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Toll-like receptor 2 deficiency promotes the generation of alloreactive Th17 cells after cardiac transplantation in mice

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ABSTRACT

The emergence of alloreactive Th17 cells that mediate allograft rejection has provided an impetus to understand the factors affecting the generation of Th17 cells in allograft transplantation. How toll-like receptor 2 (TLR2) signalling regulates the generation of Th17 cells upon alloantigen stimuli remains unclear. In this study, we used a mouse model of cardiac allograft transplantation to investigate whether TLR2 signalling influences the development of Th17 cells. Here, we demonstrate that the TLR2-deficient recipient mice show high Th17 cells, both in spleens and allografts, as well as higher infiltrating inflammatory leukocytes in cardiac allografts compared to wild-type control recipient mice. mRNA expression of IL-17, IL-6, TNF-α, CCR6 and CCL20 within the allografts is markedly increased in TLR2-deficient recipient mice compared to wild-type recipient mice. In addition, TLR2 deficiency leads to upregulation of Signal transducer and activator of transcription 3 (STAT3) phosphorylation in both spleens and allografts. In an in vitro experiment, a mixed lymphocyte reaction was assessed, which further confirmed that TLR2 deficiency leads to a significant increase in the generation of Th17 cells compared with wild-type controls. Furthermore, IL-6 secreted by the dendritic cells of TLR2-deficient mice contributes to driving the generation of these Th17 cells. These results suggest that TLR2 signalling is important in regulating the development of Th17 cells after cardiac allograft transplantation.

1. Introduction

Heart transplantation is the most effective therapeutic solution for end-stage heart disease. With the development of immunosuppressants, the long-term survival rate of transplant patients is gradually increasing, but immune rejection is still the main cause of failure of heart transplantation therapy. The innate immune system is now recognized to play an important role in allograft transplantation. Toll-like receptors (TLRs) recognize a variety of pathogen-associated molecular patterns, such as lipopolysaccharide (LPS), bacterial lipoproteins (BLPs), and zymosan. In addition, TLRs can also be activated by damage-associated molecular patterns such as high-mobility group box chromosomal protein 1 (HMGB1), heat shock protein (HSP)-60 and -70, glucose regulated protein (GRP)-94 and fibrinogen [1]. TLRs orchestrate the innate immune response and the subsequent adaptive immune response through antigen presentation, activation of dendritic cells and regulation of T cells [2]. TLRs play an important role in many facets of transplantation biology, including rejection and tolerance, ischemia/reperfusion injury (IRI), and infections after transplantation [3–6].

TLR2 signaling has been reported to be involved in various conditions, such as infection, cancer, and autoimmune inflammation in both humans and mice [7–13]. Bacterial lipoproteins (BLPs) that are expressed by all bacteria are strong activators of TLR2, and the BLP-stimulated TLR2/myeloid differentiation factor 88 (MyD88) pathway mediates both apoptosis and nuclear factor-kappa B (NF-κB) activation [14]. In addition to its pro-inflammatory effects, the TLR2 signalling pathway has been found to regulate inflammation in a variety of disease models. In a mouse model, TLR2 deficiency aggravates lung injury

Abbreviations: TLR, toll-like receptor; IL, interleukin; WT, wild-type; CYA, cyclosporine A; TAC, tacrolimus; FACS, fluorescence-activated cell sorting; BMDCs, bone-marrow-derived dendritic cells; MST, mean survival time; HMGB1, high mobility group box 1; MLC, mixed lymphocyte culture; STAT3, signal transducer and activator of transcription 3

