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Non-hematopoietic STAT6 induces epithelial tight junction dysfunction and promotes intestinal inflammation and tumorigenesis

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Enhanced gut permeability due to dysregulated epithelial tight junction is often associated with inflammatory bowel diseases (IBD), which have a greater risk for developing colorectal cancer. STAT6 activation was detected in inflamed colonic epithelium of active IBD patients, suggesting a role of epithelial STAT6 in colitis development. Here, we demonstrated that non-hematopoietic STAT6, but not hematopoietic STAT6, triggered DSS-induced colitis and subsequent tumorigenesis. This could be due to the enhancing-effect of STAT6 on gut permeability and microbiota translocation via interruption of epithelial tight junction integrity. Mechanistically, long-myosin light-chain kinase (MLCK1) was identified as a target of STAT6, leading to epithelial tight junction dysfunction and microbiota-driven colitis. Furthermore, neutralization of IL-13, which was primarily derived from type 2 innate lymphoid cells (ILC2) in a microbiota-dependent way, inhibited epithelial STAT6 activation and improved gut permeability and DSS-induced colitis. Importantly, pharmacological inhibition of STAT6 reduces murine intestinal tumor formation, and tumoral p-STAT6 levels positively correlated to the clinical stage and poor prognosis of human colorectal cancer. Thus, our study reveals a direct role of STAT6 in the disruption of epithelial tight junction integrity and colitis development, and suggests STAT6 as a potential therapeutic and prophylactic target for IBD and colitis-associated cancer.

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INTRODUCTION

Inflammatory bowel disease (IBD), clinically consisting of ulcerative colitis (UC) and Crohn's disease (CD), is a complex disorder resulting from the breakdown of gut homeostasis caused by dysregulated interactions between intestinal epithelial cells (IECs), immune cells and gut microbiota.^{1,2} IBD is also a strong predisposing factor for colorectal cancer, referred to as colitis-associated cancer (CAC).^{3,4} Compared with colorectal cancer without detectable pre-existing intestinal inflammation, CAC often occurs at an earlier age, and is more aggressive with poor response to treatment and high mortality.⁴

Epithelial tight junction is a critical component of mucosal barrier integrity that maintains intestinal homeostasis by regulating paracellular permeability.⁵ Increased intestinal permeability alone is not sufficient to induce colitis, but rather can increase the susceptibility in mice to develop colitis under certain conditions.^{6,7} Accumulating evidence demonstrates that compromised epithelial integrity could result in persistent exposure to luminal microbiota, thereby leading to excessive intestinal inflammation characterized by recruitment of leukocytes, particularly neutrophils, release of inflammatory mediators, and ultimately mucosal

damage.^{2,8} Clinical evidence suggests increased intestinal permeability as a risk factor for intestinal disease, as increased permeability may be present many years before CD onset in human subjects, and lesional mucosa from UC patients are featured by altered tight junction structure and epithelial permeability.^{9,10} Long-myosin light-chain kinase (MLCK1) is predominantly expressed in IECs and mediates tight junction permeability by driving actomyosin contraction through Myosin light chain (MLC) phosphorylation.¹¹ Clinical and experimental studies have demonstrated that MLCK is closely involved in IBD as an upstream effector to cause disrupted tight junction and resultant increased permeability.^{12–14} Despite tumor necrosis factor (TNF α) being shown to activate MLCK1 expression,¹³ little is known about whether other immunological factors regulate MLCK1 to influence epithelial integrity.

STAT6 is well-known to mediate Th2 cell immunity following interleukin (IL)-4 and IL-13 activation, playing important roles in the clearance of intestinal parasites and allergic disease.^{15,16} It is also long noted that STAT6 promotes tumor growth by inducing immunosuppressive microenvironment.¹⁷ However, the role of STAT6 in the development of colitis and CAC is poorly understood.

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Although STAT6 has been mainly demonstrated to function in immune cells, there is a growing appreciation of its role in non-immune cells, including goblet cells and smooth muscle cells.¹⁶ STAT6 activation was detected in both IECs and lamina propria leukocytes (LPLs) from UC patients.^{18,19} Interestingly, p-STAT6 staining was more commonly detected in lesional colon tissues from active IBD patients than those from CAC,^{18,19} suggesting that STAT6 might serve as a causal factor for CAC by mediating pre-existing intestinal inflammation favoring colonic tumorigenesis. However, it is unclear how STAT6 in IECs and immune cells contributes to colitis development and subsequent tumorigenesis, respectively.

In this study, we demonstrated that STAT6 deficiency in non-immune cells rendered the protection against colitis and colitis-driven tumorigenesis. Mechanistically, we revealed that STAT6 directly upregulated MLCK expression to induce dysregulated tight junction function and microbiota translocation, thereby creating a colonic inflammatory milieu to promote colitis and tumorigenesis. Finally, we demonstrated that STAT6 inhibitor significantly inhibited murine colon tumor formation and growth, and tumoral p-STAT6 levels were positively correlated with the clinical stage and poor prognosis of patients with colorectal cancer.

