 DOI: 10.1002/eji.201948430 Eur. J. Immunol. 2020. 50: 1034–1043

European Journal of Immunology

Allergy and inflammation

Research Article

IL-9 exerts biological function on antigen-experienced murine T cells and exacerbates colitis induced by adoptive transfer

Magali de Heusch, Valérie Steenwinckel*, Perrine M. Cochez, Jamila Louahed, Guy Warnier, Muriel M. Lemaire, Jean-Christophe Renauld** and Laure Dumoutier***

Experimental Medicine Unit, de Duve Institute, Université catholique de Louvain, Brussels, Belgium

IL-9 is involved in various T cell-dependent inflammatory models including colitis, encepahlitis, and asthma. However, the regulation and specificity of IL-9 responsiveness by T cells during immune responses remains poorly understood. Here, we addressed this question using two different models: experimental colitis induced by transfer of naive $CD4^+CD45RB^{high}$ T cells into immunodeficient mice, and OVA-specific T cell activation. In the colitis model, constitutive IL-9 expression exacerbated inflammation upon transfer of $CD4^+CD45RB^{high}$ T cells from WT but not from *Il9r*$^{-/-}$ mice, indicating that IL-9 acts directly on T cells. Suprisingly, such naïve $CD4^+CD45RB^{high}$ T cells failed to express the *Il9r* or respond to IL-9 in vitro, in contrast with $CD4^+CD45RB^{low}$ T cells. By using OVA-specific T cells, we observed that T cells acquired the capacity to respond to IL-9 along with CD44 upregulation, after long-lasting (5 to 12 days) in vivo antigenic stimulation. *Il9r* expression was associated with Th2 and Th17 phenotypes. Interestingly, in contrast to the IL-2 response, antigen restimulation downregulated IL-9 responsiveness. Taken together, our results demonstrate that IL-9 does not act on naïve T cells but that IL-9 responsiveness is acquired by $CD4^+$ T cells after in vivo activation and acquisition of memory markers such as CD44.

Keywords: adoptive transfer · colitis · IL-9 · Th2 · Th17

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Introduction

IL-9 was originally described as a T cell and mast cell growth factor produced by murine T cell clones [1]. It is now considered as an inflammatory cytokine that acts on various cell types, promoting mast cell differentiation, B cell activation, eosinophilia, ILC2 survival, and mucus production by lung epithelial cells (for review see [2–5]). Until the last decade, Th2 were considered the main source of IL-9 and the activity of this cytokine was largely studied in the context of Th2-associated diseases such as airway inflammation and helminth infection. Since 2008, different groups discovered the existence of a subset of T helper cells, called Th9, and

Correspondence: Laure Dumoutier
e-mail: laure.dumoutier@uclouvain.be

*These authors contributed equally to this work.
**These authors share senior co-authorship.

a subset of innate lymphoid cells, called ILC2, that produce IL-9 [6–8].

ILC2 are involved in allergic immunity as well as tissue homeostasis in skin, lung, and gut [9]. They produce IL-9 and this cytokine is crucial for their survival, as mice deficient for *Il9r* display lower number of ILC2s in the lung after infection [10]. The other main source of IL-9 is Th9 cells, which are involved in different disorders such as allergic lung inflammation, EAE, and cancer [11]. More recently, some reports have shown that Th9 cells also play a role in inflammatory bowel disease [12].

Pleiotropic activities of IL-9 in the in the intestinal mucosa have been well described and studied. IL-9 is required for oral antigen-induced intestinal and systemic anaphylaxis [13]. IL-9 induces mucosal mastocytosis and is involved in host protective immunity against intestinal nematode infection [14], and is also involved in innate immune response of the gut epithelium, inducing the expression of bactericidal and antiparasitic factors as well as expansion of Paneth cells [15]. In patients with active ulcerative colitis, it has been shown that IL-9 expression was significantly elevated in the mucosae. These patients also had increased numbers of mucosal T cells expressing PU.1, a transcription factor associated with Th9, and increased numbers of epithelial cells expressing IL-9R [16–18]. Moreover, several papers have demonstrated that the transfer of Th9 into lymphopenic *Rag2*$^{-/-}$ recipient mice worsens colitis [6,19]. Increased expression of IL-9 and IL-9R was also observed in the mouse model of oxazolone-induced colitis, contributing to the pathogenesis of chemical-induced colitis by impairing barrier functions [20]. In addition, the attenuation of colitis observed in *Il9*-deficient mice was associated with lower levels of Th2-type cytokines, suggesting that IL-9 also promotes their production by T cells [16]. Similarly, pulmonary IL-9 overexpression induced Th2 differentiation leading to immune pathology, again suggesting it acts on Th2 cells either directly or indirectly [21]. IL-9 also acts on Th17, as the neutralization of IL-9 decreases EAE, and this correlates with a decrease in infiltrating Th17 cells. In addition, it was shown that IL-9 mediates Th17 differentiation in vitro and this was regulated by STAT1 and STAT3 [22]. However, the mode of action of IL-9 and whether this cytokine acts directly on T cells in vivo remains poorly understood.

In the present study, we investigated the role of IL-9 on T cells, using the adoptive transfer colitis model and OVA-specific immunization. First, we show that IL-9 acts directly on T cells, as it does not exacerbate colitis induced by transfer of CD4$^+$CD45RB^{high} T cells from *Il9r*$^{-/-}$ mice into *Rag*2$^{-/-}$ mice. However, the original CD4$^+$CD45RB^{high} naïve T cells failed to respond to IL-9 in vitro, indicating that IL-9 responsiveness was acquired during the development of colitis. Using OVA-specific T cells, we could confirm that IL-9 acts directly on CD4$^+$ T cells, more precisely, in vivo antigen-experienced Th2 and Th17 that express memory markers such as CD44, but that antigen restimulation downregulated IL-9 responsiveness, in contrast with IL-2 responsiveness, which was increased upon antigen exposure.

Results

Transfer of CD4$^+$CD45RB^{high} cells overexpressing IL-9 aggravates colitis in SCID recipient mice

