. It remains however possible that the differential effects of IL-18Rα deficiency between the two studies at least partially depends on quantitative or qualitative differences in other colonic commensal bacteria. Relevant to that point, the IL-18Rα^{−/−} and WT animals used in the study by Harrison et al. were co-housed, while our animals were littermates. We hypothesize that yet to be identified commensals present in our animal facility are stimulating Th17 differentiation via pathways that cannot be blocked by IL-18Rα signaling.

Regarding the regulation of IL-18Rα expression, our results suggest that IL-18Rα induction on CD4^{+} T cells is not simply a consequence of late stage T activation but rather of the environment to which activated T cells localize. High IL-18Rα expression was detected primarily on CD4^{+} T cells subsequent to their localization to the intestine, irrespective of the route of immunization, indicating that the intestinal environment preferentially retains IL-18Rα^{hi} cells, or promotes the expression of IL-18Rα expression and/or preferential survival of these T cells. Interestingly, the significant proportion of IL-18Rα[hi] OT-II cells detected at another mucosal site (lungs) after i.p. (Fig. 2E and F) but not oral (data not shown) immunization, suggests that factors driving high IL-18Rα expression could also be present at multiple mucosal sites. Further studies are required to define the mechanism underlying the preferential accumulation of IL-18Rα^{+} T cells in the intestine and putative regulators of IL-18Rα expression.

In conclusion, we demonstrate a role for IL-18Rα signaling in CD4^{+} T cells in the generation of IFN-γ producing CD4^{+} T cells during colitis and in cytokine driven innate CD4^{+} T cell responses in both the steady state and inflammatory setting. Despite these findings we show that IL-18Rα signaling in CD4^{+} T cells is dispensable for the induction of T-cell mediated colitis.

## Material and methods

### Mice

C57BL/6 mice were purchased from Taconic Farms (Germantown, NY). B6.Cg- Tg(TcraTcrb)425Cbn/J (OT-II), IL-18Ra^{−/−} and Rag-1^{−/−} mice were purchased from Jackson Laboratory (Bar Harbor, ME, USA). All mice were maintained at the Biomedical Center, Lund or Clinical Research Center, Malmö. Animal experiments were performed in accordance with the Lund/Malmö Animal Ethics Committee in Lund, Sweden.

### Reagents and cytokines

RPMI-1640, PBS, Hank's Balanced Salt Solution (HBSS), fetal calf serum (FCS), hepes buffer, EDTA, sodium pyruvate, β-mercaptoethanol, penicillin, streptomycin, and gentamycin were from Invitrogen (Carlsbad, CA). Brefeldin A, PMA, OVA grade V and VI, collagenase IV, DTT and LPS were from Sigma-Aldrich (St. Louis, MO, USA). Ionomycin was from Calbiochem (San Diego, CA, USA). DNase I and Liberase TM were from Roche (Mannheim, Germany). Violet cell tracer proliferation kit and live/dead fixable dead cell stain kits were from Molecular Probes Inc. (Eugene, OR, USA). Recombinant mouse (rm) IL-12, IL-15, IL-18 and TL1a were from R&D systems (Minneapolis, MN, USA). R848 was from InvivoGen (Toulouse, France). Purified anti-CD40 (FGK4.5) mAb was from BioXCell (West Lebanon, NH, USA).

### Flow cytometry and antibodies

Flow cytometry was performed as previously described [45]. For intracellular cytokine and FoxP3 staining cells were fixed and permeabilized with "Foxp3 staining buffer set" from eBioscience (San Diego, CA, USA). Cells were analyzed on an LSR II (BD Biosciences, San Jose, CA, USA). Flow cytometry data were analyzed using FlowJo software (TreeStar, Inc., Ashland, OR, USA).