RESULTS

Non-hematopoietic STAT6 is required for DSS-induced colitis

We first detected p-STAT6-positive staining in colonic epithelium and lamina propria at different time point post dextran sulfate sodium (DSS) (Fig. 1a). Upregulated p-STAT6 levels in IECs (CD45⁺EpCAM⁺) and CD45⁺LPLs were confirmed by western blotting and flow cytometry (Fig. 1b, c). Whole-body ablation of STAT6 significantly attenuated DSS-induced colitis (Supplementary Fig. S1a–d), which was consistent with a recent study.²⁰ We then aimed to determine the respective contribution of STAT6 in immune cells and non-immune cells to DSS-induced colitis by generating BM chimeric mice. WT→STAT6^{-/-} and STAT6^{-/-}→STAT6^{-/-} mice were similarly protective from DSS-induced colitis, as evidenced by lesser body weight loss and disease activity index (DAI) scores, but longer colon length on day 7 when compared to STAT6^{-/-}→WT and WT→WT mice, which developed colitis in similar extent of severity (Fig. 1d–f). Further histological analysis revealed WT→STAT6^{-/-} and STAT6^{-/-}→STAT6^{-/-} mice had obviously reduced inflammatory infiltrates accompanying by better preserved structural integrity and more numbers of PAS-positive goblet cells (Fig. 1g). Although previously reported promoting effect of STAT6 on mucus secretion,²¹ it could be explained by better preserved goblet cells secondary to less epithelium injury in these chimeras. Colonic neutrophilia is a hallmark of human IBD and DSS-induced colitis.²² Furthermore, lack of non-hematopoietic STAT6 caused significant reductions in levels of pro-inflammatory cytokines and neutrophil infiltration (Fig. 1h, i), with no effect on other immune cell types (Supplementary Fig. S1e). Similarly, STAT6^{-/-} mice had attenuated colonic inflammation (Supplementary Fig. S1f, g). Interestingly, STAT6 deficiency did not affect the activation state of colonic macrophages, as frequencies of total macrophages and CD206⁺M2-like macrophages, as well as the expression of related effector molecules were comparable in DSS-exposed colons of WT and STAT6^{-/-} mice (Supplementary Fig. S1h). These results together demonstrate that STAT6 in non-hematopoietic cells, but not hematopoietic cells, are responsible for DSS-induced colitis.

Co-housing has no effect on DSS-induced colitis in WT mice and STAT6^{-/-} mice

To investigate whether attenuated colitis in STAT6^{-/-} mice was dependent on microbiota, which may be altered by lack of STAT6, WT, and STAT6^{-/-} mice (1:1 per cage) were co-housed for 1 month followed by DSS treatment. There were no differences in the severity of DSS-induced colitis and colonic levels of

pro-inflammatory cytokines between co-housed and separately housed WT and STAT6^{-/-} mice (Supplementary Fig. S2a–d). These results demonstrate that co-housing with STAT6^{-/-} mice fails to confer protection to WT mice against DSS-induced colitis, suggesting that attenuated colitis in STAT6^{-/-} mice is not a result of altered gut microbiota.

Non-hematopoietic STAT6 increases gut permeability preceding overt mucosal inflammation

Exposure of luminal bacteria due to disrupted epithelial barrier could cause excessive innate inflammatory responses, leading to colitis development.^{5,23} STAT6 was previously shown to mediate IL-13-induced permeability of colon epithelial cell monolayers.¹⁹ We therefore speculated that STAT6 activation in IECs may directly increase gut permeability and microbiota translocation, contributing to DSS-induced colitis. WT→STAT6^{-/-} and STAT6^{-/-}→STAT6^{-/-} mice had significantly lower colon 16S rDNA content and serum levels of FITC-dextran than STAT6^{-/-}→WT and WT→WT mice on day 7 post DSS (Fig. 2a, b). Consistently, WT→STAT6^{-/-} and STAT6^{-/-}→STAT6^{-/-} mice had significantly lower numbers of bacteria in the colonic LP (Fig. 2c). Furthermore, lack of non-hematopoietic STAT6 reduced bacterial number in mesenteric lymph nodes (MLN), spleen, and blood (Fig. 2c), suggesting a less dissemination into secondary lymphoid organs and periphery. We then attempted to determine whether improved gut permeability in DSS-exposed STAT6^{-/-} mice was a cause rather than a consequence of less inflammatory microenvironment, since TNF-α and IFN-γ have been shown to influence gut permeability during intestinal inflammation.^{24,25} To this end, we compared gut permeability between WT and STAT6^{-/-} mice on day 3 post DSS when there was no detectable colonic inflammation (Fig. S3a–d), but upregulated STAT6 activation (Fig. 1a, b). As shown in Fig. 2d–f, colon 16S rDNA content, serum levels of FITC-dextran and bacteria counts in LP were reduced in STAT6^{-/-} mice. No bacteria counts were detected in secondary lymphoid organs and periphery in both WT and STAT6^{-/-} at this early time point (Fig. 2f). Epithelial apoptosis also could impair epithelial barrier integrity. We found comparable numbers of TUNEL-positive apoptotic cells in colon sections of WT and STAT6^{-/-} mice (Fig. 2g), suggesting an apoptosis-independent mechanism. Collectively, these results suggest that non-hematopoietic STAT6 activation directly increases gut permeability, thereby contributing to microbiota-driven colitis.

STAT6 transcriptionally regulates MLCK1 and alters epithelial tight junction integrity

Intercellular tight junctions consisting of multiple proteins are critical for the maintenance of epithelial integrity.^{5,26} We found that non-hematopoietic STAT6 caused significantly higher levels of zonula occludens-1 (ZO-1) and occludin, lower claudin-2 and comparable claudin-1 in colons on day 7 (Supplementary Fig. S4), suggesting a better preserved epithelial tight junction integrity. Given that excessive colonic inflammation could cause dysfunctional tight junction, we chose to examine the colons on day 3 post DSS to determine whether STAT6 directly regulated epithelial tight junction proteins and screened the potential target. RNA sequencing was performed to analyze differential gene expression of tight junction proteins in colons of WT and STAT6^{-/-} mice on day 3 (GSE113541). STAT6 deficiency caused altered gene expression of various tight junction proteins (Fig. 3a). Of note, *Mylk* that encodes MLCK1 was most affected by STAT6 deficiency (about 2.5-fold reduction) (Fig. 3a). Immunohistochemistry (IHC) staining confirmed much more pronounced change in MLCK1 expression than other known important tight junction proteins in STAT6^{-/-} mice on day 3 (Fig. 3b). To confirm the direct act of STAT6 on tight junction proteins in IECs, we performed intestinal epithelial organoid culture by isolating intestinal crypts from colons of WT and STAT6^{-/-} mice followed by IL-13 stimulation.