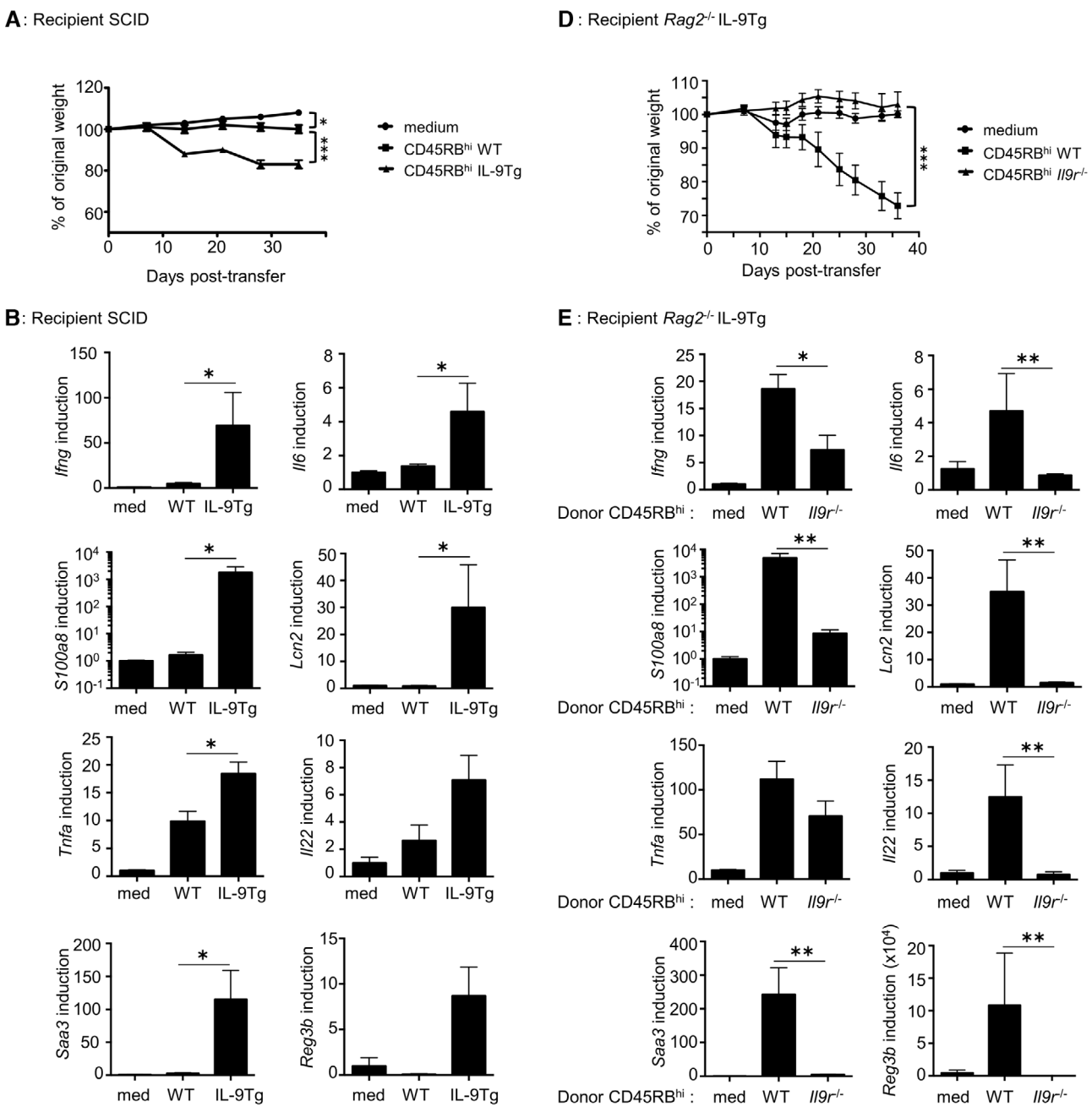
We confirmed first that IL-9 regulates colitis by transferring pathogenic CD4$^+$CD45RB^{high} T cells that did or did not overexpress the cytokine, into SCID animals. SCID recipients were of the BALB/c strain, whereas the IL-9 transgenic (Tg) mice were made on the FVB background; therefore, to prevent GvHD, we generated F1 animals by crossing IL-9 Tg-FVB mice with BALB/c mice. These F1 donor mice were designated either IL-9 Tg/c or FVB/c depending on the presence or absence of the IL-9 transgene, respectively. CD4$^+$CD45RB^{high} T cells either from FVB/c or from IL-9 Tg/c mice were transferred into SCID mice.

Mice given IL-9-overexpressing T cells developed severe colitis, and lost significantly more body-weight than mice reconstituted with WT CD4$^+$CD45RB^{high} T cells (Fig. 1A). Animals injected with CD4$^+$CD45RB^{high} IL-9 Tg cells also appeared more affected, suffering from diarrhea, anal prolapsus, and for some animals, skin inflammation in the periocular regions.

The expression of several cytokines, neutrophilic markers, and acute phase reactants was increased in SCID transferred with naive T cells that overexpressed IL-9 as compared to those reconstituted with WT naive T cells. We noted higher mRNA induction of *Il6*, *Tnf* and *Ifng* as well as inflammatory markers such as *S100a8*, *Lcn2*, and *Saa3* (Fig. 1B). These observations demonstrate a detrimental role for IL-9 in colitits induced by adoptive transfer of naive T cells.

In addition, histopathological analysis revealed that architecture of the colonic sections appeared completely disturbed by inflammatory infiltrating cells when injected cells came from IL-9 Tg/c mice (Fig. 1C, right panel). This loss of architecture was accompanied by glandular elongation and mucin depletion. The thickness of the mucosal wall was increased (bars, same magnification for the pictures) and ulcerations of the epithelium were observed (arrow, right panel). In contrast, the inflammatory infiltrate observed in SCID mice transferred with CD4$^+$ CD45RB^{high} from FVB/c animals, did not disrupt the general structure of the mucosa to the same extent (central panel).

This effect does not result from differences in the ontogeny of CD4$^+$CD45RB^{high} cells from IL-9 Tg/c versus FVB/c donors because IL-9 overexpression by *Rag2*$^{-/-}$ recipient mice also exacerbates colitis induced by WT CD4$^+$CD45RB^{high} FVB/c T cells. As illustrated in Supporting Information Fig. 1A, *Rag2*$^{-/-}$ recipient mice overexpressing IL-9 developed severe colitis, and lost significantly more body-weight as compared to control *Rag2*$^{-/-}$ receiving the same lymphocytes. In addition, IL-9 Tg *Rag2*$^{-/-}$ mice injected with T lymphocytes showed increased expression of *Il22*, *S100a*8, *Lcn*, and *Saa3* compared to control *Rag2*$^{-/-}$ (Supporting Information Fig. 1B). Disease severity was comparable in *Rag2*$^{-/-}$ IL-9 Tg mice with WT T cell transfer, and *Rag2*$^{-/-}$ mice with IL-9 Tg T cell transfer (data not shown).



IL-9 exacerbates colitis by acting directly on transferred CD4+ CD45RB^{high} T cells in vivo

IL-9 may act on effector T cells either directly or indirectly via other cells (e.g., mast cells, ILC2s, or intestinal epithelial cells) in the recipient environment. To test for a direct effect on transferred T cells, we used IL-9 Tg mice backcrossed with *Rag2*$^{-/-}$ mice. IL-9 Tg recipient mice reconstituted with WT CD4$^+$ CD45RB^{high} T cells developed severe colitis, and lost significantly more bodyweight as compared to mice receiving the *Il9r*$^{-/-}$ CD4$^+$ CD45RB^{high} T cells (Fig. 1D). Moreover, the expression of *Il6*, *Il22*, and *Ifng* as well as inflammatory markers (*S100a8, Lcn2, Saa3*, and *Reg3b)* was significantly higher in colons of mice reconstituted with WT CD4$^+$ CD45RB^{high} T cells than in those treated with *Il9r*$^{-/-}$ cells (Fig. 1E), suggesting that the effect of IL-9 in vivo requires the capacity of transferred T cells to respond to this cytokine.

CD4+ CD45RB^{high} T cells require in vivo activation to respond to IL-9 stimulation

To study the capacity of transferred T cells to respond to IL-9, we first monitored *Il9r* expression by quantitative PCR (qPCR) on mRNA from sorted CD4$^+$CD45RB^{high} or CD4$^+$CD45RB^{low} T cells. As illustrated in Fig. 2A, CD4$^+$CD45RB^{low} T cells expressed *Il9r*, but CD4$^+$CD45RB^{high} T cells showed little-to-no expression. To confirm lack of functional receptor expression in these cells, we monitored STAT3 phosphorylation in response to IL-9. Sorted CD4$^+$CD45RB^{high} and CD4$^+$CD45RB^{low} T cells were stimulated with IL-9 or with IL-10 and IFN-β as positive controls, and blotted for phosphorylated versus total STAT3. While CD4$^+$CD45RB^{low} T cells responded to IL-9 stimulation, little-to-no STAT3 phosphorylation was observed with CD4$^+$CD45RB^{high} T cells (Fig 2B). Intra- and extracellular FACS staining (for P-STAT3, CD4, and CD45RB) of spleen cells showed that STAT3 was phosphorylated in about 7% of CD4$^+$CD45RB^{low} cells upon IL-9 stimulation, whereas only 2% of CD4$^+$CD45RB^{high} cells responded (compared to a background of 1.5% in unstimulated cells or cells from *Il9r*$^{-/-}$ mice) (Fig. 2C).