*Antibodies*: anti-CD3 (17A2), -IL-18Rα (BG/IL18RA), rat IgG1 isotype control (RTK2071), -IFN-γ (XMG1.2), -IL-17A (TC11-18H10.1), -GM-CSF (MP1-22E9), and -CD25 (PC61) were from Biolegend (San Diego, CA, USA). Anti-CD4 (RM4-5 or GK1.5), -CD45RB (C363.16A), -CD45.2 (104), -TNF-α (MP6-XT22), -Foxp3 (FJK-16s), -CD62L (MEL-14) were from eBioscience. Anti-CD3 (145-2C11), -CD44 (IM7), -CD45.1 (A20) and PE-CF594-streptavidin were from BD biosciences. Anti-DR3 (goat polyclonal) and -IL-15Rα (888220) were from R&D systems.

### Cell isolation

Cell suspensions from MLN, spleen, siLP, and cLP were prepared as described previously [46], replacing collagenase VIII with Liberase TM (0.2-0.3 WunchU/mL). For isolation of cLP CD4^{+} T cells, cell suspensions were further enriched on 40/70 Percoll gradients (Amersham Pharmacia Biotech, Sweden), MACS-enriched using anti-CD4 microbeads and autoMACS-pro separator (Miltenyi biotec, Germany).

### Adoptive transfer experiments

Naïve CD4^{+} T cells were isolated from OT-II mice using the EasySep[TM] Mouse Naïve CD4^{+} T Cell Isolation Kit (Stemcell Technologies, Vancouver, Canada) and labeled with cell trace violet cell proliferation kit (Molecular Probes) according to manufacturer's instructions. C57BL/6 mice were injected i.v. with 0.5 × 10^{6} OT-II.CD45.1.CD45.2 cells and immunized one day later with

OVA grade V (50 mg/mL) and R848 (20 µg/mL) by oral gavage in 200 µL of PBS or with OVA grade VI (2.5 mg/mL), anti-CD40 mAb (125 µg/mL), and LPS (100 µg/mL) by intra-peritoneal injection in 200 µL of PBS.

## Cultures and cytokine measurements

Intestinal LPMC ($4 \times 10^6$ cells in 0.5 mL/well) or MACS-enriched CD4$^+$ T cells ($2 \times 10^5$ cells in 200 µL/well) were cultured in complete RPMI medium supplemented with IL-12 (2 ng/mL) and IL-18 (50 ng/mL), or IL-12/IL-18 together with IL-15 (25 ng/mL), and TL1a (100 ng/mL). After 48h, cytokine levels in culture supernatants were determined by Bio-Plex technology (Bio-rad, Hercules, CA, USA) or using cytometric bead array (CBA) flex set kits (BD Biosciences) or in CD4$^+$ T cells by intracellular cytokine staining (brefeldin A (10 µg/mL) was included during the last 3 h of culture). To determine the proportion of Th1/Th17 cells within intestinal CD4$^+$ T cells, LPMC were stimulated for 4 h with PMA (50 ng/mL) and ionomycin (500 ng/mL) in the presence of brefeldin A prior to intracellular cytokine analysis.

## CD45RB$^{high}$ transfer model of colitis

Six- to eight-week-old, sex matched RAG-1$^{-/-}$ mice were injected i.p. with FACS-sorted CD4$^+$CD25$^-$CD45RB$^{hi}$ T cells ($3 \times 10^5$ cells/mouse) from IL-18Rα$^{-/-}$ mice or WT littermates controls, as previously described [47]. Mice were sacrificed after 6 to 7 weeks and MLN and colon taken for analysis. Samples of proximal, mid, and distal colon were collected, fixed in formalin, embedded in paraffin, before preparing cross-sections and further staining with hematoxylin and eosin. The degree of inflammation on microscopic colon section was graded in a blinded fashion to avoid bias, according to a previously described scoring system [48]. Each sample was graded semi-quantitatively from zero to three for four criteria: (i) crypt length, degree of epithelial hyperplasia, and goblet cell depletion; (ii) leukocyte infiltration in the lamina propria; (iii) area of tissue affected; and (iv) presence of markers of severe inflammation such as crypt abscesses, submucosal inflammation, and ulcers. Scores for each criterion were added to give an overall inflammation score for each sample of 0–12. Scores from proximal, mid, and distal colon were averaged to obtain inflammation scores for the colon.