Fig. 1 Non-hematopoietic STAT6 is required for DSS-induced colitis. **a** Representative photomicrographs of IHC staining of phosphorylated-STAT6 (p-STAT6) in colons. Scale bar, 50 μ m, 400 $\times$. **b**, **c** Representative western blots (**b**), representative flow cytometry data (left) and quantification (right) of p-STAT6 levels in IECs and LPLs (**c**) ($n = 3$). **d–f** Body weight curve (**d**), colon length (**e**), and disease activity index (DAI) (**f**) of bone marrow (BM) chimeric mice on day 7 post DSS. **g** Representative photomicrographs of H&E and PAS-staining colon sections (Scale bar = 200 μ m), as well as histopathological score and quantification of goblet cells (100 $\times$ HPF). **h** TNF- α , IL-6, and IFN- γ concentrations in the colon tissue homogenates. **i** The percentages of neutrophil (CD11b⁺Gr-1⁺) in CD45⁺ leukocytes from enzymatically digested colon tumor tissues. The data were presented as mean $\pm$ S.E.M. ($n = 5–7$ per group). Results are representative of three independent experiments. One-way or two-way ANOVA test

Consistently, MLCK1 was most affected by IL-13 stimulation (Fig. 3c), which was completely blunted in STAT6^{-/-} intestinal epithelial organoids (Fig. 3d). Moreover, IL-13 upregulated MLCK1 expression at both gene and protein levels in human intestinal epithelial cell line Caco2, which was completely abrogated by STAT6 knockdown (Fig. 3e, g). IL-13 stimulation significantly reduced transepithelial electrical resistance (TER) in Caco-2 cells during different time points investigated, which was completely abrogated by STAT6 knockdown (Fig. 3f). These data suggest that MLCK1 induced by IL-13-STAT6 signaling could mediate the increased permeability of monolayer epithelial cells. We then evaluated whether IL-13 induced MLCK1 expression was functional by examining the phosphorylation of MLC in Caco-2 cells. As shown in Fig. 3g, obviously increased phosphorylated MLC was detected in IL-13-stimulated Caco-2 cells, and such effect was abrogated when STAT6 was knocked down (Fig. 3g). ROCK1 was known to activate MLC to induce actin-myosin contractility, thereby increasing epithelial permeability.²⁷ Interestingly, IL-13 stimulation obviously upregulated ROCK1 protein levels, which

was completely abrogated by STAT6 (Fig. 3g). Furthermore, obviously increased F-actin expression as determined by phalloidin staining was observed in Caco2 cells following IL-13 stimulation, which was absent when STAT6 was knocked down (Fig. 3h), suggesting increased actomyosin contractility may contribute to the increased epithelial permeability caused by activation of IL-13-STAT6 signaling. Collectively, these results suggest that STAT6 activation primarily transcriptionally upregulates MLCK1 expression, thereby leading to dysregulated epithelial tight junction and increased epithelial permeability.

IL-13 mediates gut permeability and DSS-induced colitis via activation of STAT6 in IECs

We next determined whether IL-13 activated epithelial STAT6 and mediated DSS-induced colitis. To this end, we intraperitoneal (i.p.) injected IL-13 neutralizing antibody to STAT6^{-/-}→WT mice in which epithelial STAT6 activation and DSS-induced colitis were preserved (Fig. 4a–e). IL-13 neutralization almost completely inhibited STAT6 activation in IECs of STAT6^{-/-}→WT mice (Fig. 4a) accompanied by

Fig. 2 IL-13-STAT6 signaling increases gut permeability preceding overt mucosal inflammation. **a, b** Colon 16S rDNA contents (**a**) and serum levels of FITC-dextran concentrations (**b**) in BM chimeric mice at 4 h after oral administration on day 7 post DSS. **c** Bacterial colony-forming units (CFU) counts on day 7 post DSS. **d–f** Colon 16S rDNA contents (**d**), serum levels of FITC-dextran (**e**), and bacterial CFU counts (**f**) in WT and *STAT6*^{-/-} mice on day 3 post DSS. **g** Representative photomicrographs of TUNEL staining (Scale bar = 100 μm) and quantification of TUNEL-positive cells (200x HPF) in colons on day 3 post DSS. The data were presented as Mean ± S.E.M. (*n* = 5–7 per group). Results are representative of three independent experiments. One-way ANOVA (**a–f**) or two-tailed Student's *t*-test (**g**)

attenuated DSS-induced colitis (Fig. 4b–e). Moreover, IL-13 neutralization significantly reduced gut permeability and luminal bacteria translocation and dissemination in *STAT6*^{-/-}→WT mice (Fig. 4f–h). Interestingly, neutralization of IL-4, another well-known STAT6 activator, had no effect on DSS-induced colitis, although both cytokines were upregulated in the colons of WT mice over time post DSS (Supplementary Fig. S5a–e). These results together demonstrated IL-13 as the upstream cytokine to activate epithelial STAT6. We found that IL-13Rα1 only used by IL-13 was expressed in IECs in a much higher level than those in LPLs while γc used by IL-4 was exclusively expressed in LPLs. IL-4Rα, the receptor shared by both cytokines, was expressed in IECs and LPLs (Supplementary Fig. S5f). The differential cell distribution of IL-13Rα1 and γc suggests IECs to be more sensitive to IL-13 stimulation, which could explain why neutralization of IL-13 rather than IL-4 effectively attenuated DSS-induced colitis.