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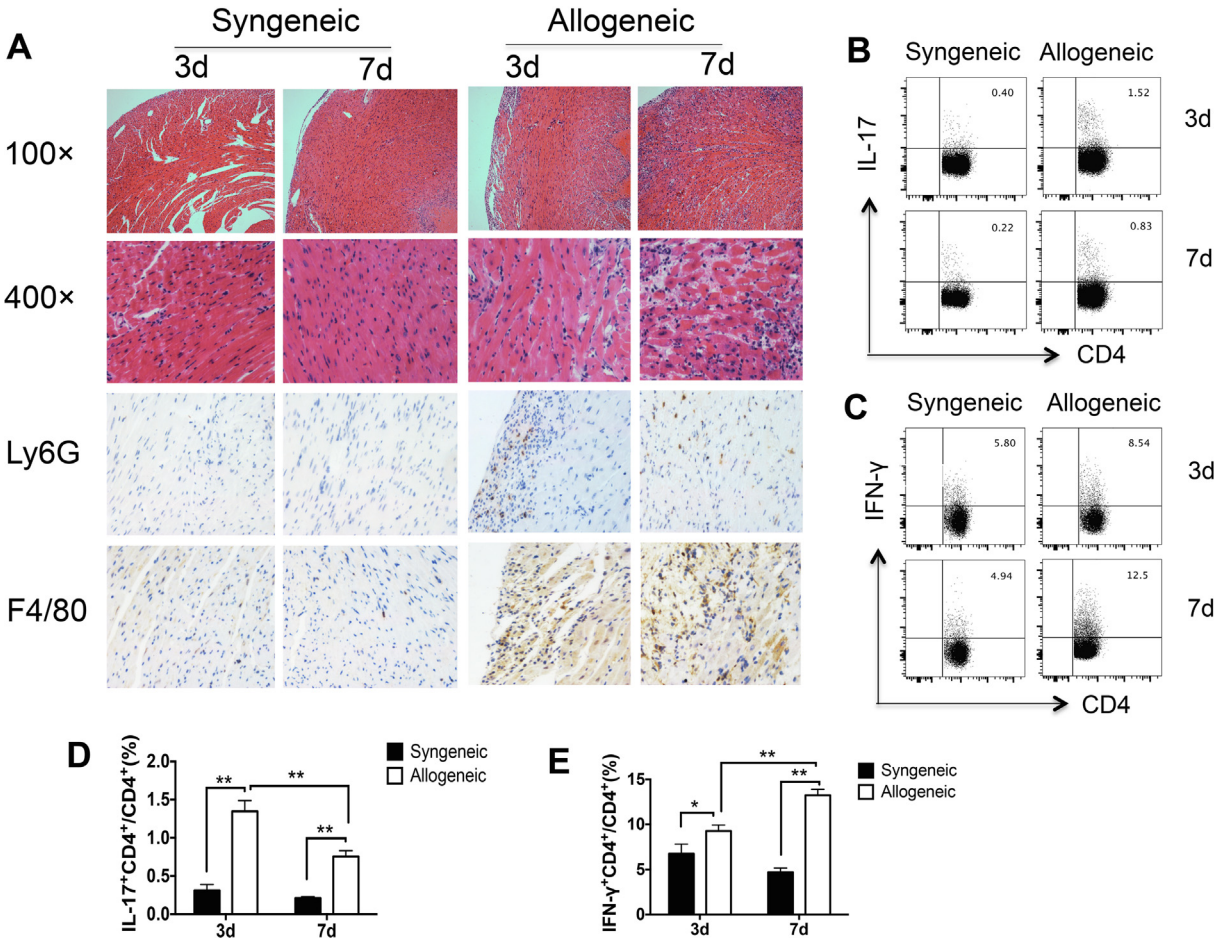

Fig. 1. Histological analysis of cardiac allografts and the kinetics for splenic alloreactive Th1 and Th17 cells. Cardiac grafts from BALB/c mice (allogeneic) or C57BL/6 (syngeneic) mice were transplanted into C57BL/6 mice. The cardiac grafts and spleens in the allogeneic or syngeneic groups were collected on the 3rd and 7th day after transplantation. (A) Histology analysis and immunostaining for neutrophils and macrophages of cardiac grafts. Pathologic changes of acute cardiac allograft rejection were examined by haematoxylin and eosin (H&E) staining (original magnification, ×100 or ×400). The sections were stained for Ly6G or F4/80 to confirm the infiltration of neutrophils or macrophages in the cardiac grafts (original magnification, ×400). (B) (D) Kinetics for Th17 cells in spleens after allogeneic and syngeneic transplantation was analysed by flow cytometry. (C) (E) Kinetics for Th1 cells in spleens after allogeneic and syngeneic transplantation was analysed by flow cytometry. Data are shown as the mean ± SD (n = 3/group). Data from one representative out of three experiments are shown. $^{*}p < 0.05$; $^{**}p < 0.01$.