As CD4$^+$CD45RB^{high} T cells seemed to be unable to respond to IL-9 before transfer, and CD4$^+$CD45RB^{low} T cells, which are

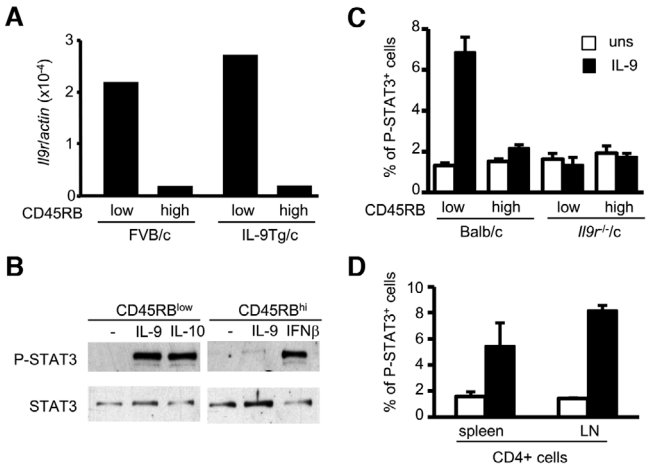


Figure 2. Il9r expression in CD4$^+$ CD45RB^{low} and RB^{high} lymphocytes and STAT3 activation by IL-9 stimulation before and after transfer. (A) T cells were isolated from FVB/c or IL-9 Tg mice (gating strategy for sorting in Supporting Information Fig. 3A). Quantitative RT-PCR was performed for *Il9r* on total RNA. Data were normalized to actin. Results are representative of two independent experiments. Six mice per group were pooled for the sorting. (B) A total of 9 × 10^3 CD45RB^{hi} and CD45Rb^{low} T cells from FVB/c mice were stimulated with control medium, IL-9 (2.5 ng/mL), IL-10 (1% Baculovirus supernatant), or IFN-β (5%, HEK293 cell supernatant) for 15 min before lysis. Total lysates were analyzed by Western blot with an antibody directed against tyrosine phosphorylated STAT3. The membrane was re-probed with anti-STAT3 antibody to control equal loading of the samples. Data are representative of three experiments. Eight mice were pooled for the sorting. (C) A total of 10^6 spleen cells from BALB/c or *Il9r*$^{-/-}$/c mice were cultured with IL-9 (black) or not (white) for 15 min and stained with A700-coupled anti-CD4, PE-coupled anti-CD45RB, and A647-coupled anti-phosphorylated STAT3 and analyzed by flow cytometry. Graph represents the percentage of phospho-STAT3 positive cells gated on CD4$^+$ CD45RB high or low cells (gating strategy in Supporting Information Fig. 3B). Mean values and SD were calculated from four samples of each group and were representative of more than 10 independent experiments. (D) *Rag2*$^{-/-}$ mice were injected with 4 × 10^5 CD4$^+$ CD45RB^{high} lymphocytes isolated from BALB/c animals. Mice were sacrificed 35 days after the cell transfer. Spleen and lymph nodes were collected and 10^6 cells were cultured with IL-9 (black) or not (white) for 15 min and stained for flow cytometry for A700-coupled anti-CD4, and A647-coupled anti-phosphorylated STAT3, results show percentage of phospho-STAT3 positive cells gated on CD4$^+$ cells. Mean values and SD were calculated from two mice of each group and are representative of seven experiments.

Figure 1. Immunodeficient mice overexpressing IL-9 develop more severe colitis mediated by IL-9 activity on transferred CD4$^+$ CD45RB^{high} T cells. (A and B) Control medium or 2 × 10^5 CD4$^+$ CD45RB^{high} lymphocytes isolated from FVB/c or IL-9 Tg/c mice were injected into SCID mice. (A) Mice were weighted weekly and graph presents percentage of original weight. Mean values and SEM were calculated from five mice per group and are representative of five independent experiments. (B) Quantitative RT-PCRs for *Ifng*, *Il6*, *Tnfa*, *Il22*, *S100a8*, *Lcn2*, *Saa3*, and *Reg3g,* performed on total RNA from colons of transferred SCID mice. Results were normalized to actin. Mean values and SEM were calculated from four to six mice of each group and are representative of two independent experiments. (C) Comparisons of the distal colonic mucosa of control SCID (left panel) and SCID mice treated 35 days before with 2 × 10^5 CD4$^+$ CD45RB^{high} T cells, from either FVB/c (central panel) or IL-9 Tg/c (right panel) mice. Bars represent the thickness of mucosa wall and the arrow points out ulceration of the epithelium. Representative sections of distal colon stained with H&E (at least four mice per group, representative of two experiments, scale bar, 100 μm and 20× magnification). (D and E) IL-9 transgenic *Rag2*$^{-/-}$ mice were injected with control medium or CD4$^+$ CD45RB^{high} lymphocytes isolated from BALB/c or *Il9r*$^{-/-}$/c animals. Mice received 4 × 10^5 cells and were sacrificed 37 days after cell transfer. (D) Mice were weighed weekly and graph presents percentage of original weight. Mean values and SEM were calculated from five mice per group and are representative of three independent experiments. (E) Quantitative RT-PCRs were performed for *Ifng*, *Il6*, *Tnfa*, *Il22*, *S100a8*, *Lcn2*, *Saa3*, and *Reg3g,* performed on total RNA from colons of transferred *Rag2*$^{-/-}$ mice. Results were normalized to *actin*. Mean values and SD were calculated from five mice of each group and are representative of three independent experiments. Statistical analyses: (A) two-way ANOVA with Bonferroni post-test; (B) Mann–Whitney test; (D) two-way ANOVA with Bonferroni post-test; (E) Mann–Whitney test ($^*p < 0.05$, $^{**}p < 0.01$; $^{***}p < 0.005$).

