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TL1A modulates the severity of colitis by promoting Th9 differentiation and IL-9 secretion

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ABSTRACT

Aims: TL1A was reported to contribute to the susceptibility to ulcerative colitis (UC). However, the molecular mechanisms of TL1A in UC development are poorly understood. We aimed to investigate the role of TL1A in colitis, and reveal the regulatory mechanism of TL1A in chronic colitis development.

Main methods: Wild-type mice and transgenic mice with overexpressing TL1A in lymphocytes were used to construct chronic DSS colitis models. To investigate the molecular mechanism in vitro, $CD4^+$ T cells were sorted from spleens and mesenteric lymph node cells to induce Th9 cells. Biopsy specimens from ulcerative colitis patients were collected for in vivo validation.

Key findings: The elevated TL1A expression in chronic DSS colitis models exacerbated intestinal inflammation. The differentiation of Th9 cells, IL-9 secretion and production of TGF-β, IL-4 and PU.1 was significantly enhanced in transgenic mice with TL1A overexpression. In vitro results showed that TL1A enhanced the Th9 cells, IL-9 and PU.1 production, while TL1A antibodies inhibited their production. In human translational studies, patients with ulcerative colitis with elevated TL1A expression also exhibited more serious inflammation with higher levels of Th9 cells, IL-9 and PU.1 expression.

Significance: We presented a possible mechanism of TL1A in UC development that TL1A may promote the differentiation of Th9 cells and enhanced IL-9 secretion by up-regulating the expression of TGF-β, IL-4 and PU.1, which provided a novel perspective to study the UC pathogenesis, and indicated that targeting of TL1A signal pathway may by a likely strategy for the treatment of chronic colitis.

1. Introduction

Inflammatory bowel disease (IBD), including ulcerative colitis (UC) and Crohn's disease (CD) is a chronic inflammatory diseases of the gastrointestinal tract with rising prevalence in the worldwide [1,2]. Recently, IBD was associated with risk variants in the human genome and dysbiosis of the gut microbiome [3]. Besides, it was also related to epithelial barrier function, and the exaggerated mucosal immune response to constituents of intestinal flora [4]. Although substantial advances in understanding of the IBD pathogenesis have been made, it still has not yet been fully elucidated.

T helper cells played a crucial role in providing protection against offending pathogens by secreting specific chemokines and cytokines [5]. However, unrestrained T helper cell responses may lead to tissue damage and chronic inflammation-mediated inflammatory disease [6]. It has known that aberrant pro-inflammatory responses induced by Th1, Th2, and Th17 subsets trigger IBD [7]. Currently, the spotlight has been drawn to a novel subset, Th9 cell, which predominantly secretes pro-inflammatory cytokine interleukin-9 (IL-9) [8,9]. Interestingly, Th9 was proved to induce inflammation and exacerbate inflammatory diseases, such as T cell–mediated inflammatory eye disease, autoimmune encephalomyelitis, allergic asthma, rheumatoid arthritis, colitis and multiple sclerosis [10–12]. These findings indicated that Th9 cells and IL-9 may become the potential therapeutic targets.

TL1A (*TNFSF15*) is a tumor necrosis factor-like molecule which has important role in host defense, innate and adaptive responses [13]. Genetic variations in the *TNFSF15* gene was proved to contribute to the susceptibility to IBD by a genome-wide association study [14]. Previously, Takedatsu et al. proposed that TL1A provides costimulatory signals to activated lymphocytes by combining with death receptor 3

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Table 1
Primers used for qRT-PCR analysis.

Gene	Primer sequence (forward)	Primer sequence (reverse)
TL1A	5′ CGGGGAGACGACCAAACAAG 3′	5′ AAGGAGAACGTGGCCCCAAGGTAG 3′
PU.1	5′ TGACTACTACTCCTTCGTGG 3′	5′ GATAAGGGAAGCACATCCGG 3′
TGF-β	5′ ACCGCAACAACGCCATCTATGAG 3′	5′ GGGGGTCAGCAGCCGGTTAC 3′
IL-4	5′ AGGGACGCCATGCACGGAGAT 3′	5′ GCACCTTGGAAGCCCTACAGACGA 3′
IL-9	5′ AACTGATGATTGTACCACACCGTGC 3′	5′ AGGACGGACACGTGATGTTCTTTAG 3′
GAPDH	5′ TCGTCCCGTAGACAAAATGG 3′	5′ TTGAGGTCAATGAAGGGGTC 3′

qRT-PCR, quantitative Reverse Transcription-Polymerase Chain Reaction.

(DR3), and thereby promotes the polarization and effector functions of Th1 and Th17 cells, eventually mediating the development of IBD [15]. Notably, TL1A was also proved to specifically enhance the differentiation of Th9 cells from Naïve T cell precursors in ocular inflammation [16]. Although the role of TL1A in multiple models of allergic diseases by enhancing differentiation of T helper cells has been demonstrated extensively, whether it also regulates the UC development through Th9 cells and IL-9 remains to be elucidated.

In the present study, we aimed to investigate the role of TL1A, and the relationship between TL1A and Th9 cells in models of chronic colitis. We demonstrated that the expression of TL1A was enhanced and TL1A overexpression could exacerbate intestinal inflammation, indicating that TL1A is a key factor in UC development. Furthermore, we showed that TL1A could enhance the differentiation of Th9 cells and production of cytokine IL-9. Additionally, PU.1, a transcription factor of the ETS family, TGF-β, and IL-4 were also up-regulated in chronic colitis. We presented a possible mechanism of TL1A regulation in chronic colitis for the first time that TL1A enhanced the differentiation of Th9 cells and IL-9 production by up-regulating TGF-β, IL-4 and PU.1 expression, and thereby mediated the UC development. Our findings provided a novel perspective to study UC pathogenesis, and indicated that targeting of TL1A signal pathway may be a likely strategy for treatment of chronic colitis.

2. Materials and methods

2.1. Mice

LCK-CD2-TL1A-GFP-transgenic (Tg) mice (age, 8–10 weeks; weight, 20–22 g) with overexpressing TL1A in lymphocytes were obtained from IBD of American Cedars-Sinai Medical Center and Immunology Research Center. Wild-type male mice were bred from Tg mice and wild-type male C57BL/6 mice (age, 8–10 weeks; weight, 20–22 g). All mice were in the C57BL/6J genetic background and were maintained under specific pathogen-free conditions. All mice were housed at room temperature (25 ± 2 °C), a relative humidity of 50 ± 10%, a 12 h light/dark cycle and free access to food and water. All animal protocols was carried out in accordance with Regulations for the Administration of Affairs Concerning Experimental Animals of People's Republic of China and approved by the Ethics Committee of Hebei Medical University.

2.2. Induction of chronic colitis, DAI and histopathological analysis

Chronic colitis was induced by four-cycles administration of 2.5% dextran sulfate sodium (DSS; 40,000–50,000 MW, MP Biomedicals, USA) drinking water on day 1–5, 8–12, 15–19 and 22–26, and distilled water during the remaining time, as described previously [15]. Mice were divided into four groups: control/Tg group, control/WT group, dextran DSS/Tg group, and DSS/WT group (n = 10 in each group). All mice were sacrificed at day 29. The disease activity index (DAI) was calculated as previously described [17]. Tissue samples were processed and stained with hematoxylin and eosin (H&E). Histopathological analysis was used to evaluate inflammation, extent, gland damage, and cell infiltration. Histological scoring for colitis was based on the established scale using inflammatory and epithelial parameters [18]. Myeloperoxidase (MPO) activity was assessed using the Myeloperoxidase Activity Assay Kit according to the manufacturer's protocol (Nanjing Jiancheng Bioengineering Institute, China).