Type 2 innate lymphoid cells (ILC2) are the major cell source for IL-13 in DSS-exposed colons, which is regulated by gut microbiota. We next attempted to identify the cell source of IL-13 in DSS-induced colitis by flow cytometric analysis of YetCre13-Rosa-YFP amplifier reporter mice. IL-13-YFP were detected in ILC2 and NKT cells, but not in other immune cell types or CD45⁻ non-immune cells (Fig. 5a). There were baseline levels of IL-13-YFP⁺ ILC2, which were markedly enhanced at days 3 and 7 post DSS (Fig. 5a, b), coincident with upregulated IL-13 levels (Supplementary Fig. S5a). The frequencies of IL-13-YFP⁺ NKT cells were much less than IL-13-YFP⁺ ILC2, and only slightly upregulated post DSS (Fig. 5a, b). Consistently, much higher IL-13 gene levels were

detected in isolated LPLs than IECs at different time point post DSS (Fig. 5c). IL-25 and IL-33, the important activators of ILC2, were also markedly upregulated in IECs post DSS (Fig. 5c). To examine whether gut microbiota was required, we treated mice with a combination of broad-spectrum antibiotics to deplete gut microflora. Antibiotic treatment completely inhibited upregulation of IL-13, as well as IL-25 and IL-33 in DSS-exposed colons (Fig. 5d). Notably, antibiotic treatment even reduced baseline levels of IL-13, IL-25, and IL-33 in colons (Fig. 5d). These results together suggest that epithelial expression of IL-25 and IL-33 is dependent on microbiota, which may result in the maintenance and activation of ILC2 and resultant IL-13 production.

Non-hematopoietic STAT6 is required by inflammation-driven intestinal tumorigenesis

Given the above data showing a critical role of non-hematopoietic STAT6 in colitis development, we speculated that non-hematopoietic STAT6 activation during colitis phase may serve as a causal factor for inflammation-driven cancer. To test our hypothesis, we used AOM + DSS model, a well-characterized experimental CAC model. WT→*STAT6*^{-/-} mice, but not *STAT6*^{-/-}→WT mice, had significantly decreased tumor numbers with smaller size and less tumor load when compared to WT→WT mice (Fig. 6a–c). Histological analysis revealed much fewer colon tumors with lower grade accompanied by much fewer Ki-67⁺ proliferating tumor cells in WT→*STAT6*^{-/-} mice (Fig. 6d, e). STAT3 is known to be a major driver of CAC by influencing the fate of epithelial cells under chronic inflammatory condition.²⁸ Significant reductions in p-STAT3⁺ cells numbers and IL-6 levels

Fig. 3 STAT6 transcriptionally regulates long-myosin light-chain kinase 1 (MLCK1) and alters epithelial tight junction integrity. **a** The heat-map picture of tight junction proteins by RNA-sequencing analysis of colons on day 3 post DSS (*n* = 3 per group). **b** Representative photomicrographs of H&E-staining and IHC staining (left, Scale bar = 100 μm) and quantification of positive staining of each protein (right, 200x HPF) in colons from WT or *STAT6*^{-/-} mice on day 3 post DSS (*n* = 5–7 per group). **c** Gene expression of tight junction proteins in colon epithelial organoid. **d** Gene expression of *Mylk*/MLCK1 in colon epithelial organoid. **e–h** Gene level of *Mylk*/MLCK1 (**e**), transepithelial electrical resistance (TER) (**f**), western blot analysis of STAT6, ROCK1, p-MLC, t-MLC, *Mylk*/MLCK1 (**g**), representative images of phalloidin staining of F-actin (**h**) in human colon epithelial cell line Caco2, which were transfected with scramble or STAT6 siRNA followed by stimulation with IL-13 (20 ng/ml). Data are represented as mean $\pm$ SEM. Data in **c** and **d** are pooled from three independent experiments, and data in **e** and **f** are represented as mean $\pm$ SEM of three replicate wells from a representative of three independent experiments with similar results. Two-tailed Student's *t*-test (**b–d**) or one-way ANOVA (**e, f**)

were found in tumor tissues of WT $\rightarrow$ *STAT6*^{-/-} mice, but not *STAT6*^{-/-} $\rightarrow$ WT mice, when compared with WT $\rightarrow$ WT mice (Fig. 6d–f). There were also significant decreases in frequencies of myeloid cells, including Gr-1⁺ cells and macrophages, as well as significant increases in IFN- γ ⁺CD4⁺ T cells and IFN- γ ⁺CD8⁺ T cells in tumors of WT $\rightarrow$ *STAT6*^{-/-} mice (Fig. 6g). In addition, we noted significant changes in tumor-infiltrating macrophages, mainly CD206⁺M2-like macrophages, and IFN- γ -expressing T cells in *STAT6*^{-/-} $\rightarrow$ WT mice, albeit in lesser extents than those observed in WT $\rightarrow$ *STAT6*^{-/-} mice (Fig. 6g), suggesting that hematopoietic STAT6 may directly mediate immunosuppression via induction of M2-like macrophage in the tumor microenvironment. Collectively, these results demonstrate that non-hematopoietic STAT6 is

critical for tumor formation by creating a more inflammatory environment.