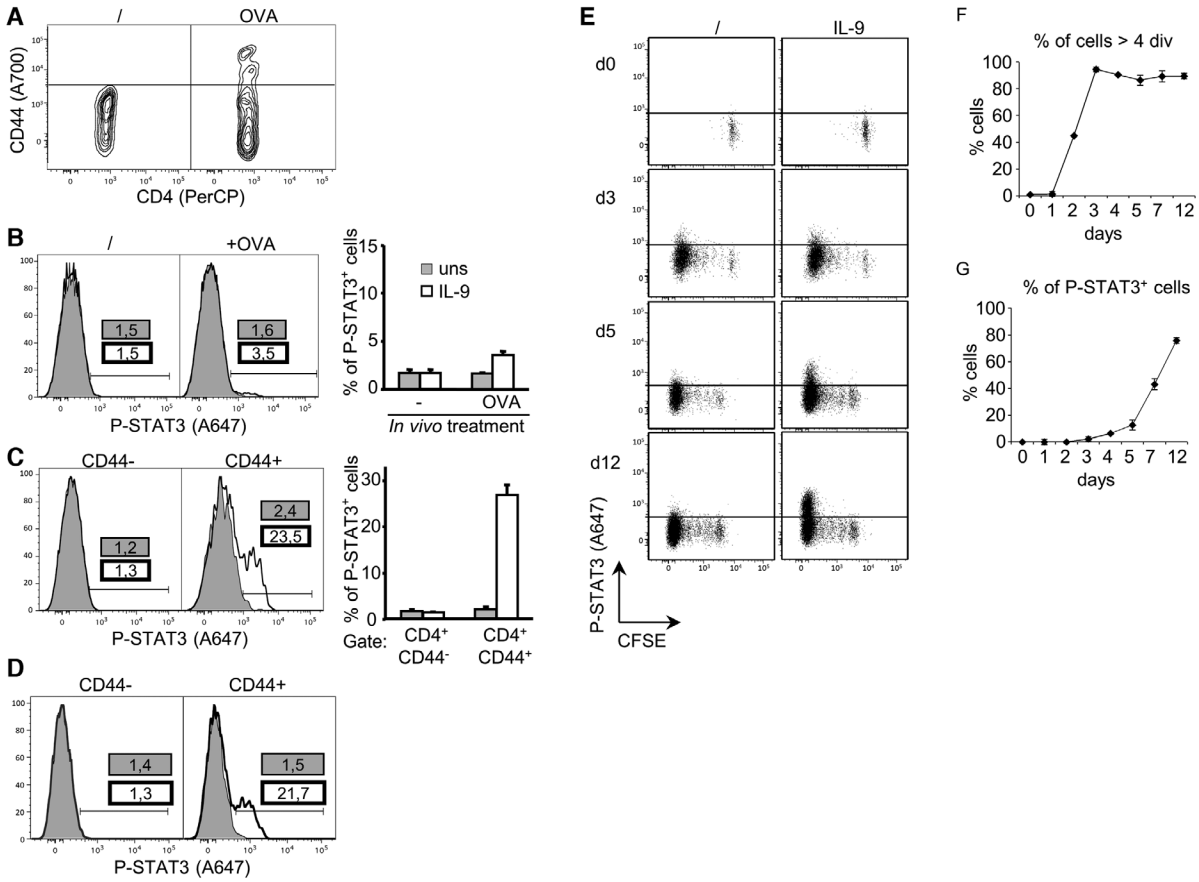


Figure 3. STAT3 activation by IL-9 stimulation in DO.11.10 Rag2$^{-/-}$ activated or naive cells. (A) CD44 expression on CD4$^+$ splenic T cells from DO.11.10 *Rag2*$^{-/-}$ mice, either naive or immunized 10 days earlier in the foodpad with chicken OVA and Alum (A700-coupled anti-CD44). Data are representative of eight experiments with two mice per group. (B) A total of 10^6 splenocytes of mice immunized or not with OVA for 10 days were cultured with IL-9 (thick line and withe columns) or not (grey filled lines and columns) for 15 min and stained for PerCP-coupled anti-CD4, A700-coupled anti-CD44, and A647-coupled anti-phosphorylated STAT3. Percentage of phospho-STAT3 positive cells gated on CD4$^+$ cells are represented. (C) Flow cytometry analysis of STAT3 phosphorylation in splenocytes of OVA immunized mice prepared in B and gated for CD4$^+$ and CD44$^+$ or CD4$^+$ and CD44$^-$ cells. (B and C) Mean values and SD were calculated from two mice of each group and were representative of eight experiments. (D) A total of 10^6 splenocytes of mice immunized or not with OVA for 40 days were cultured with IL-9 (thick line and withe columns) or not (grey filled lines and columns) for 15 min and stained for CD4, CD44, and phosphorylated STAT3, percentage of phospho-STAT3 positive cells gated on CD4$^+$ cells are represented. Mean values and SD were calculated from two mice of each group and were representative of three experiments. (E–G) A total of 5×10^6 cells from spleen and LN of DO.11.10 *Rag2*$^{-/-}$ mice were stained with CFSE before IP injection into *Rag2*$^{-/-}$ mice. Two days later, mice were immunized in the foodpad with chicken OVA and Alum for the indicated period of time. (E) Draining LN of immunized mice were stimulated with IL-9 or not for 15 minutes. STAT3 phosphorylation and CFSE staining were analyzed by flow cytometry at day 0, 3, 5, and 12. Data are representative of four experiments with three mice per group. (F and G) Kinetics of the percentage of cells with more than four divisions based on CFSE staining (F) and kinetics of the percentage of cells with a positive staining for phospho-STAT3 (G) at days 0,1, 2, 3, 4, 5, 7, and 12. Mean values and SD were calculated from three mice of each group and are representative of four experiments.

capable of responding, are considered to be experienced T cells, we assessed if transferred CD4$^+$CD45RB^{high} T cells could acquire this capacity in vivo during disease development. *Rag2*$^{-/-}$ mice were reconstituted with CD4$^+$CD45RB^{high} T cells and sacrificed 35–45 days later, when they presented symptoms of colitis. Lymph nodes and spleen cells were cultured for 15 min with or without IL-9 followed by intra- and extracellular FACS staining (for P-STAT3 and CD4). As shown in Fig. 2D, STAT3 is phosphorylated in ≈6–8% of CD4$^+$ cells from spleen or lymph nodes upon IL-9 stimulation (compared to a background of about 1.5% in unstimulated cells), showing that transferred CD4$^+$ T cells acquire the capacity to respond to IL-9 in vivo, during colitis development. CD4$^+$CD45RB^{high} T cells are mainly composed of naive cells and are not responsive to IL-9 but acquire IL-9 responsiveness in vivo during colitis development.

To analyze in more details how naive CD4$^+$ T cells acquire IL-9 responsiveness upon antigen stimulation in vivo, we took advantage of the DO11.10 *Rag2*$^{-/-}$ mice model. These mice have no B and T lymphocytes except for a single population of CD4$^+$ T cells all with the same OVA-specific TCR (recognizing chicken OVA peptide containing amino acids 323 to 339, in the context of I-A^d). As shown in Fig. 3A, CD4$^+$ T cells from these mice express low levels of CD44 (activation marker), with a CD44^{high} population appearing upon immunization (10 days earlier) with OVA in Alum in the footpad. Splenocytes from OVA-immunized versus naive DO11.10 *Rag2*$^{-/-}$ mice were stimulated in vitro with

or without IL-9, and analyzed for STAT3 phosphorylation, and OVA-TCR and CD44 expression by flow cytometry. IL-9 was able to induce STAT3 phosphorylation in T cells from OVA-immunized DO11.10 *Rag2*$^{-/-}$ mice (3.5% compared to 1.5% of background in non-stimulated cells, Fig. 3B), but not in those from their naive counterparts. P-STAT3 positive cells were only detected in the CD44^{high} and not in the CD44^{low} OVA-TCR population from immunized mice (Fig. 3C). Altogether, these data show that naïve T lymphocytes (CD4^{+}CD45RB^{high} or CD44^{neg}) need to be activated in vivo in order to respond to IL-9.

CD4^{+} IL-9 sensitive T cells are late, in vivo-activated, highly divided, long lasting cells

The data presented in Fig. 3B and C showed that 10 days after immunization, CD44^{+} activated T cells responded to IL-9. In order to determine whether this IL-9 responsive state was transitory or long lasting, we used the same protocol as previously described, but immunized the mice 40 days (instead of 10) before analysis. As shown in Fig. 3D, 40 days after immunization, CD4^{+}CD44^{+} T cells still responded to IL-9, whereas CD4^{+}CD44^{-} cells did not. In order to determine the kinetics of IL-9 reponse-acquisition by T cells and their proliferation state over the first 10 days, we transferred naive CFSE-stained DO11.10 *Rag2*$^{-/-}$ cells into *Rag2*$^{-/-}$ mice, immunized the recipients with OVA, and assessed for IL-9 responsiveness at different times post-immunization. We observed that IL-9 began to induce STAT3 phosphorylation in cells 5 days post-immunization, and that these cells expressed low levels of CFSE (Fig. 3E and G). At this time, at least 90% of T cells have divided more than four times (Fig. 3F). These observations demonstrate that IL-9 acts on highly divided CD44^{+} T cells and indicate that IL-9 responsiveness requires antigen exposure.

To define whether IL-9 acts on activated or on memory T cells, we assessed IL-9 responsiveness after a boost of T cells in vivo. Naive DO11.10 *Rag2*$^{-/-}$ mice were immunized with OVA and 14 days later, T cells were reactivated with the antigen or not. STAT3 phosphorylation induced by IL-9 did not increase, (indeed, was lower) in the boosted T cell (d14+2) as compared to T cells that had not been restimulated (d14) (Fig. 4). In contrast, IL-2 responsiveness, monitored by STAT5 phosphorylation, increased when T cells were reactivated. These results suggest that expression of IL-9R decreased upon T cell reactivation. This was confirmed by CD3 restimulation of T cells in vitro (Supporting Information Fig. 2). Spleen cells from OVA-immunized DO11.10 *Rag2*$^{-/-}$ mice expressed high levels of *Il9r* mRNA in contrast to anti-CD3 restimulated cells, which expressed no *Il9*r. Together, these data suggest that IL-9 acts on memory T cells rather than activated cells.