## Statistical analysis

All single column comparisons were performed using nonparametric Student's *t*-test and all multiple column analyses were performed using ANOVA followed by "Bonferroni's Multiple Comparison Test" (*$p<0.05$, **$p<0.01$, ***$p<0.001$). Statistical analysis was performed using GraphPad Prism software (GraphPad, La Jolla, CA, USA).

## References

1 **Stoll, S., Jonuleit, H., Schmitt, E., Muller, G., Yamauchi, H., Kurimoto, M., Knop, J. et al.,** Production of functional IL-18 by different subtypes of murine and human dendritic cells (DC): DC-derived IL-18 enhances IL-12-dependent Th1 development. *Eur. J. Immunol.* 1998. **28:** 3231–3239.

2 **Takeuchi, M., Nishizaki, Y., Sano, O., Ohta, T., Ikeda, M. and Kurimoto, M.,** Immunohistochemical and immuno-electron-microscopic detection of interferon-gamma-inducing factor ("interleukin-18") in mouse intestinal epithelial cells. *Cell Tissue Res.* 1997. **289:** 499–503.

3 **Sedimbi, S. K., Hagglof, T. and Karlsson, M. C.,** IL-18 in inflammatory and autoimmune disease. *Cell. Mol. Life Sci.* 2013. **70:** 4795–4808.

4 **Sugawara, S., Uehara, A., Nochi, T., Yamaguchi, T., Ueda, H., Sugiyama, A., Hanzawa, K. et al.,** Neutrophil proteinase 3-mediated induction of bioactive IL-18 secretion by human oral epithelial cells. *J. Immunol.* 2001. **167:** 6568–6575.

5 **Born, T. L., Thomassen, E., Bird, T. A. and Sims, J. E.,** Cloning of a novel receptor subunit, AcPL, required for interleukin-18 signaling. *J. Biol. Chem.* 1998. **273:** 29445–29450.

6 **Krumm, B., Xiang, Y. and Deng, J.,** Structural biology of the IL-1 superfamily: key cytokines in the regulation of immune and inflammatory responses. *Protein Sci.* 2014. **23:** 526–538.

7 **Robinson, D., Shibuya, K., Mui, A., Zonin, F., Murphy, E., Sana, T., Hartley, S. B. et al.,** IGIF does not drive Th1 development but synergizes with IL-12 for interferon-gamma production and activates IRAK and NFkappaB. *Immunity* 1997. **7:** 571–581.

8 **Dinarello, C. A., Novick, D., Kim, S. and Kaplanski, G.,** Interleukin-18 and IL-18 binding protein. *Front. Immunol.* 2013. **4:** 289.

9 **Pizarro, T. T., Michie, M. H., Bentz, M., Woraratanadharm, J., Smith, M. F., Jr., Foley, E., Moskaluk, C. A. et al.,** IL-18, a novel immunoregulatory cytokine, is up-regulated in Crohn's disease: expression and localization in intestinal mucosal cells. *J. Immunol.* 1999. **162:** 6829–6835.

10 **Monteleone, G., Trapasso, F., Parrello, T., Biancone, L., Stella, A., Iuliano, R., Luzza, F. et al.,** Bioactive IL-18 expression is up-regulated in Crohn's disease. *J. Immunol.* 1999. **163:** 143–147.

11 **Holmkvist, P., Roepstorff, K., Uronen-Hansson, H., Sanden, C., Gudjonsson, S., Patschan, O., Grip, O. et al.,** A major population of mucosal

memory CD4+ T cells, coexpressing IL-18Ralpha and DR3, display innate lymphocyte functionality. *Mucosal Immunol.* 2015. **8**: 545–558.

