IL-36γ signaling controls the induced regulatory T cell–Th9 cell balance via NFκB 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-36γ signals through IL-36 receptor, myeloid differentiation primary response gene 88, and nuclear factor-κBp50 in CD4+ T cells to potently inhibit Foxp3-expressing induced regulatory T cell (Treg) 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-36γ were protected from Th cell–driven intestinal inflammation and exhibited increased colonic Treg cells and diminished Th9 cells. Our findings thus reveal a fundamental contribution for the IL-36/IL-36R axis in regulating the Treg–Th9 cell balance with broad implications for Th cell–mediated disorders, such as inflammatory bowel diseases and particularly ulcerative colitis.

INTRODUCTION

CD4+ 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.1–3 Regulatory Th cells expressing Foxp3 (Treg) can develop intrathymically or in the periphery and are potently immunosuppressive and help to maintain immunological homeostasis.2 Effector Th cells (Teff), on the other hand, can be grouped into several general categories (Th1, Th2, Th9, Th17, Th22, and Th17i) 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 Th cells into either Treg or Teff lineages.5 For example, in the presence of IL-2 and transforming growth factor-β (TGFβ) naive Th cells differentiate into induced Treg cells (iTreg) while the combination of interleukin (IL)-6 plus TGFβ promotes Th17 and inhibits iTreg differentiation.6–8 Alternatively, IL-4 can promote the differentiation of Th2 cells while the addition of TGFβ can induce reprogramming into Th9 cells.9–11 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.12,13 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.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 Treg 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 Th 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+ Th cell differentiation into specific lineages remains incompletely defined. In the present report, we investigated the role of the IL-36γ/IL-36R axis in controlling the balance of Treg and Teff 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-κBp50 (NFκBp50) in CD4+ T cells to potently inhibit Treg development, while concomitantly promoting Th9 differentiation via a IL-2-STAT5- and IL-4-STAT6-dependent pathway. Additionally, mice deficient in IL-36γ demonstrated that signaling through IL-36R employs myeloid differentiation primary response gene 88 (MyD88) and nuclear factor-κBp50 (NFκBp50) in CD4+ T cells to potently inhibit Treg development, while concomitantly promoting Th9 differentiation via a IL-2-STAT5- and IL-4-STAT6-dependent pathway. Additionally, mice deficient in IL-36γ expressed IL-36γ and IL-36R signaling were protected from Th cell–dependent intestinal inflammation and exhibited increased colonic Treg and diminished Th9 cells. Collectively, these data highlight IL-36R signaling as a regulator of the Treg-Th9 balance in vitro and in vivo with functional implications in the regulation of intestinal inflammation.

RESULTS

IL-36γ abrogates Treg induction via IL-36R-mediated signaling in CD4+ T cells

To investigate the contribution of the IL-36/IL-36R axis in CD4+ Th cell differentiation, we first explored whether IL-36 ligands could modulate Foxp3 induction in responding T cells using a naive CD4+ T cell–dendritic cell (DC) in vitro coculture system in the presence of IL-36γ. Intriguingly, compared with other IL-1 family members tested, IL-36 ligands—IL-36α, IL-36β, and IL-36γ—all potently abrogated the induction of Foxp3-expressing Treg 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-36γ in the mouse intestine during colitis, we focused specifically on IL-36γ and asked whether it was acting on CD4+ T cells or DCs to inhibit Treg differentiation. To do so, we employed a co-culture system whereby CD4+ T cells or DCs were isolated from wild-type (WT) or IL-36γ-deficient (IL-36γ−/−) mice. Of note, the effect of IL-36γ in the presence of IL-36γ was not affected by the irradiation of DCs (see Supplementary Figure S4). We also confirmed the ability of IL-36γ to suppress Treg cell induction in the absence of DCs by using purified naive CD4+ T cells, which were isolated from WT mice or OT-II mice (see Supplementary Figure S5a,b).

Overall, these findings demonstrate that IL-36γ inhibits Foxp3+ Treg 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 Treg cells is MyD88 and NFκBp50 dependent

IL-36γ expression in CD4+ T cells was involved in the suppression of Treg cell development mediated by IL-36γ (Figure 2c,d). As signaling through IL-36γ can lead to NFκB activation, we next explored whether NFκBp50 was involved in IL-36γ-mediated suppression of Treg induction. To address this question, we employed a co-culture system whereby CD4+ T cells were isolated from p50-deficient (p50−/−) mice. Remarkably, deficiency of NFκBp50 specifically in CD4+ T cells fully restored Treg induction in the presence of IL-36γ (Figure 2e,f). Altogether, our data indicate that IL-36γ suppresses Treg differentiation via MyD88- and NFκBp50-dependent signaling in CD4+ T cells.