IL-9 acts on Th2 and Th17 memory cells

To define which memory T helper subsets respond to IL-9, naive T cells were differentiated in vitro into Th1, Th2, Th17, and iTreg populations. These in vitro differentiated T cells, expressing IFN-γ, IL-4, IL-17, or FoxP3, did not respond to IL-9 (data not shown) until transferred into *Rag2*$^{-/-}$ mice (Fig. 5A-B). IL-9 was able to induce the phosphorylation of STAT3 in TCR^{+}CD44^{+} cells from *Rag2*$^{-/-}$ reconstituted with Th2 and Th17 but not Th1 and Treg cells (Fig. 5B). mRNA expression of *Il9r* was examined in LN cell populations after in vivo differentiation: splenocytes from DO11.10 *Rag2*$^{-/-}$ mice were transferred into *Rag2*$^{-/-}$ mice, before immunization with OVA. After 14 days, expression of *Il9r* was measured in different subpopulations of sorted CD4^{+} cells from lymph nodes: CD44^{-}, CD44^{+}DN (CCR6^{-} and CXCR3^{-}), CD44^{+}CCR6^{+}, and CD44^{+}CXCR3^{+}. *Il9r* was highly expressed in CD44^{+}DN (likely containing Th2) and CD44^{+}CCR6^{+} (essentially Th17) (Fig. 5C). In contrast, *Il9r* was not expressed by CD44^{+}CXCR3^{+} (the source of Th1). Altogether, these results demonstrate that while naïve T cells do not respond to IL-9, this cytokine plays a role in Th2 and Th17 memory T cells.

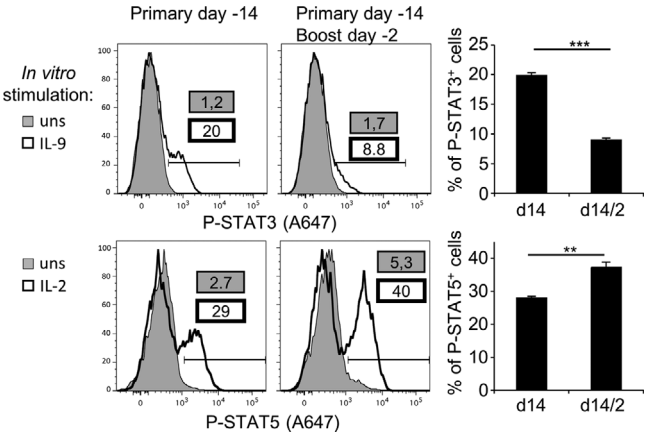


Figure 4. IL-9 responsiveness on activated and divided T cells after primary or secondary response. After 14 days of OVA immunization, DO.11.10 *Rag2*$^{-/-}$ mice received a second immunization of OVA (day 14/2) or not (day 14). Draining lymph nodes were stimulated with IL-9, IL-2 (black thick line), or left unstimulated (grey filled line) for 15 min. STAT3 and STAT5 phosphorylation were analyzed by flow cytometry and one representative sample is shown (gating strategy in Supporting Information Fig. 3C). Mean values and SD were calculated from two mice of each group and are representative of six experiments.

Discussion

In the present study, we show that naive T cells become responsive to IL-9 upon specific antigen stimulation in vivo, a process that requires 5–12 days and is paired with cell divisions and CD44 upregulation. The fact that antigen re-exposure downregulated IL-9R expression suggests that IL-9 responsiveness is a feature associated with memory rather than activated T cells, and that IL-9 does not act as an autocrine facor in contrast with IL-2, whose receptor is upregulated by antigen restimulation (Fig. 4). The observations from the OVA model provide an explanation for the apparent paradox of the colitis model where IL-9 exacerbates inflammation induced by adoptive transfer of CD4^{+}CD45RB^{high} (naive) T lymphocytes that do not express the IL-9 receptor. We showed that IL-9 directly acts on T cells, which acquire IL-9 responsiveness upon in vivo activation during the colitis when

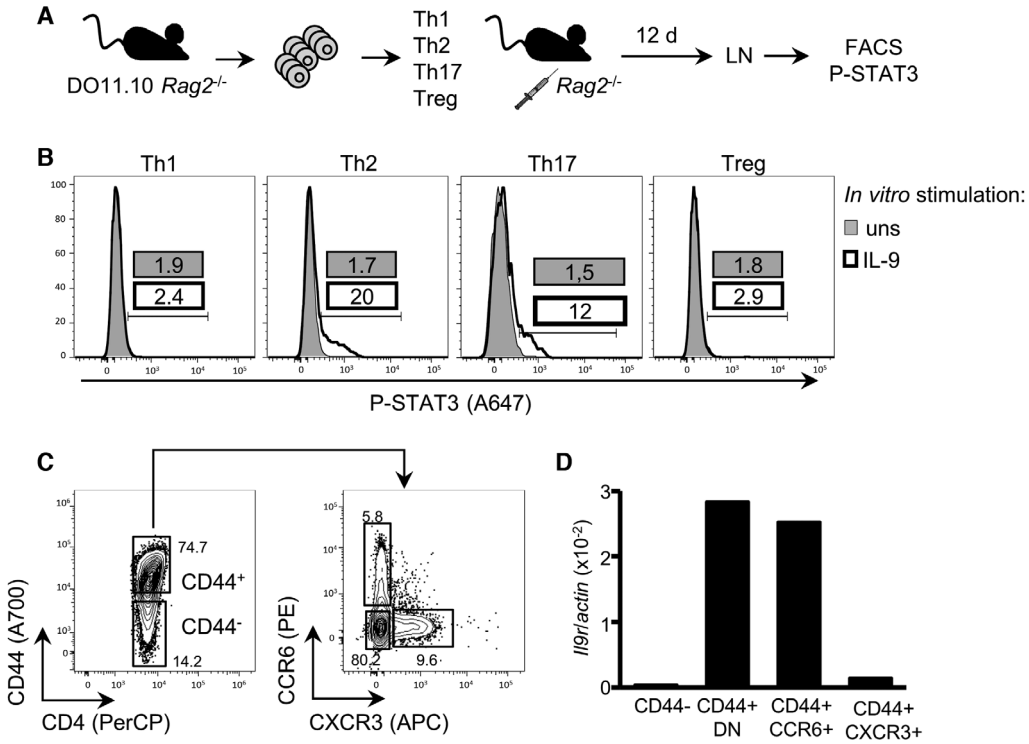


Figure 5. IL-9 responsiveness on different subtypes of T cells differentiated and activated in vivo. (A) Purified CD5$^+$ DO.11.10 *Rag2*$^{-/-}$ splenocytes were differentiated 4 days in vitro with coated anti-CD3, and soluble anti-CD28, in polarizing conditions: Th1 (anti-IL-4 and IL12), Th2 (anti-IFN-γ and IL-4), Th17 (TGF-ß, IL-6, IL-23, anti-IL-4, and anti-IFN-γ) or Treg (anti-IL-4, anti-IFN-γ, IL-2, and TGF-ß). On day 4 of culture, cells were washed and rested for 24 h. (B) Differentiated cells were transferred (5×10^6 cells i.p.) in *Rag2*$^{-/-}$ mice, 12 days later, lymph nodes were harvested, cells were stimulated with IL-9 (tick line) or not (grey filled lines) for 15 min, stained for PE-coupled anti-TCR, A700-coupled anti-CD44, and A647-coupled anti-phosphorylated STAT3 and analyzed by flow cytometry (Gating strategy in Supporting Information Fig. 3D). Results show percentage of phospho-STAT3 positive cells gated on TCR$^+$CD44$^+$ cells. Data are representative of four experiments for Th1 and Th2 and two for Th17 and Treg (four mice per condition). (C) *Rag2*$^{-/-}$ mice were transferred i.p. with DO.11.10 *Rag2*$^{-/-}$ splenocytes and immunized 2 days later with OVA/Alum s.c. into the footpad. 14 days later, draining LNs were collected and cells were stained for flow cytometry with anti-CD4, anti CD44, CCR6, and CXCR3 antibodies. CD4$^+$ cells were sorted as indicated for CD44$^-$, CD44$^+$ CCR6$^+$, CD44$^+$ CXCR3$^+$, or CD44$^+$ double negative (DN) (for CCR6 and CXCR3) cells and (D) total RNA was tested for IL-9 receptor expression, normalized with actin. Results are representative of four independent experiments. Five mice were pooled for sorting.

