

# IL-36 $\gamma$ signaling controls the induced regulatory T cell–Th9 cell balance via NF $\kappa$ B activation and STAT transcription factors

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Regulatory and effector T helper (Th) cells are abundant at mucosal surfaces, especially in the intestine, where they control the critical balance between tolerance and inflammation. However, the key factors that reciprocally dictate differentiation along these specific lineages remain incompletely understood. Here we report that the interleukin-1 (IL-1) family member IL-36 $\gamma$  signals through IL-36 receptor, myeloid differentiation primary response gene 88, and nuclear factor- $\kappa$ Bp50 in CD4<sup>+</sup> T cells to potently inhibit Foxp3-expressing induced regulatory T cell (T<sub>reg</sub>) development, while concomitantly promoting the differentiation of Th9 cells via a IL-2-STAT5- (signal transducer and activator of transcription factor 5) and IL-4-STAT6-dependent pathway. Consistent with these findings, mice deficient in IL-36 $\gamma$  were protected from Th cell–driven intestinal inflammation and exhibited increased colonic T<sub>reg</sub> cells and diminished Th9 cells. Our findings thus reveal a fundamental contribution for the IL-36/IL-36R axis in regulating the T<sub>reg</sub>–Th9 cell balance with broad implications for Th cell–mediated disorders, such as inflammatory bowel diseases and particularly ulcerative colitis.

## INTRODUCTION

CD4<sup>+</sup> T helper (Th) cells are a critical component of the adaptive immune system that can differentiate into distinct regulatory and effector lineages thus influencing autoimmune diseases, inflammatory disorders, infectious diseases, and cancer.<sup>1–3</sup> Regulatory Th cells expressing Foxp3 (T<sub>reg</sub>) can develop intrathymically or in the periphery and are potently immunosuppressive and help to maintain immunological homeostasis.<sup>2</sup> Effector Th cells (T<sub>eff</sub>), on the other hand, can be grouped into several general categories (Th1, Th2, Th9, Th17, Th22, and T<sub>FH</sub>) based on dominant signature cytokines produced and associated master transcription factors expressed.<sup>4</sup> Interestingly, specific cytokines and factors are involved in dictating differentiation of naive Th cells into either T<sub>reg</sub> or T<sub>eff</sub> lineages.<sup>5</sup> For example, in the presence of IL-2 and transforming growth factor- $\beta$  (TGF $\beta$ ) naive Th cells differentiate into induced T<sub>reg</sub> cells (iT<sub>reg</sub>) while the combination of interleukin (IL)-6 plus TGF $\beta$  promotes Th17 and inhibits iT<sub>reg</sub>

differentiation.<sup>6–8</sup> Alternatively, IL-4 can promote the differentiation of Th2 cells while the addition of TGF $\beta$  can induce reprogramming into Th9 cells.<sup>9–11</sup> Thus the local cytokine milieu present during Th cell priming dramatically influences specific lineage commitment.

The IL-1 family of cytokines have recently emerged as critical regulators of adaptive immune cell function and plasticity, particularly at mucosal surfaces.<sup>12,13</sup> IL-1 signaling was recently shown to be involved in overriding retinoic acid-mediated Foxp3 induction while inducing protective Th17 responses during *Citrobacter rodentium* infection.<sup>14</sup> Another IL-1 family member, IL-33, acts as an alarmin that is released during tissue damage and can bind to the IL-33 receptor ST2 on T<sub>reg</sub> cells to induce their stability and immunosuppressive function in the intestine.<sup>15</sup> Thus IL-1 family members can be released in the local environment following tissue damage, or in response to infection, and potently dictate Th cell differentiation and function that ultimately aids in resolution of inflammation and

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host protection. However, the role of “novel” IL-1 family members, such as IL-36, in regulating CD4<sup>+</sup> Th cell differentiation into specific lineages remains incompletely defined.<sup>16</sup> In the present report, we investigated the role of the IL-36 $\gamma$ /IL-36R axis in controlling the balance of T<sub>reg</sub> and T<sub>eff</sub> lineages, with particular focus on how this pathway regulates Th cell-dependent intestinal inflammation. Our results demonstrate that signaling through IL-36R employs myeloid differentiation primary response gene 88 (MyD88) and nuclear factor- $\kappa$ Bp50 (NF $\kappa$ Bp50) in CD4<sup>+</sup> T cells to potentially inhibit iT<sub>reg</sub> development, while concomitantly promoting Th9 differentiation via a IL-2-STAT5- and IL-4-STAT6-dependent pathway. Additionally, mice deficient in IL-36 $\gamma$ -IL-36R signaling were protected from Th cell-dependent intestinal inflammation and exhibited increased colonic iT<sub>regs</sub> and diminished Th9 cells. Collectively, these data highlight IL-36R signaling as a regulator of the iT<sub>reg</sub>-Th9 balance *in vitro* and *in vivo* with functional implications in the regulation of intestinal inflammation.

## RESULTS

### IL-36 $\gamma$ abrogates iT<sub>reg</sub> induction via IL-36R-mediated signaling in CD4<sup>+</sup> T cells

To investigate the contribution of the IL-36/IL-36R axis in CD4<sup>+</sup> Th cell differentiation, we first explored whether IL-36 ligands could modulate Foxp3 induction in responding T cells using a naive CD4<sup>+</sup> T cell-dendritic cell (DC) *in vitro* co-culture system in the presence of  $\alpha$ CD3 $\epsilon$ , TGF $\beta$ , and IL-2 (iT<sub>reg</sub> condition).<sup>17</sup> Intriguingly, compared with other IL-1 family members tested, IL-36 ligands—IL-36 $\alpha$ , IL-36 $\beta$ , and IL-36 $\gamma$ —all potentially abrogated the induction of Foxp3-expressing iT<sub>reg</sub> cells in a dose-dependent manner (Figure 1a–c; see Supplementary Figure S1a online). Given that all three IL-36 ligands were behaving similarly, combined with the preferential expression of IL-36 $\gamma$  in the mouse intestine during colitis,<sup>18</sup> we focused specifically on IL-36 $\gamma$  and asked whether it was acting on CD4<sup>+</sup> T cells or DCs to inhibit iT<sub>reg</sub> differentiation. To do so, we employed a co-culture system whereby CD4<sup>+</sup> T cells or DCs were isolated from wild-type (WT) or IL-36R-deficient mice. Interestingly, the expression of IL-36R by CD4<sup>+</sup> T cells, but not DCs, was essential for the iT<sub>reg</sub>-inhibiting ability of IL-36 $\gamma$  in this assay (Figure 1d,e). We next investigated whether IL-36 $\gamma$  was acting to inhibit iT<sub>reg</sub> differentiation via the induction of autocrine/paracrine signaling, including IL-6 that is known to potentially block *de novo* Foxp3 expression and promote Th17 differentiation.<sup>6,8</sup> Notably, inhibition of iT<sub>reg</sub> cells mediated by IL-36 $\gamma$  was not reversible by antibody (Ab)-mediated neutralization of IL-1 $\beta$ , IL-6, and IL-12/23p40 (Figure 2a,b) or IL-4, IL-5, IL-9, IL-13, IL-22, and interferon- $\gamma$  (IFN $\gamma$ ; see Supplementary Figure S2a,b), although we cannot formally confirm complete neutralization in our specific culture conditions. As recent studies have implicated that the glucocorticoid-induced tissue necrosis factor receptor-related protein (GITR)/GITR ligand axis is suppressing Foxp3<sup>+</sup> iT<sub>reg</sub> differentiation,<sup>19,20</sup> we also examined whether

this pathway could be involved in the inhibition of iT<sub>reg</sub> differentiation mediated by IL-36 $\gamma$ . Notably, Ab-mediated blockade of GITR ligand was also unable to reverse the effects of IL-36 $\gamma$  on suppressing iT<sub>reg</sub> differentiation (see Supplementary Figure S3a,b). Of note, the effect of IL-36 $\gamma$  on iT<sub>reg</sub> inhibition was not affected by the irradiation of DCs (see Supplementary Figure S4). We also confirmed the ability of IL-36 $\gamma$  to suppress iT<sub>reg</sub> cell induction in the absence of DCs by using purified naive CD4<sup>+</sup> T cells, which were isolated from WT mice or OT-II mice (see Supplementary Figure S5a,b).

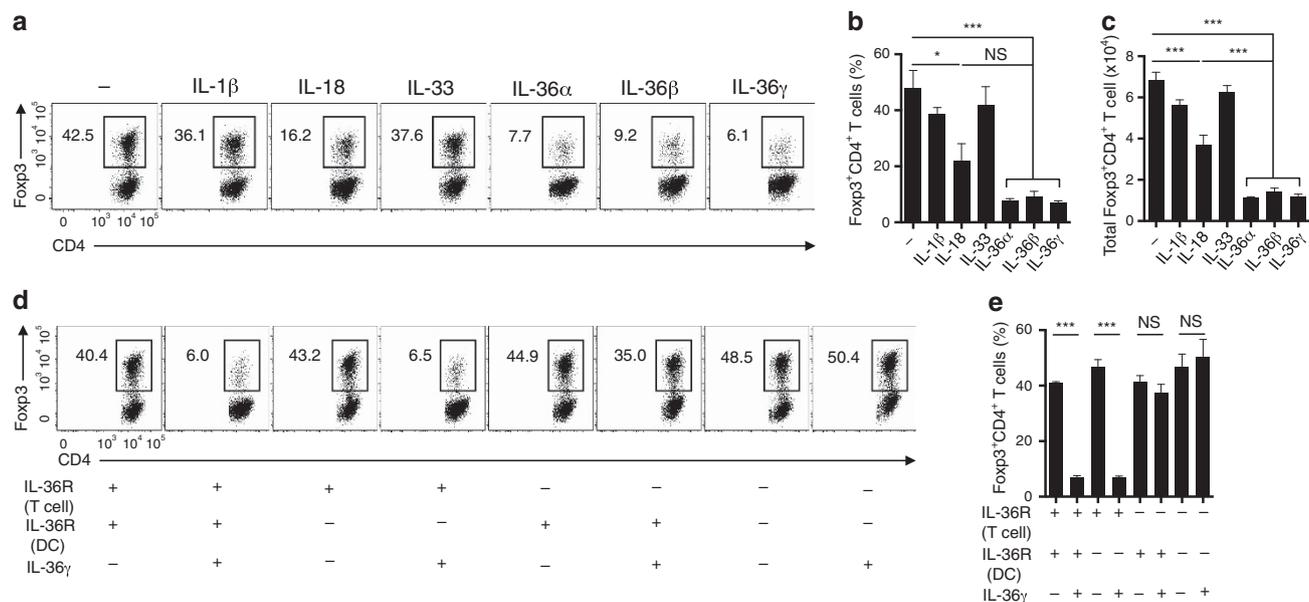
Overall, these findings demonstrate that IL-36 $\gamma$  inhibits Foxp3<sup>+</sup> iT<sub>reg</sub> differentiation via IL-36R-mediated signaling in CD4<sup>+</sup> cells independent of well-defined inflammatory cytokines or GITR/GITR ligand induction.

### IL-36 $\gamma$ -mediated suppression of iT<sub>reg</sub> cells is MyD88 and NF $\kappa$ Bp50 dependent

Given that MyD88 is a key adaptor molecule employed for signaling downstream of several IL-1 family member cytokines receptors, including IL-1R, IL-18R, IL-33R, and IL-36R,<sup>12</sup> we performed T cell and DC (T/DC) co-cultures whereby CD4<sup>+</sup> T cells were isolated from MyD88-sufficient (WT) or -deficient (*Myd88*<sup>-/-</sup>) mice in order to assess whether deletion of MyD88 specifically in CD4<sup>+</sup> T cells restored iT<sub>reg</sub> differentiation in the presence of IL-36 $\gamma$ . Notably, MyD88 expression in CD4<sup>+</sup> T cells was involved in the suppression of iT<sub>reg</sub> cell development mediated by IL-36 $\gamma$  (Figure 2c,d). As signaling through IL-36R can lead to NF $\kappa$ B activation,<sup>21</sup> we next explored whether NF $\kappa$ Bp50 was involved in IL-36 $\gamma$ -mediated suppression of iT<sub>reg</sub> differentiation. To address this question, we employed a co-culture system whereby CD4<sup>+</sup> T cells were isolated from p50-sufficient (WT) or -deficient (*p50*<sup>-/-</sup>) mice. Remarkably, deficiency of NF $\kappa$ Bp50 specifically in CD4<sup>+</sup> T cells fully restored iT<sub>reg</sub> induction in the presence of IL-36 $\gamma$  (Figure 2e,f). Altogether, our data indicate that IL-36 $\gamma$  suppresses iT<sub>reg</sub> differentiation via MyD88- and NF $\kappa$ Bp50-dependent signaling in CD4<sup>+</sup> T cells.