IL-36γ alters NFκB signaling and acetylation of the Foxp3 locus during Treg cell differentiation

We next investigated whether IL-36γ may be mediating its effects on inhibiting Treg cells by interfering with signaling downstream of TGFβ receptor, specifically SMAD3, as it is known to bind the conserved noncoding sequence 1 (CNS1) in the Foxp3 locus and positively regulate Foxp3 expression. Flow cytometric analyses revealed that IL-36γ did not inhibit TGFβ-induced phosphorylation of SMAD2/3 (Figure 3a) or nuclear translocation of SMAD3 (Figure 3b). As deficiency of NFκBp50 in CD4+ T cells fully restored Treg 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 Figure 3b, upon CD4+ T-cell activation under neutral conditions, IL-36γ promoted nuclear translocation of NFκB p65 and p50, but not p105. Under Treg conditions, p65 nuclear translocation was also observed; however, this nuclear translocation was similar to IL-36γ. Interestingly, NFκBp50 nuclear translocation was observed under Treg conditions and this pathway could be involved in the inhibition of Treg differentiation mediated by IL-36γ. Notably, Ab-mediated blockade of GITR ligand was also unable to reverse the effects of IL-36γ on suppressing Treg differentiation (see Supplementary Figure S3a,b). Of note, the effect of IL-36γ on Treg inhibition was not affected by the irradiation of DCs (see Supplementary Figure S4). We also confirmed the ability of IL-36γ to suppress Treg cell induction in the absence of DCs by using purified naive CD4+ T cells, which were isolated from WT mice or OT-II mice (see Supplementary Figure S5a,b).

Overall, these findings demonstrate that IL-36γ inhibits Foxp3+ Treg differentiation via IL-36R-mediated signaling in CD4+ cells independent of well-defined inflammatory cytokines or GITR/GITR ligand induction.
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 iTreg cell differentiation. As the IL-36γ–MyD88–p50 axis could epigenetically alter the chromatin status in CD4⁺ T cells under iTreg 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 iTreg conditions (Figure 3c). Collectively, these data suggest that IL-36γ activates NFκBp50 in CD4⁺ T cells and modulate histone acetylation status at the Foxp3 locus during iTreg cell differentiation in vitro.