caused by mechanical ventilation and enhances pulmonary levels of IL-6, IL-1β and keratinocyte-derived chemokine [15]. TLR2 ligands attenuate cardiac dysfunction in cecal ligation and puncture (CLP)-induced sepsis via a phosphoinositide 3-kinase (PI3K)-dependent mechanism, which may be a negative feedback regulator of inflammation [16]. A previously reported study suggests that TLR2 signaling regulates innate and adaptive immunity to *Paracoccidioides brasiliensis* infection. TLR2 deficiency leads to an unrestrained inflammatory response in the lungs with increased Th17 cells and diminished regulatory T cells. This study suggested a link between TLR2 signaling and the development of Th17 cells [17]. It is unclear whether TLR2 signaling regulates the generation of alloreactive Th17 cells in the context of allograft transplantation.

The TLR2 signaling is activated by damage-associated molecular patterns such as HMGB1 and HSP-60 after organ transplantation [18]. Previous studies have shown that the expression of TLR2 and TLR4 of the allografts and on peripheral blood mononuclear cells is increased in acute rejection after cardiac transplantation, liver transplantation and small intestine transplantation. These studies suggest that TLR2 and TLR4 may promote transplant rejection [19–21]. However, in another study, increased TLR2 expression in G-CSF-mobilized donor grafts hardly affected alloreactive T cell responses and had no effect on acute GVHD [22]. Therefore, the role of TLR2 in allogeneic transplantation is still controversial and worth exploring.

Our laboratory [23–25] and others [26,27] demonstrated that IL-17 and Th17 cells promote allograft rejection by enhancing neutrophil infiltration. In this study, we sought to determine the role of TLR2 signaling in the development of alloreactive Th17 cells using a cardiac allograft transplantation model in mice. Our results provide evidence of a solid link between TLR2 and Th17 cells in the context of heart allograft transplantation.

2. Materials and methods

2.1. Animals

C57BL/6J and BALB/c mice were purchased from Charles River Laboratories (Beijing, China). C57/B6.129-Tlr2$^{tmIkir/JNju}$ (TLR2$^{-/-}$) mice were purchased from Biomedical Research Institute (Nanjing, China). Only female mice were used in the studies. Mice were kept in specific pathogen-free facilities, and all of the experiments were performed in accordance with the guidelines of the Tongji Medical College Animal Care and Use Committee. The study protocols were specifically reviewed and approved by this ethics committee.