### IL-36 $\gamma$ alters NF $\kappa$ B signaling and acetylation of the Foxp3 locus during iT<sub>reg</sub> cell differentiation

We next investigated whether IL-36 $\gamma$  may be mediating its effects on inhibiting iT<sub>reg</sub> cells by interfering with signaling downstream of TGF $\beta$  receptor, specifically SMAD3, as it is known to bind the conserved noncoding sequence 1 (CNS1) in the Foxp3 locus and positively regulate Foxp3 expression.<sup>22</sup> Flow cytometric analyses revealed that IL-36 $\gamma$  did not inhibit TGF $\beta$ -induced phosphorylation of SMAD2/3 (Figure 3a) or nuclear translocation of SMAD3 (Figure 3b). As deficiency of NF $\kappa$ Bp50 in CD4<sup>+</sup> T cells fully restored iT<sub>reg</sub> induction in the presence of IL-36 $\gamma$ , we next examined how IL-36 $\gamma$  modulates the nuclear translocation of specific NF $\kappa$ B family members. As shown in Figure 3b, upon CD4<sup>+</sup> T-cell activation under neutral conditions, IL-36 $\gamma$  promoted nuclear translocation of NF $\kappa$ B p65 and p50, but not p105. Under iT<sub>reg</sub> conditions, p65 nuclear translocation was also observed; however, this nuclear translocation was similar  $\pm$  IL-36 $\gamma$ . Interestingly, NF $\kappa$ Bp50 nuclear translocation was observed under iT<sub>reg</sub> conditions and



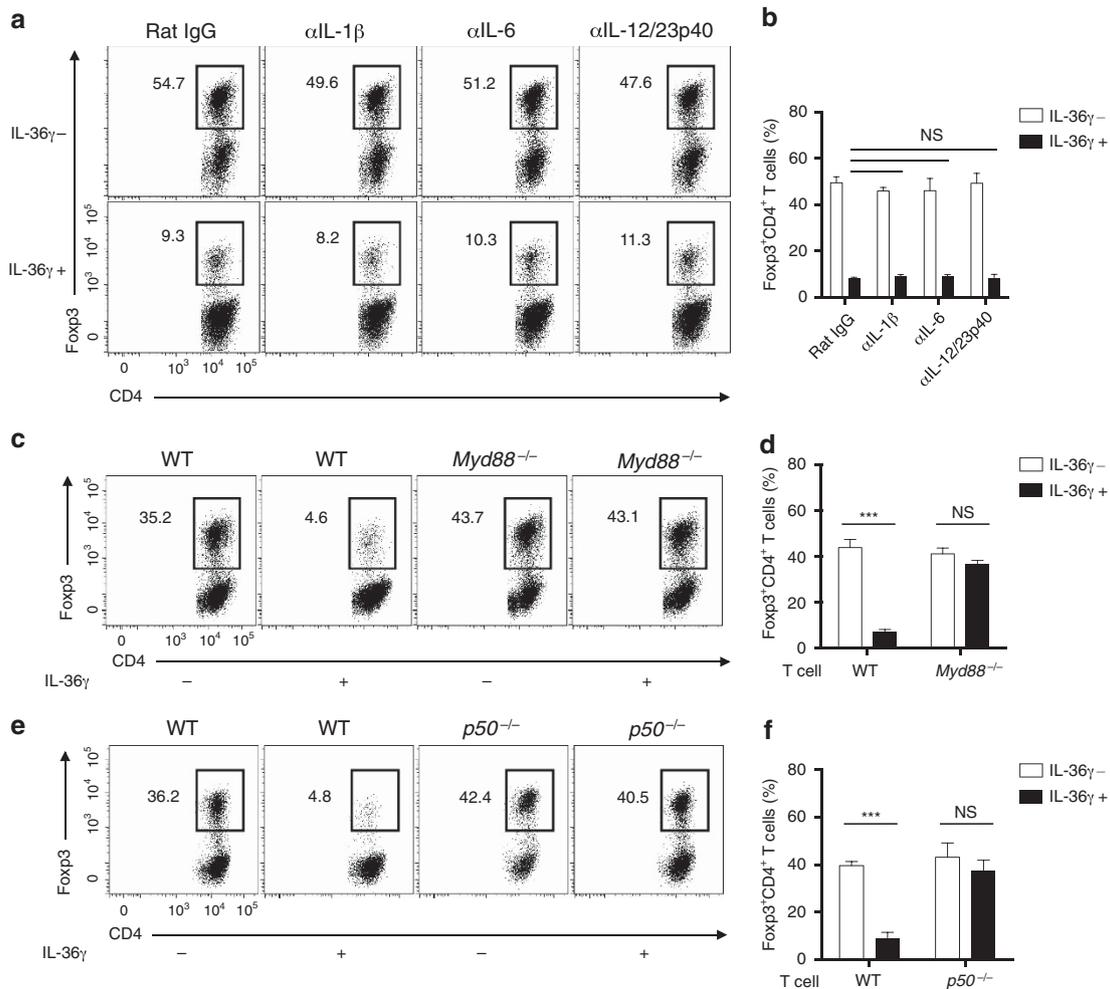
**Figure 1** Interleukin (IL)-36 $\gamma$  abrogates induced regulatory T-cell induction via IL-36R-mediated signaling in CD4<sup>+</sup> T cells. (a–c) Fcpx3 expression by CD4<sup>+</sup> T cells co-cultured with dendritic cells (DCs) for 4 days in the presence of the indicated IL-1 family member. Representative dot plots are shown in a, the frequencies of Fcpx3<sup>+</sup> T cells among total CD4<sup>+</sup> T cells are shown in (b) and the total numbers of Fcpx3<sup>+</sup> CD4<sup>+</sup> T cells are shown in (c). (d, e) Fcpx3 expression by CD4<sup>+</sup> T cells co-cultured with DCs for 4 days in the presence or absence of IL-36 $\gamma$  using the indicated cells from wild-type (WT; IL-36R “+”) and/or *Il1rl2*<sup>-/-</sup> (IL-36R “-”) mice. Representative dot plots are shown in (d). The frequencies of Fcpx3<sup>+</sup> T cells among total CD4<sup>+</sup> T cells are shown in (e). All data are representative of three independent experiments with three replicates. One-way analysis of variance and Tukey’s Multiple Comparison Test was used to determine significance. Error bars indicate mean  $\pm$  s.e.m. \* $P$ <0.05, \*\* $P$ <0.01, \*\*\* $P$ <0.001; NS, not significant.

the addition of IL-36 $\gamma$  further augmented nuclear translocation. Together, these results suggest that IL-36 $\gamma$ -enhanced NF $\kappa$ Bp50 nuclear translocation may alter the overall ratio of nuclear p50 to p65, resulting in impaired iT<sub>reg</sub> cell differentiation. As the IL-36 $\gamma$ -MyD88-p50 axis could epigenetically alter the chromatin status in CD4<sup>+</sup> T cells under iT<sub>reg</sub> conditions,<sup>19</sup> we next examined whether IL-36 $\gamma$  modulates histone acetylation at the Fcpx3 locus, which includes the promoter, CNS1, and CNS2 regions. Indeed, we observed significant decreases in histone H3 acetylation (H3Ac) status at the Fcpx3 promoter, CNS1 and CNS2 regions under iT<sub>reg</sub> conditions in the presence of IL-36 $\gamma$  (Figure 3c). Collectively, these data suggest that IL-36 $\gamma$  activates NF $\kappa$ Bp50 in CD4<sup>+</sup> T cells and modulate histone acetylation status at the Fcpx3 locus during iT<sub>reg</sub> cell differentiation *in vitro*.

### IL-36 $\gamma$ promotes Th9 differentiation in a MyD88- and NF $\kappa$ Bp50-dependent manner

Having defined the ability of IL-36 $\gamma$  to inhibit iT<sub>reg</sub> cell differentiation, we next explored which Th cell lineages IL-36 $\gamma$  may be favoring. To do so, we performed gene expression profiling of CD4<sup>+</sup> Th cells stimulated with  $\alpha$ CD3 $\epsilon$  in the presence or absence of IL-36 $\gamma$  by using the T/DC co-culture system. Notably, under iT<sub>reg</sub> conditions, IL-9 was identified as one of the top genes induced by IL-36 $\gamma$  among the analyzed gene set (Figure 4a,b; see Supplementary Figure S6). We also performed parallel experiments but under neutral conditions (no addition of either TGF $\beta$ 1 or IL-2). Similar to results

observed using iT<sub>reg</sub> culture conditions, IL-9 was the top gene induced by IL-36 $\gamma$  under neutral conditions (Figure 4a,c; see Supplementary Figure S6). The ability of IL-36 $\gamma$  to potentially induce IL-9 expression in a dose-dependent manner was additionally confirmed by quantitative real-time PCR (qPCR) and enzyme-linked immunosorbent assay (Figure 4d,e; see Supplementary Figure S1b). Interestingly, the induction of IL-9 mediated by IL-36 $\gamma$  was more than fivefold greater than that of IL-1 $\beta$ , which has been reported to induce IL-9,<sup>23</sup> and IL-18 and IL-33 in both iT<sub>reg</sub> and neutral conditions (Figure 4e). We also tested whether IL-9 production by CD4<sup>+</sup> T cells was controlled via IL-36R expression on T cells or DCs by performing the co-culture experiments as in Figure 1d. Similar to the requirement for IL-36R expression on CD4<sup>+</sup> T cells in mediating iT<sub>reg</sub> inhibition, the expression of IL-36R by CD4<sup>+</sup> T cells, but not DCs, was involved in the IL-9-inducing ability of IL-36 $\gamma$  (Figure 4f). Additionally, we also confirmed that the irradiation of DCs does not affect the ability of IL-36 $\gamma$  to induce IL-9 production in this setting (see Supplementary Figure S4). Next we investigated the mechanism via which IL-36 $\gamma$  induced IL-9 production from CD4<sup>+</sup> T cells. As IL-36 $\gamma$  abrogated iT<sub>reg</sub> differentiation via a MyD88- and NF $\kappa$ Bp50-dependent signaling pathway, we asked whether IL-36 $\gamma$ -induced IL-9 production was also dependent upon MyD88 and NF $\kappa$ Bp50. Indeed, using CD4<sup>+</sup> T cells isolated from *Myd88*<sup>-/-</sup> or *p50*<sup>-/-</sup> mice, a near complete abrogation of IL-36 $\gamma$ -induced IL-9 production was observed in neutral conditions as well as in iT<sub>reg</sub> conditions (Figure 4g). We further examined whether the addition of IL-36 $\gamma$  could



**Figure 2** Interleukin (IL)-36 $\gamma$ -mediated suppression of induced regulatory T cells is myeloid differentiation primary response gene 88 (MyD88) and nuclear factor- $\kappa$ Bp50 dependent. (a, b) Fopx3 expression by CD4<sup>+</sup> T cells co-cultured with dendritic cells (DCs) for 4 days in the presence or absence of IL-36 $\gamma$  supplemented with the indicated neutralizing antibodies. Representative dot plots (a) and the frequencies of Fopx3<sup>+</sup> T cells among total CD4<sup>+</sup> T cells (b) are shown. (c, d) Fopx3 expression by CD4<sup>+</sup> T cells from wild-type (WT) and/or *Myd88*<sup>-/-</sup> mice co-cultured with WT DCs for 4 days in the presence or absence of IL-36 $\gamma$ . Representative dot plots (c) and the frequencies of Fopx3<sup>+</sup> T cells among total CD4<sup>+</sup> T cells (d) are shown. (e, f) Fopx3 expression by CD4<sup>+</sup> T cells from WT and/or *p50*<sup>-/-</sup> mice co-cultured with WT DCs for 4 days in the presence or absence of IL-36 $\gamma$ . Representative dot plots (e) and the frequencies of Fopx3<sup>+</sup> T cells among total CD4<sup>+</sup> T cells (f) are shown. Data are representative of three independent experiments (a, b) and from two independent experiments (c–f) with three replicates (b) or six replicates (d, f). One-way analysis of variance and Tukey's Multiple Comparison Test was used to determine significance. Error bars indicate mean  $\pm$  s.e.m. \* $P$  < 0.05, \*\* $P$  < 0.01, \*\*\* $P$  < 0.001; NS, not significant.