IL-36γ promotes Th9 differentiation in a MyD88- and NFκBp50-dependent manner

Having defined the ability of IL-36γ to inhibit iTreg cell differentiation, we next explored which Th cell lineages IL-36γ may be favoring. To do so, we performed gene expression profiling of CD4⁺ Th cells stimulated with sCD3ε in the presence or absence of IL-36γ by using the T/DC co-culture system. Notably, under iTreg conditions, IL-9 was identified as one of the top genes induced by IL-36γ 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 TGFβ1 or IL-2). Similar to results observed using iTreg culture conditions, IL-9 was the top gene induced by IL-36γ under neutral conditions (Figure 4a,c; see Supplementary Figure S6). The ability of IL-36γ to potently 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-36γ was more than fivefold greater than that of IL-1β, which has been reported to induce IL-9,²³ and IL-18 and IL-33 in both iTreg and neutral conditions (Figure 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 Figure 1d. Similar to the requirement for IL-36R expression on CD4⁺ T cells in mediating iTreg inhibition, the expression of IL-36γ by CD4⁺ T cells, but not DCs, was involved in the IL-9-inducing ability of IL-36γ (Figure 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 (see Supplementary Figure S4). Next we investigated the mechanism via which IL-36γ induced IL-9 production from CD4⁺ T cells. As IL-36γ abrogated iTreg differentiation via a MyD88- and NFκBp50-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 iTreg conditions (Figure 4g). We further examined whether the addition of IL-36γ could...
modulate the expression of transcription factors known to be required for Th9 differentiation, specifically PU.1 and IRF4. 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 (see Supplementary Figure S7a,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 Th cell–polarizing conditions. Consistent with our data from T/DC co-cultures, IL-36γ significantly induced the differentiation of IL-9-producing cells under Th0 conditions (see Supplementary Figure S8a,b). Remarkably, IL-36γ also significantly augmented Th9 cell differentiation under Th9 conditions (see Supplementary Figure S8a,c). Intriguingly, the ability of IL-36γ to potentilly induce IL-9-producing T cells was also observed under other Th cell–polarizing conditions, including Th2 and iTreg conditions but to a far lesser extent under Th1 and Th17 conditions (see Supplementary Figure S8a). Thus these findings demonstrate that IL-36γ is a potent inducer of IL-9 production and augments Th9 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. 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 (Figure 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 assessed by 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 (Figure 5i). Consistent with these data, IL-36γ-induced pSTAT6 was also significantly reduced in Il4⁻/⁻ and Stat6⁻/⁻ mice. As IL-2 signaling activates STAT5, which is involved in the development of Th9 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 Figure 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 (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-36γ-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. 26 As shown in Figure 5f–g, IL-4 (positive control) significantly induced pSTAT6 within 45 min as compared with 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 (Figure 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 manner, demonstrating that STAT6 is also involved in IL-36γ-induced IL-9 production (Figure 5h). Similarly, STAT6 inhibition abrogated IL-36γ-induced IL-4 production (Figure 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 (Figure 5i).
CD4⁺ T cells (Figure 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 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, and more recently, Th9 cells have been shown to have a central role in disease pathogenesis in this colitis model as well as in UC. 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 with WT control mice (Figure 6a,b), although we did not observe differences in survival rate (see Supplementary Figure S9a). As IL-36γ mRNA expression was significantly higher than that of IL-36α and IL-36β in total colonic tissue of oxazolone-treated mice (see Supplementary Figure S10), we next examined mice deficient in IL-36γ.
**Figure 5** Interleukin (IL)-36γ 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\(^+\) T cells and dendritic cells (DCs) were co-cultured for 4 days under neutral conditions in the presence of IL-36\(γ\) 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\(^+\) T cells in the presence of IL-2 or IL-36\(γ\) (100 ng ml\(^{-1}\)). FACS-sorted naive CD4\(^+\) T cells and DCs were co-cultured for the indicated times in the presence of IL-2 or IL-36\(γ\) 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 naive CD4\(^+\) T cells and DCs from wild-type (WT) mice were co-cultured for 1 day in the presence of IL-36\(γ\) and/or anti-IL-2 neutralizing Abs and pSTAT5 expression in CD4\(^+\) T cells was analyzed. Representative histograms and MFI for pSTAT5 among CD4\(^+\) T cells are shown. (e) FACS-sorted naive CD4\(^+\) 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\(^+\) T cells in the presence of IL-4 or IL-36\(γ\) (100 ng ml\(^{-1}\)). FACS-sorted naive CD4\(^+\) T cells and DCs were co-cultured for the indicated times in the presence of IL-4 or IL-36\(γ\) and pSTAT6 expression in CD4\(^+\) T cells was analyzed. Representative histograms (f), the frequencies of pSTAT6\(^+\) cells and MFI for pSTAT6 (g) are shown. (h) FACS-sorted naive CD4\(^+\) 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\(^+\) T cells and DCs obtained from the indicated mouse strains were co-cultured for 4 days in the presence of IL-36\(γ\) and IL-9 protein in the supernatant was assessed by ELISA. (j) FACS-sorted naive CD4\(^+\) T cells and DCs from the indicated mouse strains were co-cultured for 3 days in the presence of IL-36\(γ\) and pSTAT6 expression in CD4\(^+\) T cells was analyzed. Representative histograms and 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 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 ± s.e.m. *P<0.05, **P<0.01, ***P<0.001; NS, not significant.
(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 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+CD45RBhi T-cell transfer model of colitis. Using this model, Rag1−/− mice transferred with IL-36R-deficient CD45RBhi cells exhibited modestly but significantly reduced weight loss and colonic inflammation when compared with Rag1−/− mice transferred with IL-36R-sufficient CD45RBhi cells (see Supplementary Figure S11a,b). Thus these results indicate that the IL-36γ/IL-36R axis has a key role in driving Th cell–dependent colonic inflammation, particularly in the Th2/9 oxazolone model.