2.2. Cardiac transplantation

C57BL/6J (WT) and C57/B6.129-Tlr2$^{tmIkir/JNju}$ (TLR2$^{-/-}$) mice

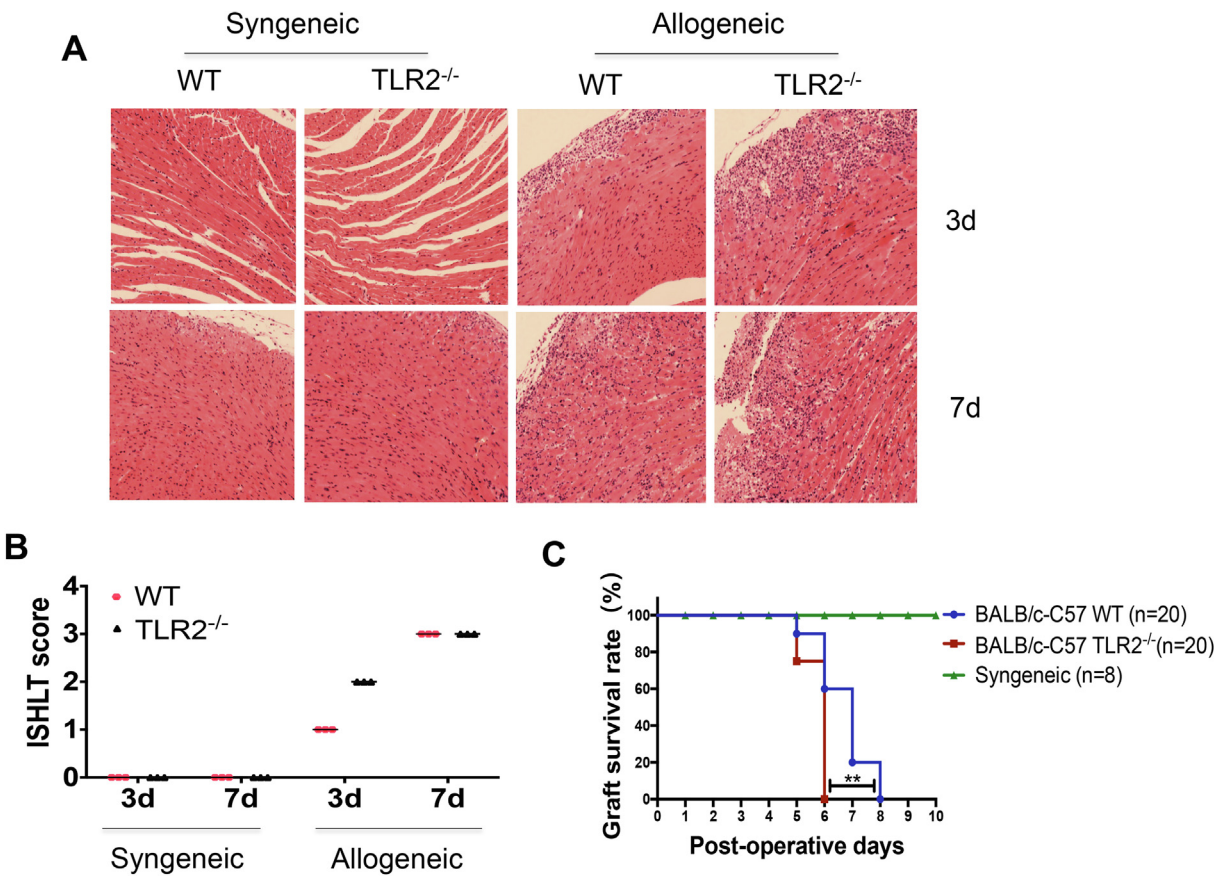

Fig. 2. TLR2 deficiency led to more severe inflammation in the early stage of acute cardiac allograft rejection. Cardiac grafts from BALB/c mice (allogeneic) or C57BL/6 (syngeneic) mice were transplanted into ($TLR2^{-/-}$ or WT) C57BL/6 mice. (A) Histology analysis of cardiac grafts. (B) International Society for Heart and Lung (ISHLT) scores of cardiac allograft rejection activity were evaluated based on H&E staining. (C) Heart allograft survival time (MST) was monitored. Graft survival in the $TLR2^{-/-}$ group (5.75 ± 0.10 days) was shorter than the WT controls (6.70 ± 0.21 days). The cardiac allografts in the $TLR2^{-/-}$ or WT groups were collected on the 3rd and 7th day after transplantation. Cell infiltration in the grafts was examined by haematoxylin and eosin (H&E) staining (original magnification, ×200). Data from one representative out of three experiments are shown. $^{**}p < 0.01$.

(8–10 weeks) were used as recipients, and BALB/c mice (6–8 weeks) were used as donors in the allogeneic graft transplantation models. In the syngeneic graft transplantation models, C57BL/6J (WT) and C57/B6.129-$Tlr2^{tm1kir/JNju}$ ($TLR2^{-/-}$) mice were used as recipients, and C57BL/6J mice were used as donors. Heterotopic cardiac transplantation and assessment of cardiac graft function were performed as described previously [23]. After surgery, cardiac impulses were assessed by daily abdominal palpation. Rejection was defined as complete cessation of cardiac contractility. Cardiac allograft survival was present as mean survival time (MST) [23].