2.3. Expression analysis

The colon tissues, splenic cells, mesenteric lymph node (MLN) cells and lamina propria mononuclear cells (LPMC) were prepared for qRT-PCR analysis. Total RNA was isolated using TRIzol reagent (Ambion, Austin, TX) and cDNA was prepared using Takara primeScript qRT-PCR Kit (TaKaRa, China). The mRNA expression of TL1A, TGF-β, IL-4 and PU.1 were analyzed by qRT-PCR. PCR primer sequences were available in Table 1. To measure expression levels, SYBR ® GreenER™ qPCR SuperMix kit (Invitrogen, Carlsbad, CA) was used following the manufacturer's protocol. Then, the RT-qPCR was performed in triplicate on an ABI 7500 real-time PCR system (Applied Biosystems, USA). GAPDH was used as endogenous control to calculate the mRNA relative expression. Data were calculated by the comparative cycle threshold (CT) ($2^{-\Delta\Delta CT}$) method.

Expression of TL1A and PU.1 was measured by Western blot using antibodies to TL1A (1:400 dilution, Santa Cruz, USA) and PU.1 (1:600 dilution, Bioworld, China), respectively. Enzyme-linked immunosorbent assay (ELISA) was used to analyze the production of TGF-β, IL-4 and IL-9 in cell supernatants according to manufacturer's protocol (MultiSciences Biotech Co., Ltd. Hangzhou, China).

2.4. Cell isolation, culture and flow cytometry assay

Isolations and culture of splenic cells, mesenteric lymph node (MLN) cells and lamina propria mononuclear cells (LPMC) were performed as previously reported [15]. Splenic cells, MLN cells and LPMC were cultured in RPMI-1640 supplemented with 10% fetal bovine serum (FBS, Gibco, USA), 25 mM HEPES (Corning, USA), 1 mM sodium pyruvate, 10 U/ml penicillin, 10 U/ml streptomycin, and 2 mM glutamine. For flow cytometry, cells were acquired on a FACSVerse flow-cytometer (BD Biosciences, San Jose, CA) and analyzed using FlowJo analysis software after 3.5–4 hour cultivation.

2.5. Immunofluorescent staining

For immunofluorescent staining, colon tissue was embedded into paraffin and then prepared into 4 μm paraffin sections. Paraffin sections were incubated with primary antibodies (1:50 dilution, 4 °C overnight) and secondary antibodies (1:500 dilution, 37 °C, 1 h), respectively. Then paraffin sections were incubated with 4′,6-Diamidino-2-phenylindole (DAPI, Beyotime Biotechnology, China) for 10 min in the dark. Finally, paraffin sections were sealed in anti-fluorescence. Images were acquired on a laser scanning confocal microscope FV12-IXCOV (OLYMPUS, Japan). Green and red fluorescence represented the positive expression of genes. The mean optical density was calculated by Image-J to analyze the positive expression.

2.6. T cell differentiation assays

Spleens and LMN cells were isolated from wild-type C57BL/6 mice and transgenic mice, respectively. Cells were sorted for $CD4^+$ T cells using the MojoSort™ Mouse CD4 Naïve T Cell Isolation Kit (Biolegend, USA) according to manufacturer's protocol. CD4 Naïve T cells were cultured at 1×10^6/mL on 96-well plate. For activation and costimulation, plates were coated with 2 μg/mL anti-mouse CD3 (Biolegend, USA) and 2 μg/mL anti-CD28 (Biolegend, USA). For Th9 cells differentiation, 3 ng/ ml TGF-β, 20 ng/mL IL-4, and 30 ng/mL IL-2 was sequentially added into the plates. After 5 days culture, Th9 cells were harvest and then analyzed by flow cytometry.

2.7. Human subjects

A total of 105 patients diagnosed with UC according to standard clinical, endoscopic, radiological, and histological findings who were treated in Hebei Medical University between Sep. 2015 and Jan. 2018 were included in the study. 30 patients with no abnormality in colonoscopy and single polyp without other diseases were set as the normal control group. Clinical course, laboratory values, tissue samples and endoscopic findings were collected. The biopsy specimens from the first available colonoscopy after UC diagnosis were used. According to the Modified Mayo Endoscopic Score (MMES), the severity of disease activity was dived into mild-moderate, and severe UC [19]. The levels of TL1A and IL-9 in serum were evaluated using ELISA according to manufacturer's protocol (MultiSciences Biotech Co., Ltd. Hangzhou, China) respectively. The number of Th9 cells in colon tissue was measured by immunofluorescence staining. The levels of TL1A and PU.1 in colon tissue were detected by immunocytochemistry. qRT-PCR was preformed to measure the mRNA expression of TL1A, IL-9 and PU.1. The protocol for this investigation was approved by Ethics Committee of Hebei Medical University (Number: 2015-P018). Written informed consent was obtained from each subject.

2.8. Statistical analysis

Statistical analysis was performed with IBM SPSS statistics 19.0 (SPSS Inc., Chicago, Illinois, USA). Results are expressed as mean ± the standard error of the mean (SEM) or standard deviation (SD) of three separate experiments, as indicated in the figure legends. The normal distribution of quantitative data was tested with Kolmogorov-Smirnov test. If the data were normally distributed, statistical significance between multiple groups was determined with one-way ANOVA followed by a Student-Newman-Keuls (SNK) post hoc test. If the data were non-normally distributed, statistical significance between multiple groups was determined with Kruskal-Wallis test followed by a Nemenyi post hoc test. Pearson correlation analysis was used to analyze the association between continuous variables. Differences were considered statistically significant when $p < 0.05$.

3. Results

3.1. TL1A exacerbated intestinal inflammation in chronic DSS colitis model

To evaluate the role of TL1A in colitis development, wild-type mice and transgenic (Tg) mice with overexpressing TL1A in lymphocytes were used to construct chronic colitis models. After construction of chronic DSS colitis model, qRT-PCR and western blot analysis were preformed to detect the expression of TL1A in colon tissues. As shown in Fig. 1A and B, TL1A was overexpressed in transgenic mice, suggesting the successful construction of overexpressing TL1A transgenic mice. Besides, compared to the control mice, the DSS-treated mice were found to have a higher expression level of TL1A (Fig. 1A and B), indicating that TL1A was involved in chronic colitis development.

To determine whether TL1A influence the intestinal inflammation, the DAI and histopathological indicators was evaluated in DSS models. During experiment, a greater weight loss was found in DSS/Tg mice compared to that in DSS/WT mice (Fig. 1C). Meanwhile, the DAI score in DSS/Tg mice was significantly higher than that in DSS/WT mice (Fig. 1D). We isolated the colonic tissue, inspection of colonic specimens showed the increased colonic mucosa hyperemia and wall thickening, and decreased colon length of DSS/Tg mice compared with the DSS/WT mice (Fig. 1E). Histopathological examination results showed that DSS-treated mice presented the colonic mucosa damage, decreased goblet cell, gland damage and increased mononuclear cell infiltration (Fig. 1F). Moreover, the histology score of DSS/Tg mice was significantly higher than that of DSS/WT mice (Fig. 1G). Additionally, compared to DSS/WT mice, increased myeloperoxidase activity was found in the colonic tissue of DSS/Tg mice (Fig. 1H). Taken together, these results indicated that the TL1A exacerbated intestinal inflammation.

