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Research Article

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OX40L promotes follicular helper T cell differentiation and function in mice with immune thrombocytopenia

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Abstract: Immune thrombocytopenia (ITP) is a hemorrhagic autoimmune disease characterized by antibody-mediated platelet injury. ITP has complicated immunopathological mechanisms that need further elucidation. It is well known that the costimulatory molecules OX40L and OX40 play essential roles in the immunological mechanisms of autoimmune diseases. Previously, we discovered that the expression of OX40L and OX40 is significantly increased in the PBMCs of ITP patients. In our present study, OX40L-induced Tfh cells exhibited an activated phenotype with elevated ICOS, PD-1, and CD40L expression *in vitro*. Moreover, aberrant OX40L-OX40 expression might promote the Tfh1-to-Tfh2 shift *in vivo*, inducing the generation of autoantibodies by enhancing the helper function of Tfh cells for B lymphocytes in a mouse model, which might accelerate the progression of ITP. Additionally, signal transduction through the OX40L-OX40 axis might be related to the activation of TRAF-NF-κB and JAK-STAT signaling pathways. Overall, OX40L-OX40 signaling is proposed as a potential novel therapeutic target for ITP.

Key words: OX40L; OX40; Immune thrombocytopenia; Follicular T helper cell; B cell

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1 Introduction

Immune thrombocytopenia (ITP) is a heterogeneous acquired disease characterized by antibody-mediated platelet injury and low baseline platelet counts (Mingot-Castellano et al., 2022). Epidemiological data show that ITP affects 2 to 4 per 100,000 persons annually, with a total incidence rate of 10 individuals in 100,000 (Ghanima et al., 2021). Patients show an increased risk of skin bruising and bleeding. Besides, low platelet counts may result in internal bleeding, particularly intracranial bleeding, which can be severe and even life-threatening to ITP patients (Ghanima et al., 2021). Currently, multiple therapeutic approaches are utilized for the clinical treatment of ITP, including thrombopoietin receptor agonists, rituximab, and splenectomy (Liu et al., 2023). However, some patients are unresponsive to multiple treatment regimens and ultimately develop refractory ITP with prolonged and severe thrombocytopenia (Liu et al., 2023). Therefore, it is important to further elucidate the pathogenesis of ITP and identify new targeted therapies for this disease.

Platelet destruction and the suppression of platelet production are aggravated by multiple humoral immune responses and immune cell abnormalities in ITP patients. It has been well recognized that the pathogenesis of

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ITP is associated with the production of antiplatelet autoantibodies (Chen et al., 2022). However, the exact underlying mechanisms of ITP remain unclear. Follicular helper T (Tfh) cells are a particular subset of $CD4^+$ T cells, which can migrate to target points and produce multiple cytokines that promote the occurrence and development of autoimmune disorders (Chen et al., 2022). The percentage of Tfh cells is increased in B cell-related autoimmune disorder patients, such as those with systemic lupus erythematosus (SLE), primary Sjögren's syndrome, and rheumatoid arthritis (RA), and exhibit features of a highly activated state (Caza et al., 2022; Sasaki et al., 2022; Pontarini et al., 2020; Wei and Niu, 2023). Another critical role of Tfh cells in autoimmune diseases is to support B lymphocyte differentiation and facilitate the production of high-affinity autoantibodies (Audia et al., 2014; Hernandez-Molina et al., 2021). Tfh cells highly express CXC chemokine receptor type 5 (CXCR5) and the classical Tfh-associated transcription factor Bcl6, and typically secrete interleukin-21 (IL-21) and several important surface markers, including inducible T-cell costimulator (ICOS), programmed cell death-1 (PD-1) and the costimulatory molecule OX40 (Jensen et al., 2022).

OX40 is a promising therapeutic target for diverse T-cell-mediated disorders (Iriki et al., 2023). In several animal models of autoimmune disease and infectious disease, an OX40 agonist was shown to ligate OX40 and convey signals to activate T cells, thereby facilitating T-cell activation and proliferation (Fu et al., 2020). Researchers revealed that treating lupus patients with soluble OX40 ligand (OX40L) enhanced the expression levels of multiple characteristic markers associated with Tfh cells *in vitro*. Moreover, in murine models, OX40L-OX40 interactions encourage $CD4^+$ T cells to accumulate in B-cell follicles and at the T-B boundary (Fu et al., 2020). The mechanism through which the OX40L-OX40 axis influences T-cell function is complex. OX40L-OX40 signals, via the tumor necrosis factor (TNF) receptor-associated factor (TRAF) signaling molecules and the NF-κB and JAK-STAT signaling pathways, can expand the immune response in T-cell subsets (Fu et al., 2020; Zhao et al., 2022). We previously reported that the peripheral blood mononuclear cells (PBMCs) of ITP patients exhibited considerably higher mRNA levels of OX40L and OX40 (Cui et al., 2019). However, the specific mechanism of OX40L-OX40 costimulation signals promoting the development of ITP is unclear.

In the present study, increased OX40L and OX40 levels were found in the spleens of ITP model mice, which might promote Tfh cell proliferation and differentiation via the TRAF-NF-κB and JAK-STAT signaling pathways, inducing the generation of autoantibodies through enhancing the helper function of Tfh cells for B lymphocytes. In addition, the secretion of proinflammatory cytokines in peripheral blood was markedly greater in ITP model mice than in normal controls. In this fashion, the OX40L-OX40 axis might activate Tfh cell-related signaling pathways to execute the proinflammatory response, aggravating the pathogenesis of ITP. Taken together, our study suggests that OX40L-OX40 might be a potential therapeutic target for ITP.

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2 Methods

2.1 Animals

We obtained 8-week-old C57BL/6J wild-type (WT) female mice from Ziyuan Experimental Animal Technology Co., Ltd., Hangzhou. The animals were maintained under a 12-hour light/dark cycle with a controlled temperature and were allowed to acclimate for 1 week before experimentation. All animal experiments were carried out based on the Guide for the Use and Care of Laboratory Animals (eight edition, 2012).

2.2 Passive ITP model

Twenty female mice were equally divided into two groups: control group (n = 10) and ITP group (n = 10). Murine thrombocytopenia was induced and maintained as previously described (Sun et al., 2018): The mice

were intraperitoneally injected with 4 μg/d/mouse agent (monoclonal platelet antibody, MWReg30 in 200 μL of PBS at pH 7.2) for 7 days, while the control mice were treated with the same amount of PBS. Whole blood samples (5 μL) were collected through the tail vein, mixed with diluents (V-3D, Mindray) and collected in EDTA-K2 tubes. The baseline platelet counts of the mice were determined before injection, and the platelet numbers were counted daily for 7 days after injection. Platelet counts were performed by an automatic hematology analyzer (BV-30Vet, Mindray, Shenzhen, China).

2.3 Histopathological study and immunofluorescence assay

The mice were sacrificed after 7 days of injection and spleen tissues were harvested for histopathological analyses. Hematoxylin and eosin (H&E) staining, immunohistochemical (IHC) staining and immunofluorescence (IF) staining, as well as imaging, were performed by Wuhan Pinuofei Biological Technology Company. The number of megakaryocytes and positively immunostained cells were determined using ImageJ software.

