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IL-36γ signaling controls the induced regulatory T cell – T_H9 cell balance via NFκB activation and STAT transcription factors

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Abstract

Regulatory and effector T helper (T_H) 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 (IL)-1 family member IL-36γ signals through IL-36 receptor, MyD88, and NFκBp50 in $CD4^+$ T cells to potently inhibit Foxp3-expressing induced regulatory T cell (T_{reg}) development, while concomitantly promoting the differentiation of T helper 9 (T_H9) cells via a IL-2-STAT5 and IL-4-STAT6 dependent pathway. Consistent with these findings, mice deficient in IL-36γ were protected from T_H cell-driven intestinal inflammation and exhibited increased colonic T_{reg} cells and diminished T_H9 cells. Our findings thus reveal a fundamental contribution for the IL-36/IL-36R axis in regulating the T_{reg}-T_H9 cell balance with broad implications for T_H cell-mediated disorders such as inflammatory bowel diseases, and particularly ulcerative colitis.

Introduction

$CD4^+$ T helper (T_H) 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.[1–3] Regulatory T_H cells

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expressing Foxp3 (T_{reg}) can develop intrathymically or in the periphery and are potently immunosuppressive and help to maintain immunological homeostasis.[2] Effector T_H cells (T_{eff}), on the other hand, can be grouped into several general categories (T_H1, T_H2, T_H9, T_H17, T_H22, and T_{FH}) based on dominant signature cytokines produced and associated master transcription factors expressed.[4] Interestingly, specific cytokines and factors are involved in dictating differentiation of naive T_H cells into either T_{reg} or T_{eff} lineages.[5] For example, in the presence of IL-2 and TGFβ naive T_H cells differentiate into induced T_{reg} cells (iT_{reg}) while the combination of IL-6 plus TGFβ promotes T_H17 and inhibits iT_{reg} differentiation. [6–8] Alternatively, IL-4 can promote the differentiation of T_H2 cells while the addition of TGFβ can induce reprograming into T_H9 cells.[9–11] Thus, the local cytokine milieu present during T_H cell priming dramatically influences specific lineage commitment.

The interleukin-1 (IL-1) family of cytokines have recently emerged as critical regulators of adaptive immune cell function and plasticity, particularly at mucosal surfaces.[12, 13] IL-1 signaling was recently shown to be involved in overriding retinoic acid-mediated Foxp3 induction while inducing protective T_H17 responses during *Citrobacter rodentium* infection.[14] 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_{reg} cells to induce their stability and immunosuppressive function in the intestine.[15] Thus, IL-1 family members can be released in the local environment following tissue damage, or in response to infection, and potently dictate T_H cell differentiation and function that ultimately aids in resolution of inflammation and host protection. However, the role of "novel" IL-1 family members, such as IL-36, in regulating $CD4^+$ T_H cell differentiation into specific lineages remains incompletely defined.[16] In the present report, we investigated the role of the IL-36γ/IL-36R axis in controlling the balance of T_{reg} and T_{eff} lineages, with particular focus on how this pathway regulates T_H cell dependent intestinal inflammation. Our results demonstrate that signaling through IL-36R employs MyD88 and NFκBp50 in $CD4^+$ T cells to potently inhibit iT_{reg} development, while concomitantly promoting T_H9 differentiation via a IL-2-STAT5 and IL-4-STAT6 dependent pathway. Additionally, mice deficient in IL-36γ-IL-36R signaling were protected from T_H cell-dependent intestinal inflammation and exhibited increased colonic iT_{regs} and diminished T_H9 cells. Collectively, these data highlight IL-36R signaling as a regulator of the iT_{reg}-T_H9 balance *in vitro* and *in vivo* with functional implications in the regulation of intestinal inflammation.

Results

IL-36γ abrogates iT_{reg} induction via IL-36R-mediated signaling in $CD4^+$ T cells

To investigate the contribution of the IL-36/IL-36R axis in $CD4^+$ T_H cell differentiation, we first explored whether IL-36 ligands could modulate Foxp3 induction in responding T cells using a naive $CD4^+$ T cell–DC *in vitro* co-culture system in the presence of αCD3ε, TGFβ and IL-2 (iT_{reg} condition).[17] Intriguingly, compared to other IL-1 family members tested, IL-36 ligands – IL-36α, IL-36β and IL-36γ – all potently abrogated the induction of Foxp3-expressing iT_{reg} cells in a dose dependent fashion (Fig. 1a–c; Supplementary Fig. 1a). Given that all three IL-36 ligands were behaving similarly, combined with the preferential expression of IL-36γ in the mouse intestine during colitis,[18] we focused specifically on

IL-36γ and asked whether it was acting on $CD4^+$ T cells or DCs to inhibit iT_{reg} differentiation. To do so, we employed a co-culture system whereby $CD4^+$ T cells or DCs were isolated from WT or IL-36R-deficient mice. Interestingly, the expression of IL-36R by $CD4^+$ T cells, but not DCs, was essential for the iT_{reg}-inhibiting ability of IL-36γ in this assay (Fig. 1d,e). We next investigated whether IL-36γ was acting to inhibit iT_{reg} differentiation via the induction of autocrine/paracrine signaling, including IL-6 which is known to potently block *de novo* Foxp3 expression and promote T_H17 differentiation.[6, 8] Notably, inhibition of iT_{reg} cells mediated by IL-36γ was not reversible by antibody-mediated neutralization of IL-1β, IL-6, IL-12/23p40 (Fig. 2a,b), or IL-4, IL-5, IL-9, IL-13, IL-22 and IFNγ (Supplementary Fig. 2a,b), although we cannot formally confirm complete neutralization in our specific culture conditions. Since recent studies have implicated the glucocorticoid-induced tissue necrosis factor receptor related protein (GITR)/GITR ligand axis is suppressing $Foxp3^+$ iT_{reg} differentiation,[19, 20] we also examined whether this pathway could be involved in the inhibition of iT_{reg} differentiation mediated by IL-36γ. Notably, antibody-mediated blockade of GITR ligand was also unable to reverse the effects of IL-36γ on suppressing iT_{reg} differentiation (Supplementary Fig. 3a,b). Of note, the effect of IL-36γ on iT_{reg} inhibition was not affected by the irradiation of DCs (Supplementary Fig. 4). We also confirmed the ability of IL-36γ to suppress iT_{reg} cell induction in the absence of DCs by using purified naive $CD4^+$ T cells which were isolated from WT mice or OT-II mice (Supplementary Fig. 5a and b).

Overall, these findings demonstrate that IL-36γ inhibits $Foxp3^+$ iT_{reg} differentiation via IL-36R-mediated signaling in $CD4^+$ cells independent of well-defined inflammatory cytokines or GITR/GITR ligand induction.

IL-36γ-mediated suppression of iT_{reg} cells is MyD88- and NFκBp50-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,[12] we performed T/DC co-cultures whereby $CD4_+$ T cells were isolated from MyD88-sufficient (WT) or deficient (*Myd88*$^{-/-}$) mice in order to assess whether deletion of MyD88 specifically in $CD4^+$ T cells restored iT_{reg} differentiation in the presence of IL-36γ. Notably, MyD88 expression in $CD4^+$ T cells was involved in the suppression of iT_{reg} cell development mediated by IL-36γ (Fig. 2c,d). Since signaling through IL-36R can lead to NFκB activation,[21] we next explored whether NFκBp50 was involved in IL-36γ-mediated suppression of iT_{reg} differentiation. To address this question we employed a co-culture system whereby $CD4^+$ T cells were isolated from p50-sufficient (WT) or deficient (*p50*$^{-/-}$) mice. Remarkably, deficiency of NFκBp50 specifically in $CD4^+$ T cells fully restored iT_{reg} induction in the presence of IL-36γ (Fig. 2e,f). Altogether, our data indicate that IL-36γ suppresses iT_{reg} differentiation via MyD88- and NFκBp50-dependent signaling in $CD4^+$ T cells.