3.2. TL1A enhanced the Th9 cells differentiation and IL-9 secretion in chronic DSS colitis model

To assess the effect of TL1A expression on Th9 cells differentiation, we performed immunofluorescence co-staining of CD4 and IL-9 in colonic tissue to measure the proportion of Th9 cells. The green fluorescence represented CD4 expression and the red fluorescence represented IL-9. As shown in Fig. 2A, the co-expression mean density of DSS/Tg mice was significant higher than that of DSS/WT mice, indicating a high proportion of Th9 cells in DSS/Tg mice. Flow cytometry assay was used to measure the proportion of $CD4^+IL\text{-}9^+$ T cells in splenic cells, MLN and LPMC. A higher proportion of $CD4^+IL\text{-}9^+$ T cells in splenic cells, MLN and LPMC was found in the DSS/Tg mice (Fig. 2B). To determine the expression of IL-9 in DSS chronic colitis model, qRT-PCR was performed to detect the IL-9 mRNA expression in colonic tissue and ELISA analysis was used to detect the secretion of IL-9 in serum, splenic cells, MLN and LPMC, respectively. As shown in Fig. 2C, the IL-9 mRNA expression in colonic tissue and IL-9 secretion was greatly enhanced in DSS/Tg mice. Taken together, these results indicated that TL1A promoted the differentiation of IL-9 producing cells, Th9 cells and enhanced IL-9 secretion in chronic colitis.

3.3. TL1A enhanced TGF-β, IL-4 secretion and PU.1 expression in chronic DSS colitis model

As previously reported, TL1A significantly enhanced Th9 cells differentiation in the presence of IL-4 and TGF-β. Thus, we speculated that TL1A may regulate the Th9 cells differentiation by TGF-β and IL-4. To assess the effect of TL1A on the production of cytokines, qRT-PCR was performed to detect the mRNA expression in colonic tissue. An increased mRNA expression of cytokines TGF-β and IL-4 was observed in DSS/Tg mice (Fig. 3A and B). ELISA analysis was used to detect the production of TGF-β and IL-4 in splenic cells, MLN and LPMC, respectively. Results showed that compared to DSS/WT mice, the production of TGF-β and IL-4 in splenic cells, MLN and LPMC of DSS/Tg mice were greatly enhanced by the TL1A overexpression (Fig. 3A and B). These results suggested that TL1A enhanced TGF-β and IL-4 production in chronic colitis, indicating that TL1A may promote the Th9 cells differentiation by up-regulating the expression of TGF-β and IL-4.

PU.1, as the ETS family transcription factor, was required for the development of IL-9-producing T cells and allergic inflammation [12]. To analysis the role of PU.1 in Th9 cells differentiation, we measure the level of PU.1 expression by qRT-PCR and western blot analysis, respectively. qRT-PCR showed that the mRNA expression of PU.1 in DSS/Tg mice was significant increased compared with the DSS/WT mice (Fig. 3C). Further, western blot analysis verified the higher expression level of PU.1 protein in DSS/Tg mice (Fig. 3C). These results suggested that TL1A enhanced PU.1 expression in chronic colitis, indicating that TL1A may promote the Th9 cells differentiation by up-regulating the

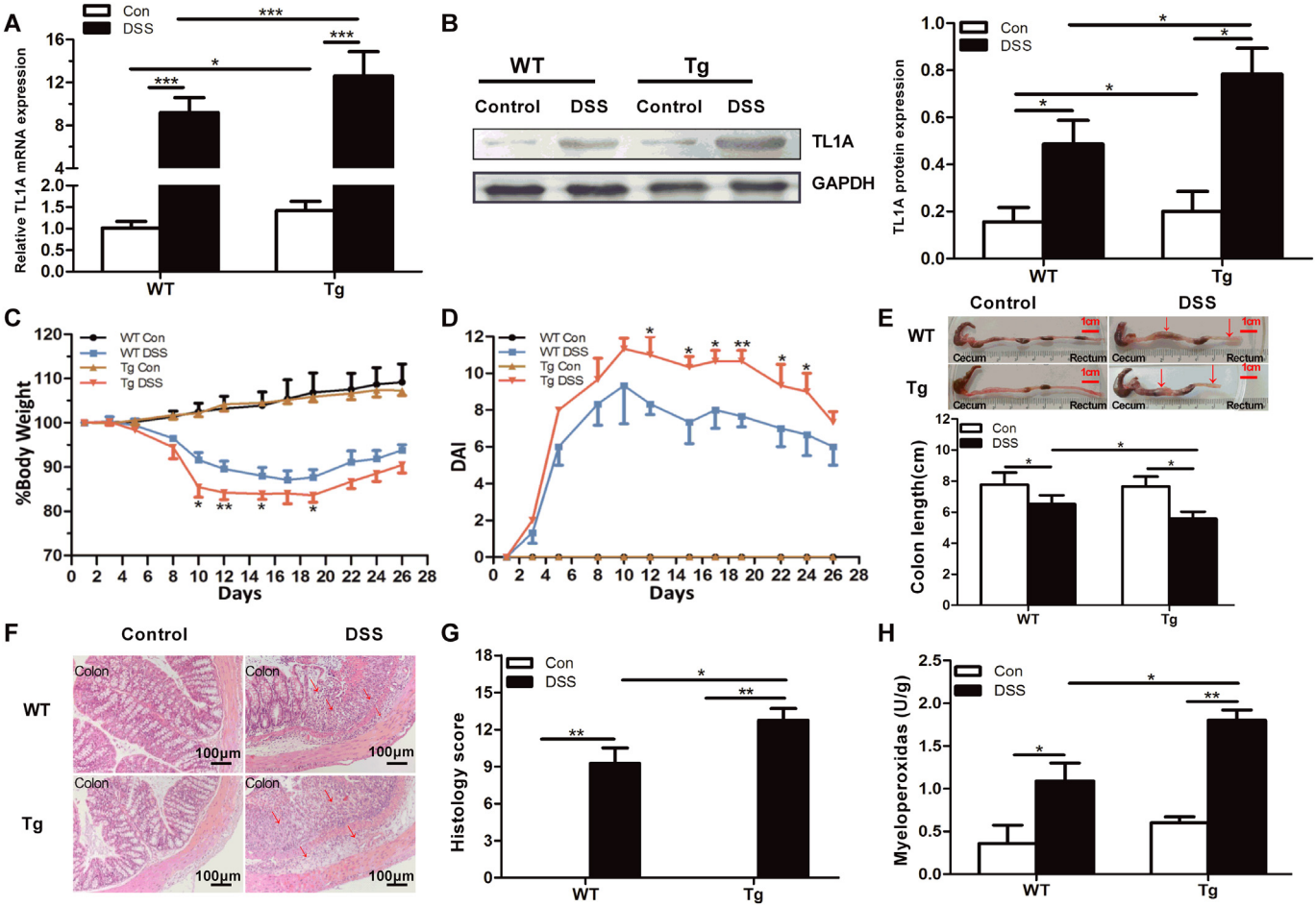

Fig. 1. TL1A overexpression in chronic colitis exacerbated intestinal inflammation. (A) TL1A mRNA expression detected by qRT-PCR analysis. (B) TL1A protein expression detected by western blot analysis. (C) Percentage of body weight change is shown for DSS-induced colitis. (D) Disease activity index were quantitated and were shown. (E) The colon length was quantitated and representative colonic specimen were shown. Red arrows indicate the inflammatory site. (F) Representative H &E-stained colon sections from C57BL/6 mice at original magnification ×100 were shown. Scale bars, 100 μm. Red arrows indicate the inflammatory site. (G) Histology score from 10 mice was quantitated and shown. (H) Myeloperoxidase activity was measured, and data are expressed as arbitrary unit (U) per gram (g) of protein. Data are expressed as mean ± SD. * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

expression of PU.1.