STAT6 inhibitor effectively suppresses colon tumor formation in *Apc*^{min/+} mice

To evaluate the potential therapeutic efficiency by targeting STAT6, we i.p. injected STAT6 inhibitor, AS1517499, to *Apc*^{min/+} mice, which spontaneously develop intestinal tumor. We confirmed the efficiency of STAT6 inhibition by showing very few p-STAT6⁺ cells in colons of AS1517499-treated *Apc*^{min/+} mice (Fig. 7b). Importantly, STAT6 inhibition significantly reduced tumor numbers and tumor load accompanied by markedly reduced p-STAT3⁺ and Ki67⁺ cells (Fig. 7a, b). These results together

Fig. 4 IL-13 activates epithelial STAT6, leading to enhanced gut permeability and DSS-induced colitis. **a** Representative flow cytometry data of p-STAT6 expression. $n = 3$. **b–e** Neutralizing IL-13 antibodies (10 μ g/mouse) were i.p. administered during DSS treatment. Body weight curve (**b**), colon length and DAI (**c**) of BM chimeric mice on day 7 post DSS. **d** Representative photomicrographs of H&E-staining colon sections and histopathological score. Scale bar = 200 μ m, 100 $\times$. **e** TNF- α and IL-6 concentrations in the colon tissue homogenates. **f–h** colon 16S rDNA content (**f**), serum levels of FITC-dextran (**g**) and bacterial CFU counts (**h**) in BM chimeric mice on day 7 post DSS. The data were presented as mean $\pm$ S.E.M. ($n = 7$ per group). Results are representative of three independent experiments. One-way ANOVA

demonstrate that pharmacological inhibition of STAT6 efficiently inhibits intestinal tumor growth.

Increased STAT6 activation is positively associated with poor prognosis of human colorectal cancer

To assess the clinical relevance of STAT6 activation to colorectal cancer progression, we performed IHC-based tissue microarray analysis (TMA) of patients with colorectal cancer ($n = 100$) by p-STAT6 staining (Fig. 7c). The clinic information was summarized in Supplementary Table S1. Compared with normal colon tissues, tumor areas had more p-STAT6⁺ cells, which positively correlated with the clinical stage of colorectal cancer (Fig. 7c). Of note, p-STAT6⁺ staining was mostly located in epithelial tumor cells during the early stages of colorectal cancer, whereas much more p-STAT6⁺ cells were found in infiltrating stromal cells, including immune cells over cancer progression during late stage III and IV (Fig. 7d). More importantly, colorectal cancer patients with higher tumoral p-STAT6 levels had significantly lower 3- and 5-year survival rate of than those with lower p-STAT6 levels (Fig. 7e).

These data demonstrate that tumoral p-STAT6 levels could be a potential indicator of the progression and prognosis of patients with colorectal cancer.

DISCUSSION

Our study here reveals a critical role of non-hematopoietic STAT6 in the induction of colitis and consequent tumorigenesis. Specifically, epithelial STAT6, which could be activated by gut-resident ILC2-producing IL-13, transcriptionally upregulates MLCK1 to cause epithelial tight junction dysfunction, thereby initiating microbiota-driven inflammatory cascade that facilitates colonic tumorigenesis (Fig. 7f).

Extensive attention has been paid to the role of STAT6 in the activation of immune cells. We here demonstrated that hematopoietic STAT6 was dispensable for DSS-induced colitis. Instead, our data strongly supported the contribution of STAT6 in non-hematopoietic cells, most likely IECs, to DSS-induced colitis by enhancing gut permeability to induce microbiota-driven colonic

Fig. 5 Type 2 innate lymphoid cells (ILC2) are major cell source of IL-13 in DSS-exposed colons, which are regulated by gut microbiota. **a** Gating strategy to identify IL-13-YFP⁺ cells in the colons. **b** The percentages of IL-13-YFP⁺NKT cells (NK1.1⁺CD3⁺) and IL-13-YFP⁺ILC2 (Lin⁻Sca-1⁺KLRG1⁺) cells ($n = 3$). **c** Gene expression of IL-13, IL-25, and IL-33 ($n = 3$). **d** IL-13 concentrations and mRNA levels of IL-25 and IL-33 in the colons. The data were presented as mean $\pm$ S.E.M ($n = 7$ per group). Results are representative of three independent experiments. One-way ANOVA

inflammation characterized by markedly increased levels of pro-inflammatory cytokines and neutrophilia. Although previous studies showed that IL-4/IL-13 induced adhesive molecules in endothelial cells via STAT6,^{29–31} IL-4/IL-13 induced adhesion of eosinophils but not neutrophils to HUVECs under flow.³² Thus, decreased neutrophilia is unlikely to be secondary to STAT6

deficiency in endothelial cells but rather could be due to less colonic inflammation in these mice. Notably, STAT6 activation caused altered tight junction protein and enhanced intestinal epithelial permeability in our model, stressing a direct effect of STAT6 on the disruption of epithelial tight junction integrity. MLCK1 is a key upstream regulator of pathophysiological tight