IL-36γ alters NFκB signaling and acetylation of the Foxp3 locus during iT_{reg} cell differentiation

We next investigated whether IL-36γ may be mediating its effects on inhibiting iT_{reg} cells by interfering with signaling downstream of TGFβ receptor, specifically SMAD3, since it is

known to bind the conserved noncoding sequence 1 (CNS1) in the Foxp3 locus and positively regulate Foxp3 expression.[22] Flow cytometric analyses revealed that IL-36γ did not inhibit TGFβ-induced phosphorylation of SMAD2/3 (Fig. 3a) or nuclear translocation of SMAD3 (Fig. 3b). Since deficiency of NFκBp50 in $CD4^+$ T cells fully restored iT_{reg} induction in the presence of IL-36γ, we next examined how IL-36γ modulates the nuclear translocation of specific NFκB family members. As shown in Fig. 3b, upon $CD4^+$ T cell activation under neutral conditions, IL-36γ promoted nuclear translocation of NFκB p65 and p50, but not p105. Under iT_{reg} conditions, p65 nuclear translocation was also observed, however, this nuclear translocation was similar +/− IL-36γ. Interestingly, NFκBp50 nuclear translocation was observed under iT_{reg} conditions and the addition of IL-36γ further augmented nuclear translocation. Together, these results suggest that IL-36γ-enhanced NFκBp50 nuclear translocation may alter the overall ratio of nuclear p50 to p65 resulting in impaired iT_{reg} cell differentiation. Since the IL-36γ - MyD88 - p50 axis could epigenetically alter the chromatin status in $CD4^+$ T cells under iT_{reg} conditions,[19] we next examined whether IL-36γ modulates histone acetylation at the Foxp3 locus, which includes the promoter, CNS1, and CNS2 regions. Indeed, we observed significant decreases in histone H3 acetylation (H3Ac) status at the Foxp3 promoter, CNS1 and CNS2 regions under iT_{reg} conditions in the presence of IL-36γ (Fig. 3c). Collectively, these data suggest that IL-36γ activates NFκBp50 in $CD4^+$ T cells and modulate histone acetylation status at the Foxp3 locus during iT_{reg} cell differentiation *in vitro*.

IL-36γ promotes T_H9 differentiation in a MyD88- and NFκBp50-dependent manner

Having defined the ability of IL-36γ to inhibit iT_{reg} cell differentiation, we next explored which T_H cell lineages IL-36γ may be favoring. To do so, we performed gene expression profiling of $CD4^+$ T_H cells stimulated with αCD3ε in the presence or absence of IL-36γ by using the T/DC co-culture system. Notably, under iT_{reg} conditions, IL-9 was identified as one of the top genes induced by IL-36γ among the analyzed gene set (Fig. 4a,b; Supplementary Fig. 6). We also performed parallel experiments but under neutral conditions (no addition of either TGFβ1 or IL-2). Similar to results observed using iT_{reg} culture conditions, IL-9 was the top gene induced by IL-36γ under neutral conditions (Fig. 4a,c; Supplementary Fig. 6). The ability of IL-36γ to potently induce IL-9 expression in a dose dependent manner was additionally confirmed by qPCR and ELISA (Fig. 4d,e; Supplementary Fig. 1b). Interestingly, the induction of IL-9 mediated by IL-36γ was more than 5-fold greater than that of IL-1β, which has been reported to induce IL-9,[23] and IL-18 and IL-33 in both iT_{reg} and neutral conditions (Fig. 4e). We also tested whether IL-9 production by $CD4^+$ T cells was controlled via IL-36R expression on T cells or DCs by performing the co-culture experiments as in Fig. 1d. Similar to the requirement for IL-36R expression on $CD4^+$ T cells in mediating iT_{reg} inhibition, the expression of IL-36R by $CD4^+$ T cells, but not DCs, was involved in the IL-9-inducing ability of IL-36γ (Fig. 4f). Additionally, we also confirmed that the irradiation of DCs does not affect the ability of IL-36γ to induce IL-9 production in this setting (Supplementary Fig. 4). Next we investigated the mechanism via which IL-36γ induced IL-9 production from $CD4^+$ T cells. Since IL-36γ abrogated iT_{reg} differentiation via a MyD88 and NF-κBp50 dependent signaling pathway, we asked whether IL-36γ-induced IL-9 production was also dependent upon MyD88 and NFκBp50. Indeed, using $CD4^+$ T cells isolated from *Myd88*$^{-/-}$ or *p50*$^{-/-}$

mice, a near complete abrogation of IL-36γ-induced IL-9 production was observed in neutral conditions as well as in iT_{reg} conditions (Fig. 4g). We further examined whether the addition of IL-36γ could modulate the expression of transcription factors known to be required for T_H9 differentiation, specifically PU.1[24] and IRF4.[25] Indeed, both PU.1 and IRF4 were significantly induced in $CD4^+$ T cells in the presence of IL-36γ as compared with the cells cultured in the absence of IL-36γ (Supplementary Fig. 7a,b). Thus, IL-36γ robustly induces IL-9 producing $CD4^+$ T cells via T cell-intrinsic MyD88- and NFκBp50-dependent signaling.

Next, we further investigated the ability of IL-36γ to induce IL-9 in the absence of DCs by using purified $CD4^+$ T cell cultures and various standard T_H cell polarizing conditions. Consistent with our data from T/DC co-cultures, IL-36γ significantly induced the differentiation of IL-9 producing cells under T_H0 conditions (Supplementary Fig. 8a,b). Remarkably, IL-36γ also significantly augmented T_H9 cell differentiation under T_H9 conditions (Supplementary Fig. 8a,c). Intriguingly, the ability of IL-36γ to potently induce IL-9 producing T cells was also observed under other T_H cell polarizing conditions including T_H2 and iT_{reg} conditions, but to a far lesser extent under T_H1 and T_H17 conditions (Supplementary Fig. 8a). Thus, these findings demonstrate that IL-36γ is a potent inducer of IL-9 production and augments T_H9 differentiation, even in the absence of DCs.

IL-36γ induces IL-9 expression via IL-2-STAT5 and IL-4-STAT6 signaling

Several factors have been reported to promote IL-9 production by $CD4^+$ T cells, including IL-1β, IL-2, IL-4, IL-21, IL-25 and TGFβ.[26] To investigate the potential involvement of these cytokines in IL-36γ-induced IL-9 production, we performed T/DC co-cultures in the presence of cytokine specific neutralizing Abs targeting IL-2, IL-4, TGFβ, IL-5, IL-13, IL-1β, 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-36γ (Fig. 5a). Although neutralization of TGFβ 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-36γ was largely IL-2 and IL-4 dependent, and are consistent with the ability of IL-36γ to induce both IL-2 and IL-4 production from responding $CD4^+$ T cells (Fig. 3a–c).

Since IL-2 signaling activates STAT5, which is involved in the development of T_H9 cells, [26] we next examined the phosphorylation of STAT5 (pSTAT5) in T/DC co-cultures in response to IL-2 (as a positive control) or IL-36γ. As shown in Fig. 5b,c, IL-36γ induced pSTAT5 by day 1 to the same level as IL-2. At day 3, IL-2 continued to increase $pSTAT5^+$ cells as did IL-36γ, albeit to a far lesser extent. We further examined whether IL-36γ-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-36γ was completely abrogated in the presence of anti-IL-2 neutralizing Abs (Fig. 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-36γ-induced IL-9 production (Fig. 5e).

Next we examined the phosphorylation of STAT6 (pSTAT6), which is required for mediating responses to IL-4 and also involved in T_H9 cell development.[26] As shown in Fig. 5f,g, IL-4 (positive control) significantly induced pSTAT6 within 45 minutes as compared to IL-36γ, however, IL-36γ 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-36γ (Fig. 5b,c). To further assess the requirement for STAT6 in IL-36γ-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 fashion, demonstrating that STAT6 is also involved in IL-36γ-induced IL-9 production (Fig. 5h). Similarly, STAT6 inhibition abrogated IL-36γ-induced IL-4 production (Fig. 5h). To further confirm the requirements for IL-4 and STAT6 in regulating IL-36γ-induced IL-9 production, we isolated $CD4^+$ T cells and DCs from either IL-4-deficient (*Il4*$^{-/-}$) or STAT6-deficient (*Stat6*$^{-/-}$) mice and performed T/DC co-cultures. Deficiency of either IL-4 or STAT6 significantly impaired IL-9 production induced by IL-36γ (Fig. 5i). Consistent with these data, IL-36γ-induced pSTAT6 was also significantly reduced in *Il4*$^{-/-}$ and *Stat6*$^{-/-}$ $CD4^+$ T cells (Fig. 5j). Taken together, these data suggest that IL-36γ induces IL-2-STAT5 signaling followed by IL-4-STAT6 signaling to drive IL-9 expression in $CD4^+$ T cells.