3.4. TL1A promoted Th9 cells differentiation and IL-9 secretion by up-regulating PU.1 in vitro

Given the findings in chronic DSS colitis models, we speculated a possible mechanism that TL1A may promote the Th9 cells differentiation an IL-9 production to modulate the severity of colitis by up-regulating the expression of TGF-β, IL-4 and PU.1. Thus, to verify the potential molecular mechanism of TL1A in IBD development and the role of TL1A in promoting Th9 cells and IL-9 production, we isolated CD4 Naïve T cells from spleen and MLN to induce the Th9 cells differentiation in vitro. CD4 Naïve T cells was sorted at appropriate purity (spleen: 90.18% ± 2.62%; MLN: 92.12% ± 2.82%) for subsequent Th9 cells differentiation (Fig.4A). As a result, a higher proportion of $CD4^+IL\text{-}9^+$ T cells was induced from the spleen and MLN cells of Tg mice (Fig.4B). Then TL1A, TL1A antibody isotypes and TL1A antibodies was used to stimulate the Th9 cells differentiation in the presence of TGF-β and IL-4. As shown in Fig. 5A and B, TL1A enhanced the Th9 cells and IL-9 production both in the WT and Tg mice, while TL1A antibodies inhibited the production. Additionally, the expression of PU.1 was significantly enhanced by TL1A, while inhibited by TL1A antibodies (Fig. 5C). These results in vitro confirmed our hypothesis that TL1A significantly enhanced the production of Th9 cells, IL-9 by up-regulating the expression of PU.1.

3.5. TL1A mediated the severity of colitis by regulating Th9 cells and PU.1 in UC patients

Given the results of mice models and the in vitro experiment, we verified whether the severity of colitis in human patients mediated by TL1A and confirmed the potential link between TL1A and Th9 cells. We collected the colonic tissue from UC patients to measure the expression levels of relevant cytokines and proteins. Firstly, TL1A was verified to be significantly increased in UC patients' serum and colonic tissue (Fig. 6A). The frequency of Th9 cells and secretion of IL-9 in UC patients were also found to be significant higher than that in the control (Fig. 6B). Meanwhile, the expression of PU.1 was also proved to be significantly enhanced in UC patients' colonic tissue (Fig. 6C). Correlations of TL1A and other cytokines were analyzed by Pearson correlation analysis. Results showed that IL-9, Th9 cells was significantly positively correlated with TL1A (IL-9 in serum: r = 0.5001, $p < 0.05$; IL-9 in colon: r = 0.7059, $p < 0.001$; Th9 cells: r = 0.4751, $p < 0.05$). In addition, IL-9 and Th9 cells was significantly positively correlated with the expression levels of PU.1 (IL-9 in colon: r = 0.6764, $p < 0.05$; Th9 cells: r = 0.4671, $p < 0.05$).

To determine whether the TL1A exacerbates intestinal inflammation in patients, we also analyzed the expression of relevant cytokines and

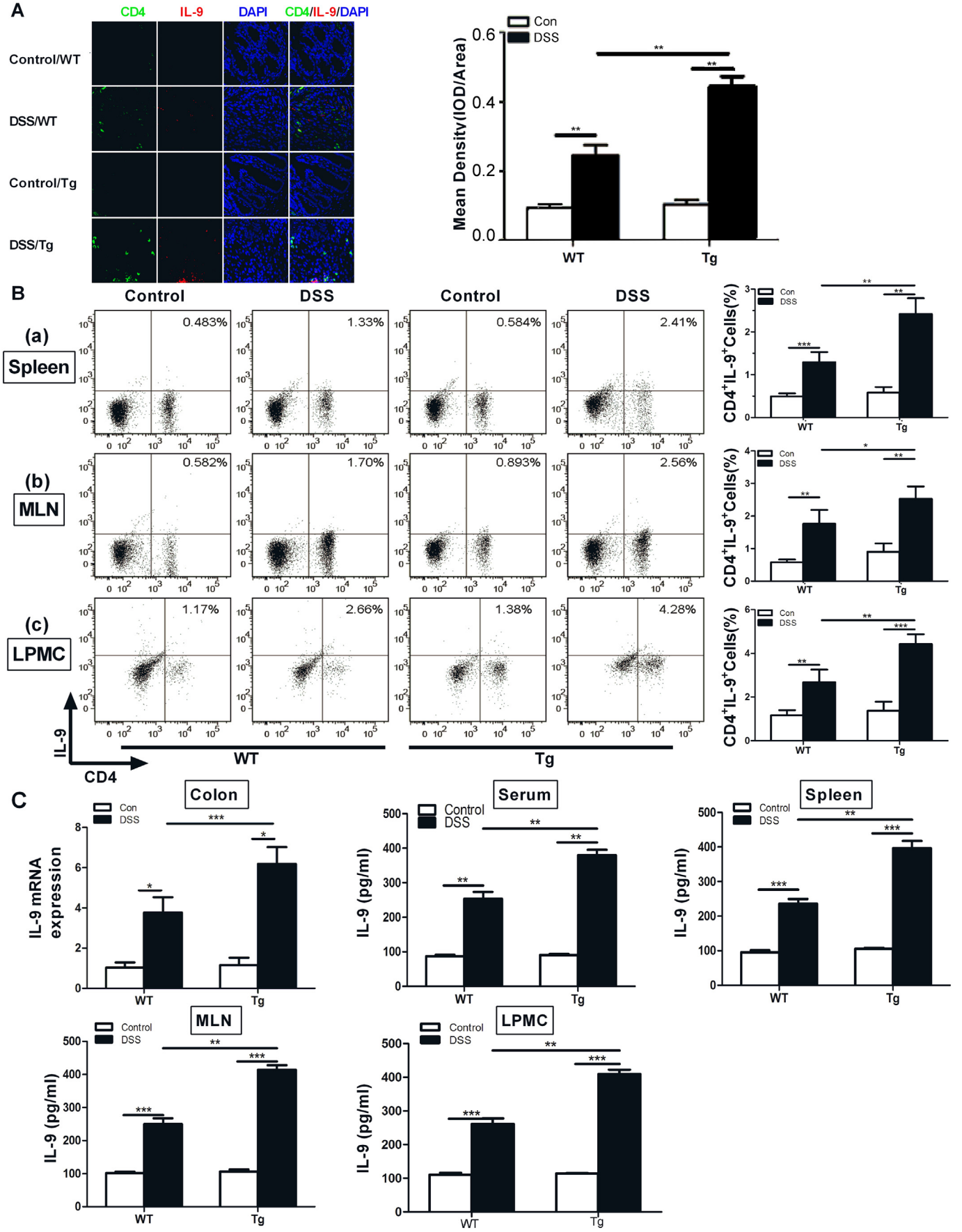

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Fig. 2. TL1A enhanced the Th9 cells differentiation and IL-9 secretion in chronic DSS colitis model. (A) Quantification of $CD4^+IL\text{-}9^+$ T cells by immunofluorescence staining (×400). Green fluorescence represented CD4 expression and red fluorescence represented IL-9. (B) Frequency of $CD4^+IL\text{-}9^+$ T cells from spleen, mesenteric lymph node (MLN) and lamina propria mononuclear (LPM) were detected by flow cytometry assay. (C) IL-9 mRNA were measured by qRT-PCR and the IL-9 secretion in serum, spleen, MLN and LPM were measured by ELISA. Data are expressed as mean ± SD. $^*p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

proteins in patient populations with varying severities of colitis according to the Modified Mayo Endoscopic Score. As shown in Fig. 7A, the expression of TL1A was significant higher in the patients with severe UC than that in the patients with mild-moderate UC. Meanwhile, the production of IL-9, Th9 cells and PU.1 was also significantly increased with the severity of UC (Fig. 7B and C). Taken together, results showed that TL1A promoted Th9 differentiation and IL-9 secretion by up-regulating PU.1 to exacerbate the intestinal inflammation in UC patients, which verified the results of animal studies.