2.4 Cell purification and cultivation

We purified splenic naïve $CD4^+$ T cells from 8-week-old female C57BL/6 WT mice using the MojoSort™ $CD4^+$ Naïve T Cell Isolation Kit (480041, BioLegend). In the expansion and activation assays, 10^5 cells were placed in 96-well plates with 200 μL of medium/well and stimulated with a T Cell Activation and Expansion Kit (130-093-627, Miltenyi Biotec). Dry powders of the OX40L recombinant protein (CM74, novoprotein) were dissolved in sterilized deionized water. The cell samples were harvested and stimulated with or without 4 mg/mL OX40L recombinant protein (CM74, novoprotein). After culture for 6 days, the culture supernatants and cell samples were subjected to ELISA and flow cytometry (FCM) analyses, respectively.

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2.5 ELISA

The levels of IL-4, IL-6, IL-10, IL-17A, IL-21, and TGF-β in the serum or culture supernatants were measured via ELISA kits (Proteintech, China). The experiments were performed in accordance with the manufacturer's protocols. The absorbance of samples was measured with a Synergy Neo2 multimode reader (BioTek) at 450 nm, and the serum concentrations of cytokines were calculated using standard curves.

2.6 Western blotting

Total proteins were extracted from the control and ITP model mice with a total protein extraction kit (BestBio). The albumin concentration of spleen samples was quantified by a BCA assay kit (BestBio). The proteins were separated via 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis at 80 V. Subsequently, the proteins were transferred to polyvinylidene fluoride membranes (Millipore) at 330 mA for 60 min and blocked with 5% (w/v) skimmed milk (Solarbio). The protein membranes were incubated with primary Abs overnight at 4℃ and then with the appropriate secondary HRP-conjugated Abs for 1 hour. Images of the protein bands were ultimately taken with the ChemiDoc MP imaging system (Bio-Rad). Protein grayscale analysis was conducted with ImageJ software.

2.7 Flow cytometry analysis

Before staining, the cells were incubated with Fc blocking antibody for 15 min, subsequently washed with stain buffer (FBS, BD Biosciences) and centrifuged at 300 × g for 5 min. The fixable viability stain 440UV (BD Biosciences) was then used to stain dead cells for 15 min. The samples were washed with stain buffer and centrifuged at 300 × g for 5 min. Subsequently, the cells were preincubated with brilliant stain buffer (BD

Biosciences) for 15 min. The cell samples were then stained separately with APC-Cy7-conjugated anti-mouse CD3, BUV496-conjugated anti-mouse CD4, FITC-conjugated anti-mouse CD8, BB700-conjugated anti-mouse CD45RA, BV421-conjugated anti-mouse CXCR5, BV605-conjugated anti-mouse OX40, BV480-conjugated anti-mouse PD-1, or BV750-conjugated anti-mouse CD40L (from BD Biosciences), and with BV711-conjugated anti-mouse CD62L, BV785-conjugated anti-mouse CCR6, or PE/Fire 810-conjugated anti-mouse-CXCR3 (from BioLegend) for 45 min. After surface staining, the samples were washed with stain buffer, centrifuged at 300 g for 5 min and resuspended in 200 μL stain buffer. The data were obtained using a Sony ID7000 Spectral Cell Analyzer (Sony Biotechnology) and analyzed with FlowJo v10.8.1 software.

2.8 Statistical analysis

All data in our study were presented as the mean ± standard deviation (SD). The significance of differences between the control and ITP groups was determined using a t test or Mann-Whitney U test depending on the normality of the data. All statistical calculations were implemented using GraphPad Prism version 8.0 software, with a P value <0.05 indicating statistical significance.

3 Results

3.1 Establishment of passive murine models of ITP (antibody injection)

In order to examine the vital role of OX40 in the immunopathological mechanisms of ITP, we established a passive ITP mouse model (Fig. 1a). First, an anti-mouse CD41 antibody was injected into the peritoneal cavity of C57BL/6 WT mice, and control mice were injected with the same volume of PBS. The mice treated with the monoclonal PLT antibody became thrombocytopenic; the PLT counts were significantly reduced within 24 h after injection and relatively gradually decreased from Day 2 to Day 7 (Fig. 1b). Meanwhile, PLT counts in the control mice remained stable throughout the study (Fig. 1b). Compared to those of the controls, the spleens of the ITP model mice exhibited abnormal appearance, with significantly greater enlargement and weight increase (Fig. 1c). In addition, histopathological examinations were performed on the spleens of the mice in the control and ITP groups. By H&E section observation, thrombocytopenic mice exhibited significant histopathological changes compared to control mice. In the spleens of mice with ITP, the lymphocytes were dense and the red blood sinuses were expanded (Fig. 1d). Compared to those in the controls, a greater percentage of infiltrating megakaryocytes in the spleen was observed in the model group (Fig. 1d). In addition, the antiplatelet agent MWReg30 induced megakaryocyte morphological aberrations, which consisted of pyknotic nuclei and cytoplasmic blebbing (Fig. 1d).

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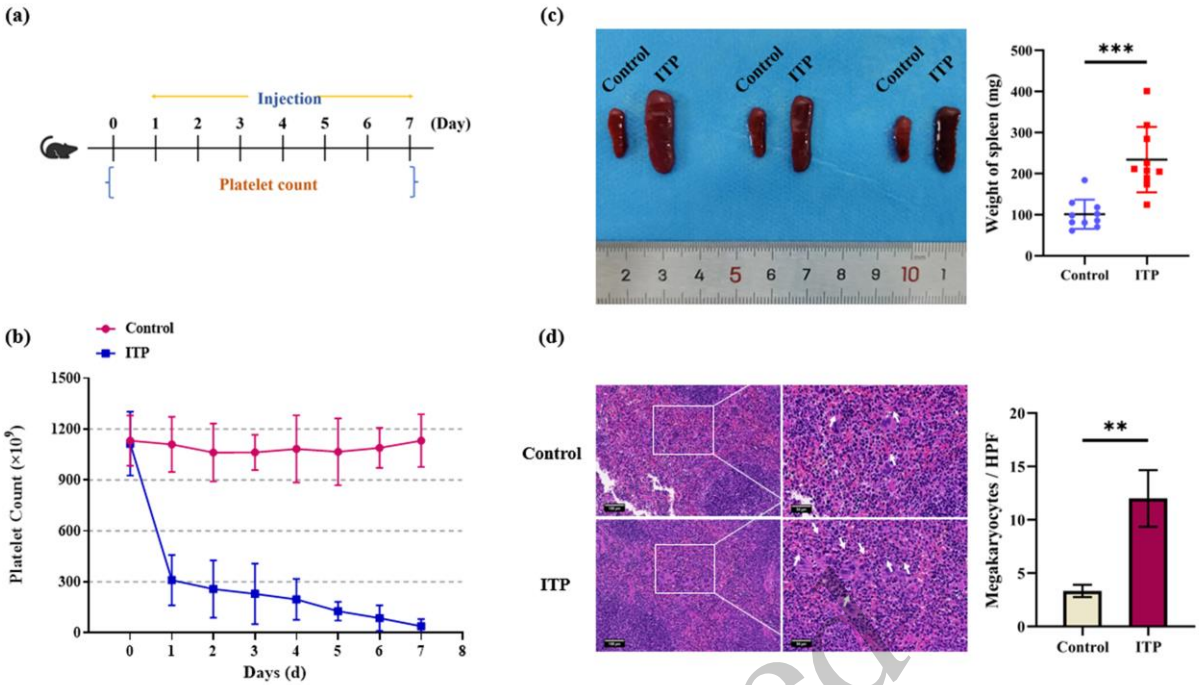