12 Camoglio, L., Juffermans, N. P., Peppelenbosch, M., te Velde, A. A., ten Kate, F. J., van Deventer, S. J. and Kopf, M., Contrasting roles of IL-12p40 and IL-12p35 in the development of hapten-induced colitis. *Eur. J. Immunol.* 2002. **32**: 261–269.

13 Kanai, T., Watanabe, M., Okazawa, A., Sato, T. and Hibi, T., Interleukin-18 and Crohn's disease. *Digestion* 2001. **63** Suppl 1: 37–42.

14 Wirtz, S., Becker, C., Blumberg, R., Galle, P. R. and Neurath, M. F., Treatment of T cell-dependent experimental colitis in SCID mice by local administration of an adenovirus expressing IL-18 antisense mRNA. *J. Immunol.* 2002. **168**: 411–420.

15 Takagi, H., Kanai, T., Okazawa, A., Kishi, Y., Sato, T., Takaishi, H., Inoue, N. et al., Contrasting action of IL-12 and IL-18 in the development of dextran sodium sulphate colitis in mice. *Scandin. J. Gastroenterol.* 2003. **38**: 837–844.

16 Salcedo, R., Worschech, A., Cardone, M., Jones, Y., Gyulai, Z., Dai, R. M., Wang, E. et al., MyD88-mediated signaling prevents development of adenocarcinomas of the colon: role of interleukin 18. *J. Experim. Med.* 2010. **207**: 1625–1636.

17 Dupaul-Chicoine, J., Yeretssian, G., Doiron, K., Bergstrom, K. S., McIntire, C. R., LeBlanc, P. M., Meunier, C. et al., Control of intestinal homeostasis, colitis, and colitis-associated colorectal cancer by the inflammatory caspases. *Immunity* 2010. **32**: 367–378.

18 Kampfer, H., Kalina, U., Muhl, H., Pfeilschifter, J. and Frank, S., Counter-regulation of interleukin-18 mRNA and protein expression during cutaneous wound repair in mice. *J. Inv. Dermat.* 1999. **113**: 369–374.

19 Keith, J. C., Jr., Albert, L., Sonis, S. T., Pfeiffer, C. J. and Schaub, R. G., IL-11, a pleiotropic cytokine: exciting new effects of IL-11 on gastrointestinal mucosal biology. *Stem Cells* 1994. **12** Suppl 1: 79–89; discussion 89-90.

20 Nowarski, R., Jackson, R., Gagliani, N., de Zoete, M. R., Palm, N. W., Bailis, W., Low, J. S. et al., Epithelial IL-18 equilibrium controls barrier function in colitis. *Cell* 2015. **163**: 1444–1456.

21 Levy, M., Thaiss, C. A., Zeevi, D., Dohnalova, L., Zilberman-Schapira, G., Mahdi, J. A., David, E. et al., Microbiota-modulated metabolites shape the intestinal microenvironment by regulating NLRP6 inflammasome signaling. *Cell* 2015. **163**: 1428–1443.

22 Smeltz, R. B., Chen, J., Hu-Li, J. and Shevach, E. M., Regulation of interleukin (IL)-18 receptor alpha chain expression on CD4(+) T cells during T helper (Th)1/Th2 differentiation. Critical downregulatory role of IL-4. *J. Exp. Med.* 2001. **194**: 143–153.

23 Tominaga, K., Yoshimoto, T., Torigoe, K., Kurimoto, M., Matsui, K., Hada, T., Okamura, H. et al., IL-12 synergizes with IL-18 or IL-1beta for IFN-gamma production from human T cells. *Internat. Immunol.* 2000. **12**: 151–160.

24 Nakanishi, K., Yoshimoto, T., Tsutsui, H. and Okamura, H., Interleukin-18 regulates both Th1 and Th2 responses. *Annu. Rev. Immunol.* 2001. **19**: 423–474.