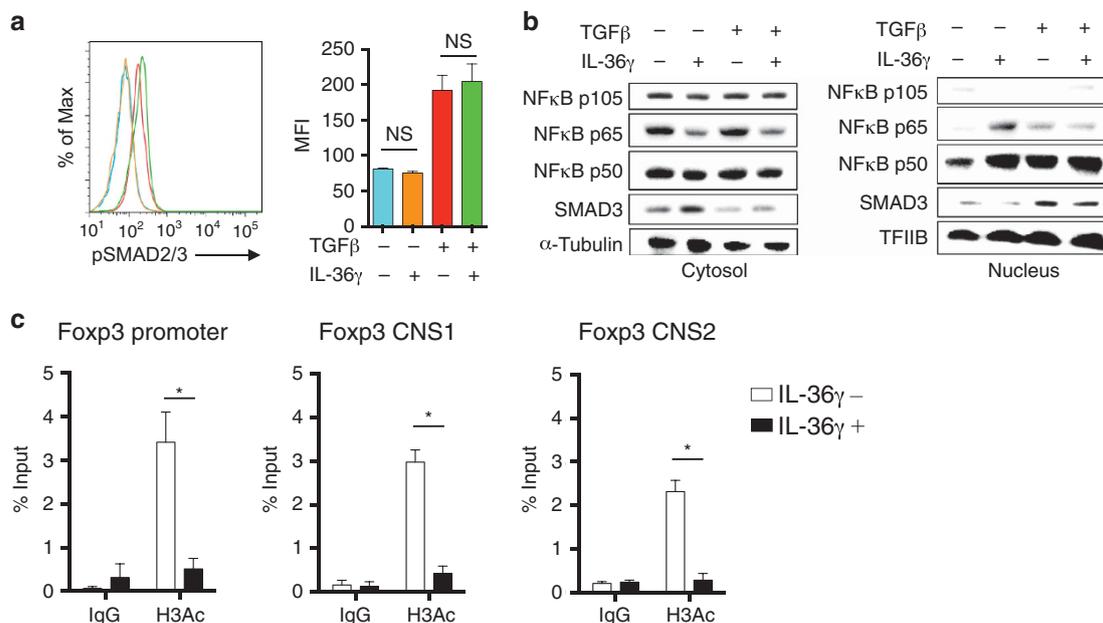
modulate the expression of transcription factors known to be required for Th9 differentiation, specifically PU.1<sup>24</sup> and IRF4.<sup>25</sup> Indeed, both PU.1 and IRF4 were significantly induced in CD4<sup>+</sup> T cells in the presence of IL-36 $\gamma$  as compared with the cells cultured in the absence of IL-36 $\gamma$  (see **Supplementary Figure S7a,b**). Thus IL-36 $\gamma$  robustly induces IL-9-producing CD4<sup>+</sup> T cells via T-cell-intrinsic MyD88- and NF $\kappa$ Bp50-dependent signaling.

Next we further investigated the ability of IL-36 $\gamma$  to induce IL-9 in the absence of DCs by using purified CD4<sup>+</sup> T-cell cultures and various standard Th cell-polarizing conditions. Consistent with our data from T/DC co-cultures, IL-36 $\gamma$  significantly induced the differentiation of IL-9-producing cells under Th0 conditions (see **Supplementary Figure S8a,b**). Remarkably, IL-36 $\gamma$  also significantly augmented Th9 cell differentiation under Th9 conditions (see **Supplementary**

**Figure S8a,c**). Intriguingly, the ability of IL-36 $\gamma$  to potentially induce IL-9-producing T cells was also observed under other Th cell-polarizing conditions, including Th2 and iT<sub>reg</sub> conditions but to a far lesser extent under Th1 and Th17 conditions (see **Supplementary Figure S8a**). Thus these findings demonstrate that IL-36 $\gamma$  is a potent inducer of IL-9 production and augments Th9 differentiation, even in the absence of DCs.

#### IL-36 $\gamma$ induces IL-9 expression via IL-2-STAT5 and IL-4-STAT6 signaling

Several factors have been reported to promote IL-9 production by CD4<sup>+</sup> T cells, including IL-1 $\beta$ , IL-2, IL-4, IL-21, IL-25, and TGF $\beta$ .<sup>26</sup> To investigate the potential involvement of these cytokines in IL-36 $\gamma$ -induced IL-9 production, we performed T/DC co-cultures in the presence of cytokine-specific



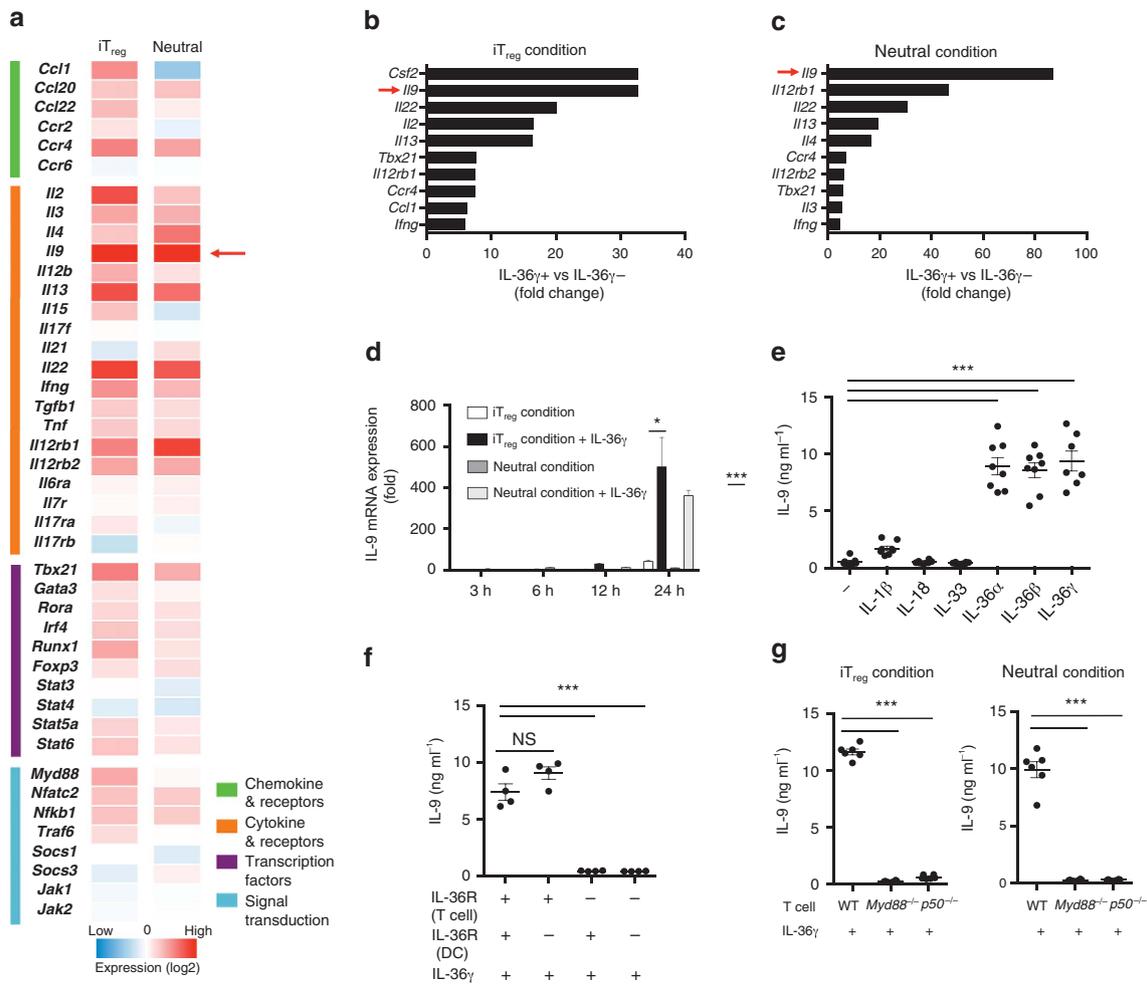
**Figure 3** Interleukin (IL)-36 $\gamma$  alters nuclear factor- $\kappa$ B (NF $\kappa$ B) signaling and acetylation of the Foxp3 locus during induced regulatory T cell (iT<sub>reg</sub>) differentiation. **(a)** FACS (fluorescence-activated cell sorter)-sorted naive CD4<sup>+</sup> T cells were co-cultured with dendritic cells (DCs) at a 10:1 T/DC cell ratio in the presence or absence of transforming growth factor- $\beta$  and IL-36 $\gamma$  for 30 min. Cells were then fixed and permeabilized for staining with anti-phospho-SMAD2/3 (pSMAD2/3) antibodies. Representative histograms for pSMAD2/3 in CD4<sup>+</sup> T cells are shown. The graph represents mean fluorescent intensity (MFI) of pSMAD2/3 among CD4<sup>+</sup> T cells. **(b)** Western blotting analysis of NF $\kappa$ B p105, p65, p50, and SMAD3 in the cytosol and nucleus in FACS-sorted naive CD4<sup>+</sup> T cells activated with the indicated cytokines for 45 min.  $\alpha$ -Tubulin and TFIIIB are shown as loading controls, respectively. **(c)** Chromatin immunoprecipitation assays for H3Ac modifications in the Foxp3 locus in naive CD4<sup>+</sup> T cells co-cultured with DCs at a 10:1 T/DC cell ratio for 4 days under iT<sub>reg</sub> conditions. Data are representative of two independent experiments (**a–c**) with three replicates (**a, c**). One-way analysis of variance and Tukey's Multiple Comparison Test was used to determine significance. Error bars indicate mean  $\pm$  s.e.m. Statistical significance is indicated by \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ ; NS, not significant.

neutralizing Abs targeting IL-2, IL-4, TGF $\beta$ , IL-5, IL-13, IL-1 $\beta$ , IL-6, IL-12/23p40, IL-21, and IL-25. Among these known IL-9-inducing cytokines tested, only neutralization of IL-2 and IL-4 significantly reduced the production of IL-9 induced by IL-36 $\gamma$  (**Figure 5a**). Although neutralization of TGF $\beta$  and IL-5 modestly reduced the induction of IL-9, the reduction was not statistically significant. These data indicate that IL-9 induction mediated by IL-36 $\gamma$  was largely IL-2 and IL-4 dependent and are consistent with the ability of IL-36 $\gamma$  to induce both IL-2 and IL-4 production from responding CD4<sup>+</sup> T cells (**Figure 3a–c**).