Fig. 1 Assessment of platelet count, splenomegaly and megakaryocytes in the spleen. (a) Flowchart of the murine passive ITP model. (b) Peripheral blood was extracted at different time points for measuring platelet count. (c) Wet weights of spleens and gross appearance of representative spleen tissues in control and ITP mice. (d) Photomicrograph of hematoxylin-eosin (HE) staining of pathological changes of the spleen in ITP mice. Significant differences are indicated by *****P*<0.01 and ******P* < 0.001. Scale bars: 84 and 168 μm.

3.2 OX40L-OX40 has abnormal expression levels in ITP model mice

The costimulatory signals of OX40L-OX40 play a major role in the pathophysiology of autoimmune diseases (Edner et al., 2020). To explore the potential roles of OX40L and OX40 in aggravating the pathogenesis of ITP, the protein levels of OX40L and OX40 in the spleens of control and ITP model mice were determined via western blotting and immunohistochemical assays. For both datasets, the results showed that the expression levels and positive immunostaining of splenic OX40L and OX40 in ITP model mice were markedly higher than those in normal controls (Figs. 2a and 2b). Interestingly, OX40-positivity was detected in the germinal center (GC) of the white pulp in the splenic lymphoid nodule of ITP model mice (Fig. 2b).

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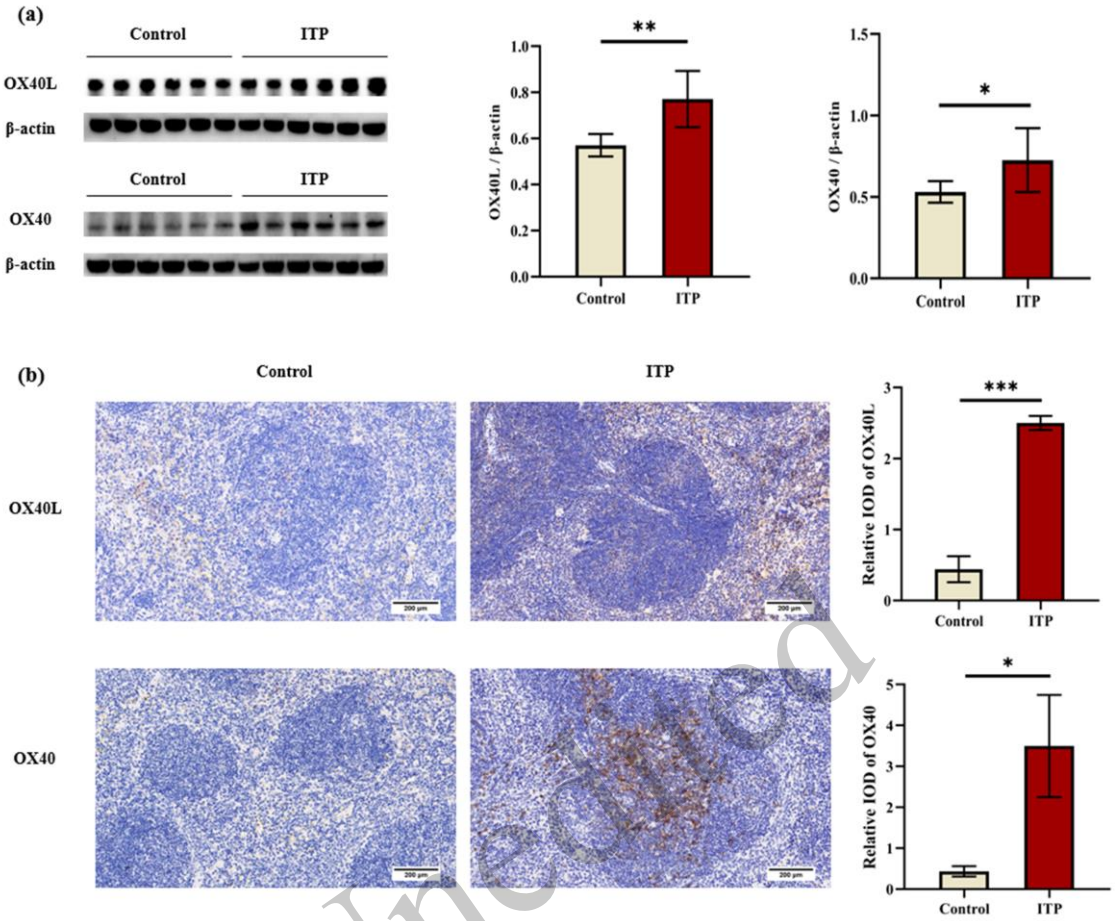

Fig. 2 Upregulated expression of OX40L and OX40 in the spleen of ITP mice. (a) The protein levels of OX40L and OX40 in the spleen of ITP mice. (b) IHC for OX40L and OX40 performed on splenic tissues. Significant differences are indicated by *P < 0.05, **P < 0.01, and ***P < 0.001. Scale bar: 200 μm.

3.3 OX40L-OX40 immunoregulatory axis mediates Tfh differentiation *in vitro*

Antiplatelet antibody-producing B lymphocytes play a critical role in the pathogenesis of ITP (Audia et al., 2021). In the GC, the maturation of high-affinity B-cell clones depend on support from Tfh cells (Liu et al., 2021). The costimulating signal of the OX40L-OX40 axis is important for T-cell activation and function. To evaluate the potential effects of OX40L and OX40 on Tfh cells, we purified splenic $CD4^+$ naïve T cells from C57BL/6 WT mice via activation with anti-CD3e/CD28 beads with or without incubation with the recombinant protein OX40L for 6 days. The phenotype and the expression of specific markers OX40, ICOS, PD-1, and CD40L on Tfh cells were examined by FCM.

Compared to those in the controls, the frequencies of $CD4^+CD45RA^-CXCR5^+$ Tfh cells in the group treated with OX40L were significantly greater after 6 days of differentiation and the expression of ICOS, PD-1 and CD40L was upregulated (Figs. 3a, 3c–3e). Meanwhile, the expression of OX40 on Tfh cells were significantly downregulated in the OX40L-stimulated group, indicating that OX40L increased binding to OX40 (Fig. 3b). Next, we examined the levels of several Tfh-related cytokines in the cell supernatant. In the OX40L-treated group, the concentrations of IL-4, IL-17A, and IL-21 were significantly increased (Fig. 3f). These data indicated that the surface-bound molecule OX40L combined with OX40 promotes Tfh cell differentiation and function *in vitro* (Fig. 3a).