Deficiency of IL-36γ or IL-36R *in vivo* ameliorates T_H cell-driven colitis

As our findings demonstrated that IL-36R signaling strongly induced T_H9 cell differentiation *in vitro*, we explored the role of the IL-36/IL-36R axis in a $T_H2/9$ cell-dependent model of colitis induced by the hapten oxazolone. Oxazolone-induced colitis is a T_H2 model of colitis resembling ulcerative colitis (UC) in humans[27] and more recently, T_H9 cells have been shown to play a central role in disease pathogenesis in this colitis model[28] as well as in UC.[29] We first used mice deficient in IL-36R (*Il1rl2*$^{-/-}$) to examine the contribution of IL-36R signaling in driving colonic inflammation in this model. In response to oxazolone treatment, *Il1rl2*$^{-/-}$ mice exhibited significantly reduced weight loss and colonic inflammation when compared to WT control mice (Fig. 6a,b), although we did not observe differences in survival rate (Supplementary Fig. 9a). Since IL-36γ mRNA expression was significantly higher than that of IL-36α and IL-36β in total colonic tissue of oxazolone-treated mice (Supplementary Fig. 10), we next examined mice deficient in IL-36γ (*Il1f9*$^{-/-}$) to confirm the contribution of this specific cytokine in regulating colonic inflammation. Similar to *Il1rl2*$^{-/-}$ mice, *Il1f9*$^{-/-}$ mice exhibited significantly reduced weight loss and colonic inflammation when compared to WT control mice, (Fig. 6c,d) but with no differences in survival rate as well (Supplementary Fig. 9b). Of note, we also tested the contribution of IL-36R receptor signaling in the $CD4^+CD45RB^{hi}$ T cell transfer model of colitis. Using this model, *Rag1*$^{-/-}$ mice transferred with IL-36R-deficient $CD45RB^{hi}$ cells exhibited modestly, but significantly reduced weight loss and colonic inflammation when compared to *Rag1*$^{-/-}$ mice transferred with IL-36R-sufficient $CD45RB^{hi}$ cells (Supplementary Fig. 11a,b). Thus, these results indicate that the IL-36γ/IL-36R axis plays a key role in driving T_H cell-dependent colonic inflammation, particularly in the $T_H2/9$ oxazolone model.

IL-36γ controls the T_{reg}-T_H9 cell balance *in vivo*

Next, we further investigated IL-36γ-mediated regulation of T_H differentiation *in vivo* during colitis. Following treatment with oxazolone, *Il1f9*$^{-/-}$ mice displayed significantly reduced IL-9 production by colonic lamina propria lymphocytes (LPL) (Fig. 7a), as well as reduced IL-9 producing $CD4^+$ T cell frequency and absolute cell number (Fig. 7b,c), when compared to WT mice. In addition, the frequency and absolute number of $Foxp3^+CD4^+$ T cells was significantly increased in *Il1f9*$^{-/-}$ colonic tissue (Fig. 7d,e). Further both the frequency and absolute number of $Helios^-Foxp3^+CD4^+$ T cells were significantly increased in *Il1f9*$^{-/-}$ colonic tissue (Fig. 7f,g). We also confirmed that *Il1rl2*$^{-/-}$ mice exhibit reduced IL-9 production as well as increased $Helios^-$ $Foxp3^+CD4^+$ T cells in this model (Supplementary Fig. 12a,b). Although the frequency of $Helios^-Foxp3^+CD4^+$ T cell was modestly higher in *Il1f9*$^{-/-}$ colonic tissue at steady state when compared to WT colonic tissue, it was not statistically significant (Supplementary Fig. 13a,b). Thus, diminished T_H9 cells and enhanced $Helios^-Foxp3^+$ T_{reg} cells observed in *Il1f9*$^{-/-}$ mice and *Il1rl2*$^{-/-}$ mice in the oxazolone model of colitis were consistent with the ability of IL-36γ and IL-36R to control the iT_{reg}-T_H9 balance *in vitro*. Since 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*. Lastly, we investigated the correlation of human IL-9 and IL-36 cytokines in datasets generated from UC and Crohn's disease (CD) patient samples. Within two different datasets, there were significant correlations between human IL-9 and IL-36α or IL-36β in UC, and a positive correlation was also observed between IL-9 and IL-36γ, although it did not reach statistical significance. Notably, these correlations between human IL-9 and IL-36 cytokines were not observed in CD (Supplementary Fig. 14). Altogether, the IL-36/IL-36R pathway appears to play a major role in regulating the T_H cell balance *in vivo* during T_H9-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_{reg} differentiation while redirecting towards IL-9 producing T_{eff} cells via a pathway involving MyD88 and NFκBp50 in $CD4^+$ 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-36γ-induced T_H9 differentiation. Importantly, the role of IL-36R signaling in controlling the iT_{reg}-T_H9 balance was further confirmed *in vivo* using the oxazolone model of colitis. In this model, mice deficient in IL-36γ-IL-36R signaling exhibited increased iT_{reg} and diminished T_H9 cells and significantly ameliorated colonic inflammation. Overall, these data highlight a fundamental contribution of the IL-36/IL-36R axis in the regulation of T_H 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 play 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.[30] These findings were further supported by evidence for increased expression of IL-36α and IL-36γ in skin psoriatic lesions in mice and humans and the fact that transgenic mice overexpressing IL-36α in keratinocytes develop skin inflammation.[31] IL-36 cytokines can also be expressed by bronchial epithelial cells in response pro-inflammatory cytokines such as TNF, IL-1β, and IL-17, as well as in response to microbial challenge.[32] Additionally, direct administration of IL-36α or IL-36γ in the lungs of mice was sufficient to induce neutrophil recruitment, inflammatory cell influx, enhanced mucus production and lung resistance.[32] In the intestine, IL-36 ligands were induced following DSS-induced intestinal damage in response to stimulation by the microbiota and IL-36R plays a fundamental role in the repair of tissue damage in this T-cell independent model of colitis.[18, 33] Consistent with these findings, IL-36α as well as IL-36γ, have been shown to be increased in human IBD, particularly, ulcerative colitis (UC), however the function of IL-36 ligands and IL-36R during human IBD remains obscure.[34, 35] 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.[36, 37] 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.[38] Following cleavage and activation, IL-36 ligands then mediate their biological effects by binding to IL-36R which, like IL-36 ligands, is expressed by numerous cell types including dendritic cells, T cells, keratinocytes and epithelial cells.[18, 31, 32]

Interestingly, we found that expression of IL-36R on $CD4^+$ T cells was involved in the effects of IL-36 ligands in regulating iT_{reg} and T_H9 cell differentiation. Another recent report defined an important contribution of IL-1R signaling directly in $CD4^+$ T cells in the control of the T_H17-iT_{reg} cell balance in the presence of retinoic acid.[14] This study is consistent with previous reports indicating that T cell-specific IL-1-MyD88 signaling is required for the induction for T_H17 cell differentiation.[39, 40] Further, the IL-1 family member, IL-33, can directly augment colonic T_{reg} function by binding to the IL-33 receptor, ST2.[15] Overall, these data highlight IL-1 family members as central mediators in the control of adaptive immune responses via direct action on T_H 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 TGFβ, it is known that cytokines such as IL-6 and IL-4 can direct naive T_H cells to T_H17 and T_H9 lineages, respectively, by blocking the generation of iT_{reg} cells.[6, 7, 10, 11] Therefore, it is intriguing that IL-36-mediated inhibition of iT_{reg} differentiation occurred independent of these cytokines. Additionally, we did not observe any inhibition of the TGFβ-signaling pathway by IL-36γ. These data suggest that signaling through IL-36R may be capable of directly inhibiting the Foxp3 transcription machinery. Although the mechanism of how NFκB signaling controls the accessibility to Foxp3 locus during iT_{reg} cell development remains unclear,[41] it is notable that a recent report provided evidence that the GITR costimulatory molecule was a potent inhibitor of iT_{reg} differentiation and an inducer of T_H9 cells through NFκBp50 activation, leading to recruitment of histone

deacetylases at the Foxp3 locus and a 'closed' chromatin structure.[19] In the present study, we demonstrated that NFκBp50, but not the GITR/GITR ligand axis, is instrumental in contributing to diminished iT_{reg} cell development and enhanced T_H9 differentiation mediated by IL-36γ, indicating that signaling through IL-36R and GITR may regulate iT_{reg} – T_H9 cell balance via unique, albeit partially overlapping mechanisms. Of note, in addition to inhibiting iT_{reg} differentiation, it remains to be elucidated whether IL-36 receptor signaling also alters the function of thymically derived nT_{reg} cells or not.