4. Discussion

Recently, several researches have confirmed that TL1A was involved in the allergic diseases and autoimmune disease by enhancing differentiation of T helper cells [15–17]. Nevertheless, there are few studies on the relationship of TL1A and Th9 cells in colitis. Accordingly, we focused on the TL1A and Th9 cells in colitis for the first time. In our present study, we used the chronic DSS colitis mice model to explore the role of TL1A in the development of UC and the potential regulatory pathways. Moreover, in vitro cell experiments and clinical sample tests were performed to verify the regulatory mechanism. As a result, we found that TL1A was up-regulated in colitis, and meanwhile may enhance the differentiation of Th9 cells and IL-9 production by up-regulating TGF-β, IL-4 and PU.1 expression to mediate the UC development.

TL1A, a member of the TNF superfamily, has known to mediate the immunopathology in many autoimmune disease models by promoting the accumulation of T cells [20,21]. Firstly, in our study, we detected the TL1A expression in different tissue and cells from colitis mice model or patients. As a result, we demonstrated that TL1A was indeed up-regulated in both DSS colitis mice model, colitis cells and patients with UC. Previously, TL1A has been widely confirmed to be enhanced in autoimmune and inflammatory diseases, such as CD [22], autoimmune nephritis [23], rheumatoid arthritis [24], and ankylosing spondylitis [25]. Our findings were consistent with these previous researches. Furthermore, the results of animals' experiments showed that TL1A could exacerbate colonic mucosa damage and promote the severity of intestinal inflammation, which was also verified in the clinical patients. The TL1A was proved to be related to the severity of intestinal inflammation in patients. As the previous reported, TL1A could modulate

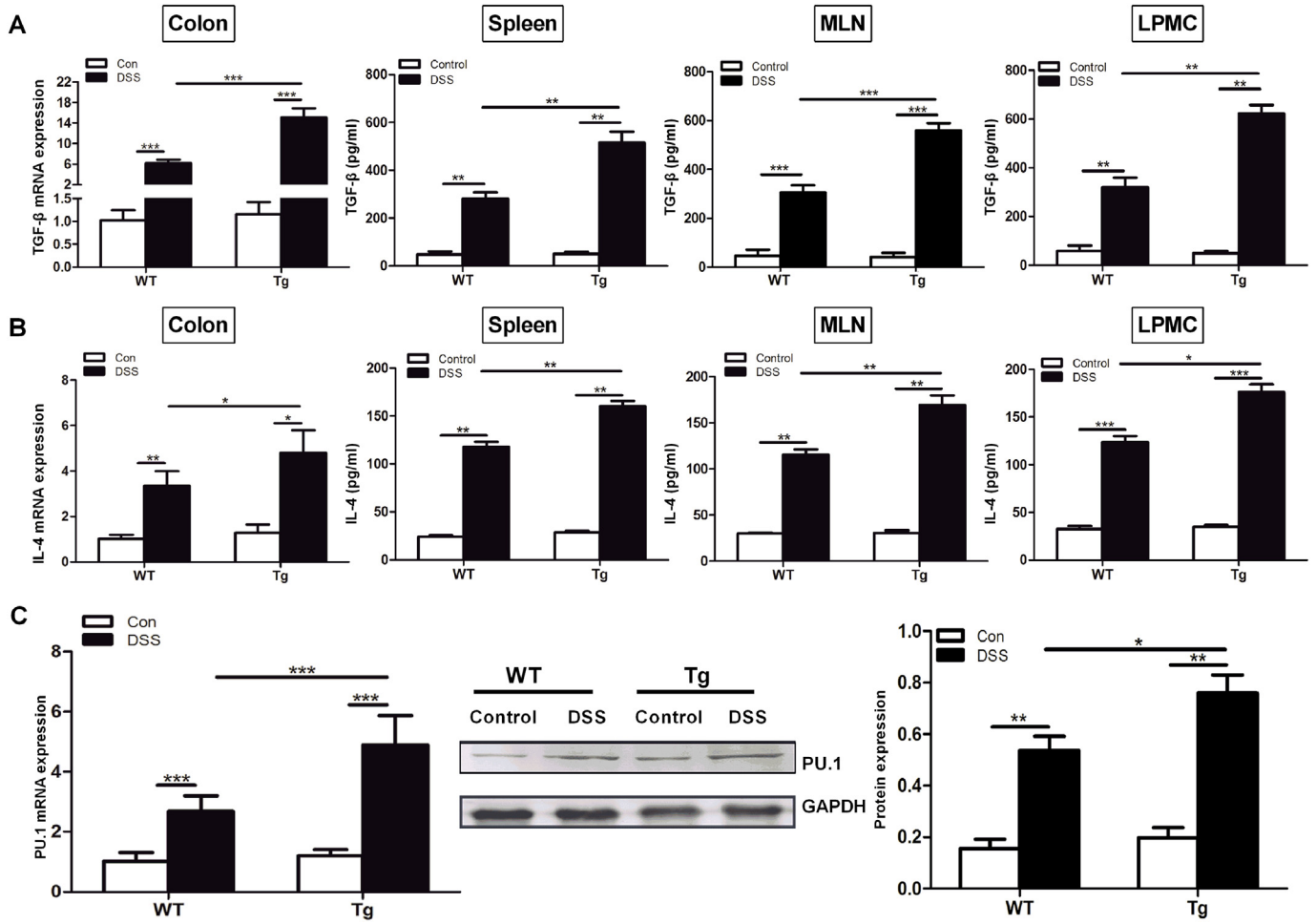

Fig. 3. TL1A enhanced TGF-β, IL-4 secretion and PU.1 expression in chronic DSS colitis model. (A) The expression of TGF-β in colonic tissue, splenic cells, MLN and LPMC. (B) The expression of IL-4 in colonic tissue, splenic cells, MLN and LPMC. (C) The expression of PU.1 in colonic tissue was measure by qRT-PCR and western blot. Data are expressed as mean ± SD. $^*p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$.