CD4$^+$CD45RB^{high} cells become CD4$^+$CD45RB^{low} [23], exactly as naïve OVA-specific T cells acquire IL-9 responsiveness 5–10 days after antigen exposure when they become CD44$^+$ (Fig. 3).

Although it was originally described as a T cell growth factor for in vitro cell clones and transformed cell lines [1,4,5], the role of IL-9 on T lymphocyte responses remains controversial. There are some indications that IL-9 may target certain subsets such as Th17 and Treg. IL-9 may collaborate with TGF-ß to promote Th17 cell differentiation in vitro [24]. In contrast to our observation that naïve T cells fail to respond to IL-9, it was shown that addition of IL-9 or anti-IL-9 during the in vitro differentiation of naïve T cells increases IL-17 production. This discrepancy might be due to the source of naive CD4 T cells, which were purified from spleens of WT mice such as Balb/c or C57Bl/6 and may vary in their status based on the purification protocols, whereas we used DO11.10 *Rag2*$^{-/-}$ mice. In the latter model, when mice are kept under strict SPF conditions, all the T cells are naïve. In vivo, a decreased level of Th17 is observed in IL-9R-deficient mice during EAE, although this may be an indirect effect.

Our results point to Th2 and Th17 memory cells as the major IL-9 responsive T cell subsets, preferentially to memory Th1 and iTregs. Interestingly, Elyaman and colleagues showed that, in contrast to inducible Tregs, freshly isolated natural Tregs express the IL-9 receptor and that IL-9 enhances their suppressive function [24]. Although we confirmed that inducible Tregs do not respond to IL-9 in our experimental setting, preliminary results from our laboratory using Foxp3-GFP knock-in mice, showed that at least a part of CD4$^+$ GFP$^+$ (FOXP3$^+$) Treg cells respond to IL-9 by STAT3 phosporylation (data not shown), suggesting that natural Tregs may also respond to IL-9 as indicated by the team of Elyaman.

To our knowledge, the observation of IL-9-induced STAT3 phosphorylation in CD4$^+$CD44^{high} T cells is the first evidence of a direct effect of IL-9 on freshly isolated activated T lymphocytes. Since T cells have to be in vivo activated in order to respond to IL-9, IL-9-responsiveness on T cells probably does not promote the initiation of autoimmune diseases, but might exacerbate their severity by acting directly on memory autoimmune cells. Unlike IL-2, it is unlikely that IL-9 acts as an autocrine growth factor. Although IL-9 is transiently produced by Th9, Th2, and Th17 cells upon antigen activation, our data show that IL-9 responsiveness is downregulated during this stage of activation. Mice given IL-9-deficient T cells develop less severe colitits than WT mice [16],

suggesting that IL-9 produced by T cells plays a crucial role in the inflammatory response. In this case, innate lymphoid cells such as ILC2, which express both high levels of IL-9 and of its receptor might play an important role in the inflammatory network. IL-9 produced upon antigen activation by T cells can act on ILC2 and induce their expansion, whereas at later stages of the immune response, when memory T cells acquire IL-9 responsiveness, ILC2 cells may produce IL-9 in response to innate stimuli such as IL-25 or IL-33, and subsequently promote the proliferation, survival, or further differentiation of memory T cells. In contrast with IL-7, which acts as a paracrine homeostatic growth factor for T lymphocytes, and with IL-2, which acts as an autocrine growth factor for activated T cells, these observations point to IL-9 as a key cytokine linking ILC2 and Th subsets that might have complementary mode of expression of IL-9 and its receptor.

Materials and methods

Mice

All mice used in this study were bred in the animal facility of the Brussels branch of the Ludwig Institute for Cancer Research under specific pathogen-free (SPF) conditions and female mice between 8 and 12 wk of age were used for this study. Handling of mice and experimental procedures were conducted in accordance with national and institutional guidelines for animal care. CB17/lcr-Prkdc^{scid}/Crl (SCID/c) mice (Charles River France Laboratories and The Jackson Laboratory) and *Rag2*$^{-/-}$ mice (Taconic, Ejby, Denmark) were maintained on isocap in closed cages in a SPF environment and were 8–12 weeks of age at the beginning of the transfer experiments. DO11.10 mice were provided by A. Radbruch (German Rheumatism Research Centre, Berlin, Germany) and backcrossed with *Rag2*$^{-/-}$ mice. BALB/c OlaHsd (BALB/c) mice were originally obtained from Harlan, the Netherlands. Tg5 IL-9-transgenic (IL-9 Tg) mice, generated in the FVB/N background and expressing high levels of IL-9 in all organs, were obtained as described previously [25]. To obtain F1 IL-9 Tg/c mice, we crossed IL-9 Tg animals with BALB/c mice. Control animals were obtained by crossing FVB mice with BALB/c mice.

To derive SCID/c IL-9 Tg/c or *Rag2*$^{-/-}$ IL-9 Tg/c offsprings, IL-9 Tg mice were bred with SCID/c mice or *Rag2*$^{-/-}$/c mice to obtain F1 animals that were backcrossed with SCID/c mice or *Rag2*$^{-/-}$/c mice. F2 mice were screened using a TS1 bioassay on serum to detect circulating IL-9 in IL-9 transgenic mice [1]. To screen for SCID or *Rag2*$^{-/-}$ in backcrossed animals, we performed an ELISA for IgM in the sera. In the present study, SCID/c IL-9 Tg animals had been backcrossed for three generations with SCID mice. The SCID/c or *Rag2*$^{-/-}$ littermates of the SCID/c IL-9 Tg or *Rag2*$^{-/-}$ IL-9 Tg/c animals were used as controls in the experiments.

The IL-9 receptor α KO mice (*Il9r*$^{-/-}$) were generated on the C57BL/c background as described previously [26]. To obtain the knock-out on the BALB/c background, they were backcrossed with BALB/c mice. After five generations, BALB/c mice were crossed with *Il9r*$^{-/-}$ mice to obtain F1 animals. Then F1 mice were crossed together to obtain littermate mice. *Il9r*$^{-/-}$ mice were screened using PCR on tail genomic DNA with primer sets corresponding to *Il9r* WT and *Il9r*$^{-/-}$ alleles [26].

Fluorescence-activated cell sorter analysis

Single-cell suspensions of splenocytes and LN cells were prepared in Hanks' medium. Cells were preincubated with 10 μg/mL purified rat anti-mouse CD16-CD32 mAb (Fc block; BD Pharmingen, San Diego, CA) for 15 min at room temperature. Biolegend (San Diego, CA) fluorochrome-coupled Abs specific for CD4 (GK1.5), TCRα/ß (IP26), DO11.10 TCR (KJ1-26), CD45RB (C363-16A), CD44 (IM7), CCR6 (29-2L17), and CXCR3 (CXCR3-173) were then added at a final concentration of 2 μg/mL and incubated for 1 h at 4°C. Stained cells were analyzed with a Fortessa instrument (BD Biosciences). Post-acquisition analysis was performed using FlowJo software (Tree Star, Ashland, OR). Flow cytometry and cell sorting experiments were performed according to the guidelines in immunological studies [27].

For STAT3 or STAT5 phosphostaining, 2×10^5 cells were left unstimulated or stimulated with IL-9 or IL-2 for 15 min at 37°C. Cells were fixed in 2% paraformaldehyde for 10 min at 37°C and then permeabilized in 90% methanol for 30 min on ice. Cells were washed twice with Hanks' medium, incubated overnight, and then stained with anti-phosphorylated STAT3-Y705, STAT5-Y (BD Biosciences), and antibody for extracellular staining. Cells were analyzed on a BD Biosciences FACSCalibur^{TM} flow cytometer (see Supporting Information Fig. 3 for gating strategies).