Although the contribution of IL-2, IL-4, and TGFβ to T_H9 differentiation is well established.[9–11], the endogenous inducers of T_H9 cells have not been fully elucidated.[26] Previous reports have demonstrated that IL-36 cytokines can induce T_H1 responses and further augment IL-9 expression by polarized T_H9 cells, as well as suppress T_H17 responses.[16, 35] However, the effect of IL-36 cytokines on naive $CD4^+$ T cells to modulate *de novo* differentiation into the iT_{reg} or T_H9 cell lineages has not been previously reported. By employing *ex vivo* cell culture system using FACS-sorted $CD4^+$ 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-36γ 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-36γ-induced IL-9 production appears to be due to neither IL-25 nor IL-1β, which has been shown to drive IL-2-IL-4-independent T_H9 responses.[23, 42] Additionally, by using various T cell polarizing conditions, we demonstrated that IL-36γ significantly induced IL-9 under T_H0, T_H2, T_H9 and iT_{reg} conditions, whereas it did not under T_H1 and T_H17 conditions. This is consistent with previous reports showing that IFNγ inhibits IL-9 production by neutralizing the effect of IL-4,[9] and IL-6 inhibits IL-9 production by regulating STAT5 activation via STAT3.[43] Overall, these data support the notion that the IL-36/IL-36R axis induces T_H9 cells via both IL-2-STAT5 and IL-4-STAT6 dependent pathways.

Accumulating evidence suggests the importance of T_H9 cells in diseases including atopic dermatitis[44, 45], asthma[46, 47], cancer,[23, 48] and IBD.[28, 29] While the term IBD comprises CD and UC, both of which the etiology remain unknown, distinct immunological dysregulation are associated with each disease.[49] Particularly, IL-9 and IL-9R were shown to be up-regulated in patients with UC.[28, 29] 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.[28] Our data reported here propose a link between IL-36 and IL-9 during T_H cell-driven intestinal inflammation resembling UC and suggest that the IL-36/IL-36R may be contributing to disease pathology in T_H9-mediated inflammatory disorders. Indeed, data from human IBD samples indicated a link between IL-36α, IL-36β and IL-9 specifically in UC. Collectively, these findings define a novel role for the IL-36 pathway in controlling the T_{reg} –T_H9 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.

Materials and Methods

Mice

C57BL/6 (WT), *Myd88*$^{-/-}$, *p50*$^{-/-}$, *Il4*$^{-/-}$, *Stat6*$^{-/-}$, B6.Cg-Tg(TcraTcrb)425Cbn/J (OT-II) and *Rag1*$^{-/-}$ mice were obtained from the Jackson Laboratories and housed in SPF conditions. IL-36R$^{-/-}$ mice (*Il1rl2*$^{-/-}$) mice were provided by Amgen. Sperm of IL-36γ$^{-/-}$ (*Il1f9*$^{-/-}$) mice was obtained from the KOMP repository (UC Davis) and heterozygous *Il1f9*$^{+/-}$ founder mice were generated by the Mouse Transgenic and Gene Targeting Core facility at Emory University. *Il1f9*$^{+/-}$ mice were subsequently bred to generate *Il1f9*$^{-/-}$ 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 antibodies (Abs) specific for CD3 (145-2C11), CD4 (L3T4), CD25 (PC61.5), CD45 (30F11), CD45RB (C363.16A), CD11c (N418), TCRβ (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), eBioscience, Biolegend and Cell Signaling Technology. Fc block (2.4G2) was purchased from BD. Dead cells were identified using the fixable Aqua dead cell staining kit (Invitrogen). 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), ionomycin (Sigma) and brefeldin A (eBioscience) for 4 hours. 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. Multi-parameter analysis was performed on a Fortessa (BD) and analyzed with FlowJo software (Tree Star). Cell sorting was performed using a SH800Z cell sorter (SONY).

T cell and dendritic cell (T/DC) co-culture

Naive CD4$^+$ T cells (CD4$^+$CD25$^-$) were purified from spleens by magnetic selection (Miltenyi Biotec) and subsequently sorted by fluorescence activated cell sorting (FACS) and cultured for 4 days in the presence of FACS-sorted CD45$^+$MHCII$^+$CD11c$^+$ DCs at a 10:1 T/DC cell ratio (DCs were not irradiated unless specified). All cultures contained purified anti-CD3ε (2μg/ml; 145-2C11; eBioscience). For iT$_{reg}$ cell induction, the cultures contained human TGFβ (5ng/ml) (Peprotech) and human IL-2 (20ng/ml) (Peprotech). The following cytokines were used in indicated experiments (100ng/ml): murine IL-1β (Peprotech), murine IL-18 (R&D), 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 (10μg/ml). In

indicated experiments the STAT5 inhibitor (CAS 285986-31-4; EMD Millipore) and STAT6 inhibitor (AS 1517499; Axon Medchem) was used.

$CD4^+$ T cell differentiation

FACS-sorted $CD4^+CD25^-$ T cells purified from spleen were activated with plate-bound anti-CD3ε (1μg/ml) and anti-CD28 (10μg/ml; 37.51; BD) and cultured for 3 days under T_H9 conditions (murine IL-4: 100 ng/ml; human TGFβ: 5ng/ml; and anti–IFNγ: 10 μg/ml), T_H2 conditions (murine IL-4; and anti–IFNγ), T_H1 conditions (murine IL-12: 1 ng/ml), T_H17 conditions (human TGFβ: 1 ng/ml; murine IL-6: 50 ng/ml; anti–IFNγ; and anti–IL-4: 10 μg/ml), and iT_{reg} conditions (human TGFβ: 5ng/ml). Recombinant proteins were purchased from Peprotech and R&D. In some experiments Ovalbumin 323–339 (OVA; Sigma) was used.

Gene expression analysis by RT^2 profiler PCR array system

RT^2 profler PCR array system (Qiagen) was used to assess gene expressions of T_H cell immune responses induced by IL-36γ. Briefly, FACS-sorted naive $CD4^+CD25^-$ T cells were co-cultured in the presence of FACS-sorted $CD45^+MHCII^+CD11c^+$ DCs for 24hr 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 RT2 First Strand Kit (Qiagen) followed by genomic DNA elimination. cDNA was applied for Mouse T_H17 response RT2 Profiler PCR Array (PAMM-073ZA; Qiagen) and then PCR amplification was performed with RT^2 SYBR Green qPCR Mastermix. Gene expression analysis was performed using **ΔΔ** C_T method by Profiler PCR Array Analysis Software (Qiagen; version 3.5). Non-detected genes (C_T 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 (ELISA)

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

RNA isolation and quantitative real-time polymerase chain reaction (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 RT-PCR and random hexamer primers (Invitrogen). qPCR was performed with SYBR Green on an StepOnePlus real-time PCR system (Applied Biosystems) and gene expression was normalized to *Gapdh*. Primers used were:

m*Il1f9* (F, TTGACTTGGACCAGCAGGTGTG;

R, GGGTACTTGCATGGGAGGATAG)

m*Il1f6* (F, TAGTGGGTGTAGTTCTGTAGTGTGC;

R, GTTCGTCTCAAGAGTGTCCAGATAT)

m*Il1f8* (F, ACAAAAAGCCTTTCTGTTCTATCAT;