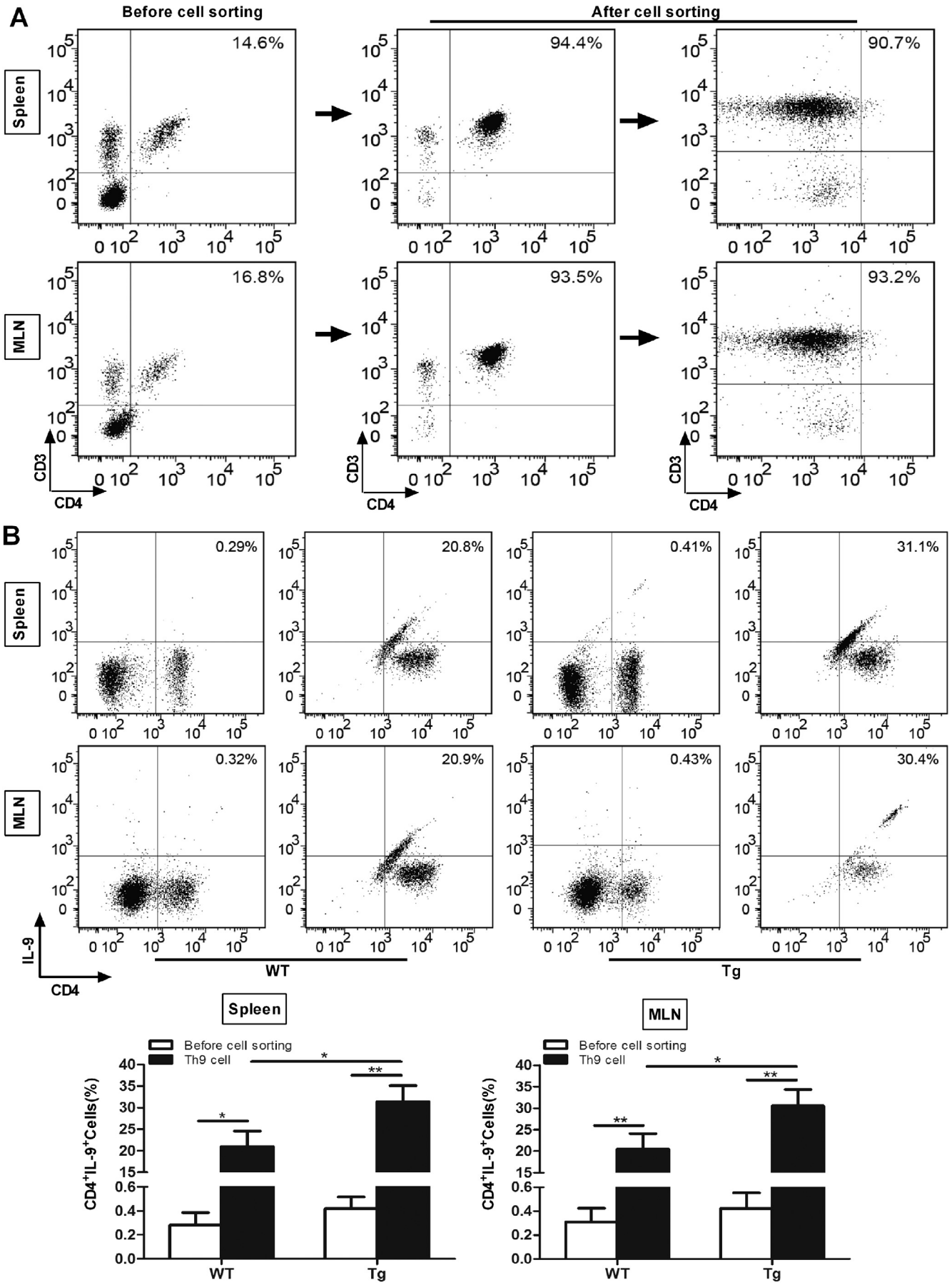

Fig. 4. $CD4^+IL\text{-}9^+$ T cells sorted from spleen and MLN cells. (A) CD4 Naïve T cells from spleen and MLN were sorted. (B) $CD4^+IL\text{-}9^+$ T cells induced from spleen and MLN cells were harvest. Frequency of $CD4^+IL\text{-}9^+$ T cells was shown at the below. Data are expressed as mean ± SD. *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$.

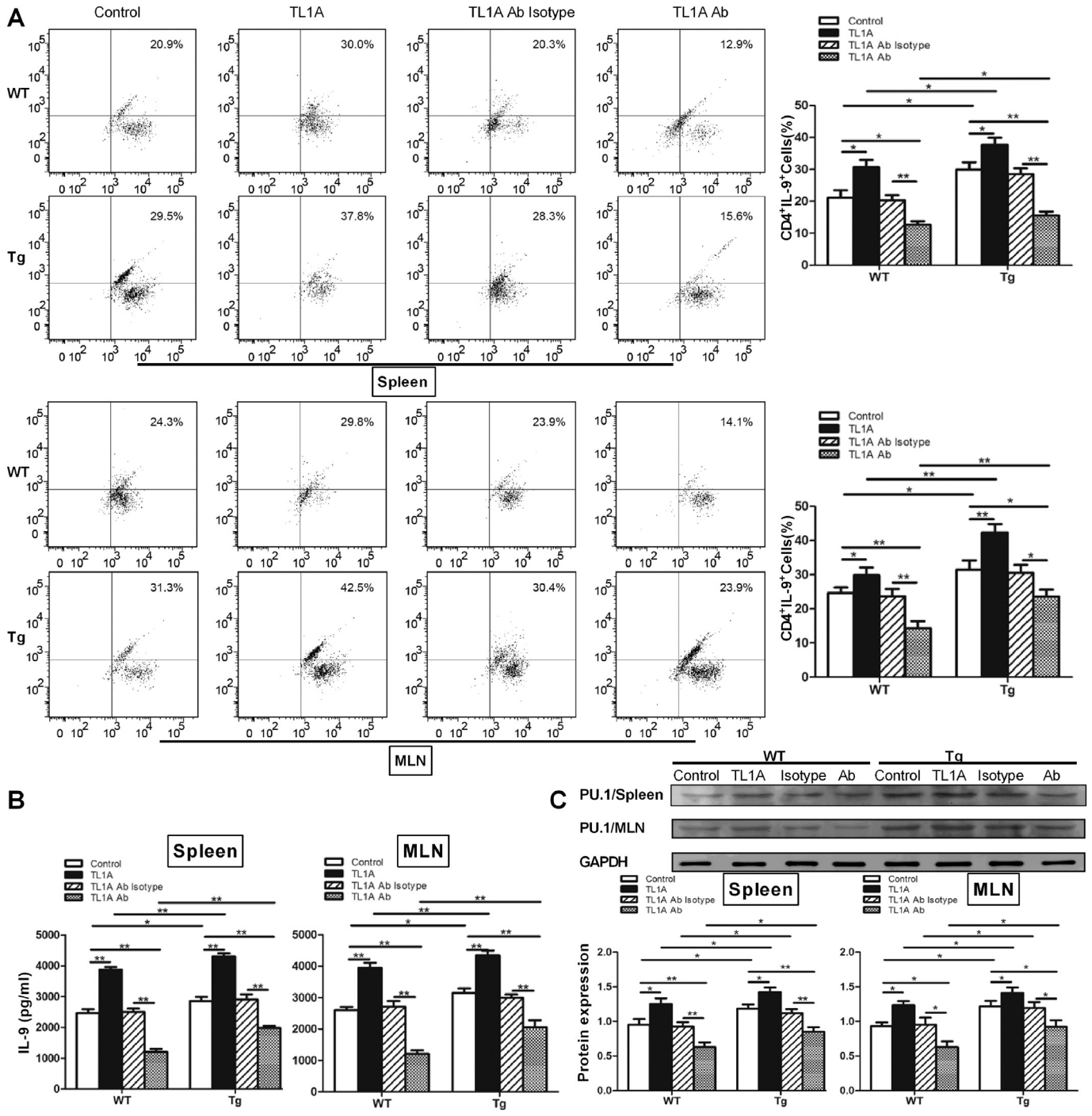

Fig. 5. TL1A promoted Th9 cells differentiation and IL-9 secretion by up-regulating PU.1 in vitro. (A) CD4 Naïve T cells isolated from the spleen and MLN of WT and Tg mice were differentiated into Th9 cells with different stimulants (TL1A, TL1A antibody and TL1A antibody isotype). (B) The IL-9 secretion and (C) PU.1 expression after Th9 cells differentiation by different stimulants (TL1A, TL1A antibody and TL1A antibody isotype). Data are expressed as mean ± SD. *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$.