Sorting of CD4$^+$CD45RB T cell populations

Spleens were removed aseptically from FVB/c, IL-9 Tg/c, BALB/c, *Il9r*$^{-/-}$/c, and DO11.10 *Rag2*$^{-/-}$ mice (8–12 wks old). Cells were resuspended and filtered through 70 μm nylon cell strainer. Before the CD4$^+$T or CD5$^+$ T (for DO11.10 *Rag2*$^{-/-}$ mice) cell enrichment with anti-mouse CD4 particles-MSC (GK1.5, BD^{TM} IMag, BD Biosciences Pharmingen) or with anti-mouse CD5 microbeads (Ly-1, Miltenyi Biotec, North Rhine-Westphalia, Germany), erythrocytes were removed by osmotic shock. Staining and selection of cells were performed following the manufacturer's protocol. In brief, cells were incubated 15 min, at 4°C, with anti-CD4 particles-MSC (10% v/v), at the concentration of 100×10^6 cells/mL, before washing, filtering through 40 μm, and resuspension in 2 mL of MACS buffer (PBS supplemented with 2 mM EDTA, 2% decomplemented fetal calf bovine serum). The CD4$^+$ T cells were separated with the possel_s protocol on AutoMACS instrument (Miltenyi Biotec). To allow the sorting of resulting enriched CD4$^+$ T cells populations into CD4$^+$CD45RB^{high} and CD4$^+$CD45RB^{low} populations, CD4$^+$ lymphocytes were stained by FITC-labeled anti-mCD4 (L3T4) and PE-labeled anti-mCD45RB (16A) antibodies (dilution 1/100, BD PharMingen) during 45 min at 4°C. The second selection step was performed on a FACSVantage SE apparatus (BD Biosciences) after washing, filtering (40 μm), and resuspension of

the cells in FACS buffer (Hank's medium supplemented with 3% decomplemented fetal calf bovine serum). The $CD4^+CD45RB^{high}$ and $CD4^+CD45RB^{low}$ populations were defined as the highest staining (40–50%) and the lowest staining (15–20%), according to the Powrie et al. protocol (see Supporting Information Fig. 3A for gating strategy). For the experiment with CSFE staining, 5×10^6 spleen cells were stained with CSFE (5 μM, Molecular probes) for 10 min before the transfer.

Cell transfer

For cell transfer experiments, SCID or *Rag2*$^{-/-}$ recipient mice were injected intraperitoneally with 1.75–2.5 $\times$ 10^5 $CD4^+CD45RB^{high}$ or $CD4^+CD45RB^{low}$ cells. Cells were injected in a volume of 200–400 μL of sterile DMEM. The control recipients were injected with an equivalent volume of medium. Mice were sacrificed ≈5 wks after the transfer, except in the *Il9r*$^{-/-}$ cell transfer experiment, in which recipient mice were sacrificed 9 wks post-transfer. For OVA immunization of *Rag2*$^{-/-}$ and DO11.10 *Rag2*$^{-/-}$ mice, OVA (20 μg chicken-egg OVA grade V; Sigma–Aldrich, St Louis, MI) was injected i.p. with 70 μL alum (Alum Imject; ThermoScientific, Waltham, MA) in PBS to reach 200 μL.

Reverse transcription-PCR

$CD4^+$ $CD45RB^{low}$ or ^{high} T cells were obtained as described above. Distal colons were removed and submerged in RNA later (Ambion, Life technologies, Carlsbad, CA) between 12 and 48 h. Total RNA was isolated using TriPure isolation reagent (Roche, Basel, Switzerland) according to the manufacturer's instructions. Reverse transcription was performed on 1–2 μg of total RNA with an oligo (dT) primer (Roche) and M-MLV RT (Invitrogen, Carlsbad, CA). Quantitative PCR amplifications were performed from cDNA corresponding to 20 ng of total RNA. Quantitative PCRs were performed using primers sets corresponding to murine *Il6, Tnf, Ifng*, *Il22*, *S100A8, Lcn2, Saa3, Reg3b*, *Il9r*, or *Actb*. The PCRs were run with either qPCR™ Mastermix core kit for SYBR® Green I or qPCR™ Mastermix core kit for Taqman probes (Eurogentec, Liége, Belgium). The sequences of the primers (final concentration: 300 nM) and probes (final concentration: 100 nM) are summarized in Supporting Information Table 1. Samples were first heated for 10 min at 95°C. cDNA was amplified as follows: 40 cycles of a two-step PCR program at 95°C for 15 s and 60°C for 1 min. Melting point analysis was carried out by heating the amplicon from 60 to 95°C with SYBR Green kit. The data were normalized to ß-actin. Consistent results were obtained with normalization to 18S RNA.

Western blot

The $CD4^+CD45RB^{high}$ and $CD4^+CD45RB^{low}$ populations were obtained as described above. A total of 9 $\times$ 10^5 cells were stimulated for 15 min at 37°C with IL-9 (2.5 ng/mL, Baculovirus supernatant), IL-10 (100 U/mL corresponding to 1% Baculovirus supernatant), IFN-β (5%, HEK293 cell supernatant), or with control medium. Cells were lysed with 200 μL of Laemmli sample buffer (BioRad, Hercules, CA) and boiled for 5 min. Eighteen microliters of cell lysate were loaded and separated on a 12% pre-cast Tris-glycine gel (Invitrogen) and transfered electrophoretically to a nitrocellulose membrane (Hybon™-C, Amersham Biosciences, Little Chalfont, UK). The membrane was blocked in 5% nonfat dry milk, washed, and probed with anti-phospho STAT3 (Tyr 705, 1/1000, Cell Signaling, Leiden, the Netherlands). An anti-rabbit HRP linked IgG (1/10 000, Cell Signaling) is used as second antibody and the signal is obtained with the SuperSignal West Pico and Femto detection kits (Thermoscientific). The blot was subsequently reprobed with an anti-STAT3 antibody (1/1000, Cell Signaling) to control equal loading of the samples.

In vitro Th cell differentiation

$CD5^+$ cells from the spleen and LN of DO11.10 *Rag2*$^{-/-}$ mice were purified as described above. Cells cultured in DMEM medium supplemented with 10% FCS were stimulated with plate-bound anti-CD3 (purified hamster anti-mouse CD3, 145-2C11 [28], 5 μg/mL) and soluble anti-CD28 antibodies (purified NA/LE hamster anti-mouse CD28, 37.51 BD Pharmingen, 2.5 μg/mL). For Th1 stimuli, cells were stimulated with IL-12 (10 ng/mL, Biolegend) and anti-IL-4 antibody (clone 11B11, 10 mg/mL). For Th2 stimuli, cells were stimulated with IL-4 (200 U/mL, R&D Systems, Minneapolis) and anti-IFN-γ (clone FxIVF3, 5 mg/mL). For Th17, cells were stimulated with TGFß (1 ng/mL, R&D Systems, Minneapolis, MN), IL-6 (produced in our laboratory), IL-23 (10 ng/mL, e-Bioscience) anti-IL-4 antibody, and anti-IFN-γ. A total of 2 $\times$ 10^6 differentiated cells were injected IP into *Rag2*$^{-/-}$ mice.

Histology

Distal colons were fixed with formalin and embedded in paraffin. Five-micron sections were cut and stained with H&E and scanned with Mirax (Zeiss, Oberkochen, Germany). Two evaluators analyzed the staining.

Statistical analysis

Statistical significance was analyzed using the InStat3 program. Mann–Whitney *U*-test was run to determine the *p*-value when comparing two groups. Differences with $p < 0.05$ were considered statistically significant. Values are presented as mean ± SEM.

Acknowledgments: This work was supported by the Belgian Programme on Interuniversity Poles of Attraction initiated by the Belgian State, Prime Minister's Office, Science Policy Programming, and by the Actions de Recherche Concertées of the Communauté

Française de Belgique. L.D. is a Research Associate with the Fonds National de la Recherche Scientifique, Belgium. M.D.H. and P.M.C. were supported by the Actions de Recherche Concertées of the Communauté Française de Belgique and by the Belgian Programme on Interuniversity Poles of Attraction. The authors are very grateful to N. Limaye for her editing assistance.

Author contributions statement: M.D.H., V.S., P.M.C., J.-C.R., and L.D. wrote the manuscript and prepared the figures. M.D.H. V.S., J.L., and M.M.L. performed the experiments. G.W. provided the mice. All the authors reviewed the manuscript.