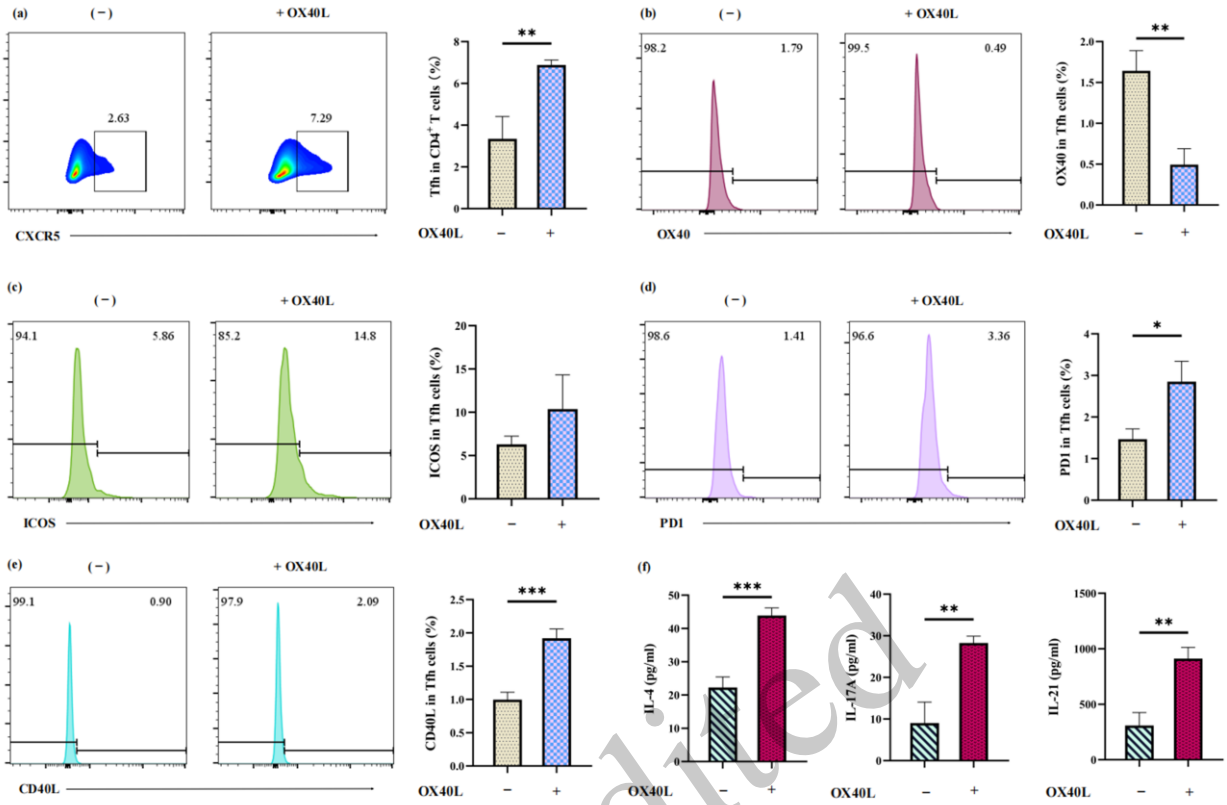

Fig. 3 The OX40L-OX40 axis promotes Tfh differentiation and development *in vitro*. Purified splenic CD4-naïve T cells were activated by anti-CD3e/CD28 beads and cultured in the presence or absence of recombinant protein OX40L for 6 days. (a) Frequency of Tfh cells among the total $CD4^+$ T cells from normal and ITP mice. (b) Percentage of OX40 expression on Tfh cells. (c) Percentage of ICOS expression on Tfh cells. (d) Percentage of PD-1 expression on Tfh cells. (e) Percentage of CD40L expression on Tfh cells. (f) Levels of IL-4, IL-17A, and IL-21 in the culture supernatants. Significant differences are indicated by *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$.

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3.4 Function of OX40L-OX40-mediated Tfh differentiation in ITP model mice

In order to further study the differentiation and function of Tfh cells in the ITP model mice, we measured the phenotype of splenic T cells and the expression levels of characteristic markers on Tfh cells in the control and ITP model mice. In the ITP group, the Tfh molecules CXCR5, Bcl6, ICOS, PD-1, and CD40L were upregulated (Fig. 4a). Splenic Tfh cells were also analyzed by FCM in the present study, and we observed a 1.3-fold increase in the percentage of $CD4^+CD45RA^-CXCR5^+$ Tfh cells in ITP model mice compared to that in controls (Fig. 4b). According to the documented gating strategy, we further investigated three Tfh cell subtypes, Tfh1 ($CXCR3^+CCR6^-$), Tfh2 ($CXCR3^-CCR6^-$) and Tfh17 ($CXCR3^-CCR6^+$) cells in the spleens of control and ITP model mice (Bonami et al., 2020). Compared to the controls, the percentage of Tfh2 cells were greater, but the percentages of Tfh1 cells were lower in the ITP model mice (Fig. 4b). The percentage of Tfh17 cells was extremely low, with no significant difference between the two groups (Fig. 4b). The Tfh2/Tfh1 cell ratio in ITP model mice was greater than that in normal mice (Fig. 4b). These findings imply that the cell populations exhibited a Tfh1 to Tfh2 shift in mice with ITP. Furthermore, the expression of the key Tfh activation markers OX40, ICOS, and PD-1 on Tfh2 cells was significantly increased in ITP model mice, while that of CD40L slightly increased, with no statistical significance (Fig. 4c). We further assessed the plasma levels of pro- and anti-inflammatory cytokines that are related to Tfh differentiation and ITP pathogenesis. As shown in Fig. 4d, in the serum of ITP model mice, the secretion of the proinflammatory cytokines IL-4, IL-6, IL-17A, and IL-21 was increased, whereas the secretion of the anti-inflammatory cytokines IL-10 and TGF-β was decreased. These

data imply that OX40L-OX40 coupling might stimulate the proliferation, expansion and function of Tfh cells and shift Tfh populations to Tfh2 cells in ITP.