25 Papadakis, K. A., Prehn, J. L., Landers, C., Han, Q., Luo, X., Cha, S. C., Wei, P. et al., TL1A synergizes with IL-12 and IL-18 to enhance IFN-gamma production in human T cells and NK cells. *J. Immunol.* 2004. **172**: 7002–7007.

26 Weaver, C. T., Harrington, L. E., Mangan, P. R., Gavrieli, M. and Murphy, K. M., Th17: an effector CD4 T cell lineage with regulatory T cell ties. *Immunity* 2006. **24**: 677–688.

27 Weaver, C. T., Hatton, R. D., Mangan, P. R. and Harrington, L. E., IL-17 family cytokines and the expanding diversity of effector T cell lineages. *Annu. Rev. Immunol.* 2007. **25**: 821–852.

28 Ahn, H. J., Maruo, S., Tomura, M., Mu, J., Hamaoka, T., Nakanishi, K., Clark, S. et al., A mechanism underlying synergy between IL-12 and IFN-gamma-inducing factor in enhanced production of IFN-gamma. *J. Immunol.* 1997. **159**: 2125–2131.

29 Yoshimoto, T., Takeda, K., Tanaka, T., Ohkusu, K., Kashiwamura, S., Okamura, H., Akira, S. et al., IL-12 up-regulates IL-18 receptor expression on T cells, Th1 cells, and B cells: synergism with IL-18 for IFN-gamma production. *J. Immunol.* 1998. **161**: 3400–3407.

30 Shale, M., Schiering, C. and Powrie, F., CD4(+) T-cell subsets in intestinal inflammation. *Immunol. Rev.* 2013. **252**: 164–182.

31 O'Donnell, H., Pham, O. H., Li, L. X., Atif, S. M., Lee, S. J., Ravesloot, M. M., Stolfi, J. L. et al., Toll-like receptor and inflammasome signals converge to amplify the innate bactericidal capacity of T helper 1 cells. *Immunity* 2014. **40**: 213–224.

32 Srinivasan, A., Salazar-Gonzalez, R. M., Jarcho, M., Sandau, M. M., Lefrancois, L. and McSorley, S. J., Innate immune activation of CD4 T cells in salmonella-infected mice is dependent on IL-18. *J. Immunol.* 2007. **178**: 6342–6349.

33 Elson, C. O., Cong, Y., McCracken, V. J., Dimmitt, R. A., Lorenz, R. G. and Weaver, C. T., Experimental models of inflammatory bowel disease reveal innate, adaptive, and regulatory mechanisms of host dialogue with the microbiota. *Immunol. Rev.* 2005. **206**: 260–276.

34 Harrison, O. J., Srinivasan, N., Pott, J., Schiering, C., Krausgruber, T., Ilott, N. E. and Maloy, K. J., Epithelial-derived IL-18 regulates Th17 cell differentiation and Foxp3(+) Treg cell function in the intestine. *Mucosal. Immunol.* 2015. **8**: 1226–1236.

35 Chang, J. T., Segal, B. M., Nakanishi, K., Okamura, H. and Shevach, E. M., The costimulatory effect of IL-18 on the induction of antigen-specific IFN-gamma production by resting T cells is IL-12 dependent and is mediated by up-regulation of the IL-12 receptor beta2 subunit. *Eur. J. Immunol.* 2000. **30**: 1113–1119.

36 Nakahira, M., Ahn, H. J., Park, W. R., Gao, P., Tomura, M., Park, C. S., Hamaoka, T. et al., Synergy of IL-12 and IL-18 for IFN-gamma gene expression: IL-12-induced STAT4 contributes to IFN-gamma promoter activation by up-regulating the binding activity of IL-18-induced activator protein 1. *J. Immunol.* 2002. **168**: 1146–1153.