Fig. 6 Non-hematopoietic STAT6 promotes inflammation-driven intestinal tumorigenesis. **a, b** Picture of colon tumors (**a**), tumor load (**b**), and the number of tumors in different size (**c**) in BM chimeric mice on day 81 of the AOM + DSS-induced CAC model. **d** Representative photomicrographs of H&E-staining (Scale bar = 2 mm or 500 μm) and IHC staining of Ki67 and p-STAT3 (Scale bar, 50 μm). **e** Quantification of Ki67⁺ cells and p-STAT3⁺ cells (200x HPF). **f** IL-6 concentrations in the colon tumor homogenates. **g** The percentages of infiltrating immune cells. The data were presented as mean ± S.E.M. ($n = 7$ per group). Results are representative of three independent experiments. One-way ANOVA

junction function, which could be activated by TNFs.^{13,33,34} Our study provided evidence identifying MLCK1 as the target of STAT6 in IECs, which serves as an upstream factor to cause epithelial tight junction dysfunction, thereby promoting colonic inflammation, as MLCK1 was screened to be most affected tight junction protein in STAT6^{-/-} mice on day 3 when there was no detectable intestinal inflammation. This was also supported by our in vitro studies showing that IL-13-induced MLCK1 expression and epithelial permeability was accompanied by increases in MLC phosphorylation and F-actin expression in Caco-2 cells in a STAT6-dependent manner. Interestingly, we found that IL-13-STAT6 activation also induced ROCK1 expression in Caco-2 cells, suggesting that, in addition to MLCK1, STAT6 may target other downstream molecules to impair epithelial tight junction integrity.

Our study demonstrated that IL-13, which was primarily derived from ILC2, was upregulated during DSS injury, and mediated epithelial STAT6 activation and DSS-induced colitis. Furthermore, upregulation of IL-13 as well as IL-25 and IL-33, the activators of ILC2s, were completely dependent on gut microbiota. This is consistent with previous studies showing that IL-25 was driven by gut microbiota.^{35,36} Our findings are in contrast to previous studies showing NKT cells as the major source for IL-13 in oxazolone (OXA)-induced colitis.^{37,38} This could be explained by

different types of immune cells activated by stimuli with different nature, mediating colitis development. Recent studies demonstrated that OXA activated AHR in IECs, which resulted in iNKT-mediated intestinal inflammation independently of microbiota.³⁸ In contrast, DSS-induced colitis is primarily mediated by the intolerance to microbiota due to disrupted epithelial barrier. Thus, it could be speculated that cytokine cues from microbiota-exposed epithelium may activate gut-resident ILC2s to produce IL-13, thereby activating STAT6 to disrupt tight junction integrity and in turn cause excessive microbiota exposure under some conditions, finally initiating a vicious cycle of inflammation.

Moreover, we demonstrated that STAT6 in non-hematopoietic cells, but not hematopoietic cells, is critical for inflammation-driven tumorigenesis. It is well-accepted that STAT3, a critical inflammatory transcription factor, is important for CAC development.³⁹ Consistent with reduced tumor formation, markedly reduced STAT3 activation and cell proliferation were observed in colon tumors of mice that lacked non-hematopoietic STAT6. Very recent studies reported that STAT6 promoted colon tumor development via induction of myeloid cell-mediated immunosuppressive microenvironment.^{20,40} Consistently, we found that, despite no effect on colitis-driven tumorigenesis, hematopoietic STAT6 deficiency resulted in fewer numbers of large-size tumor

Fig. 7 STAT6 inhibitor effectively suppresses murine intestinal tumor formation and tumoral p-STAT6 levels is positively associated with poor prognosis of human colorectal cancer. **a** *Apc*^{min/+} mice were i.p. injected with STAT6 inhibitor AS1517499 (10 mg/kg) or DMSO every 2 days starting from 8th week and lasting 8 weeks. The tumor number (up) and tumor load (down) from the small intestines or colons of *Apc*^{min/+} mice. **b** Representative photomicrographs of IHC staining (up, Scale bar = 50 μ m) and quantification of p-STAT6⁺, p-STAT3⁺, and Ki67⁺ cells (down, 400x HPF). (*n* = 7 per group for **a**, **b**. Results are representative of three independent experiments. **c** Representative photomicrographs of p-STAT6 staining (scale bar = 500 μ m and 100 μ m) and quantification of p-STAT6⁺ cells (200x HPF) in colon tumors from the different clinical TNM stage of patients with colorectal cancer. *n* = 100. **d** Quantification of the numbers of p-STAT6⁺ epithelial cells (ECs) and p-STAT6⁺ non-ECs (200x HPF) in colorectal cancer patients with different stages. **e** The relationship between tumoral p-STAT6 expression and overall survival was evaluated in the patients. *n* = 100, *p* = 0.0197, Kaplan–Meier method followed with log-rank test. **f** Schematics of a proposed model. The data were presented as mean $\pm$ S.E.M. Two-tailed Student's *t*-test (**a**, **b**) or one-way ANOVA (**c**, **d**)

accompanied by decreased M2-like macrophages but increased anti-tumor T-cell immunity. Overall, we propose that STAT6 contributes to CAC development through distinct mechanism during the different stage: increasing gut permeability to create an inflammatory environment favoring tumor transformation at colitis stage and inducing tumor immunosuppression to promote tumor growth during CAC stage. Importantly, we demonstrated that the important role of STAT6 in CAC development could be translated into the therapeutic potential, as administration of STAT6 inhibitor efficiently suppressed murine intestinal tumor

formation and growth, and increased p-STAT6 levels were found in human colon tumors, which was positively associated with the clinical stage and poor prognosis of patients with colorectal cancer.