R, CCATGTTGGATTTACTTCTCAGACT)

m*Il9* (F, CATCAGTGTCTCTCCGTCCCAACTGATG;

R, GATTTCTGTGTGGCATTGGTCAG)

m*Gapdh* (F, TGGCAAAGTGGAGATTGTTGCC;

R, AAGATGGTGATGGGCTTCCCG)

Western blot analysis

Following cytokine stimulations, proteins were extracted from $CD4^+$ 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 % SDS-PAGE gel, transferred to polyvinylidene difluoride (PVDF) membrane. The membrane was blocked with blocking buffer (TBS containing 0.1% Tween 20 (TBS-T) and 5% nonfat dry milk). After 3 washes with TBS-T, the membrane was incubated overnight with primary antibodies at 1: 1,000–1: 4,000 dilutions in blocking buffer at 4 °C. After 3 washes with TBS-T, the membrane was incubated with corresponding secondary antibody at 1: 4,000 dilution in blocking buffer for 1 h. After 3 washes with TBS-T, the proteins were visualized using Amersham ECL Prime Detection Reagent (GE Healthcare). The primary antibodies used were: α-Tubulin (sc-69969, Santa Cruz); NF-κB p65 (sc-8008, Santa Cruz); NF-κB p105/p50 (ab32360, Abcam); SMAD3 (ab28379, Abcam); TFIIB (sc-225, Santa Cruz). The secondary antibodies used were: anti-mouse IgG (#7076, Cell Signaling Technology); anti-rabbit IgG (#7074, Cell Signaling Technology).

Chromatin Immunoprecipitation (ChIP) assay

FACS-sorted naive $CD4^+CD25^-$ T cells were co-cultured in the presence of FACS-sorted $CD45^+MHCII^+CD11c^+$ DCs for 4days at a 10:1 T/DC cell ratio under iT_{reg} conditions in the presence or absence of IL-36γ. ChIP assays were performed with EZ-ChIP kit (17–371, EMD Millipore) as previously described.[50] 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 antibody (06–599, EMD Millipore). The precipitated DNA was subjected to qPCR using specific primers for Foxp3 locus.[19]

Oxazolone colitis

Oxazolone colitis was induced as previously described.[27] Briefly, in order to presensitize mice, a 2 × 2 cm 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 100μl of 1% oxazolone in 50% ethanol under general anesthesia with isoflurane.

$CD4^+CD45RB^{hi}$-induced colitis

FACS-sorted $CD4^+CD25^-CD45RB^{hi}$ naive T cells from WT mice or IL-$36R^{-/-}$ mice ($5x10^5$) were injected i.p. into *Rag1*$^{-/-}$ recipients. Colons were dissected from recipient mice 6 weeks post-transfer 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) of was graded in a blinded fashion from 0 to 4 or 0 to 3 respectively.

Microarray analysis

Two microarray datasets were downloaded from the Gene Expression Omnibus database (http://www.ncbi.nlm.nih.gov/geo/), including GSE36807 (Montero 2013) and GSE16879 (Arijs 2009). Expression data from GSE36807 included colonic specimens from 15 ulcerative colitis patients and 13 Crohn's disease patients. Microarray data from GSE16879 included samples from 24 ulcerative colitis patients and 37 Crohn's disease patients prior to infliximab treatment. These datasets contained gene expression data derived from the Affymetrix U133_plus2 platform. 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 GraphPad Prism software, version 6.0b (Graphpad Software). Student's *t* test or One-way ANOVA 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 IBM SPSS Statistics 19 software.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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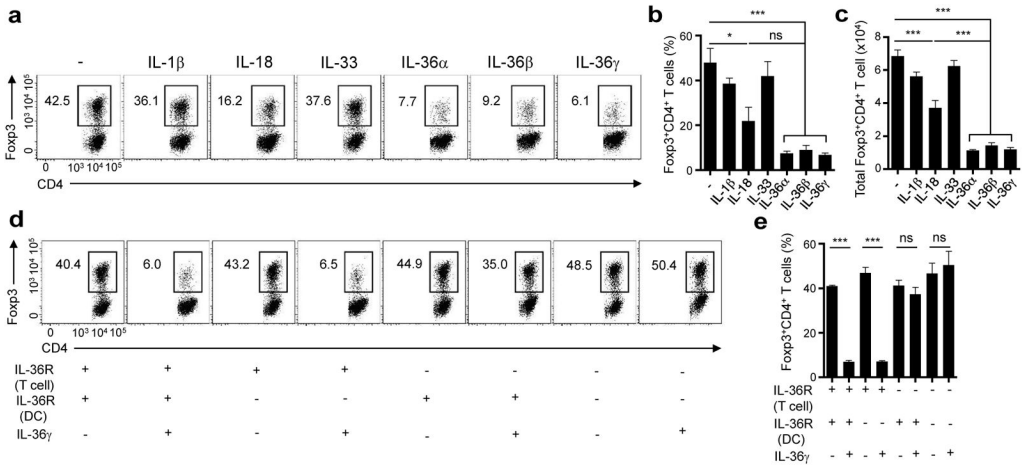

Figure 1. IL-36γ abrogates iT_{reg} induction via IL-36R-mediated signaling in $CD4^+$ T cells

(a–c) Foxp3 expression by $CD4^+$ T cells co-cultured with DCs for 4 days in the presence of indicated IL-1 family member. Representative dot plots are shown in (a), the frequencies of $Foxp3^+$ T cells among total $CD4^+$ T cells are shown in (b) and the total numbers of $Foxp3^+CD4^+$ T cells are shown in (c). **(d–e)** Foxp3 expression by $CD4^+$ T cells co-cultured with DCs for 4 days in the presence or absence of IL-36γ using the indicated cells from WT (IL-36R "+") and/or *Il1rl2*$^{-/-}$ (IL-36R "−") mice. Representative dot plots are shown in (d). The frequencies of $Foxp3^+$ T cells among total $CD4^+$ T cells are shown in (e). All data are representative of three independent experiments with 3 replicates. One-way ANOVA and Tukey's Multiple Comparison Test was used to determine significance. Error bars indicate mean ± s.e.m. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$; ns, not significant.