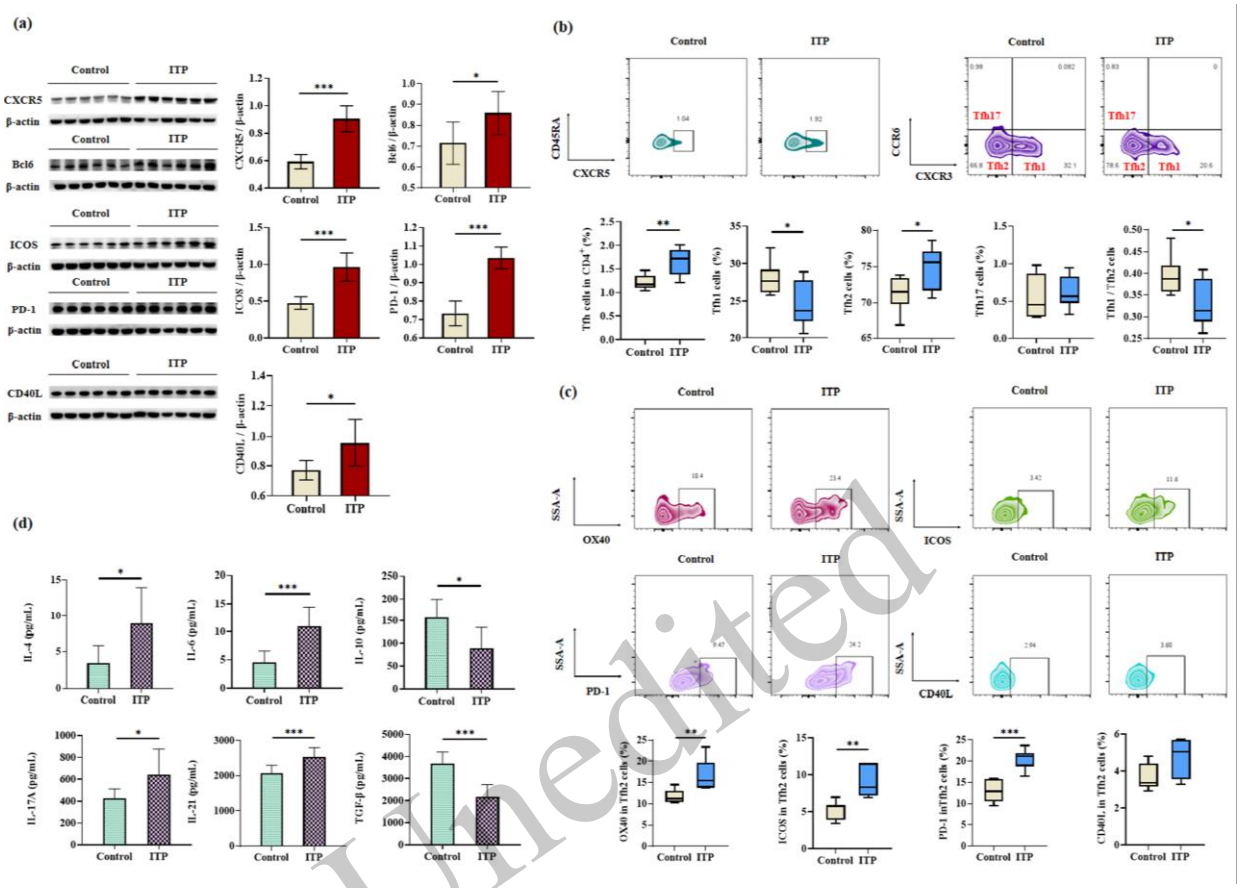

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Fig. 4 Differentiation and function of Tfh subtypes in ITP mice. (a) Expression of key molecules (CXCR5, Bcl6, ICOS, PD-1, and CD40L) of Tfh cells in the spleen of normal and ITP mice. (b) Levels of proinflammatory cytokines (IL-4, IL-6, IL-17A, and IL-21) and anti-inflammatory cytokines (TGF-β and IL-10) in the serum of normal and ITP mice. (c) Frequencies of splenic $CXCR5^+$ $CD45RA^-$ Tfh cell and Tfh subtypes in normal and ITP mice. (d) Expression of ICOS, PD-1, and CD40L on Tfh2 cells. Significant differences are indicated by *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$.

3.5 OX40 colocalizes with Tfh and B cells during the migration of B cells to germinal centers in ITP model mice

In order to determine the localization of OX40, we stained spleen tissues from control and ITP model mice with anti-CD4, anti-CXCR5, anti-CD19, and anti-OX40 antibodies. In the spleens of ITP model mice, OX40 was highly expressed in the GCs of the white pulp and colocalized with CD4 and CXCR5 (Fig. 5a and 5b). These results suggest that OX40L-OX40 coupling might contribute to aberrant Tfh cell activation in ITP. Splenic $CD4^+CD19^+$ B cells from normal mice were found mainly in the marginal zone, whereas $CD4^+CD19^+$ B cells from the spleens of ITP model mice were abundantly distributed in the GCs of white pulp and colocalized with CD4, CXCR5 and OX40 (Fig. 5b). Taken the above findings together, we speculated that the aberrant expression of OX40L and OX40 increases Tfh cell differentiation and helper function. Subsequently, Tfh cells may interact with B lymphocytes, which promotes their migration to germinal centers and their maturation into antibody-produced plasma cells. The OX40L-OX40 axis play a pivotal role in ITP pathogenesis; however, the specific regulatory mechanism of Tfh cells' helper function on B lymphocytes in ITP, regulated by this axis, needs to be further explored.