The currently most efficacious IBD treatment is anti-TNF α antibodies, to which, however, only one-third of the patients are responsive. Extensive studies have demonstrated the dual role of STAT3 in mediating tissue repair as well as in enhancing tumor growth,^{41,42} suggesting inhibition of STAT3 signaling may enhance colitis-associated mucosal injury, although it could be

beneficial for treating colorectal cancer. Instead, our study revealed the promoting effects of STAT6 on both colitis and colorectal cancer, and moreover stressed the important contribution of STAT6 in non-hematopoietic cells, most likely IECs, to colonic inflammation and subsequent colonic tumorigenesis via induction of MLCK1-dependent epithelial tight junction dysfunction and microbiota translocation. Given that increased intestinal permeability is observed in people with high risk for IBD, and IBD patients have greater risk for developing colorectal cancer, STAT6 may have potential as a promising prophylactic or therapeutic target for colitis and colorectal cancer, at least in a subset of patients with elevated mucosal STAT6 activation.

METHODS

Animals and cell lines

BALB/c mice were purchased from Chinese Academy of Sciences (Shanghai, China). C.129S2-*Stat6*^{tm1Gru/J}, C.129S4 (B6)-*Il13*^{tm1(YFP/cre)Lky/J}, C57BL/6J *Apc*^{Min/J}, B6.Cg-Gt(*ROSA*)26Sor^{tm32/J} were purchased from The Jackson Laboratory. C.129S4 (B6)-*Il13*^{tm1(YFP/cre)Lky/J} mice were crossed with B6.Cg-Gt(*ROSA*)26Sor^{tm32/J} mice to generate YetCre13-Rosa-YFP amplifier reporter mice. All mice were kept and bred in a specific pathogen-free environment in the animal facility of Shanghai Medical College, Fudan University. Animal experiments were approved by the Animal Care and Use Committee at Fudan University. Mice were given 3% DSS (36,000–50,000 MW; MP Biomedicals) for 7 days to induce colitis. IL-13 was neutralized by intraperitoneally (i.p.) injecting αIL-13 (10 μg/mouse, R&D Systems) on day 1 and 4 during DSS treatment. The disease activity index (DAI) was scored according to the average of three parameters: stool consistency (0, 2, 4), fecal blood (0, 2, 4), and percentage weight loss (0–4). To establish CAC model, mice were i.p. administrated with AOM (12 mg/kg) (day 0 and day 20) followed 3% DSS for 5 days (day 7, day 27, and day 48). For *Apc*^{min} spontaneous intestinal tumor, the average tumor size was determined by averaging diameters of all tumors present. Tumor load was determined as the sum of the diameters of all tumors presented. For generating bone marrow chimeras mice, recipient WT and *STAT6*^{−/−} mice were irradiated twice (5 Gy/mouse each time) with a 4 h interval followed by intravenous injection with 10⁶ bone marrow cells of donor mice. The human IEC line Caco-2 was obtained from ATCC (Manassas, VA) and cultured by Eagle's Minimum Essential Medium with 10% fetal bovine serum. The cell lines were tested to exclude mycoplasma contamination before experiments.

Isolation of colonic cells

LPLs and IECs were isolated as previously described.³⁷ Briefly, colons were removed and cut into 2 mm and stirred in Hank's buffered salt solution (HBSS) containing 5 mM EDTA at 37 °C twice for 30 min each. The remaining tissues were further cut and digested by 1 mg/ml collagenase type VI, 10U DNase I (Sigma) for 1 h in 37 °C. The single-cell suspension of digested colon tissues was then subjected to 75%/44% gradient Percoll to purify IEC and LPLs.

Flow cytometry

For cell surface staining, single-cell suspension was pre-incubated with anti-CD16/32 and then stained with the appropriate fluorochrome-labeled antibodies that were listed in Supplementary Table S2. For p-STAT6 staining, cells were fixed and permeabilized, and then stained with fluorochrome-labeled STAT6 antibodies. Flow cytometer data were acquired using a Celesta flow cytometer (BDSciences) and analyzed by Flowjo software (Treestar). The antibodies were listed in Supplementary Table S2.

Enzyme-linked immunosorbent assay (ELISA)

The whole-colon tissues were homogenized in 2 ml phosphate-buffered saline (PBS) with protease inhibitors (#78429, Thermo Scientific). The supernatants were collected for ELISA analysis. The

protein levels were normalized to the length of colon that was collected for homogenization. The concentrations of IL-6, TNF-α, IFN-γ, IL-4, and IL-13 were measured using Mouse ELISA Due Set kits from R&D Systems as listed in Supplementary Table S2.

Quantitative real-time PCR (qPCR)

RNA was isolated from colon tissues or cells by TRIzol (Invitrogen) and reverse-transcribed into cDNA by PrimeScript RT Master Mix (#A360, TaKaRa). qPCR was performed by Applied Biosystems 7500 using Power SYBR Green Master kit (#RR820A, TaKaRa). The relative expression of target gene was calculated using the 2^{−ΔC_t} method. Fold change of target gene expression were calculated by normalization to control group. The primer sequences are shown in Supplementary Table S3.

Histology and microscopy analysis

Paraffin-embedded colon tissue sections were stained with hematoxylin and eosin (H&E) or Periodic Acid Schiff (PAS). TdT-mediated dUTP nick end labeling (TUNEL) was performed to determine the apoptotic cells in colon tissue sections. IHC staining of p-STAT6, Ki67, p-STAT3, Claudin-1, Claudin-2, ZO-1, Occludin1, and MLCK1 were performed using corresponding antibodies as listed in Supplementary Table S2. The F-actin was determined by phalloidin staining according to the manufacturer's protocols (#ab176753, Abcam). The values of density were quantified by Image-Pro Plus v6.2 software and cells that were positive for nuclei p-STAT6, Ki67, p-STAT3, or TUNEL were counted in randomly chosen five 200x high-power magnification (HPF).