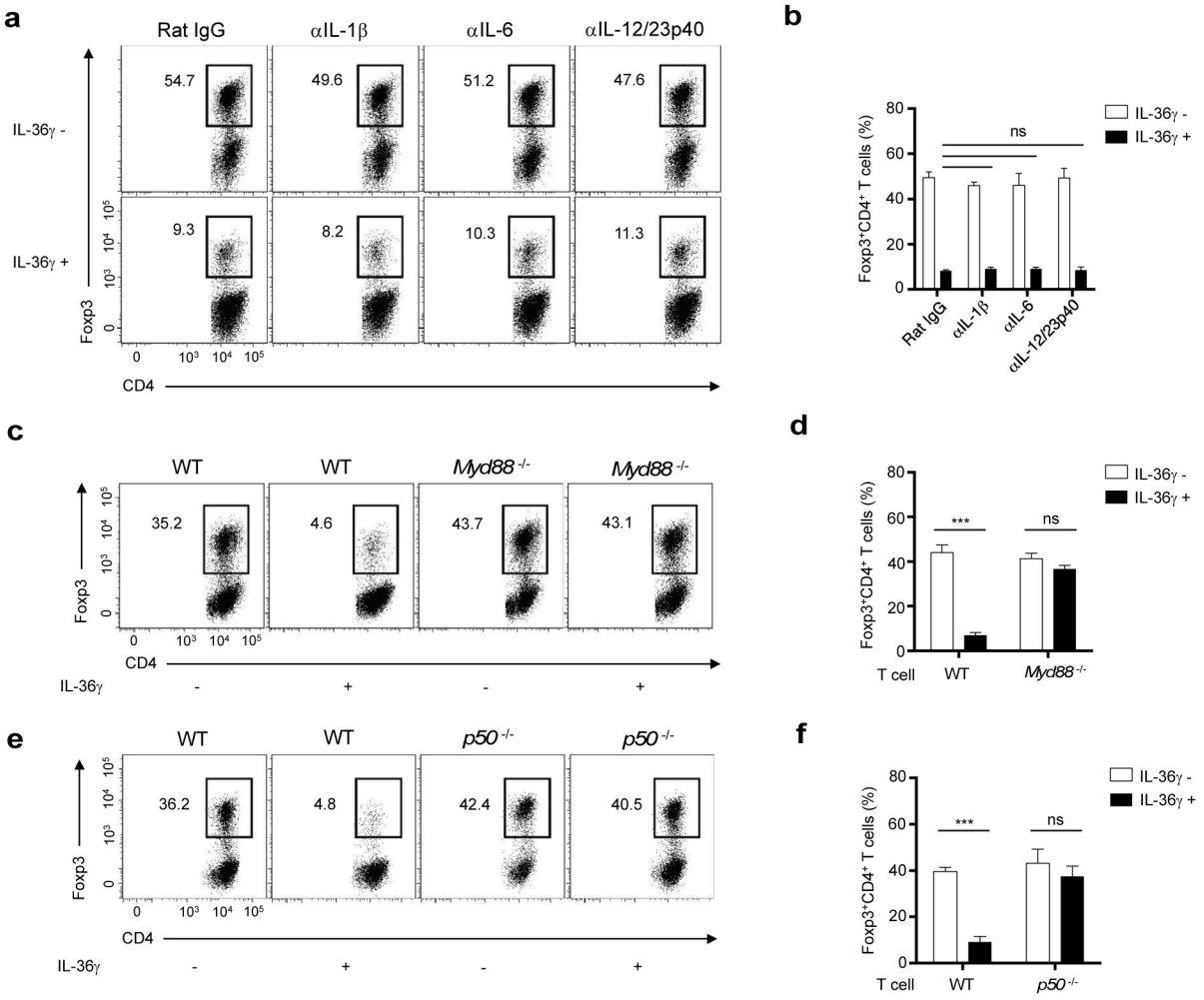

Figure 2. IL-36γ-mediated suppression of iT_{reg} cells is MyD88- and NFκBp50-dependent

(a–b) Foxp3 expression by $CD4^+$ T cells co-cultured with DCs for 4 days in the presence or absence of IL-36γ supplemented with indicated neutralizing Abs. Representative dot plots (a) and the frequencies of $Foxp3^+$ T cells among total $CD4^+$ T cells (b) are shown. **(c–d)** Foxp3 expression by $CD4^+$ T cells from WT and/or *Myd88*$^{-/-}$ mice co-cultured with WT DCs for 4 days in the presence or absence of IL-36γ. Representative dot plots (c) and the frequencies of $Foxp3^+$ T cells among total $CD4^+$ T cells (d) are shown. **(e–f)** Foxp3 expression by $CD4^+$ T cells from WT and/or *p50*$^{-/-}$ mice co-cultured with WT DCs for 4 days in the presence or absence of IL-36γ. Representative dot plots (e) and the frequencies of $Foxp3^+$ T cells among total $CD4^+$ T cells (f) are shown. Data are representative of three independent experiments (a–b) and from two independent experiments (c–f) with 3 replicates (b) or 6 replicates (d,f). One-way ANOVA and Tukey's Multiple Comparison Test was used to determine significance. Error bars indicate mean ± s.e.m. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$; ns, not significant.

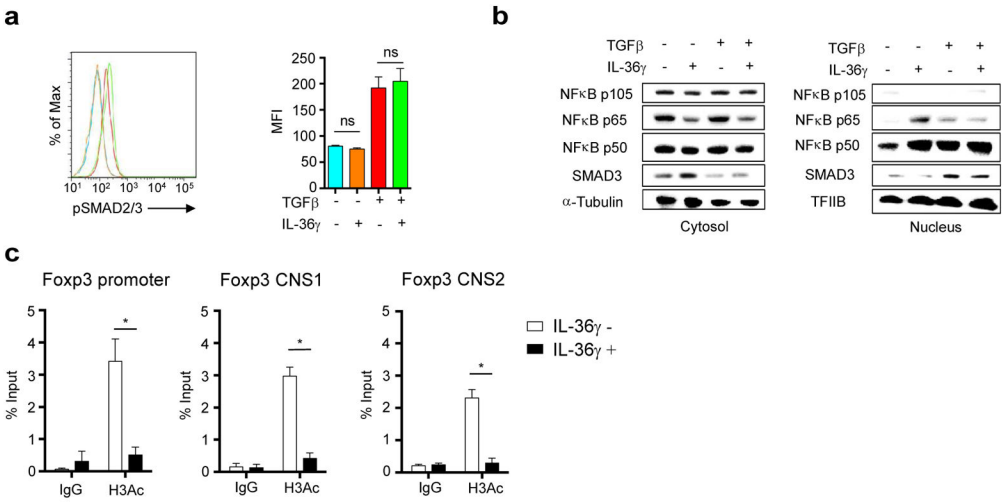

Figure 3. IL-36γ alters NFκB signaling and acetylation of the Foxp3 locus during iT_{reg} cell differentiation

(**a**) FACS-sorted naïve $CD4^+$ T cells were co-cultured with DCs at a 10:1 T/DC cell ratio in the presence or absence of TGFβ and IL-36γ for 30min. Cells were then fixed and permeabilized for staining with anti-phospho-SMAD2/3 (pSMAD2/3) Abs. Representative histograms for pSMAD2/3 in $CD4^+$ T cells are shown. The graph represents mean fluorescent intensity (MFI) of pSMAD2/3 among $CD4^+$ T cells. (**b**) Western blot analysis of NFκB p105, p65, p50 and SMAD3 in the cytosol and nucleus in FACS-sorted naive $CD4^+$ T cells activated with the indicated cytokines for 45min. α-Tublin and TFIIB are shown as loading controls, respectively. (**c**) ChIP assays for H3Ac modifications in the Foxp3 locus in naive $CD4^+$ T cells co-cultured with DCs at a 10:1 T/DC cell ratio for 4 days under iT_{reg} conditions. Data are representative of two independent experiments (a–c) with 3 replicates (a,c). One-way ANOVA and Tukey's Multiple Comparison Test was used to determine significance. Error bars indicate mean ± s.e.m. Statistical significance is indicated by $*P < 0.05$, $**P < 0.01$, $***P < 0.001$; ns, not significant.