2.3. Histological analysis and cardiac allograft rejection score

For histological examination, on the 3rd and 7th day after transplantation, the grafts were removed and fixed in 4% paraformaldehyde. Paraffin-embedded sections (5 μm) were stained with haematoxylin and eosin (H&E). The severity of cardiac rejection was measured by the International Society for Heart and Lung (ISHLT) score (revised): Grade 0R (no rejection); Grade 1R (mild rejection); Grade 2R (moderate rejection); Grade 3R (severe rejection) [28].

2.4. Immunohistochemical analysis

Paraffin sections (5 μm) of grafts were incubated with rabbit polyclonal anti-LY6G antibody (1:500, Servicebio, Wuhan, China) or rabbit polyclonal anti-F4/80 antibody (1:500, Servicebio) overnight at 4 °C and then were stained with the Streptavidin/Peroxidase Histostain™-Plus Kit (ZSGQ-BIO, Beijing, China) according to the manufacturer's instructions.

2.5. Quantitative real-time PCR

Total RNA samples from cardiac grafts or spleens removed on the 3rd day after transplantation were isolated using RNAiso Plus (TAKARA, Dalian, China). cDNA was synthesized from 4 μg RNA using Revert Aid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, Waltham, MA USA). mRNA levels for IL-17A, IL-6, TNF-α, CCL20, CCR6, IFN-γ, IL-10 and TGF-β in the cardiac grafts, as well as IL-17A, IL-6, TGF-β and SOCS3 in the spleens were detected by real-time PCR using FastStart universal SYBR Green Master Mix (Roche Pharma, AG, Germany). GAPDH was used as the normalization control. The $2^{-\triangle\triangle CT}$ method was used to calculate the relative mRNA levels. The primers are as follows:

GAPDH, 5′-TTCACCACCATGGAGAAGGC-3′, 5′-GGCATGGACTGTGGTCATGA-3′; IL-17, 5′-AGGACGCGCAAACATGAGTC-3′5′-TTGGACACGCTGAGCTTTGG-3′; IL-6, 5′-GAGACTTCCATCCAGTTGCC-3′, 5′-AAGTGCATCATCGTTGTTCATACA-3′; TNF-α, 5′-CATCTTCTCAAAATTCGAGTGACAA-3′, 5′-TGGGAGTAGACAAGGTACAACCC-3′; CCL20, 5′-GGTACTGCTGGCTCACCTCT-3′, 5′-CTTCATCGGCCATCTGTCTT-3′; CCR6, 5′-CACACCTGTGAGAGGAAGCA-3′, 5′-CTAGTGCAGATGTCGGGAGAG-3′; IFN-γ, 5′-TCAAGTGGCATAGATGTGGAAGAA-3′, 5′-TGGCTCTGCAGGATTTTCATG-3′; IL-10, 5′-GCTCCTAGAGCTGCGGACT-3′, 5′-TGTTGTCCAGCTGGTCCTTT-3′; TGF-β, 5′-GGACACACAGTACAGCAAGGTC-3′, 5′-TCAGCTGCACTTGCAGGAG-3′; SOCS3, 5′-TGTCACCGA