the proximal migration and severity of gut mucosal inflammation and induce the fibrostenosis [26,27]. One possible explanation is that TL1A overexpression led to an accelerated the effector function of T-helper and thereby the effector phase of the disease is reached earlier [17]. Another possible explanation proposed by Shih, D. Q et al. and Barrett, R. et al. is that TL1A may be related to a pro-fibrogenic gene to induce the fibrosis process [17,27]. In addition, TL1A may facilitate the secretion of inflammatory cytokines to modulate the severity of gut mucosal inflammation. So far, although we cannot directly make clear the regulation mechanism of TL1A to the severity of UC, these findings about TL1A in UC pathogenesis indicated that TL1A may be a therapeutic target for UC and would be medically useful to the treatment of UC patients.

Despite IL-9 and IL-9 producing cells, Th9 cells was reported to be involved the development of allergic diseases, rheumatoid arthritis, and other autoimmune diseases [10], the role of Th9 in the pathogenesis of UC has not attracted enough attention from researchers [28]. IL-9 production has been found to be enhanced in the intestinal epithelial cells from patients with active UC [29,30]. Thus, in current study we measured the IL-9 and Th9 cells and found an enhanced production in

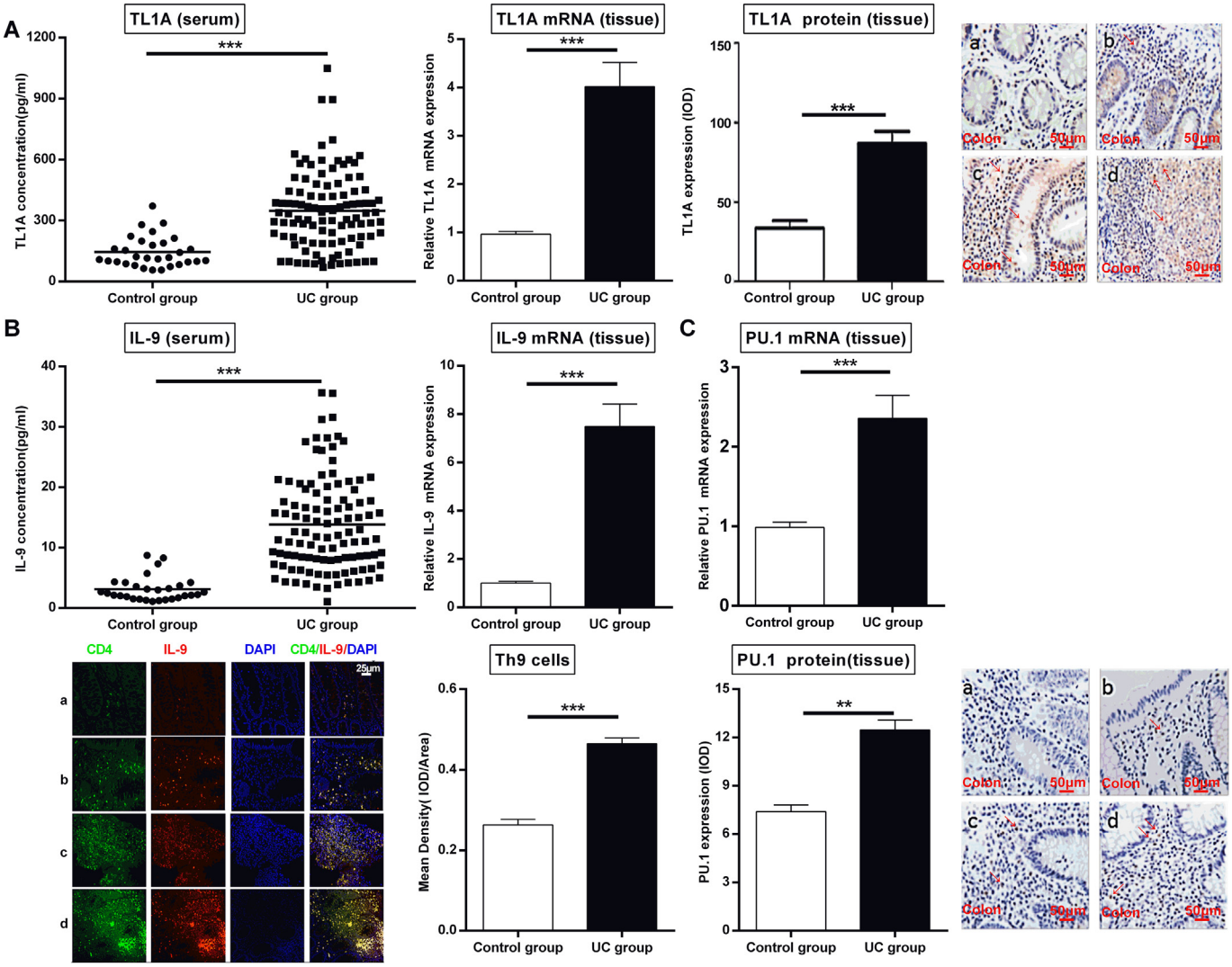

Fig. 6. Increased production of TL1A and revelent cytokines in UC patients. (A) The TL1A expression was enhanced both in the colonic tissue and serum of the UC patients. Representative colon sections by immunocytochemistry from control and UC patients were shown at the right. Red arrows indicate the TL1A. (B) The IL-9 expression and Th9 cells was enhanced the UC patients. Representative of immunofluorescence staining colon sections from control and UC patients were shown at bottom. Quantification of $CD4^{+}IL\text{-}9^{+}$ T cells by immunofluorescence staining (×400). Green fluorescence represented CD4 expression and red fluorescence represented IL-9. (C) The PU.1 expression was enhanced in UC patients. Representative colon sections by immunocytochemistry from control and UC patients were shown at the right. Red arrows indicate the PU.1. Data are expressed as mean ± SEM. $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

the chronic DSS colitis models and UC patients, suggesting that activation of Th9 cells was involved in the development of chronic colitis. Next, we further explored the regulation relationship between TL1A and Th9 cells. In previous study on the allergic lung disease mice models, Th9 differentiation and IL-9 production was confirmed to be induced by TL1A in the presence of TGF-β and IL-4 [16]. In line with this previous report, we found that increased IL-9 production and Th9 differentiation was detected in the TL1A overexpression mice and UC patients. Meanwhile, the in vitro researches have further showed that in the presence of TGF-β and IL-4 TL1A could divert CD4 Naïve T cells to become the IL-9 producing cells. Additionally, TL1A have also been shown to promote the differentiation of Th-17, Th-1 and Th-2 cells [15]. However, Richard et al. previously proposed that TL1A promotion of the Th9 differentiation was independent of IL-4 [16], which has not been proved in our present study. Therefore, further study focused on the detailed mechanism of TL1A was still needed.