As IL-2 signaling activates STAT5, which is involved in the development of Th9 cells,<sup>26</sup> we next examined the phosphorylation of STAT5 (pSTAT5) in T/DC co-cultures in response to IL-2 (as a positive control) or IL-36 $\gamma$ . As shown in **Figure 5b,c**, IL-36 $\gamma$  induced pSTAT5 by day 1 to the same level as IL-2. At day 3, IL-2 continued to increase pSTAT5<sup>+</sup> cells as did IL-36 $\gamma$ , albeit to a far lesser extent. We further examined whether IL-36 $\gamma$ -induced STAT5 activation was mediated via enhanced IL-2 production by performing T/DC co-cultures in the presence or absence of anti-IL-2-neutralizing Abs. Indeed, the phosphorylation of STAT5 mediated by IL-36 $\gamma$  was completely abrogated in the presence of anti-IL-2-neutralizing Abs (**Figure 5d**). Moreover, we performed T/DC co-cultures in the presence of the STAT5 selective inhibitor (STAT5i), CAS 285986-31-4. Pharmacological inhibition of STAT5 also significantly abrogated IL-9

production in a dose-dependent manner, suggesting that STAT5 is indeed required for IL-36 $\gamma$ -induced IL-9 production (**Figure 5e**).

Next we examined the phosphorylation of STAT6 (pSTAT6), which is required for mediating responses to IL-4 and also involved in Th9 cell development.<sup>26</sup> As shown in **Figure 5f,g**, IL-4 (positive control) significantly induced pSTAT6 within 45 min as compared with IL-36 $\gamma$ ; however, IL-36 $\gamma$  induced pSTAT6 gradually by day 1 and more so at day 3, which is in contrast to the rapid pSTAT5 activation in the presence of IL-36 $\gamma$  (**Figure 5b,c**). To further assess the requirement for STAT6 in IL-36 $\gamma$ -induced IL-9 expression, we performed T/DC co-cultures in the presence of the STAT6 selective inhibitor (STAT6i), AS1517499. Pharmacological inhibition of STAT6 significantly reduced IL-9 production in a dose-dependent manner, demonstrating that STAT6 is also involved in IL-36 $\gamma$ -induced IL-9 production (**Figure 5h**). Similarly, STAT6 inhibition abrogated IL-36 $\gamma$ -induced IL-4 production (**Figure 5h**). To further confirm the requirements for IL-4 and STAT6 in regulating IL-36 $\gamma$ -induced IL-9 production, we isolated CD4<sup>+</sup> T cells and DCs from either IL-4-deficient (*Il4*<sup>-/-</sup>) or STAT6-deficient (*Stat6*<sup>-/-</sup>) mice and performed T/DC co-cultures. Deficiency of either IL-4 or STAT6 significantly impaired IL-9 production induced by IL-36 $\gamma$  (**Figure 5i**). Consistent with these data, IL-36 $\gamma$ -induced pSTAT6 was also significantly reduced in *Il4*<sup>-/-</sup> and *Stat6*<sup>-/-</sup>



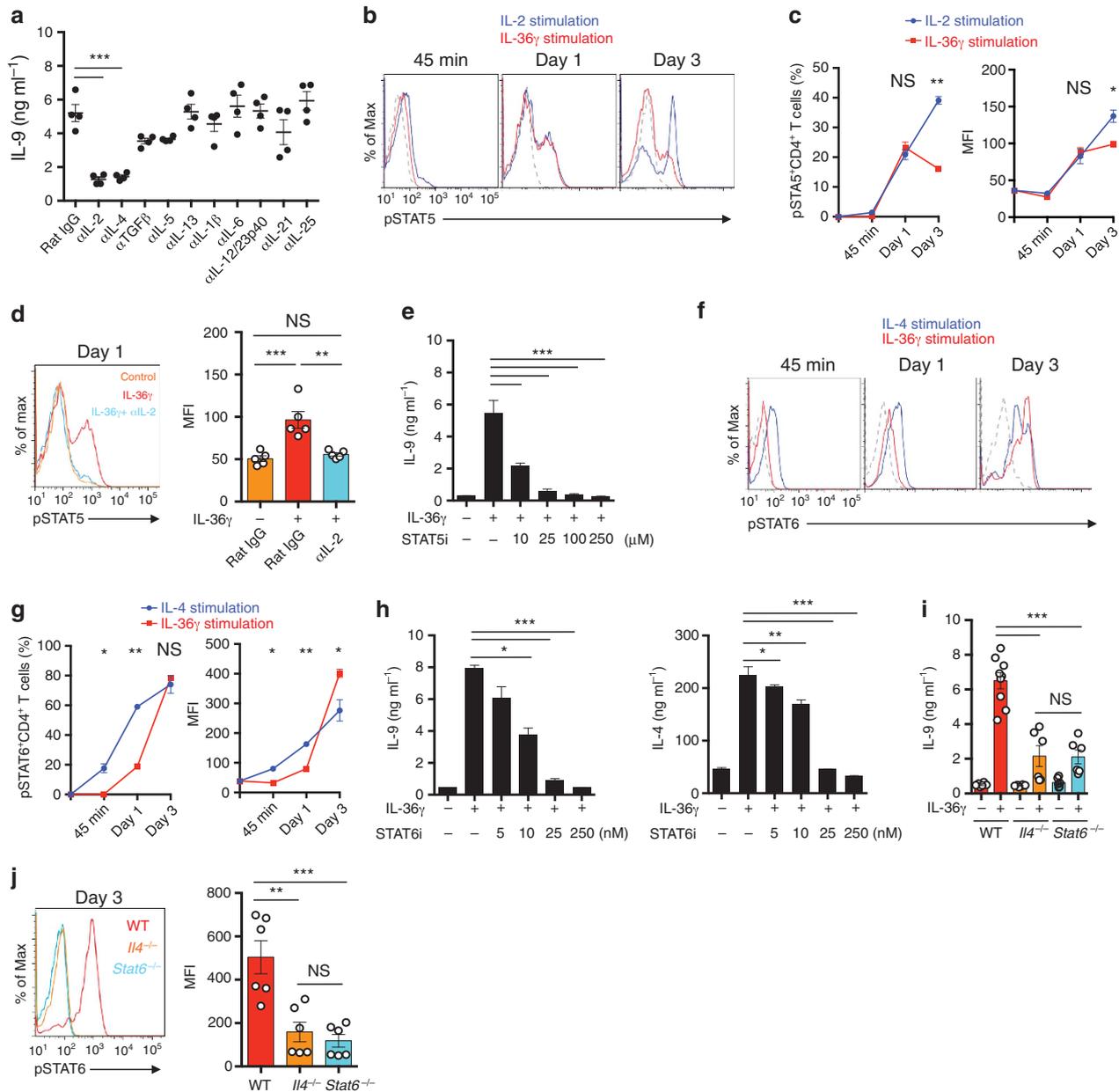
**Figure 4** Interleukin (IL)-36 $\gamma$  promotes IL-9 expression in a myeloid differentiation primary response gene 88 (MyD88)- and nuclear factor- $\kappa$ Bp50 (NF $\kappa$ Bp50)-dependent manner. **(a–c)** Gene expression profiles induced by IL-36 $\gamma$  were analyzed by PCR array analyses. RNA was isolated from CD4<sup>+</sup> T cells co-cultured with dendritic cells (DCs) for 24 h in the presence or absence of IL-36 $\gamma$ . Change in gene expression (log<sub>2</sub>) under induced regulatory T cell (iT<sub>reg</sub>) conditions (–5.0 to 5.0) and neutral conditions (–6.4 to 6.4) expressed as a heatmap **(a)**. Bar graphs show top differentially upregulated transcripts in IL-36 $\gamma$ -treated cells vs. non-treated cells under iT<sub>reg</sub> conditions **(b)** and neutral conditions **(c)**. **(d)** FACS (fluorescence-activated cell sorter)-sorted naive CD4<sup>+</sup> T cells were co-cultured with DCs for the indicated periods under iT<sub>reg</sub> conditions or neutral conditions in the presence or absence of IL-36 $\gamma$ . IL-9 mRNA expression was assessed by quantitative real-time PCR with three replicates. **(e)** FACS-sorted naive CD4<sup>+</sup> T cells and DCs were co-cultured for 4 days under iT<sub>reg</sub> conditions or neutral conditions in the presence of the indicated IL-1 family members. IL-9 protein in the supernatant was assessed by enzyme-linked immunosorbent assay (ELISA). **(f)** FACS-sorted naive CD4<sup>+</sup> T cells and DCs were co-cultured using the indicated cells from wild-type (WT; IL-36R<sup>+/+</sup>) and/or *Il1rl2*<sup>-/-</sup> (IL-36R<sup>-/-</sup>) in the presence of IL-36 $\gamma$ . IL-9 protein in the supernatant was assessed by ELISA. **(g)** Similarly, FACS-sorted naive CD4<sup>+</sup> T cells from indicated mouse strains and WT DCs were co-cultured for 4 days in the presence of IL-36 $\gamma$  and IL-9 protein in the supernatant was assessed by ELISA. Data are representative of two independent PCR array experiments for each condition **(a–c)** or from four **(e, f)** and two **(d, g)** independent experiments. One-way analysis of variance and Tukey's Multiple Comparison Test was used to determine significance. Error bars indicate mean  $\pm$  s.e.m. \*\*\* $P$ <0.001, \*\* $P$ <0.01, \* $P$ <0.05; NS, not significant.

CD4<sup>+</sup> T cells (**Figure 5**). Taken together, these data suggest that IL-36 $\gamma$  induces IL-2-STAT5 signaling followed by IL-4-STAT6 signaling to drive IL-9 expression in CD4<sup>+</sup> T cells.

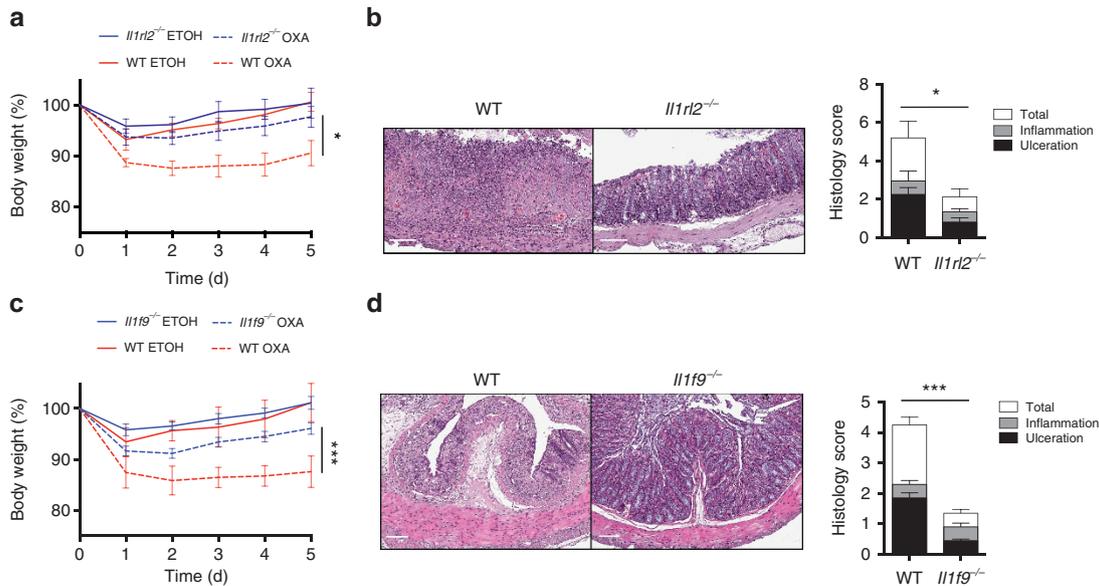
#### Deficiency of IL-36 $\gamma$ or IL-36R *in vivo* ameliorates Th cell-driven colitis