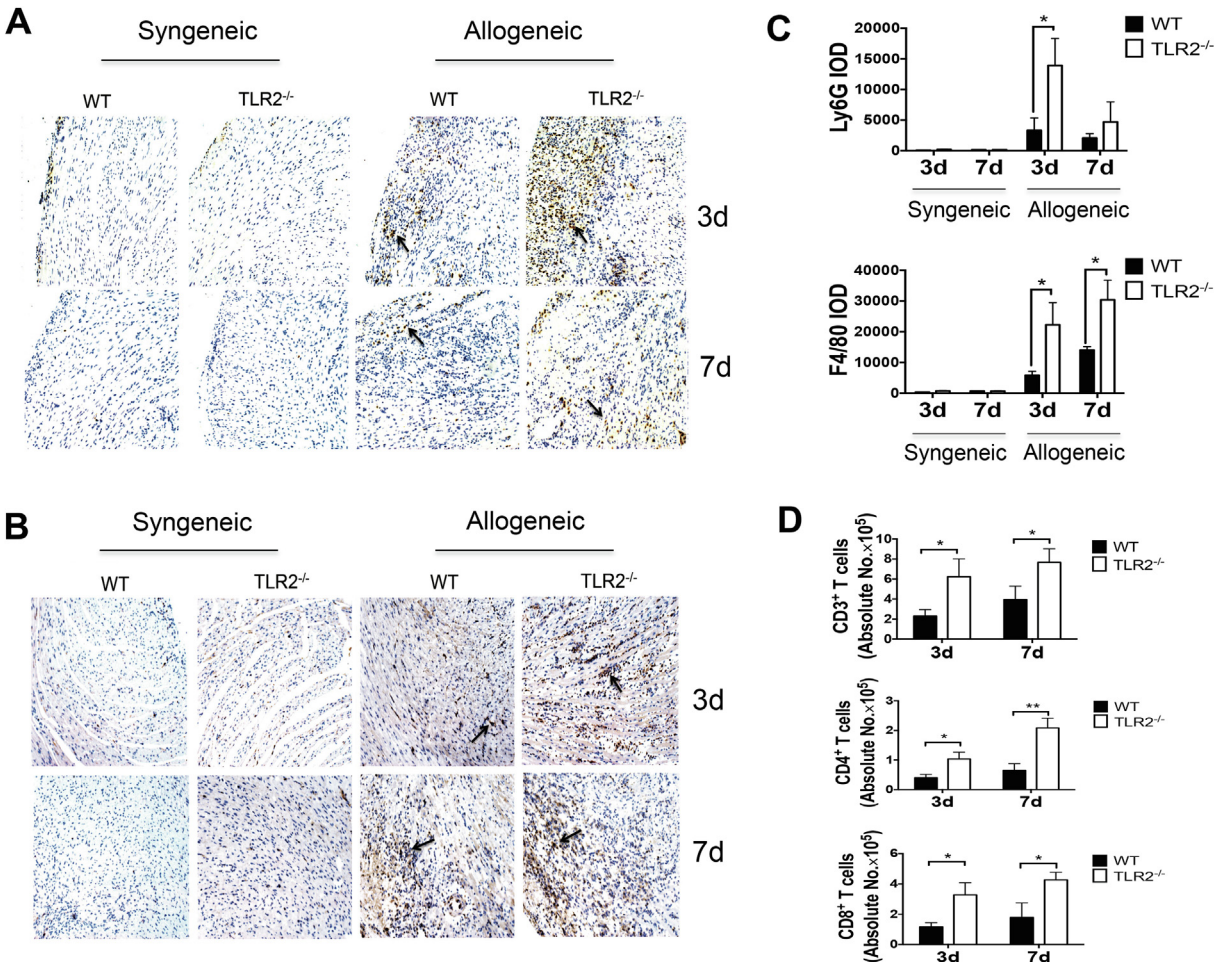

Fig. 3. Neutrophil, macrophage and T cell infiltration in allografts was increased in TLR2-deficient mice. Cardiac grafts from BALB/c mice (allogeneic) or C57BL/6 (syngeneic) mice were transplanted into (TLR2$^{-/-}$ or WT) C57BL/6 mice. The cardiac grafts in the TLR2$^{-/-}$ or WT groups were collected on the 3rd and 7th day after transplantation. (A) Immunostaining for neutrophils was performed. The sections were stained for Ly6G to confirm the infiltration of neutrophils in the cardiac grafts (original magnification, ×200). (B) Immunostaining for macrophages was performed. The sections were stained for F4/80 to confirm the infiltration of monocytes/macrophages in the cardiac grafts (original magnification, ×200). (C) Image-Pro Plus 6.0 was used to analyse the integrated optical density (IOD) by counting the neutrophils and monocytes/macrophages. (D) The absolute numbers of CD3$^+$ T cells, CD4$^+$ T cells and CD8$^+$ T cells in cardiac allografts were counted. The infiltrating inflammatory cells from allografts were collected and activated with PMA (100 ng/ml) and ionomycin (1 μg/ml) in the presence of GolgiStop (1 μl/ml) for 4.5 h, followed by flow cytometry analysis. Data are shown as the mean ± SD (n = 3–5/group). These data are representative of three independent experiments. $^*p < 0.05$; $^{**}p < 0.01$.