37 Mavropoulos, A., Sully, G., Cope, A. P. and Clark, A. R., Stabilization of IFN–gamma mRNA by MAPK p38 in IL-12- and IL-18-stimulated human NK cells. *Blood* 2005. **105**: 282–288.

38 Micallef, M. J., Ohtsuki, T., Kohno, K., Tanabe, F., Ushio, S., Namba, M., Tanimoto, T. et al., Interferon-gamma-inducing factor enhances T helper 1 cytokine production by stimulated human T cells: synergism with interleukin-12 for interferon-gamma production. *Eur. J. Immunol.* 1996. **26**: 1647–1651.

39 Yang, J., Zhu, H., Murphy, T. L., Ouyang, W. and Murphy, K. M., IL-18-stimulated GADD45 beta required in cytokine-induced, but not TCR-induced, IFN-gamma production. *Nat. Immunol.* 2001. **2**: 157–164.

40 Ito, H. and Fathman, C. G., CD45RBhigh CD4+ T cells from IFN-gamma knockout mice do not induce wasting disease. *J. Autoimmun.* 1997. **10**: 455–459.

41 Simpson, S. J., Shah, S., Comiskey, M., de Jong, Y. P., Wang, B., Mizoguchi, E., Bhan, A. K. et al., T cell-mediated pathology in two models of experimental colitis depends predominantly on the interleukin 12/Signal transducer and activator of transcription (Stat)-4 pathway, but is not conditional on interferon gamma expression by T cells. *J. Exp. Med.* 1998. **187**: 1225–1234.

42 Feng, T., Qin, H., Wang, L., Benveniste, E. N., Elson, C. O. and Cong, Y., Th17 cells induce colitis and promote Th1 cell responses through IL-17 induction of innate IL-12 and IL-23 production. *J. Immunol.* 2011. **186**: 6313–6318.

43 **Harbour, S. N., Maynard, C. L., Zindl, C. L., Schoeb, T. R. and Weaver, C. T.**, Th17 cells give rise to Th1 cells that are required for the pathogenesis of colitis. *Proc. Natl. Acad. Sci. USA* 2015. **112**: 7061–7066.

44 **Sujino, T., Kanai, T., Ono, Y., Mikami, Y., Hayashi, A., Doi, T., Matsuoka, K. et al.**, Regulatory T cells suppress development of colitis, blocking differentiation of T-helper 17 into alternative T-helper 1 cells. *Gastroenterology* 2011. **141**: 1014–1023.

45 **Persson, E. K., Uronen-Hansson, H., Semmrich, M., Rivollier, A., Hagerbrand, K., Marsal, J., Gudjonsson, S. et al.**, IRF4 transcription-factor-dependent CD103(+)CD11b(+) dendritic cells drive mucosal T helper 17 cell differentiation. *Immunity* 2013. **38**: 958–969.

46 **Jaensson, E., Uronen-Hansson, H., Pabst, O., Eksteen, B., Tian, J., Coombes, J. L., Berg, P. L. et al.**, Small intestinal CD103+ dendritic cells display unique functional properties that are conserved between mice and humans. *J. Experim. Med.* 2008. **205**: 2139–2149.

47 **Kitamura, K., Farber, J. M. and Kelsall, B. L.**, CCR6 marks regulatory T cells as a colon-tropic, IL-10-producing phenotype. *J. Immunol.* 2010. **185**: 3295–3304.

48 **Coccia, M., Harrison, O. J., Schiering, C., Asquith, M. J., Becher, B., Powrie, F. and Maloy, K. J.**, IL-1beta mediates chronic intestinal inflammation by promoting the accumulation of IL-17A secreting innate lymphoid cells and CD4(+) Th17 cells. *J. Exp. Med.* 2012. **209**: 1595–1609.

*Full correspondence:* Dr. Aymeric Rivollier, Section of Immunology and Vaccinology, National Veterinary Institute, Technical University of Denmark, Frederiksberg, Denmark
Fax. +46-46-2224218
e-mail. ayri@vet.dtu.dk