As our findings demonstrated that IL-36R signaling strongly induced Th9 cell differentiation *in vitro*, we explored the role of the IL-36/IL-36R axis in a Th2/9 cell-dependent model of colitis induced by the hapten oxazolone. Oxazolone-induced colitis is a Th2 model of colitis resembling ulcerative colitis (UC) in humans,<sup>27</sup> and more recently, Th9 cells have been

shown to have a central role in disease pathogenesis in this colitis model<sup>28</sup> as well as in UC.<sup>29</sup> We first used mice deficient in IL-36R (*Il1rl2*<sup>-/-</sup>) to examine the contribution of IL-36R signaling in driving colonic inflammation in this model. In response to oxazolone treatment, *Il1rl2*<sup>-/-</sup> mice exhibited significantly reduced weight loss and colonic inflammation when compared with WT control mice (**Figure 6a,b**), although we did not observe differences in survival rate (see **Supplementary Figure S9a**). As IL-36 $\gamma$  mRNA expression was significantly higher than that of IL-36 $\alpha$  and IL-36 $\beta$  in total colonic tissue of oxazolone-treated mice (see **Supplementary Figure S10**), we next examined mice deficient in IL-36 $\gamma$



**Figure 5** Interleukin (IL)-36 $\gamma$  induces T helper 9 (Th9) differentiation via IL-2-STAT5 (signal transducer and activator of transcription factor 5) and IL-4-STAT6 signaling. (a) FACS (fluorescence-activated cell sorter)-sorted naive CD4<sup>+</sup> T cells and dendritic cells (DCs) were co-cultured for 4 days under neutral conditions in the presence of IL-36 $\gamma$  supplemented by indicated neutralizing antibodies (Abs) for specific cytokines and IL-9 protein in the supernatant was assessed by enzyme-linked immunosorbent assay (ELISA). (b, c) Flow cytometric analysis for phospho-STAT5 (pSTAT5) in CD4<sup>+</sup> T cells in the presence of IL-2 or IL-36 $\gamma$  (100 ng ml<sup>-1</sup>). FACS-sorted naive CD4<sup>+</sup> T cells and DCs were co-cultured for the indicated times in the presence of IL-2 or IL-36 $\gamma$  and pSTAT5 expression in CD4<sup>+</sup> T cells was analyzed. Representative histograms (b), the frequencies of pSTAT5<sup>+</sup> cells and mean fluorescent intensity (MFI) for pSTAT5 (c) are shown. (d) FACS-sorted naive CD4<sup>+</sup> T cells and DCs from wild-type (WT) mice were co-cultured for 1 day in the presence of IL-36 $\gamma$  and/or anti-IL-2 neutralizing Abs and pSTAT5 expression in CD4<sup>+</sup> T cells was analyzed. Representative histograms and MFI for pSTAT5 among CD4<sup>+</sup> T cells are shown. (e) FACS-sorted naive CD4<sup>+</sup> T cells and DCs were co-cultured for 4 days in the presence of the indicated concentration of a STAT5 selective inhibitor. IL-9 protein quantification in the supernatant of T/DC co-cultures was assessed by ELISA. (f, g) Flow cytometric analysis for phospho-STAT6 (pSTAT6) in CD4<sup>+</sup> T cells in the presence of IL-4 or IL-36 $\gamma$  (100 ng ml<sup>-1</sup>). FACS-sorted naive CD4<sup>+</sup> T cells and DCs were co-cultured for the indicated times in the presence of IL-4 or IL-36 $\gamma$  and pSTAT6 expression in CD4<sup>+</sup> T cells was analyzed. Representative histograms (f), the frequencies of pSTAT6<sup>+</sup> cells and MFI for pSTAT6 (g) are shown. (h) FACS-sorted naive CD4<sup>+</sup> T cells and DCs were co-cultured for 4 days in the presence of the indicated concentration of a STAT6 selective inhibitor. IL-9 and IL-4 protein quantification in the supernatant of T/DC co-cultures was assessed by ELISA. (i) FACS-sorted naive CD4<sup>+</sup> T cells and DCs obtained from the indicated mouse strains were co-cultured for 4 days in the presence of IL-36 $\gamma$  and IL-9 protein in the supernatant was assessed by ELISA. (j) FACS-sorted naive CD4<sup>+</sup> T cells and DCs from the indicated mouse strains were co-cultured for 3 days in the presence of IL-36 $\gamma$  and pSTAT6 expression in CD4<sup>+</sup> T cells was analyzed. Representative histograms and MFI for pSTAT6 among CD4<sup>+</sup> T cells are shown. Data are representative of four (a), three (f, g) and two (b–e, h–j) independent experiments with three replicates unless specified. Student's *t*-test or one-way analysis of variance and Tukey's Multiple Comparison Test was used to determine significance. Error bars indicate mean  $\pm$  s.e.m. \**P* < 0.05, \*\**P* < 0.01, \*\*\**P* < 0.001; NS, not significant.



**Figure 6** Deficiency of interleukin (IL)-36 $\gamma$  or IL-36R *in vivo* ameliorates oxazolone-induced colitis. (a, b) Body weight of wild-type (WT) and *Il1r2*<sup>-/-</sup> mice during 5 days after treatment with oxazolone ( $n=10$  for the oxazolone-treated group,  $n=5$  for the ETOH-treated group) (a), and representative hematoxylin/eosin-stained colon sections and histology scores of the colon sections (b) are shown. Bars, 100  $\mu\text{m}$ . (c, d) Body weight of WT and *Il1f9*<sup>-/-</sup> mice during 5 days after treatment with oxazolone ( $n=10$  for the oxazolone-treated group,  $n=5$  for the ETOH-treated group) (c), and representative hematoxylin/eosin-stained colon sections and histology scores of the colon sections (d) are shown. Bars, 100  $\mu\text{m}$ . Data are cumulative of four independent experiments. Student's *t*-test or one-way analysis of variance and Tukey's Multiple Comparison Test was used to determine significance. Error bars indicate mean  $\pm$  s.e.m. \* $P<0.05$ , \*\* $P<0.01$ , \*\*\* $P<0.001$ ; NS, not significant.

(*Il1f9*<sup>-/-</sup>) to confirm the contribution of this specific cytokine in regulating colonic inflammation. Similar to *Il1r2*<sup>-/-</sup> mice, *Il1f9*<sup>-/-</sup> mice exhibited significantly reduced weight loss and colonic inflammation when compared with WT control mice (Figure 6c,d) but with no differences in survival rate as well (see Supplementary Figure S9b). Of note, we also tested the contribution of IL-36R receptor signaling in the CD4<sup>+</sup>CD45RB<sup>hi</sup> T-cell transfer model of colitis. Using this model, *Rag1*<sup>-/-</sup> mice transferred with IL-36R-deficient CD45RB<sup>hi</sup> cells exhibited modestly but significantly reduced weight loss and colonic inflammation when compared with *Rag1*<sup>-/-</sup> mice transferred with IL-36R-sufficient CD45RB<sup>hi</sup> cells (see Supplementary Figure S11a,b). Thus these results indicate that the IL-36 $\gamma$ /IL-36R axis has a key role in driving Th cell-dependent colonic inflammation, particularly in the Th2/9 oxazolone model.

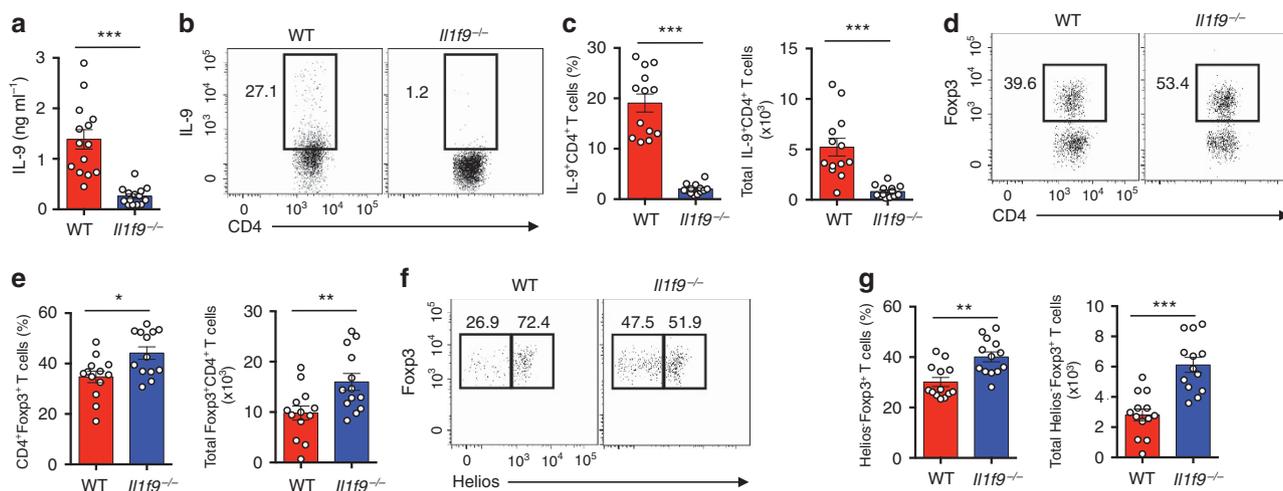
### IL-36 $\gamma$ controls the T<sub>reg</sub>-Th9 cell balance *in vivo*

Next we further investigated IL-36 $\gamma$ -mediated regulation of Th differentiation *in vivo* during colitis. Following treatment with oxazolone, *Il1f9*<sup>-/-</sup> mice displayed significantly reduced IL-9 production by colonic lamina propria lymphocytes (Figure 7a), as well as reduced IL-9-producing CD4<sup>+</sup> T-cell frequency and absolute cell number (Figure 7b,c), when compared with WT mice. In addition, the frequency and absolute number of Foxp3<sup>+</sup>CD4<sup>+</sup> T cells was significantly increased in *Il1f9*<sup>-/-</sup> colonic tissue (Figure 7d,e). Further, both the frequency and absolute number of Helios<sup>-</sup>Foxp3<sup>+</sup>CD4<sup>+</sup> T cells were significantly increased in *Il1f9*<sup>-/-</sup> colonic tissue (Figure 7f,g). We also confirmed that *Il1r2*<sup>-/-</sup> mice exhibit reduced IL-9 production as

well as increased Helios<sup>-</sup>Foxp3<sup>+</sup>CD4<sup>+</sup> T cells in this model (see Supplementary Figure S12a,b). Although the frequency of Helios<sup>-</sup>Foxp3<sup>+</sup>CD4<sup>+</sup> T cell was modestly higher in *Il1f9*<sup>-/-</sup> colonic tissue at steady state when compared with WT colonic tissue, it was not statistically significant (Supplementary Figure S13a,b). Thus diminished Th9 cells and enhanced Helios<sup>-</sup>Foxp3<sup>+</sup> T<sub>reg</sub> cells observed in *Il1f9*<sup>-/-</sup> mice and *Il1r2*<sup>-/-</sup> mice in the oxazolone model of colitis were consistent with the ability of IL-36 $\gamma$  and IL-36R to control the iT<sub>reg</sub>-Th9 balance *in vitro*. As many cell types have been shown to express IL-36R and respond to IL-36 ligands, it is possible that non-T cells expressing IL-36R may also mediate effects in the oxazolone model of colitis, and future studies employing cell-lineage-specific deletion of IL-36R are warranted to address the relative contribution of various cell types *in vivo*. Finally, we investigated the correlation of human IL-9 and IL-36 cytokines in data sets generated from UC and Crohn's disease (CD) patient samples. Within two different data sets, there were significant correlations between human IL-9 and IL-36 $\alpha$  or IL-36 $\beta$  in UC, and a positive correlation was also observed between IL-9 and IL-36 $\gamma$ , although it did not reach statistical significance. Notably, these correlations between human IL-9 and IL-36 cytokines were not observed in CD (see Supplementary Figure S14). Altogether, the IL-36/IL-36R pathway appears to have a major role in regulating the Th cell balance *in vivo* during Th9-mediated intestinal inflammation in mice and correlates with IL-9 expression in human UC.