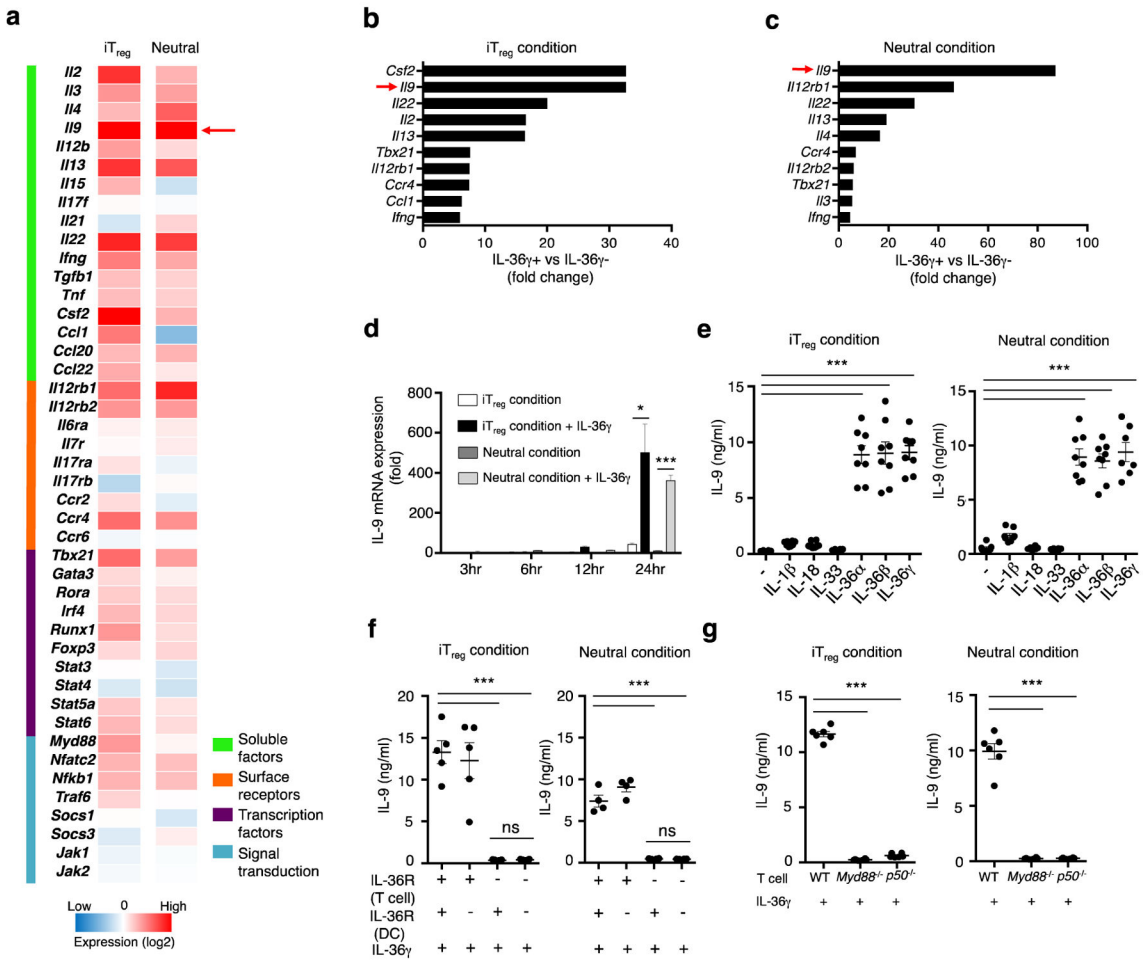

Figure 4. IL-36γ promotes IL-9 expression in a MyD88- and NFκBp50-dependent manner

(a–c) Gene expression profiles induced by IL-36γ were analyzed by PCR array analyses. RNA was isolated from $CD4^+$ T cells co-cultured with DCs for 24hrs in the presence or absence of IL-36γ. Change in gene expression (log_2) under iT_{reg} conditions (−5.0 to 5.0) and neutral conditions (−6.4 to 6.4) expressed as a heatmap (a). Bar graphs show top differentially up-regulated transcripts in IL-36γ treated cells versus non-treated cells under iT_{reg} conditions (b) and neutral conditions (c). **(d)** FACS-sorted naïve $CD4^+$ T cells were co-cultured with DCs for indicated periods under iT_{reg} conditions or neutral conditions in the presence or absence of IL-36γ. IL-9 mRNA expression was assessed by qPCR with 3 replicates. **(e)** FACS-sorted naïve $CD4^+$ T cells and DCs were co-cultured for 4 days under iT_{reg} conditions or neutral conditions in the presence of indicated IL-1 family members. IL-9 protein in supernatant was assessed by ELISA. **(f)** FACS-sorted naïve $CD4^+$ T cells and DCs were co-cultured using the indicated cells from WT (IL-36R "+") and/or *Il1rl2*$^{-/-}$ (IL-36R "−") in the presence of IL-36γ. IL-9 protein in supernatant was assessed by ELISA. **(g)** Similarly FACS-sorted naïve $CD4^+$ T cells from indicated mouse strains and WT DCs were co-cultured for 4 days in the presence of IL-36γ and IL-9 protein in 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

ANOVA and Tukey's Multiple Comparison Test was used to determine significance. Error bars indicate mean ± s.e.m. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$; ns, not significant.

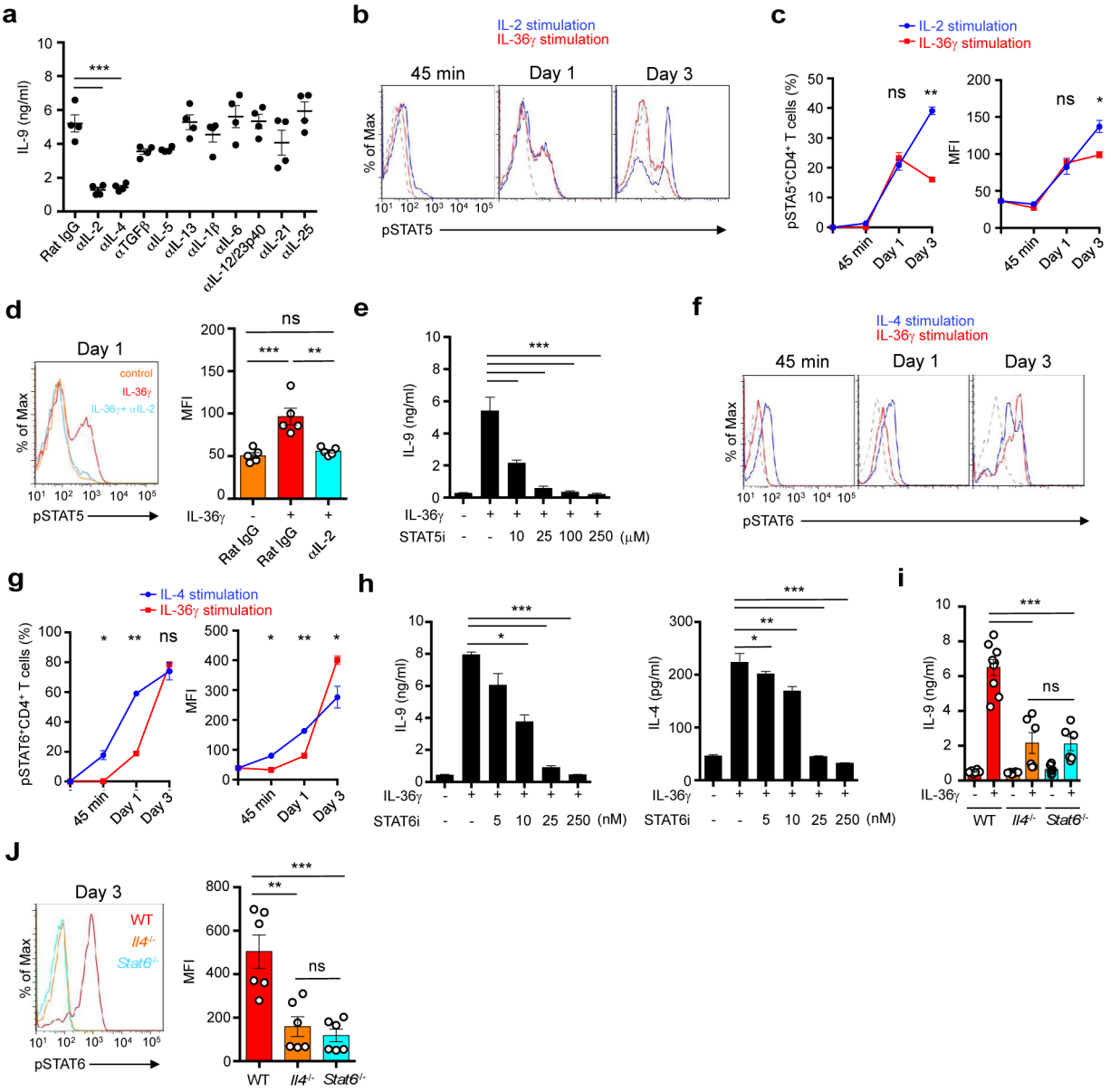

Figure 5. IL-36γ induces T_H9 differentiation via IL-2-STAT5 and IL-4-STAT6 signaling