IL-36γ controls the Treg–Th9 cell balance in vivo

Next we further investigated IL-36γ–mediated regulation of Th differentiation in vivo during colitis. Following treatment with oxazolone, Il1f9−/− mice displayed significantly reduced IL-9 production by colonic lamina propria lymphocytes (Figure 7a), as well as reduced IL-9-producing CD4+ T-cell frequency and absolute cell number (Figure 7b,c), when compared with WT mice. In addition, the frequency and absolute number of Foxp3+ CD4+ T cells was significantly increased in Il1f9−/− colonic tissue (Figure 7d,e). Further, both the frequency and absolute number of Helios−Foxp3+ CD4+ T cells were significantly increased in Il1f9−/− colonic tissue (Figure 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 (see Supplementary Figure S12a,b). Although the frequency of Helios−Foxp3+ CD4+ T cell was modestly higher in Il1f9−/− 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−Foxp3+ Treg 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 iTreg–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-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 (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 iTreg differentiation
while redirecting toward IL-9-producing T<sub>eff</sub> cells via a pathway involving MyD88 and NFκB50 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-36γ-induced Th9 differentiation. Importantly, the role of IL-36R signaling in controlling the iTreg–Th9 balance was further confirmed in vivo using the oxazolone model of colitis. In this model, mice deficient in IL-36γ-IL-36 signaling exhibited increased iTreg<sub>ab</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 an anti-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 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.18,33,34 Consistent with these findings, IL-36α as well as IL-36γ 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.34-36 It is important to note that beyond expression, IL-36 ligands must 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.18,31,32

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 iTreg<sub>ab</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–iTreg<sub>ab</sub> 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 Th17 cell differentiation.39,40 Further, the IL-1 family member, IL-33, can directly augment colonic Treg<sub>ab</sub> function by binding to the IL-33 receptor.
receptor, ST2. 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 TGFβ, 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 iTreg cells. Therefore, it is intriguing that IL-36-mediated inhibition of iTreg 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 iTreg cell development remains unclear, it is notable that a recent report provided evidence that the GITR costimulatory molecule was a potent inhibitor of iTreg differentiation and an inducer of Th9 cells through NFκB activation, leading to recruitment of histone deacetylases at the Foxp3 locus and a "closed" chromatin structure. In the present study, we demonstrated that NFκB signaling, but not the GITR/GITR ligand axis, is instrumental in contributing to diminished iTreg cell development and enhanced Th9 differentiation mediated by IL-36γ, indicating that signaling through IL-36R and GITR may regulate iTreg-Th9 cell balance via unique, albeit partially, overlapping mechanisms. Of note, in addition to inhibiting iTreg differentiation, it remains to be elucidated whether IL-36 receptor signaling also alters the function of thymically derived iTreg cells or not.

Although the contribution of IL-2, IL-4, and TGFβ to Th9 differentiation is well established, the endogenous inducers of Th9 cells have not been fully elucidated. 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. However, the effect of IL-36 cytokines on naive CD4+ T cells to modulate de novo differentiation into the iTreg or Th9 cell lineages has not been previously reported. By employing ex vivo cell culture system using FACS (fluorescence activated cell sorting)-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 Th9 responses. Additionally, by using various T-cell-polarizing conditions, we demonstrated that IL-36γ significantly induced IL-9 under Th0, Th2, Th9, and iTreg conditions, whereas it did not under Th1 and Th17 conditions. This is consistent with previous reports showing that IFNγ inhibits IL-9 production by neutralizing the effect of IL-4, and IL-6 inhibits IL-9 production by regulating STAT5 activation via STAT3.

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, asthma, cancer, and IBD. Although the term IBD comprises CD and UC, both of which the etiology remain unknown, distinct immunological dysregulation are associated with each disease. Particularly, IL-9 and IL-9R were shown to be upregulated in patients with UC. 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. 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-36x, IL-36β, and IL-9 specifically in UC. Collectively, these findings define a novel role for the IL-36 pathway in controlling the Treg-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−/−, p50−/−, Il4−/−, Stat6−/−, B6.Cg-Tg(TcraTcrb)425Cmn/J (OT-II), and Rag1−/− mice were obtained from Jackson Laboratories (Bar Harbor, ME) and housed in specific pathogen-free conditions. IL-36R−/− mice (Il1f9−/−) mice were provided by Agen (Thousand Oaks, CA). 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 (Atlanta, GA) 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 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, 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 (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+ T cells (CD4+CD25−) 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+ MHCIi+ CD11c+ DCs at a 10:1 T/DC cell ratio (DCs were not irradiated unless specified). All cultures contained purified anti-CD3e (2 μg/ml; 145-2C11; eBioscience). For iTreg cell induction, the cultures contained TGF

Western blotting 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% 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); NFKB p105/p50 (ab32360, Abcam, Cambridge, UK); SMAD3 (ab28379, Abcam); and TIF1β (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+ CD25− T cells were co-cultured in the presence of FACS-sorted CD45+ MHCIi+ CD11c+ DCs for 4 days at a 10:1 T/DC cell ratio under iTreg 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.

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Oxazolone colitis. Oxazolone colitis was induced as previously described. Briefly, in order to presensitize mice, a 2 x 2 cm2 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.

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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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