### DISCUSSION

In the present study, we provide evidence that signaling through IL-36R dramatically inhibited iT<sub>reg</sub> differentiation



**Figure 7** Interleukin (IL)-36 $\gamma$  controls the regulatory T cell–T helper 9 cell balance *in vivo*. (a) Two days after rectal challenge with oxazolone, colonic lamina propria (LP) cells were isolated from wild-type (WT) and *Il1f9*<sup>-/-</sup> mice. Isolated colonic LP cells were cultured *ex vivo* for 36 h and IL-9 protein quantification in the supernatant was assessed by enzyme-linked immunosorbent assay. (b, c) IL-9 expression among CD4<sup>+</sup> T cells isolated from the colons of oxazolone-treated WT and *Il1f9*<sup>-/-</sup> mice was analyzed by flow cytometry. Representative dot plots (b), the frequencies of IL-9<sup>+</sup> CD4<sup>+</sup> T cells among total CD4<sup>+</sup> T cells and total numbers of IL-9<sup>+</sup> CD4<sup>+</sup> T cells in WT and *Il1f9*<sup>-/-</sup> colons (c) are shown. (d, e) Foxp3 expression among CD4<sup>+</sup> T cells from WT and *Il1f9*<sup>-/-</sup> colons was analyzed by flow cytometry. Representative dot plots are shown in (d). Frequencies among CD4<sup>+</sup> T cell and total number of Foxp3<sup>+</sup> CD4<sup>+</sup> T cells from WT and *Il1f9*<sup>-/-</sup> colons are shown in (e). (f, g) Helios expression among Foxp3<sup>+</sup> CD4<sup>+</sup> T cells from WT and *Il1f9*<sup>-/-</sup> colons was analyzed by flow cytometry. Representative dot plots are shown in (f). Frequencies of Helios<sup>-</sup> cells among Foxp3<sup>+</sup> CD4<sup>+</sup> T cells and total number of Helios<sup>-</sup> Foxp3<sup>+</sup> CD4<sup>+</sup> T cells from WT and *Il1f9*<sup>-/-</sup> colons are shown in (g). Data are cumulative of four independent experiments. Student's *t*-test was used to determine significance. Error bars indicate mean  $\pm$  s.e.m. \**P*<0.05, \*\**P*<0.01, \*\*\**P*<0.001; NS, not significant.

while redirecting toward IL-9-producing T<sub>eff</sub> cells via a pathway involving MyD88 and NF $\kappa$ Bp50 in CD4<sup>+</sup> T cells. IL-36R signaling potently induced STAT5 phosphorylation via IL-2 signaling and STAT6 phosphorylation via IL-4 signaling and both pathways were required for maximal IL-36 $\gamma$ -induced Th9 differentiation. Importantly, the role of IL-36R signaling in controlling the iT<sub>reg</sub>-Th9 balance was further confirmed *in vivo* using the oxazolone model of colitis. In this model, mice deficient in IL-36 $\gamma$ -IL-36R signaling exhibited increased iT<sub>reg</sub> and diminished Th9 cells and significantly ameliorated colonic inflammation. Overall, these data highlight a fundamental contribution of the IL-36/IL-36R axis in the regulation of Th cell differentiation and intestinal inflammation in mice.

The contribution of the IL-36/IL-36R axis to regulating regulatory and effector T cells extends beyond the intestine to other mucosal surfaces. The IL-36/IL-36R axis has been shown to have a pro-inflammatory role at barrier surfaces, including the skin and lungs. Pioneering studies linked missense mutations in *IL36RN*, a gene encoding IL-36 receptor antagonist (IL-36RA), to a rare and life-threatening form of skin inflammation in humans termed generalized pustular psoriasis.<sup>30</sup> These findings were further supported by evidence for increased expression of IL-36 $\alpha$  and IL-36 $\gamma$  in skin psoriatic lesions in mice and humans and the fact that transgenic mice overexpressing IL-36 $\alpha$  in keratinocytes develop skin inflammation.<sup>31</sup> IL-36 cytokines can also be expressed by bronchial epithelial cells in response pro-inflammatory cytokines such as TNF, IL-1 $\beta$ , and IL-17, as well as in response to microbial challenge.<sup>32</sup> Additionally, direct administration of IL-36 $\alpha$  or IL-36 $\gamma$  in the lungs of mice was sufficient to induce neutrophil

recruitment, inflammatory cell influx, enhanced mucus production, and lung resistance.<sup>32</sup> In the intestine, IL-36 ligands were induced following dextran sodium sulfate-induced intestinal damage in response to stimulation by the microbiota and IL-36R has a fundamental role in the damage and repair phases of this T-cell-independent model of colitis.<sup>18,33,34</sup> Consistent with these findings, IL-36 $\alpha$  as well as IL-36 $\gamma$  have been shown to be increased in human inflammatory bowel disease (IBD), particularly, UC; however, the function of IL-36 ligands and IL-36R during human IBD remains obscure.<sup>34,35</sup> It is important to note that beyond expression, IL-36 ligands must further undergo proteolytic cleavage at the N-terminus to acquired optimal biological activity.<sup>36,37</sup> Recent evidence suggests that the neutrophil-granule-derived proteases cathepsin G, elastase, and proteinase-3 are responsible for cleavage of IL-36 ligands, which results in a dramatic increase in biological activity.<sup>38</sup> Following cleavage and activation, IL-36 ligands then mediate their biological effects by binding to IL-36R which, similar to IL-36 ligands, is expressed by numerous cell types, including DCs, T cells, keratinocytes, and epithelial cells.<sup>18,31,32</sup>

Interestingly, we found that expression of IL-36R on CD4<sup>+</sup> T cells was involved in the effects of IL-36 ligands in regulating iT<sub>reg</sub> and Th9 cell differentiation. Another recent report defined an important contribution of IL-1R signaling directly in CD4<sup>+</sup> T cells in the control of the Th17–iT<sub>reg</sub> cell balance in the presence of retinoic acid.<sup>14</sup> This study is consistent with previous reports indicating that T-cell-specific IL-1-MyD88 signaling is required for the induction for Th17 cell differentiation.<sup>39,40</sup> Further, the IL-1 family member, IL-33, can directly augment colonic T<sub>reg</sub> function by binding to the IL-33

receptor, ST2.<sup>15</sup> Overall, these data highlight IL-1 family members as central mediators in the control of adaptive immune responses via direct action on Th cells and underscores the unique and non-redundant functions of IL-1, IL-33, and IL-36 ligands in this process.

In the presence of TGF $\beta$ , it is known that cytokines such as IL-6 and IL-4 can direct naive Th cells to Th17 and Th9 lineages, respectively, by blocking the generation of iT<sub>reg</sub> cells.<sup>6,7,10,11</sup> Therefore, it is intriguing that IL-36-mediated inhibition of iT<sub>reg</sub> differentiation occurred independent of these cytokines. Additionally, we did not observe any inhibition of the TGF $\beta$ -signaling pathway by IL-36 $\gamma$ . These data suggest that signaling through IL-36R may be capable of directly inhibiting the Foxp3 transcription machinery. Although the mechanism of how NF $\kappa$ B signaling controls the accessibility to Foxp3 locus during iT<sub>reg</sub> cell development remains unclear,<sup>41</sup> it is notable that a recent report provided evidence that the GITR costimulatory molecule was a potent inhibitor of iT<sub>reg</sub> differentiation and an inducer of Th9 cells through NF $\kappa$ Bp50 activation, leading to recruitment of histone deacetylases at the Foxp3 locus and a “closed” chromatin structure.<sup>19</sup> In the present study, we demonstrated that NF $\kappa$ Bp50, but not the GITR/GITR ligand axis, is instrumental in contributing to diminished iT<sub>reg</sub> cell development and enhanced Th9 differentiation mediated by IL-36 $\gamma$ , indicating that signaling through IL-36R and GITR may regulate iT<sub>reg</sub>-Th9 cell balance via unique, albeit partially, overlapping mechanisms. Of note, in addition to inhibiting iT<sub>reg</sub> differentiation, it remains to be elucidated whether IL-36 receptor signaling also alters the function of thymically derived nT<sub>reg</sub> cells or not.

Although the contribution of IL-2, IL-4, and TGF $\beta$  to Th9 differentiation is well established,<sup>9–11</sup> the endogenous inducers of Th9 cells have not been fully elucidated.<sup>26</sup> Previous reports have demonstrated that IL-36 cytokines can induce Th1 responses and further augment IL-9 expression by polarized Th9 cells, as well as suppress Th17 responses.<sup>16,34</sup> However, the effect of IL-36 cytokines on naive CD4<sup>+</sup> T cells to modulate *de novo* differentiation into the iT<sub>reg</sub> or Th9 cell lineages has not been previously reported. By employing *ex vivo* cell culture system using FACS (fluorescence activated cell sorting)-sorted CD4<sup>+</sup> T cells and DCs, here we identified a link between IL-36R signaling, activation of IL-2-STAT5 and IL-4-STAT6 pathways, and IL-9 production. We showed that IL-36 $\gamma$  induces endogenous IL-2 and IL-4 in the T/DC co-culture and that STAT5 and STAT6 phosphorylation is dependent upon each cytokine production, respectively, although we cannot exclude that the effects of blocking IL-2-STAT5 may be due to downstream events. Notably, IL-36 $\gamma$ -induced IL-9 production appears to be due to neither IL-25 nor IL-1 $\beta$ , which has been shown to drive IL-2-IL-4-independent Th9 responses.<sup>23,42</sup> Additionally, by using various T-cell-polarizing conditions, we demonstrated that IL-36 $\gamma$  significantly induced IL-9 under Th0, Th2, Th9, and iT<sub>reg</sub> conditions, whereas it did not under Th1 and Th17 conditions. This is consistent with previous reports showing that IFN $\gamma$  inhibits IL-9 production by neutralizing the effect of IL-4,<sup>9</sup> and IL-6 inhibits IL-9

production by regulating STAT5 activation via STAT3.<sup>43</sup> Overall, these data support the notion that the IL-36/IL-36R axis induces Th9 cells via both IL-2-STAT5- and IL-4-STAT6-dependent pathways.

Accumulating evidence suggests the importance of Th9 cells in diseases, including atopic dermatitis,<sup>44,45</sup> asthma,<sup>46,47</sup> cancer,<sup>23,48</sup> and IBD.<sup>28,29</sup> Although the term IBD comprises CD and UC, both of which the etiology remain unknown, distinct immunological dysregulation are associated with each disease.<sup>49</sup> Particularly, IL-9 and IL-9R were shown to be upregulated in patients with UC.<sup>28,29</sup> During colitis, IL-9 can inhibit epithelial cell proliferation and increase intestinal permeability via IL-9R expressed in epithelial cells, suggesting that IL-9 signaling may regulate barrier function in UC.<sup>28</sup> Our data reported here propose a link between IL-36 and IL-9 during Th cell-driven intestinal inflammation resembling UC and suggest that the IL-36/IL-36R may be contributing to disease pathology in Th9-mediated inflammatory disorders. Indeed, data from human IBD samples indicated a link between IL-36 $\alpha$ , IL-36 $\beta$ , and IL-9 specifically in UC. Collectively, these findings define a novel role for the IL-36 pathway in controlling the T<sub>reg</sub>-Th9 cell balance during intestinal inflammation and provide the foundation for exploring whether manipulating this pathway may be beneficial in the treatment of IBD and other inflammatory conditions.

## METHODS

**Mice.** C57BL/6 (WT), *Myd88*<sup>-/-</sup>, *p50*<sup>-/-</sup>, *Il4*<sup>-/-</sup>, *Stat6*<sup>-/-</sup>, B6.Cg-Tg(TcraTcrb)425Cbn/J (OT-II), and *Rag1*<sup>-/-</sup> mice were obtained from Jackson Laboratories (Bar Harbor, ME) and housed in specific pathogen-free conditions. IL-36R<sup>-/-</sup> mice (*Il1rl2*<sup>-/-</sup>) mice were provided by Amgen (Thousand Oaks, CA). Sperm of IL-36 $\gamma$ <sup>-/-</sup> (*Il1f9*<sup>-/-</sup>) mice was obtained from the KOMP repository (UC Davis) and heterozygous *Il1f9*<sup>+/-</sup> founder mice were generated by the Mouse Transgenic and Gene Targeting Core facility (Atlanta, GA) at Emory University. *Il1f9*<sup>+/-</sup> mice were subsequently bred to generate *Il1f9*<sup>-/-</sup> mice. Unless otherwise stated, mice were used at 6–12 weeks of age. Experiments were carried out using age- and gender-matched groups. Animal protocols were approved by the Institutional Animal Care and Use Committee of Georgia State University.