At present, the mechanism of Th9 cells activation regulated by TL1A has not yet been fully elucidated. TGF-β and IL-4, as the key cytokines induced differentiation of Th9 cells, had marked capacities to enhance IL-9 secretion [28,31]. Thus, in the chronic DSS colitis models with overexpressing TL1A we detected the levels of TGF-β and IL-4. Obviously, the results showed an increased levels of TGF-β and IL-4 in chronic DSS colitis mice. Thus, based on the previous findings, we could speculate that TL1A may enhance the production of TGF-β and IL-4 to participate in the differentiation and activation of Th9 cells. In addition, the increased levels of TGF-β and IL-4 in TL1A overexpression mice also proved our speculation that TL1A facilitates the secretion of inflammatory cytokines to modulate the severity of gut mucosal inflammation. Taken together, our studies proposed a preliminary possible mechanism that TL1A overexpression could up-regulate the production of TGF-β and IL-4 to activate Th9 cells differentiation, and thereby exacerbate intestinal inflammation.

PU.1 is a critical transcription factor for the Th9 differentiation and IL-9 expression [12,29,32]. Gerlach K et al. have showed that the transcription factor PU.1 was expressed at a higher levels in patients with ulcerative colitis, which suggested that PU.1 plays an important role in regulating Th9 cells in IBD [29]. Here we studied the PU.1 expression in the colitis models and UC patients, and observed an

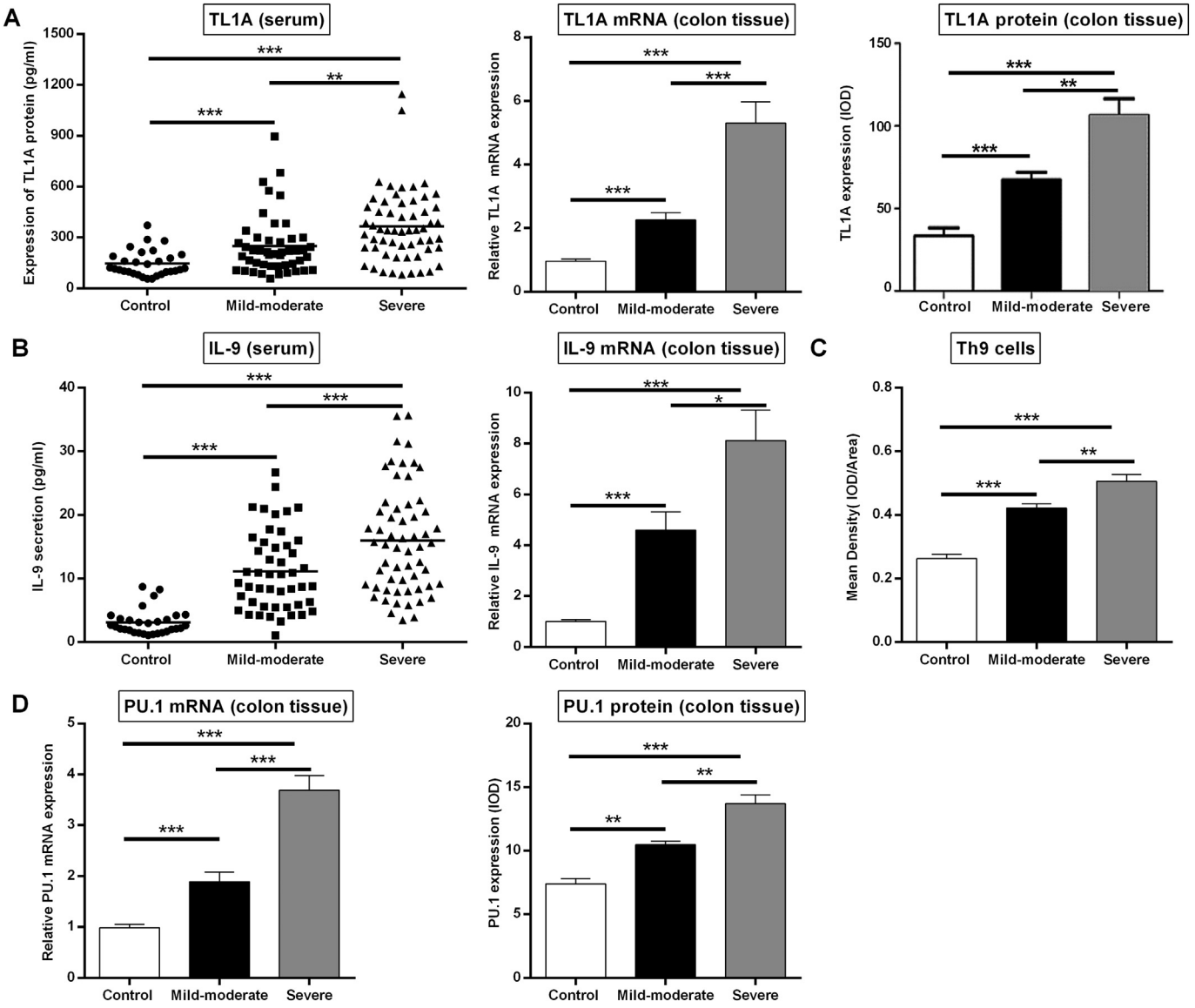

Fig. 7. TL1A and revelent cytokines increased with the severity of UC. (A) The expression of TL1A in patients with varying degrees of colitis severity. 105 patients with UC were in UC group, and 30 patient with no abnormality in colonoscopy were in control. (B) The expression of IL-9, (C) mean density of Th9 cells, and (D) expression of PU.1 in patients with varying degrees of colitis severity. Data are expressed as mean ± SEM. $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$.

enhanced PU.1 expression in the colitis compared to that in control subjects. Consistent with the previous report [29], our result confirmed that PU.1 expression was indeed related to the IBD development. Besides, IL-9 and Th9 cells were significantly positively correlated with the expression levels of PU.1. Thus, given that published studies, we speculated that PU.1 modulated the development of IBD by promoting the differentiation of Th9 cells. Notably, we also found that PU.1 expression was enhanced in the TL1A overexpression mice, which suggested that TL1A could up-regulate the PU.1 expression. In summary, our data suggested that TL1A overexpression could up-regulate the production of PU.1 to activate Th9 cells differentiation, and thereby exacerbate intestinal inflammation.

5. Conclusion

This study for the first time investigated the regulatory relationship between TL1A and Th-9 cells in chronic colitis both in vivo and in vitro, respectively. The TL1A was verified to be up-regulated in chronic colitis and enhanced TL1A expression could exacerbate intestinal inflammation, suggesting that TL1A indeed involved the development of chronic colitis. Further, we proposed a possible mechanism that TL1A may promote the differentiation of Th9 cells and enhanced IL-9 secretion by up-regulating the expression of TGF-β, IL-4 and PU.1. These findings indicated that TL1A and Th9 cells may be useful as therapeutic targets for chronic colitis. The investigations on detailed mechanism of TL1A remain to be further researched.

Declaration of Competing Interest

The authors declare that they have no conflict of interest.

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Ethical approval

All applicable international, national, and/or institutional guidelines for the care and use of animals were followed. The protocol for this investigation in patients was approved by Ethics Committee of Hebei Medical University.

Informed consent

Written informed consent was obtained from each subject.

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