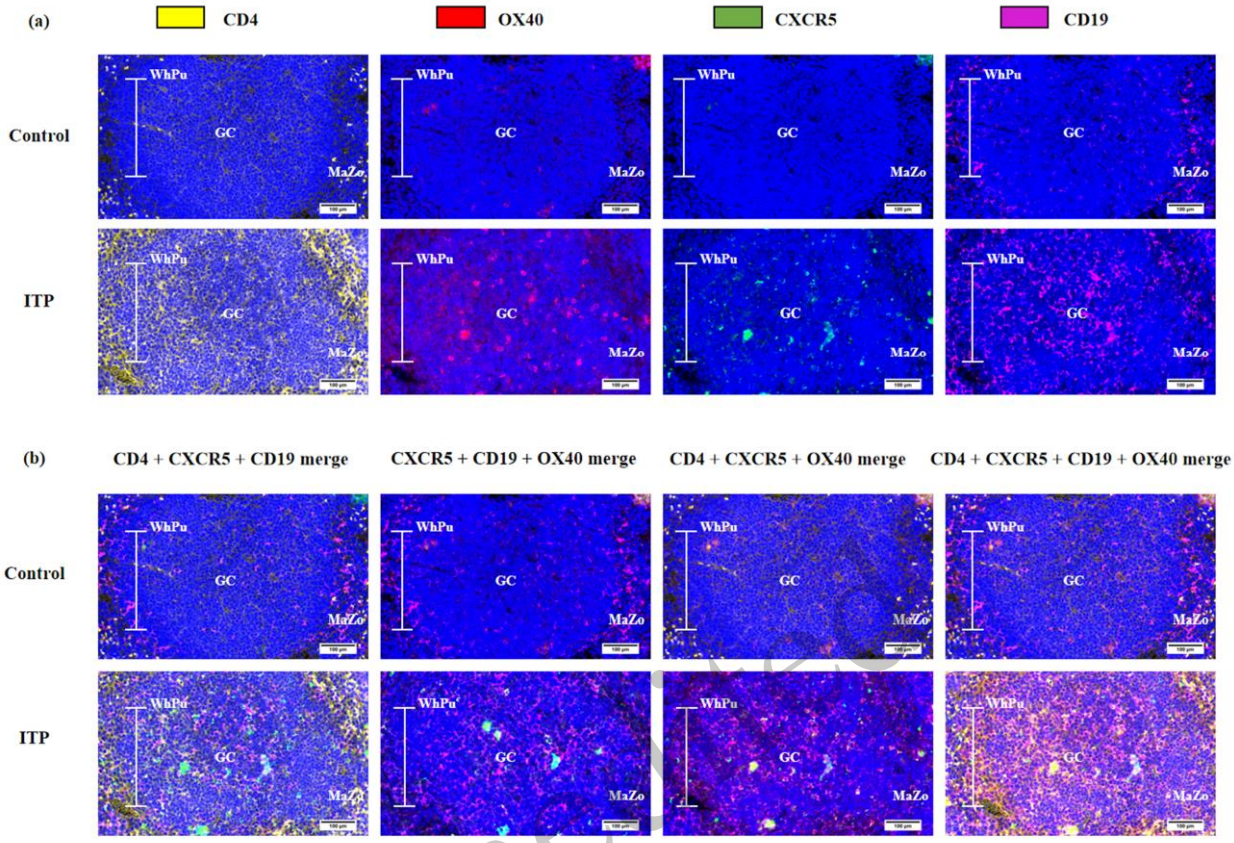

Fig. 5 Expression and localization of OX40, Tfh cells and B cells in the spleen of normal and ITP mice. (a) Individual markers CD4 (yellow), OX40 (red), CXCR5 (green), and CD19 (pink) of images. (b) Composite images of multiple markers. WhPu, GC and MaZo represent white pulp, germinal center and marginal zone, respectively. Scale bar: 100 μm.

3.6 Signaling pathways are associated with Tfh differentiation in ITP model mice

We confirmed that the OX40L-OX40 axis plays an essential role in Tfh cell differentiation and development in ITP, but the downstream signaling pathways of OX40L-OX40 in Tfh-mediated ITP remained to be discovered. Previously, the signaling mechanism of OX40 was considered to be associated with the activation of the TRAF-NF-κB pathway (Pedros et al., 2018). To assess the possible regulatory mechanisms of the OX40L-OX40 axis in ITP, we extended our study by investigating the expression of TRAF proteins and the activation of NF-κB pathway in an ITP mouse model. Among the six members of the TRAF protein family (TRAF1-TRAF6), TRAF2, TRAF3, TRAF4, and TRAF5 were significantly upregulated in the ITP mouse model (Fig. 6a). The protein levels of TRAF1 and TRAF6 were not significantly different between the control and ITP groups (Fig. 6a). Moreover, the NF-κB pathway was activated in ITP model mice, as evidenced by a significantly increased ratio of p-P65 (phospho-P65)/P65 (Fig. 6a). Crosstalk between the JAK-STAT pathway and TRAF-mediated pathway has been reported in previous studies (Nagashima et al., 2018). Therefore, to assess the activation of the JAK-STAT pathway, we further evaluated the expression and phosphorylation of JAK kinases and STAT family members in ITP model mice. Compared to that in normal mice, the p-JAK3/JAK3 ratio was significantly lower, whereas the p-Tyk2/Tyk2 ratio was notably greater in ITP model mice (Fig. 6b). The p-JAK1/JAK1 and p-JAK2/JAK2 ratios were not significantly different between the two groups (Fig. 6b). In the ITP mouse model, western blotting analysis demonstrated notable increase in the p-STAT3/STAT3 and p-STAT6/STAT6 ratios and a significant decrease in the p-STAT2/STAT2 and

p-STAT5/STAT5 ratios (Fig. 6c). Taken together, the above data suggest that OX40 might activate the downstream TRAF-NF-κB signaling pathway and crosstalk with the JAK-STAT pathway, regulating Tfh differentiation in ITP model mice.