**Flow cytometry.** Fluorescence dye-labeled Abs specific for CD3 (145-2C11), CD4 (L3T4), CD25 (PC61.5), CD45 (30F11), CD45RB (C363.16A), CD11c (N418), TCR $\beta$  (H57-597), MHC-II (M5/114.15.2), Helios (22F6), FoxP3 (NRRF-30), IL-9 (RM9A4), pSMAD2/3 (O72-670), pSTAT5 (SRBCZX), pSTAT6 (18/P-Stat6), PU.1 (9G7), and IRF4 (3E4) were purchased from Becton Dickinson (BD, Franklin Lakes, NJ), eBioscience (San Diego, CA), Biolegend (San Diego, CA) and Cell Signaling Technology (Danvers, MA). Fc block (2.4G2) was purchased from BD. Dead cells were identified using the fixable Aqua Dead Cell Staining Kit (Thermo Fisher Scientific, Waltham, MA). Intracellular staining for Helios, IRF4, and Foxp3 was performed using a Foxp3 staining buffer set (eBioscience). Intracellular staining of IL-9 was performed after restimulation of cells with phorbol-12-myristate 13-acetate (Sigma, St Louis, MO), ionomycin (Sigma), and brefeldin A (eBioscience) for 4 h. Stimulated cells were fixed and permeabilized and then stained with Abs specific for IL-9. Detection of pSMAD2/3 was performed according to the BD Phosflow protocol. For intracellular detection of pSTAT5, pSTAT6, and PU.1, cells were fixed by 1.6% paraformaldehyde and incubated for 10 min at room temperature. Cells were then permeabilized by ice-cold methanol and stored at -80 °C before staining. Multiparameter analysis was

performed on a Fortessa (BD) and analyzed with the FlowJo software (Tree Star, Ashland, OR). Cell sorting was performed using a SH800Z cell sorter (SONY, Tokyo, Japan).

**T/DC co-culture.** Naive CD4<sup>+</sup> T cells (CD4<sup>+</sup>CD25<sup>-</sup>) were purified from spleens by magnetic selection (Miltenyi Biotec, Bergisch Gladbach, Germany) and subsequently sorted by FACS and cultured for 4 days in the presence of FACS-sorted CD45<sup>+</sup>MHCII<sup>+</sup>CD11c<sup>+</sup> DCs at a 10:1 T/DC cell ratio (DCs were not irradiated unless specified). All cultures contained purified anti-CD3ε (2 μg ml<sup>-1</sup>; 145-2C11; eBioscience). For iT<sub>reg</sub> cell induction, the cultures contained human TGFβ (5 ng ml<sup>-1</sup>) (Peprotech, Rocky Hill, NJ) and human IL-2 (20 ng ml<sup>-1</sup>) (Peprotech). The following cytokines were used in the indicated experiments (100 ng ml<sup>-1</sup>): murine IL-1β (Peprotech), murine IL-18 (R&D, Minneapolis, MN), murine IL-33 (Peprotech), murine IL-36α, murine IL-36β and murine IL-36γ (R&D). Neutralizing Abs specific for IL-1β (B122), IL-2 (JES6-1A12), IL-4 (11B11), IL-5 (TRFK5), IL-6 (MP5-20F3), IL-9 (D9302C12), IL-12/23p40 (C17.8), IL-13 (ebio1316H), IL-21 (FFA21), IL-22 (IL22JOP), IL-25 (35B), TGFβ-1,2,3 (1D11), and IFNγ (XMG1.2) purchased from eBioscience, Biolegend, and R&D were used (10 μg ml<sup>-1</sup>). In the indicated experiments, the STAT5 inhibitor (CAS 285986-31-4; EMD Millipore, Billerica, MA) and STAT6 inhibitor (AS 1517499; Axon Medchem, Reston, VA) was used.

**CD4<sup>+</sup> T-cell differentiation.** FACS-sorted CD4<sup>+</sup>CD25<sup>-</sup> T cells purified from the spleen were activated with plate-bound anti-CD3ε (1 μg ml<sup>-1</sup>) and anti-CD28 (10 μg ml<sup>-1</sup>; 37.51; BD) and cultured for 3 days under Th9 conditions (murine IL-4: 100 ng ml<sup>-1</sup>; human TGFβ: 5 ng ml<sup>-1</sup>; and anti-IFNγ: 10 μg ml<sup>-1</sup>), Th2 conditions (murine IL-4; and anti-IFNγ), Th1 conditions (murine IL-12: 1 ng ml<sup>-1</sup>), Th17 conditions (human TGFβ: 1 ng ml<sup>-1</sup>; murine IL-6: 50 ng ml<sup>-1</sup>; anti-IFNγ; and anti-IL-4: 10 μg ml<sup>-1</sup>), and iT<sub>reg</sub> conditions (human TGFβ: 5 ng ml<sup>-1</sup>). Recombinant proteins were purchased from Peprotech and R&D. In some experiments, Ovalbumin 323–339 (Sigma) was used.

**Gene expression analysis by RT<sup>2</sup> profiler PCR array system.** RT<sup>2</sup> profiler PCR array system (Qiagen, Hilden, Germany) was used to assess gene expression levels of Th cell immune responses induced by IL-36γ. Briefly, FACS-sorted naive CD4<sup>+</sup>CD25<sup>-</sup> T cells were co-cultured in the presence of FACS-sorted CD45<sup>+</sup>MHCII<sup>+</sup>CD11c<sup>+</sup> DCs for 24 h at a 10:1 T/DC cell ratio. RNA was obtained from the cultured cells by using the RNeasy mini kit (Qiagen) and then cDNA was generated using the RT<sup>2</sup> First Strand Kit (Qiagen) followed by genomic DNA elimination. cDNA was applied for Mouse Th17 response RT<sup>2</sup> Profiler PCR Array (PAMM-073ZA; Qiagen) and then PCR amplification was performed with RT<sup>2</sup> SYBR Green qPCR Mastermix. Gene expression analysis was performed using ΔΔ C<sub>T</sub> method by the Profiler PCR Array Analysis Software (Qiagen; version 3.5). Non-detected genes (C<sub>T</sub> value >35) were excluded and gene expression was normalized to the mean of five housekeeping gene sets according to Qiagen's recommendations.

**Enzyme-linked immunosorbent assay.** Cytokine protein secretion was measured in cell-free supernatants using enzyme-linked immunosorbent assay kits for mouse IL-4 and IL-9 (eBioscience) according to the manufacturer's protocol.

**RNA isolation and qPCR.** Total RNA was isolated from murine cells and tissues using the Qiagen RNeasy Mini Kit and QIAcube with on-column DNase digestion. cDNA was generated using the Superscript First-Strand Synthesis System for reverse transcriptase-PCR and random hexamer primers (Thermo Fisher Scientific). qPCR was performed with SYBR Green on an StepOnePlus real-time PCR system (Applied Biosystems, Foster City, CA) and gene expression was normalized to *Gapdh*.

Primers used were:

*mIl1f9* (F, 5'-TTGACTTGACCAGCAGGTGTG-3';

R, 5'-GGGTACTTGCATGGGAGGATAG-3')

*mIl1f6* (F, 5'-TAGTGGGTGTAGTTCTGTAGTGTGC-3';

R, 5'-GTTCTCTCAAGAGTGTCCAGATAT-3')

*mIl1f8* (F, 5'-ACAAAAAGCCTTTCTGTTCTATCAT-3';

R, 5'-CCATGTTGGATTTACTTCTCAGACT-3')

*mIl9* (F, 5'-CATCAGTGTCTCCGTCCTCCCAACTGATG-3';

R, 5'-GATTCTGTGTGGCATTGGTCAG-3')

*mGapdh* (F, 5'-TGGCAAAGTGGAGATTGTTGCC-3';

R, 5'-AAGATGGTGATGGGCTTCCC-3')

**Western blotting analysis.** Following cytokine stimulations, proteins were extracted from CD4<sup>+</sup> T cells and separated into cytosol and nuclear fractions using NE-PER Nuclear and Cytoplasmic Extraction Reagents (Thermo Fisher Scientific) according to the manufacturer's instruction. The samples were separated on 8% or 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis gel, transferred to polyvinylidene difluoride membrane. The membrane was blocked with blocking buffer (TBS containing 0.1% Tween 20 (TBS-T) and 5% nonfat dry milk). After three washes with TBS-T, the membrane was incubated overnight with primary Abs at 1:1,000–1:4,000 dilutions in blocking buffer at 4 °C. After three washes with TBS-T, the membrane was incubated with corresponding secondary Ab at 1:4,000 dilution in blocking buffer for 1 h. After washes with TBS-T, the proteins were visualized using Amersham ECL Prime Detection Reagent (GE Healthcare, Chicago, IL). The primary Abs used were: α-Tubulin (sc-69969, Santa Cruz); NFκB p65 (sc-8008, Santa Cruz, Dallas, TX); NFκB p105/p50 (ab32360, Abcam, Cambridge, UK); SMAD3 (ab28379, Abcam); and TFIIB (sc-225, Santa Cruz). The secondary Abs used were: anti-mouse IgG (#7076, Cell Signaling Technology); anti-rabbit IgG (#7074, Cell Signaling Technology).

**Chromatin immunoprecipitation (ChIP) assay.** FACS-sorted naive CD4<sup>+</sup>CD25<sup>-</sup> T cells were co-cultured in the presence of FACS-sorted CD45<sup>+</sup>MHCII<sup>+</sup>CD11c<sup>+</sup> DCs for 4 days at a 10:1 T/DC cell ratio under iT<sub>reg</sub> conditions in the presence or absence of IL-36γ. ChIP assays were performed with the EZ-ChIP Kit (17–371, EMD Millipore) as previously described.<sup>50</sup> Briefly, chromatin DNA was obtained from the cultured cells after fixation with formaldehyde and fragmented by sonication to a mean length of 500 bp and was immunoprecipitated with control or anti-acetyl-histone H3 Ab (06-599, EMD Millipore). The precipitated DNA was subjected to qPCR using specific primers for Foxp3 locus.<sup>19</sup>

**Oxazolone colitis.** Oxazolone colitis was induced as previously described.<sup>27</sup> Briefly, in order to presensitize mice, a 2 × 2 cm<sup>2</sup> field of the abdominal skin was shaved, and 100 μl of a 3% solution of oxazolone (4-ethoxymethylene-2-phenyl-2-oxazoline-5-one; Sigma) in 100% ethanol was applied. Five days after presensitization, mice were challenged intrarectally with 100 μl of 1% oxazolone in 50% ethanol under general anesthesia with isoflurane.

**CD4<sup>+</sup>CD45RB<sup>hi</sup>-induced colitis.** FACS-sorted CD4<sup>+</sup>CD25<sup>-</sup>CD45RB<sup>hi</sup> naive T cells from WT mice or IL-36R<sup>-/-</sup> mice (5 × 10<sup>5</sup>) were injected intraperitoneally into *Rag1*<sup>-/-</sup> recipients. Colons were dissected from recipient mice 6 weeks posttransfer when clinical signs of chronic colitis were evident.

**Histology.** Colons were gently flushed clean and fixed in 10% formalin. Paraffin embedding, sectioning, hematoxylin/eosin staining, slide scanning, and assessment of histology were performed at the University of Michigan Pathology Core. The degree of inflammation (infiltration of immune cells) and epithelial damage (epithelial injury and ulceration) was graded in a blinded manner from 0 to 4 or 0 to 3, respectively.