AGAACCAGGCAATG-3′, 5′-GGACCTCGACGGAGAGCTGTAG-3′.

2.6. Flow cytometry

Mononuclear cells in cardiac allografts were isolated by digestion in RPMI 1640 medium containing 10% FBS, 2 mg/ml collagenase and 0.1 mg/ml DNase by shaking (200 rpm) at 37 °C for 90 min. The resulting cells were re-suspended in 40% Percoll and centrifuged at 2200 rpm for 30 min; then, the cells at the bottom of the solution were collected and used for flow cytometry. Mononuclear cells isolated from the cardiac allograft and spleen were stimulated for 4.5 h with 100 ng/ml PMA (Sigma-Aldrich, St Louis, MO, USA), 1 μg/ml ionomycin (Sigma) and 1 μl/ml GolgiStop (BD Biosystems, San Jose, CA, USA). After stimulation, the cells were stained with surface markers (CD3, CD4, CD25, CD11C and/or F4/80) for 30 min at 4 °C. Subsequently, the cells were fixed and permeabilized with Cytofix/Cytoperm (BD) or TF Fix/Perm Buffer (BD) for 40 min at 4 °C and then incubated with intracellular Abs (IL-17A, IFN-γ and/or IL-6) or anti-Foxp3. All antibodies were purchased from BioLegend (San Diego, CA, USA) except for IL-6 (BD). Flow cytometry data were collected by BD LSRFortessa cytometer and analysed by FlowJo X.

2.7. Mixed lymphocyte culture

BALB/c bone marrow derived dendritic cells (BMDCs) were cultured under stimulation with GM-CSF (PeproTech, London, UK) and IL-4 (PeproTech) as described previously [29]. After 7 days, lipopolysaccharide (LPS) (1 μg/ml) was used to stimulate the cells for 48 h. The BMDCs were inactivated by mitomycin C (Sigma-Aldrich) before the co-culture experiments. T cells were isolated from spleen cells by using the CD3ε MicroBead Kit for mice (Miltenyi Biotec, Bergisch Gladbach, Germany) as per the kit's instruction. For the mixed lymphocyte culture (MLC), 2×10^6 CD3$^+$ T cells from C57BL/6 wt or TLR2$^{-/-}$ mice were cultured with 2.5×10^5 BMDCs from BALB/c mice in 24-well plates containing TGF-β (2.5 ng/ml) (Peprotech) and/or IL-6 (20 ng/ml) (Peprotech) for 4 days. For the other experiment with a mixed lymphocyte culture, 2.5×10^6 spleen cells from C57BL/6 wt or TLR2$^{-/-}$ mice were cultured with 2.5×10^5 BMDCs from BALB/c mice in 24-well plates for 4 days. The supernatants were collected for the detection of IL-6 by an ELISA kit (BioLegend) and the cells were collected for analysis of Th17 cells by flow cytometry. To observe the effect of IL-6 on Th17 cells, anti-IL-6 antibody (10 μg/ml, BioLegend) or rat IgG (10 μg/ml, BioLegend) were used in the cultures, and flow cytometry was used to analyse the