Bacterial 16S rDNA measurement

Extraction of bacterial genomic DNA was performed as previously described.⁴³ Briefly, the luminal feces were flushed with 3 ml sterile PBS. The remaining colons were flushed vigorously to collect the bacteria, which were adherent to the mucosal surface and the inner mucus layer with 2 ml sterile PBS. Genomic DNA was extracted from the bacterial pellets with the Qiagen Stool Kit (Qiagen, #51504). 16S rDNA primer sequences were used: -For (5'-ACTCCTACGGGAGGAGCAGCT-3') and -Rev (5'-ATTACCGCGGCTGCTGG C -3').

Epithelial permeability assay

Mice were gavaged with FITC-Dextran (60 mg/kg per mouse, 4 kDa, 46944, Sigma-Aldrich). Fluorescence was measured in the serum with a fluorimeter after 4 h (Excitation 490 nm; Emission 520 nm). Serial dilutions of FITC were used as standards ranging from 0–20 μg/ml.

Bacterial translocation

Colons, the whole spleen and MLN were subsequently homogenized in 500 μl sterile PBS. Blood and homogenates of colons, spleen, MLN were serially diluted and plated onto blood agar medium overnight followed by enumeration of bacterial colony-forming units CFU for each sample.²³

RNA-seq and analysis

Total RNAs from colon tissue were isolated with trizol and the RNA-seq library was prepared by the Beijing Genomics Institute. Sequence reads were obtained using BGISEQ500 and successfully mapped to mouse genome. Reads counts were normalized based on RPKM (Fragments Per Kilobase per Million mapped reads). Genes encoding tight junction protein with at least 50 FPKM expression value were used. Heatmaps were drawn using multiple array viewer. The accession number for the RNA-seq is: GSE113541.

Colon organoid culture

Murine colon crypts were isolated following procedures described previously.⁴⁴ In brief, the colon was washed and scraped by a Cell

Scraper. The colon was cut and incubated in liberate buffer (5 mM EDTA, 1 mM DTT in HBSS) for 15 min on 37 °C. The tissue was further digested with collagenase XI (50 ng/ml) and Dispase I (20 ng/ml) in HBSS for 15 min on 37 °C. Crypts were released by vortexing followed by passing through a 70- μ m strainer (BD Biosciences). Crypt-containing fractions were collected and resuspended in Advanced DMEM/F12 (Thermo Fisher Scientific). Colon organoids were grown in 50 μ l droplets of Matrigel overlaid with Advanced DMEM/F12 containing B27, Noggin (100 ng/ml), N-acetylcysteine (2 μ M), mouse EGF (50 ng/ml, Peprotech). Five days later, the organoids were stimulated for 6 h.

Western blotting and RNA Interference

Cells or colonic biopsy specimens were homogenized in 2 ml iced PBS buffer with protease inhibitor cocktail (Roche). Aliquots of 10 μ g protein were separated by polyacrylamide gel electrophoresis and transferred to a poly vinylidene difluoride transfer membrane. The antibodies used for western blotting were shown in Supplementary Table S2. Small-interfering RNA (siRNA) targeting human *STAT6* (5'-AAACGAGAGUG UUAUAACUGUTT-3') was synthesized by GenePharma (GenePharma). Human *STAT6* siRNA or scramble siRNA was transfected into Caco2 cells by using Lipofectamine RNAiMAX according to the manufacturer's protocols (Thermo Fisher Scientific, #13778030). Transfected Caco2 were then treated with IL-13 (20 ng/ml) for 24 h.

Transepithelial electrical resistance (TER) measurement

The integrity of tight junctions was monitored by measuring transepithelial electrical resistance (TER) using a Millicell Electrical Resistance System (Merck Millipore). TER was measured as ohms (Ω) $\times$ cm², and fluid resistance was subtracted. The Caco2 cell monolayers transduced with *STAT6* siRNA or Scramble siRNA were grown in the upper chamber of 24-well transwell plate (pore size, 0.4 μ m, surface area, 0.33 cm², Millipore) for 2 weeks until the TER value reached maximal polarity (> 330 Ω /cm²). Then, these cells were treated with or without IL13 (20 ng/ml, Peprotech). The TER values were measured at 6, 12, and 24 h after IL-13 stimulation. The TER values for the cell-free control wells were subtracted from the obtained values to remove background values. The assay was tested at least three times in triplicate.

Human samples

Formalin fixed paraffin-embedded human colon cancer specimens were randomly collected between January 2003 and December 2005 ($N = 100$) at Shanghai General Hospital, Shanghai Jiaotong University. The study protocol was approved by the Institute Review Board of Shanghai General Hospital, Shanghai Jiaotong. Follow-up data were summarized at the end of December, 2014. Overall survival (OS) was defined as the interval between the dates of surgery and death. For survived patients, the data were censored at the date of death or the last follow-up. TMAs were constructed by Shanghai Guge Co, Ltd.

Statistical analysis

The data were presented as mean $\pm$ S.E.M. Two groups' comparisons were performed by two-tailed Student's *t*-tests. Multiple-group comparisons were performed by one-way or two-way ANOVA followed by a Bonferroni correction. A Pearson correlation was used to evaluate the association. Survival was determined by the Kaplan–Meier method and calculated by log-rank test. Statistical analyses were performed by Graph Prism 7. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, ns = not significant

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AUTHOR CONTRIBUTIONS

R.H. and Y.L., designed experiments. Y.L., B.L., and X.Y. conducted experiments. Y.Z., L.J., and Z.F. provided reagents. T.L., T.S., and B.D. acquired and analyzed data. X.Y. and Y.C. provided and analyzed the human data. R.H. and Y.L. wrote the paper.

ADDITIONAL INFORMATION

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