**Microarray analysis.** Two microarray data sets were downloaded from the Gene Expression Omnibus database (<http://www.ncbi.nlm.nih.gov/geo/>), including GSE36807 and GSE16879. Expression data from GSE36807 included colonic specimens from 15 UC patients and 13 CD

patients. Microarray data from GSE16879 included samples from 24 UC patients and 37 CD patients prior to infliximab treatment. These data sets contained gene expression data derived from the Affymetrix U133\_plus2 platform (Affymetrix, Santa Clara, CA). For microarray analysis, expression and raw expression data (CEL files) were summarized and normalized using the Robust Multi-array Average algorithm from the Bioconductor library for the R statistical programming system.

**Statistical analysis.** All statistical analyses except for human microarray analysis were performed with the GraphPad Prism software, version 6.0b (Graphpad Software, La Jolla, CA). Student's *t*-test or one-way analysis of variance and Tukey's Multiple Comparison Test was used to determine significance. \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ ; NS, not significant. For human microarray data, Spearman's rank correlation coefficient analysis was performed with the IBM SPSS Statistics 19 software (Armonk, NY).

**SUPPLEMENTARY MATERIAL** is linked to the online version of the paper at <http://www.nature.com/mi>

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#### AUTHOR CONTRIBUTIONS

A.H. and T.L.D. conceived the idea for this project and designed the experiments. A.H. performed most of the experiments and analyzed the data. K.M. and S.O. performed the western blotting analysis and the microarray analysis, respectively. H.A., V.L.N., and S.W.Y. provided technical support. A.N. supervised pathological analysis. J.E.K., J.D.L., and A.T.G. provided reagents, mice and critical discussion. H.A., A.N., and A.T.G. critically read the manuscript. A.H. and T.L.D. wrote the manuscript.

#### DISCLOSURE

The authors declared no conflict of interest.

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#### REFERENCES

- Zenewicz, L.A., Antov, A. & Flavell, R.A. CD4 T-cell differentiation and inflammatory bowel disease. *Trends Mol. Med.* **15**, 199–207 (2009).
- Josefowicz, S.Z., Lu, L.F. & Rudensky, A.Y. Regulatory T cells: mechanisms of differentiation and function. *Annu. Rev. Immunol.* **30**, 531–564 (2012).
- Saito, T. *et al.* Two FOXP3CD4 T cell subpopulations distinctly control the prognosis of colorectal cancers. *Nat. Med.* **22**, 679–684 (2016).
- Kanno, Y., Vahedi, G., Hirahara, K., Singleton, K. & O'Shea, J.J. Transcriptional and epigenetic control of T helper cell specification: molecular mechanisms underlying commitment and plasticity. *Annu. Rev. Immunol.* **30**, 707–731 (2012).
- Mucida, D. *et al.* Reciprocal TH17 and regulatory T cell differentiation mediated by retinoic acid. *Science* **317**, 256–260 (2007).
- Bettelli, E. *et al.* Reciprocal developmental pathways for the generation of pathogenic effector TH17 and regulatory T cells. *Nature* **441**, 235–238 (2006).
- Mangan, P.R. *et al.* Transforming growth factor-beta induces development of the T(H)17 lineage. *Nature* **441**, 231–234 (2006).
- Veldhoen, M., Hocking, R.J., Atkins, C.J., Locksley, R.M. & Stockinger, B. TGFbeta in the context of an inflammatory cytokine milieu supports *de novo* differentiation of IL-17-producing T cells. *Immunity* **24**, 179–189 (2006).
- Schmitt, E. *et al.* IL-9 production of naive CD4+ T cells depends on IL-2, is synergistically enhanced by a combination of TGF-beta and IL-4, and is inhibited by IFN-gamma. *J. Immunol.* **153**, 3989–3996 (1994).
- Veldhoen, M. *et al.* Transforming growth factor-beta "reprograms" the differentiation of T helper 2 cells and promotes an interleukin 9-producing subset. *Nat. Immunol.* **9**, 1341–1346 (2008).
- Dardalhon, V. *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.* **9**, 1347–1355 (2008).
- Garlanda, C., Dinarello, C.A. & Mantovani, A. The interleukin-1 family: back to the future. *Immunity* **39**, 1003–1018 (2013).
- Ohne, Y. *et al.* IL-1 is a critical regulator of group 2 innate lymphoid cell function and plasticity. *Nat. Immunol.* **17**, 646–655 (2016).
- Basu, R. *et al.* IL-1 signaling modulates activation of STAT transcription factors to antagonize retinoic acid signaling and control the TH17 cell-iTreg cell balance. *Nat. Immunol.* **16**, 286–295 (2015).
- Schiering, C. *et al.* The alarmin IL-33 promotes regulatory T-cell function in the intestine. *Nature* **513**, 564–568 (2014).
- Vigne, S. *et al.* IL-36 signaling amplifies Th1 responses by enhancing proliferation and Th1 polarization of naive CD4+ T cells. *Blood* **120**, 3478–3487 (2012).
- Chen, W. *et al.* Conversion of peripheral CD4+ CD25- naive T cells to CD4+ CD25+ regulatory T cells by TGF-beta induction of transcription factor Foxp3. *J. Exp. Med.* **198**, 1875–1886 (2003).
- Medina-Contreras, O. *et al.* Cutting edge: IL-36 receptor promotes resolution of intestinal damage. *J. Immunol.* **196**, 34–38 (2016).
- Xiao, X. *et al.* GTR subverts Foxp3(+) Tregs to boost Th9 immunity through regulation of histone acetylation. *Nat. Commun.* **6**, 8266 (2015).
- Kim, I.K. *et al.* Glucocorticoid-induced tumor necrosis factor receptor-related protein co-stimulation facilitates tumor regression by inducing IL-9-producing helper T cells. *Nat. Med.* **21**, 1010–1017 (2015).
- Towne, J.E., Garka, K.E., Renshaw, B.R., Virca, G.D. & Sims, J.E. Interleukin (IL)-1F6, IL-1F8, and IL-1F9 signal through IL-1Rrp2 and IL-1RAcP to activate the pathway leading to NF-kappaB and MAPKs. *J. Biol. Chem.* **279**, 13677–13688 (2004).
- Schlenker, S.M., Weigmann, B., Ruan, Q., Chen, Y. & von Boehmer, H. Smad3 binding to the foxp3 enhancer is dispensable for the development of regulatory T cells with the exception of the gut. *J. Exp. Med.* **209**, 1529–1535 (2012).
- Vegran, F. *et al.* The transcription factor IRF1 dictates the IL-21-dependent anticancer functions of TH9 cells. *Nat. Immunol.* **15**, 758–766 (2014).
- Chang, H.C. *et al.* The transcription factor PU.1 is required for the development of IL-9-producing T cells and allergic inflammation. *Nat. Immunol.* **11**, 527–534 (2010).
- Staudt, V. *et al.* Interferon-regulatory factor 4 is essential for the developmental program of T helper 9 cells. *Immunity* **33**, 192–202 (2010).
- Kaplan, M.H., Hufford, M.M. & Olson, M.R. The development and *in vivo* function of T helper 9 cells. *Nat. Rev. Immunol.* **15**, 295–307 (2015).
- Heller, F., Fuss, I.J., Nieuwenhuis, E.E., Blumberg, R.S. & Strober, W. Oxazolone colitis, a Th2 colitis model resembling ulcerative colitis, is mediated by IL-13-producing NK-T cells. *Immunity* **17**, 629–638 (2002).
- Gerlach, K. *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.* **15**, 676–686 (2014).
- Nallewew, N. *et al.* IL-9 and its receptor are predominantly involved in the pathogenesis of UC. *Gut* **64**, 743–755 (2015).
- Marrakchi, S. *et al.* Interleukin-36-receptor antagonist deficiency and generalized pustular psoriasis. *N. Engl. J. Med.* **365**, 620–628 (2011).
- Blumberg, H. *et al.* Opposing activities of two novel members of the IL-1 ligand family regulate skin inflammation. *J. Exp. Med.* **204**, 2603–2614 (2007).
- Gabay, C. & Towne, J.E. Regulation and function of interleukin-36 cytokines in homeostasis and pathological conditions. *J. Leukoc. Biol.* **97**, 645–652 (2015).
- Scheibe, K. *et al.* IL-36R signalling activates intestinal epithelial cells and fibroblasts and promotes mucosal healing *in vivo*. *Gut pii*, gutjnl-2015-310374; doi: 10.1136/gutjnl-2015-310374 (2016). [Epub ahead of print].
- Russell, S.E. *et al.* IL-36alpha expression is elevated in ulcerative colitis and promotes colonic inflammation. *Mucosal Immunol.* **9**, 1193–1204 (2016).

35. Nishida, A. *et al.* Increased expression of interleukin-36, a member of the interleukin-1 cytokine family, in inflammatory bowel disease. *Inflamm. Bowel Dis.* **22**, 303–314 (2016).
36. Towne, J.E. *et al.* Interleukin-36 (IL-36) ligands require processing for full agonist (IL-36alpha, IL-36beta, and IL-36gamma) or antagonist (IL-36Ra) activity. *J. Biol. Chem.* **286**, 42594–42602 (2011).
37. Afonina, I.S., Muller, C., Martin, S.J. & Beyaert, R. Proteolytic processing of interleukin-1 family cytokines: variations on a common theme. *Immunity* **42**, 991–1004 (2015).
38. Henry, C.M., Sullivan, G.P., Clancy, D.M., Afonina, I.S., Kulms, D. & Martin, S.J. Neutrophil-derived proteases escalate inflammation through activation of IL-36 family cytokines. *Cell Rep.* **14**, 708–722 (2016).
39. Chung, Y. *et al.* Critical regulation of early Th17 cell differentiation by interleukin-1 signaling. *Immunity* **30**, 576–587 (2009).
40. Schenten, D. *et al.* Signaling through the adaptor molecule MyD88 in CD4+ T cells is required to overcome suppression by regulatory T cells. *Immunity* **40**, 78–90 (2014).
41. Oh, H. & Ghosh, S. NF-kappaB: roles and regulation in different CD4(+) T-cell subsets. *Immunol. Rev.* **252**, 41–51 (2013).
42. Angkasekwinai, P., Chang, S.H., Thapa, M., Watarai, H. & Dong, C. Regulation of IL-9 expression by IL-25 signaling. *Nat. Immunol.* **11**, 250–256 (2010).
43. Olson, M.R., Verdan, F.F., Hufford, M.M., Dent, A.L. & Kaplan, M.H. STAT3 impairs STAT5 activation in the development of IL-9-secreting T cells. *J. Immunol.* **196**, 3297–3304 (2016).
44. Yao, W., Tepper, R.S. & Kaplan, M.H. Predisposition to the development of IL-9-secreting T cells in atopic infants. *J. Allergy Clin. Immunol.* **128**, 1357–1360 e1355 (2011).
45. Schlapbach, C. *et al.* Human TH9 cells are skin-tropic and have autocrine and paracrine proinflammatory capacity. *Sci. Transl. Med.* **6**, 219ra218 (2014).
46. Wilhelm, C. *et al.* An IL-9 fate reporter demonstrates the induction of an innate IL-9 response in lung inflammation. *Nat. Immunol.* **12**, 1071–1077 (2011).
47. Jones, C.P., Gregory, L.G., Causton, B., Campbell, G.A. & Lloyd, C.M. Activin A and TGF-beta promote T(H)9 cell-mediated pulmonary allergic pathology. *J. Allergy Clin. Immunol.* **129**, 1000–1010 e1003 (2012).
48. Purwar, R. *et al.* Robust tumor immunity to melanoma mediated by interleukin-9-producing T cells. *Nat. Med.* **18**, 1248–1253 (2012).
49. Kaser, A., Zeissig, S. & Blumberg, R.S. Inflammatory bowel disease. *Annu. Rev. Immunol.* **28**, 573–621 (2010).
50. Tanaka, H. *et al.* Epigenetic Thpok silencing limits the time window to choose CD4(+) helper-lineage fate in the thymus. *EMBO J.* **32**, 1183–1194 (2013).