Conflict of interest: The authors state no commercial or financial conflict of interest.

References

1 **Uyttenhove, C., Simpson, R. J. and Van Snick, J.**, Functional and structural characterization of P40, a mouse glycoprotein with T-cell growth factor activity. *Proc. Natl. Acad. Sci. U. S. A.* 1988. **85**: 6934–6938.

2 **Renauld, J.-C. and Van Snick, J.**, Interleukin-9. In Thompson, A. (Ed.) *The Cytokine Handbook*, 4th Edn. Academic Press, London 2003, pp 347–362.

3 **Goswami, R. and Kaplan, M. H.**, A brief history of IL-9. *J. Immunol.* 2011. **186**: 3283–3288.

4 **Knoops, L. and Renauld, J. C.**, IL-9 and its receptor: from signal transduction to tumorigenesis. *Groundwater* 2004. **22**: 207–215.

5 **Chakraborty, S., Kubatzky, K. F. and Mitra, D. K.**, An update on interleukin-9: from its cellular source and signal transduction to its role in immunopathogenesis. *Int. J. Mol. Sci.* 2019. **20**.

6 **Dardalhon, V., Awasthi, A., Kwon, H., Galileos, G., Gao, W., Sobel, R. A., Mitsdoerffer, M. et al.**, IL-4 inhibits TGF-beta-induced Foxp3+ T cells and, together with TGF-beta, generates IL-9+ IL-10+ Foxp3(-) effector T cells. *Nat. Immunol.* 2008. **9**: 1347–1355.

7 **Schmitt, E. and Bopp, T.**, Discovery and initial characterization of Th9 cells: the early years. *Semin. Immunopathol.* 2017. **39**: 5–10.

8 **Walker, J. A. and McKenzie, A. N.**, Development and function of group 2 innate lymphoid cells. *Curr. Opin. Immunol.* 2013. **25**: 148–155.

9 **Kim, D. H. and Van Dyken, S. J.**, ILC2s in high definition: decoding the logic of tissue-based immunity. *Trends Immunol.* 2019.

10 **Turner, J. E., Morrison, P. J., Wilhelm, C., Wilson, M., Ahlfors, H., Renauld, J. C., Panzer, U. et al.**, IL-9-mediated survival of type 2 innate lymphoid cells promotes damage control in helminth-induced lung inflammation. *J. Exp. Med.* 2013. **210**: 2951–2965.

11 **Chen, J., Guan, L., Tang, L., Liu, S., Zhou, Y., Chen, C., He, Z. et al.**, T helper 9 cells: a new player in immune-related diseases. *DNA Cell Biol.* 2019. **38**: 1040–1047.

12 **Vyas, S. P. and Goswami, R.**, A decade of Th9 cells: role of Th9 cells in inflammatory bowel disease. *Front. Immunol.* 2018. **9**: 1139.

13 **Osterfeld, H., Ahrens, R., Strait, R., Finkelman, F. D., Renauld, J. C. and Hogan, S. P.**, Differential roles for the IL-9/IL-9 receptor alpha-chain pathway in systemic and oral antigen-induced anaphylaxis. *J. Allergy Clin. Immunol.* 2010. **125**: 469–476 e462.

14 **Faulkner, H., Humphreys, N., Renauld, J. C., Van Snick, J. and Grencis, R.**, Interleukin-9 is involved in host protective immunity to intestinal nematode infection. *Eur. J. Immunol.* 1997. **27**: 2536–2540.

15 **Steenwinckel, V., Louahed, J., Lemaire, M. M., Sommereyns, C., Warnier, G., McKenzie, A., Brombacher, F. et al.**, IL-9 promotes IL-13-dependent paneth cell hyperplasia and up-regulation of innate immunity mediators in intestinal mucosa. *J. Immunol.* 2009. **182**: 4737–4743.

16 **Gerlach, K., Hwang, Y., Nikolaev, A., Atreya, R., Dornhoff, H., Steiner, S., Lehr, H. A. et al.**, TH9 cells that express the transcription factor PU.1 drive T cell-mediated colitis via IL-9 receptor signaling in intestinal epithelial cells. *Nat. Immunol.* 2014. **15**: 676–686.

17 **Nalleweg, N., Chiriac, M. T., Podstawa, E., Lehmann, C., Rau, T. T., Atreya, R., Krauss, E. et al.**, IL-9 and its receptor are predominantly involved in the pathogenesis of UC. *Gut* 2014. **64**: 743–755.

18 **Weigmann, B. and Neurath, M. F.**, Th9 cells in inflammatory bowel diseases. *Semin. Immunopathol.* 2017. **39**: 89–95.

19 **Veldhoen, M., Uyttenhove, C., van Snick, J., Helmby, H., Westendorf, A., Buer, J., Martin, B. et al.**, Transforming growth factor-beta 'reprograms' the differentiation of T helper 2 cells and promotes an interleukin 9-producing subset. *Nat. Immunol.* 2008. **9**: 1341–1346.

20 **Gerlach, K., McKenzie, A. N., Neurath, M. F. and Weigmann, B.**, IL-9 regulates intestinal barrier function in experimental T cell-mediated colitis. *Tissue Barriers* 2015. **3**: e983777.

21 **Temann, U. A., Ray, P. and Flavell, R. A.**, Pulmonary overexpression of IL-9 induces Th2 cytokine expression, leading to immune pathology. *J. Clin. Invest.* 2002. **109**: 29–39.

22 **Li, H., Nourbakhsh, B., Cullimore, M., Zhang, G. X. and Rostami, A.**, IL-9 is important for T-cell activation and differentiation in autoimmune inflammation of the central nervous system. *Eur. J. Immunol.* 2011. **41**: 2197–2206.

23 **Lee, W. T., Yin, X. M. and Vitetta, E. S.**, Functional and ontogenetic analysis of murine $CD45R^{hi}$ and $CD45R^{lo}$ $CD4^+$ T cells. *J. Immunol.* 1990. **144**: 3288–3295.

24 **Elyaman, W., Bradshaw, E. M., Uyttenhove, C., Dardalhon, V., Awasthi, A., Imitola, J., Bettelli, E. et al.**, IL-9 induces differentiation of TH17 cells and enhances function of FoxP3+ natural regulatory T cells. *Proc. Natl. Acad. Sci. U. S. A.* 2009. **106**: 12885–12890.

25 **Renauld, J. C., van der Lugt, N., Vink, A., van Roon, M., Godfraind, C., Warnier, G., Merz, H. et al.**, Thymic lymphomas in interleukin 9 transgenic mice. *Oncogene* 1994. **9**: 1327–1332.

26 **Steenwinckel, V., Louahed, J., Orabona, C., Huaux, F., Warnier, G., McKenzie, A., Lison, D. et al.**, IL-13 mediates in vivo IL-9 activities on lung epithelial cells but not on hematopoietic cells. *J. Immunol.* 2007. **178**: 3244–3251.

27 **Cossarizza, A., Chang, H. D., Radbruch, A., Acs, A., Adam, D., Adam-Klages, S., Agace, W. W. et al.**, Guidelines for the use of flow cytometry and cell sorting in immunological studies (second edition). *Eur. J. Immunol.* 2019. **49**: 1457–1973.

28 **Leo, O., Foo, M., Sachs, D. H., Samelson, L. E. and Bluestone, J. A.**, Identification of a monoclonal antibody specific for a murine T3 polypeptide. *Proc. Natl. Acad. Sci. U. S. A.* 1987. **84**: 1374–1378.

Abbreviations: **qPCR**: quantitative PCR · **SPF**: specific pathogen-free · **Tg**: transgenic

Full correspondence: Laure Dumoutier, de Duve Institute, Avenue Hippocrate, 74, B-1200 Brussels, Belgium
e-mail: laure.dumoutier@uclouvain.be

The peer review history for this article is available at https://publons.com/publon/10.1002/eji.201948430

Received: 1/10/2019
Revised: 29/1/2020
Accepted: 3/3/2020
Accepted article online: 4/3/2020