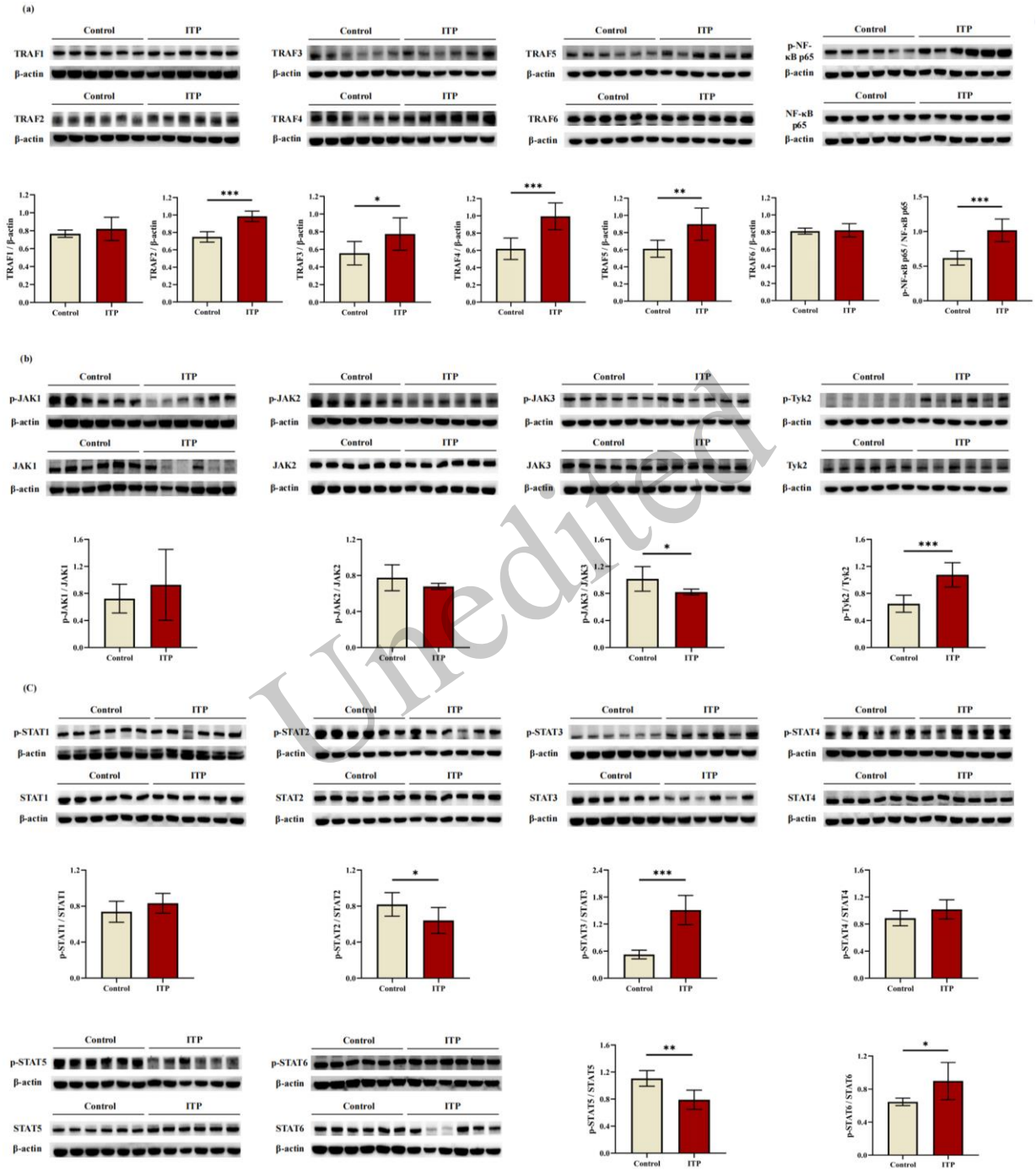

Fig. 6 Activation of TRAF-NF-κB and JAK-STAT signal pathways in normal and ITP mice. (a) Expression of TRAFs protein and phosphorylation of NF-κB p65 in the spleen of normal and ITP mice. (b) Phosphorylation of JAKs in the spleen of normal and ITP mice. (c) Phosphorylation of STATs in the spleen of normal and ITP mice. Significant differences are indicated by *$P < 0.05$, **$P < 0.01$ and ***$P < 0.001$.

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4 Discussion

ITP is a complicated autoimmune disease characterized by impaired platelet generation and exacerbated platelet injury (Mingot-Castellano et al., 2022). Some ITP patients with prolonged and severe thrombocytopenia are insensitive to current therapies, and these patients also have an increased risk of bleeding and complications (Vianelli et al., 2023; Cines, 2023). Therefore, the identification of novel therapeutic methods for ITP is necessary.

The OX40L-OX40 axis has been proposed as a promising target for treating autoimmunity (Furue et al., 2021). A previous study indicated the crucial role of the OX40L-OX40 interaction in the immunopathological mechanisms of autoimmune diseases, such as SLE, RA and allergic asthma (Cui et al., 2019). In an SLE mouse model, the expression of OX40 on several $CD4^+$ T-cell subsets was enhanced in kidney and spleen tissues and this was correlated with disease severity (Sitrin et al., 2017). OX40L expression tended to increase in RA patients, and it also increased in an age-dependent manner in the serum of healthy volunteers (An et al., 2019). In our previous study, to explore the clinical significance of OX40L and OX40 expression in ITP patients, we collected samples from fifty-four newly diagnosed ITP patients and 24 healthy controls (HCs). Compared with HCs, the expression of OX40L and OX40 mRNA in PBMCs were significantly upregulated in ITP patients, accompanied by significantly elevated $OX40^+CD4^+$ T cells' frequencies and plasma sOX40L levels (Cui et al., 2019). In the present study, we discovered that splenic OX40L and OX40 were significantly upregulated in mice with ITP, similar to the findings of previous studies (Cui et al., 2019). On this basis, we inferred that the abnormal expression of OX40L and OX40 might contribute to the development of ITP. Numerous studies have shown that the costimulatory signals of OX40L-OX40 can support Tfh cell development and survival and enhance the ability of Tfh cells to support B lymphocytes (Fu et al., 2021). Jacquemin (2015) discovered that OX40L and OX40 are involved in the abnormal Tfh response in patients with SLE (Jacquemin et al., 2015). Recently, many studies have shown that abnormal OX40L and OX40 expression on Tregs and Tfr cells impairs the ability of Tfh cell-dependent B lymphocyte activation (Jacquemin et al., 2018). In our survey, we found that OX40L increased the expression of multiple Tfh cell molecules (ICOS, PD-1, and CD40L) and promoted effector cytokine secretion (IL-4, IL-17A, and IL-21), likely enhancing the helper function of Tfh cells for B cells *in vitro*. Consistent with the *in vivo* results, in the ITP mouse model, the percentage of $OX40^+$ Tfh cells increased, and this change was accompanied by the high expression of characteristic molecules strongly related to Tfh cell differentiation and function. These data implied that upregulated OX40-mediated abnormal Tfh activity might play a major pathogenic role in ITP.

In order to explore the interaction between OX40L-OX40, Tfh cells and B cells in ITP, we conducted immunofluorescence staining of spleen tissues from control and ITP model mice. The immunofluorescence images showed that splenic $CD4^+CD19^+$ B cells in ITP model mice were concentrated in the germinal center of the white pulp and colocalized with Tfh cells and OX40, while $CD4^+CD19^+$ B cells in normal mice were distributed in the marginal zone. OX40L-OX40 costimulatory signals play critical roles in improving the function of T cells, enhancing their survival and preventing their apoptosis (Fu et al., 2021). Jacquemin (2018) discovered that OX40L-OX40 could induce the expression of multiple molecules on human-derived Tfh cells and promote them to become functional B cell helpers *in vitro* (Jacquemin et al., 2018). A previous study considered that OX40L-OX40 interaction could promote both GC Tfh and GC B cell differentiation, and accelerate B cells differentiate into plasma cells with the help of GC Tfh cells (Fu et al., 2021). Antiplatelet autoantibodies produced by B lymphocytes are the main pathogenic factors for ITP; they not only increase platelet destruction but also impair the ability of megakaryocytes to generate platelets (Wang et al., 2020). The majority of antiplatelet antibodies are specifically generated by long-lived plasma cells in the spleen and the bone marrow (Vernava and Schmitt, 2023). In the resting state, B lymphocytes are mainly located in the T-cell zone and the marginal zone in the spleen (Victora and Nussenzweig, 2022). After antigen stimulation, B cells are activated and migrate to GCs, where Tfh cells help B lymphocytes differentiate into long-lived plasma cells to produce antiplatelet antibodies against glycoproteins (like GPIIb/IIIa and GPIb/IX) on thrombocytes and

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megakaryocytes (Victora and Nussenzweig, 2022; Vernava and Schmitt, 2023). Antiplatelet antibodies can target glycoproteins, especially the GPIIb/IIIa, through interactions with Fc gamma receptors (FcγRs) and complement receptors, inducing platelet phagocytosis (Audia et al., 2021). Indeed, a study reported that splenic Tfh cells were increased and this was correlated with increased germinal center and plasma cell in ITP patients (Audia et al., 2014). *In vitro*, the activation of Tfh cells promoted the differentiation of splenic B cells into plasma cells and induced the secretion of antiplatelet antibodies in ITP patients (Audia et al., 2014). These data suggested that upregulated OX40 signaling might induce abnormal Tfh activity, promoting the establishment of GCs and regulating the migration, differentiation and maturation of antibody-secreting B lymphocytes. Overall, OX40 signaling likely contributes to the production of autoantibodies by enhancing the helper function of Tfh cells for B lymphocytes, resulting in the acceleration of ITP progression.