(a) FACS-sorted naïve $CD4^+$ T cells and DCs were co-cultured for 4 days under neutral conditions in the presence of IL-36γ supplemented by indicated neutralizing Abs for specific cytokines and IL-9 protein in supernatant was assessed by ELISA. **(b–c)** Flow cytometry analysis for phospho-STAT5 (pSTAT5) in $CD4^+$ T cells in the presence of IL-2 or IL-36γ (100ng/ml). FACS-sorted naïve $CD4^+$ T cells and DCs were co-cultured for indicated times in the presence of IL-2 or IL-36γ and pSTAT5 expression in CD4 T^+ cells was analyzed. Representative histograms (b), the frequencies of $pSTAT5^+$ cells and mean fluorescent intensity (MFI) for pSTAT5 (c) are shown. **(d)** FACS-sorted naïve $CD4^+$ T cells and DCs from WT mice were co-cultured for 1 day in the presence of IL-36γ and/or anti-IL-2 neutralizing Abs and pSTAT5 expression in $CD4^+$ T cells was analyzed. Representative histograms and mean fluorescent intensity (MFI) for pSTAT5 among $CD4^+$ T cells are

shown. **(e)** FACS-sorted naïve $CD4^+$ T cells and DCs were co-cultured for 4 days in the presence of indicated concentration of a STAT5 selective inhibitor. IL-9 protein quantification in supernatant of T/DC co-cultures was assessed by ELISA. **(f–g)** Flow cytometry analysis for phospho-STAT6 (pSTAT6) in $CD4^+$ T cells in the presence of IL-4 or IL-36γ (100ng/ml). FACS-sorted naïve $CD4^+$ T cells and DCs were co-cultured for indicated times in the presence of IL-4 or IL-36γ and pSTAT6 expression in CD4 T^+ cells was analyzed. Representative histograms (f), the frequencies of $pSTAT6^+$ cells and mean fluorescent intensity (MFI) for pSTAT6 (g) are shown. **(h)** FACS-sorted naïve $CD4^+$ T cells and DCs were co-cultured for 4 days in the presence of 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 naïve $CD4^+$ T cells and DCs obtained from indicated mouse strains were co-cultured for 4 days in the presence of IL-36γ and IL-9 protein in supernatant was assessed by ELISA. **(j)** FACS-sorted naïve $CD4^+$ T cells and DCs from indicated mouse strains were co-cultured for 3 days in the presence of IL-36γ and pSTAT6 expression in $CD4^+$ T cells was analyzed. Representative histograms and mean fluorescent intensity (MFI) for pSTAT6 among $CD4^+$ T cells are shown. Data are representative of four (a), three (f,g) and two (b–e, h–j) independent experiments with 3 replicates unless specified. Student's *t* test or One-way ANOVA and Tukey's Multiple Comparison Test was used to determine significance. Error bars indicate mean ± s.e.m. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$; ns, not significant.

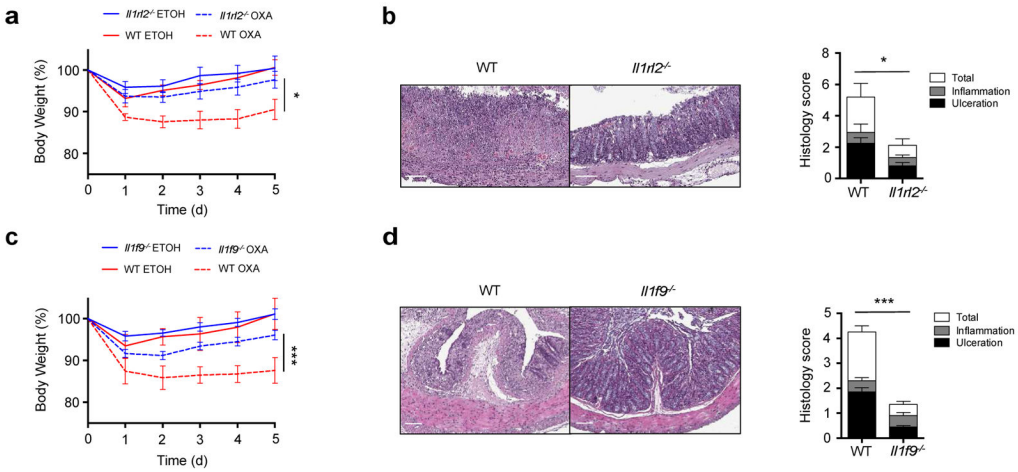

Figure 6. Deficiency of IL-36γ or IL-36R *in vivo* ameliorates oxazolone-induced colitis

(a–b) Body weight of WT and *Il1rl2*$^{-/-}$ mice during 5 days after treatment with oxazolone (n=10 for oxazolone treated group, n=5 for ETOH treated group) (a), representative hematoxylin/eosin stained colon sections and histology scores of the colon sections (b) are shown respectively. Bars, 100 μm. **(c–d)** Body weight of WT and *Il1f9*$^{-/-}$ mice during 5 days after treatment with oxazolone (n=10 for oxazolone treated group, n=5 for ETOH treated group) (c), representative hematoxylin/eosin stained colon sections and histology scores of the colon sections (d) are shown respectively. Bars, 100 μm. Data are cumulative of four independent experiments. Student's *t* test or One-way ANOVA and Tukey's Multiple Comparison Test was used to determine significance. Error bars indicate mean ± s.e.m. *$P <$ 0.05, **$P < 0.01$, ***$P < 0.001$; ns, not significant.

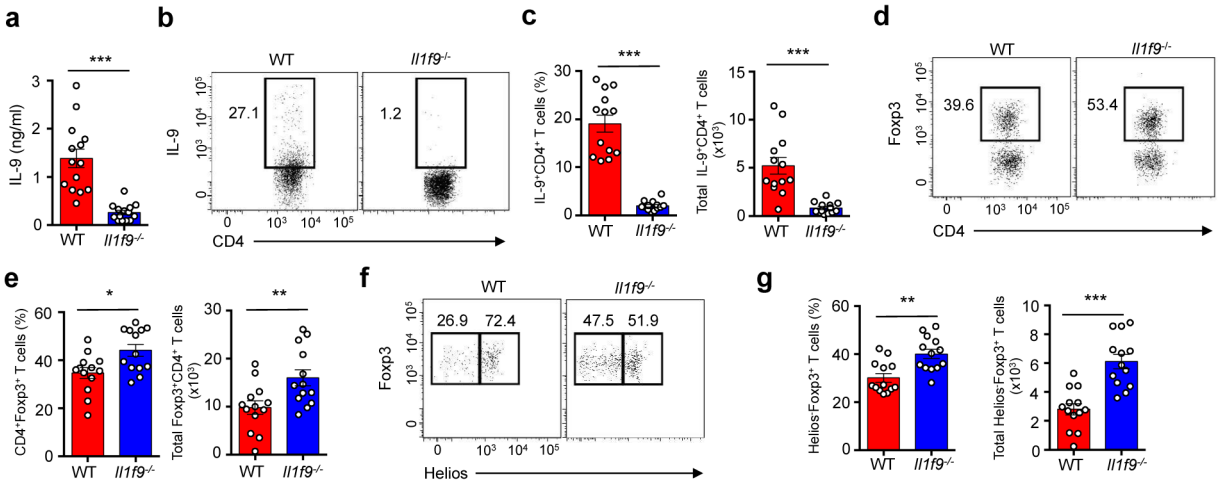

Figure 7. IL-36γ controls the T_{reg} – T_H9 cell balance *in vivo*

(a) 2 days after rectal challenge with oxazolone, colonic LP cells were isolated from WT and *Il1f9*$^{-/-}$ mice. Isolated colonic LP cells were cultured *ex vivo* for 36hrs and IL-9 protein quantification in supernatant was assessed by ELISA. **(b–c)** IL-9 expression among CD4$^+$ T cells isolated from colons of oxazolone treated WT and *Il1f9*$^{-/-}$ mice was analyzed by flow cytometry. Representative dot plots (b), the frequencies of IL-9$^+$CD4$^+$ T cells among total CD4$^+$ T cells and total numbers of IL-9$^+$CD4$^+$ T cells in WT and *Il1f9*$^{-/-}$ colons (c) are shown. **(d–e)** Foxp3 expression among CD4$^+$ T cells from WT and *Il1f9*$^{-/-}$ colons was analyzed by flow cytometry. Representative dot plots are shown in (d). Frequencies among CD4$^+$ T cell and total number of Foxp3$^+$CD4$^+$ T cells from WT and *Il1f9*$^{-/-}$ colons are shown in (e). **(f–g)** Helios expression among Foxp3$^+$CD4$^+$ T cells from WT and *Il1f9*$^{-/-}$ colons was analyzed by flow cytometry. Representative dot plots are shown in (f). Frequencies of Helios$^-$ cells among Foxp3$^+$CD4$^+$ T cells and total number of Helios$^-$Foxp3$^+$CD4$^+$ T cells from WT and *Il1f9*$^{-/-}$ 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 ± s.e.m. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$; ns, not significant.