Because of the important effect of Tfh cells on ITP pathogenesis, our study further explored the subtype distribution of Tfh cells in an ITP animal model. The results indicated that the percentages of Tfh1 and Tfh2 cells in the spleens of ITP model mice decreased and increased, respectively, and the ratio of Tfh2/Tfh1 cells increased. The percentage of Tfh17 cells was extremely low in the present study, so further analysis of this subset was not performed. Similar trends have been observed in other autoimmune diseases in other studies. For instance, Xu (2019) reported that AAV patients exhibit a Tfh1-to-Tfh2 shift, with a decrease in Tfh1 cells and an increase in Tfh2 cells (Xu et al., 2019). Moreover, in ankylosing spondylitis (AS) patients, a high degree of disease activity was associated with elevated Tfh2 effector memory levels (Shesternya et al., 2022). Patients with juvenile dermatomyositis also had an increased ratio of Tfh2/Tfh1 cells, with Tfh1 cells incapable of assisting naïve B lymphocytes (Schmitt et al., 2014; Morita et al., 2011). These findings illustrate that changes in the subtype composition of Tfh cells (Tfh1 to Tfh2 shift) might participate in the progression of ITP and that Tfh2 cells likely play a major role in promoting B lymphocyte differentiation and development.

In order to further verify the molecular mechanisms of OX40L-OX40 signaling in ITP, the downstream interacting proteins and phosphorylation pathways of OX40 were explored, and the expression of TRAF2, TRAF3, TRAF4, and TRAF5 was significantly increased in the ITP mouse model. OX40 is a TRAF-dependent TNFRSF family member associated with the differentiation and function of Tfh cells (Pedros et al., 2018). OX40L binding of OX40 results in the recruitment of TRAF2, TRAF3 and TRAF5 (Fu et al., 2021). Previous studies have reported that OX40 ligation by OX40L can promote the assembly of a unique complex TRAF-RIP-IKKα/β/γ that activates NF-κB1 to provide survival signals to T cells (So et al., 2011). NF-κB activation plays an essential role in T-cell responses, and TRAFs are viewed as strong activators of NF-κB for T-cell immunity (So et al., 2011). Researchers discovered that activated OX40 signaling proteins can interact with TRAF2 and TRAF5, promoting the NF-κB pathway to positively regulate Tfh differentiation (Nagashima et al., 2018; Furue et al., 2021). Moreover, TRAF3 also plays an important role in the conduction of OX40 signaling, whereas TRAF3 might also inhibit OX40 activation and Tfh development (Nagashima et al., 2018). TRAF6 could induce the activation of the non-canonical NF-κB pathway in the presence of OX40 ligation (Xiao et al., 2012). Aberrant ubiquitination of TRAF6 can also facilitate the activation of the NF-κB pathway; however, TRAF6 levels were not significantly different between the control and ITP groups in our study (Yang et al., 2023). He (2018) suggested that NF-κB expression significantly increases in ITP patients, and overexpressed NF-κB co-incubation with $CD34^+$ cells can result in a reduction in megakaryocytic differentiation and thrombopoiesis *in vitro*, indicating the important role of the NF-κB signal in ITP pathogenesis (He et al., 2018). Certainly, we observed increased phosphorylation of the NF-κB p65 protein in the spleens of ITP model mice. Nonetheless, more experiments should be conducted to further clarify the role of OX40L-OX40 axis in mediating TRAF-NF-κB to regulate ITP pathogenesis. Additionally, the JAK-STAT pathway, which is generated by the cytokine receptors IL-6 and IL-21, influences Tfh cell development, and interactions with TRAF-mediated pathways can facilitate the generation of the integrated signals required to induce and maintain Tfh differentiation (Nagashima et al., 2018). In this study, the levels of IL-6 and IL-21 in the serum of ITP model mice were increased. The p-Tyk2/Tyk2, p-STAT3/STAT3, and p-STAT6/STAT6 ratios were notably raised, while the p-JAK3/JAK3, p-STAT2/STAT2, and p-STAT5/STAT5 ratios were

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significantly decreased. Thus, we speculated that the OX40-OX40 axis might activate the TRAF-NF-κB pathway in ITP, promoting Tfh differentiation. Subsequently, the secretion of the Tfh-related cytokines IL-6 and IL-21 was increased, which acted on cytokine receptors of Tfh cells, inducing the activation of the JAK-STAT signaling pathway, promoting Tfh differentiation. However, the regulatory mechanism of the OX40L-OX40 downstream signaling pathway in ITP is complex and needs further exploration by experiments.

In the present work, we constructed a passive ITP mice model of MWReg30. Compared to the controls, MWReg30 induced a significant reduction in the PLT counts of ITP model mice, leading to spleens exhibiting enlargement, weight increase and aberrant histopathological changes. MWReg30 was discovered by Burstein (1992) and has been widely used to construct passive ITP animal models (Burstein et al., 1992; Guo et al., 2018). Guo et al. found that MWReg30 could reduce the PLT counts and significantly increase the number of bone marrow megakaryocyte counts (Guo et al., 2018). Similarly, we observed a higher concentration infiltration of megakaryocytes in the spleen of ITP model mice. Yu (2018) used the passive ITP mice model to validate the PGRN deficiency impairing proliferation of Tregs (Yu et al., 2018). A recent study established another efficient murine model of ITP through immunized CD41-KO mice (Li et al., 2019). Different from the ITP mouse model constructed with MWReg30, the mature megakaryocytes in bone marrow were obviously decreased in this novel ITP murine model (Li et al., 2019). In the future, we aim to develop more efficient murine ITP models to make our conclusions more robust and explore the complicated pathophysiology of ITP.

5 Conclusions

Our study demonstrated that upregulated OX40L- and OX40-mediated abnormal Tfh activity plays a major pathogenic role in ITP. Aberrant OX40L-OX40 signaling might activate the TRAF-NF-κB and JAK-STAT signaling pathways. We speculated that the interactions between the two signaling pathways likely promote the Tfh1-to-Tfh2 shift, inducing the generation of autoantibodies by enhancing the helper function of Tfh cells for B cells, which results in the acceleration of the pathogenetic progression of ITP. Overall, our results suggest that the costimulation signaling pathway of OX40L-OX40 might be a potential therapeutic target for ITP. As a limitation of this study, we only used a passive ITP mice model; therefore, additional investigations of the OX40L-OX40 axis mediating Tfh cell differentiation and function in ITP pathogenesis should be conducted in other efficient murine ITP models and clinical samples of ITP patients.

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Data availability statement

All data supporting the conclusions during present study are included within the article.

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Author contributions

Ziyin YANG, Dawei CUI, and Jue XIE conceived and designed the study. Ziyin YANG and Lei HAI performed most of the experiments with help from Siwen WU and Yan LV. Ziyin YANG, Lei HAI, and Xiaoyu CHEN contributed to data analyses. Ziyin YANG, Dawei CUI, and Jue XIE wrote the manuscript. All authors read and approved the final manuscript.

Compliance with ethics guidelines

The authors in present study declare that they have no competing interests.

All the animal experiments were consented of the Animal Ethics Committee of the First Affiliated Hospital, College of Medicine, Zhejiang University (No. 2019621, Feb 27, 2019).

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