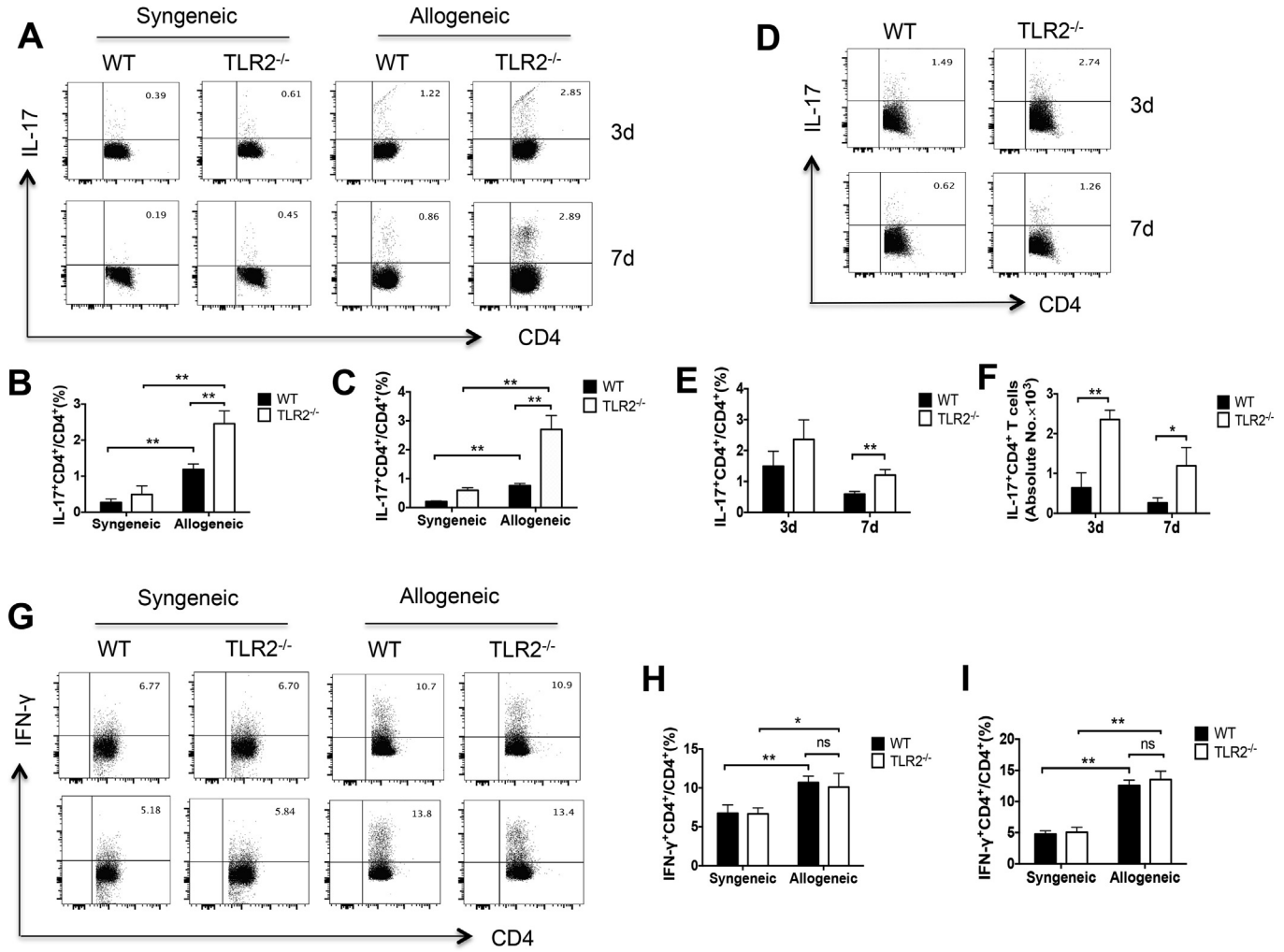

Fig. 4. The production of Th17 cells was significantly increased in TLR2-deficient mice. Cardiac grafts from BALB/c mice (allogeneic) or C57BL/6 (syngeneic) mice were transplanted into ($TLR2^{-/-}$ or WT) C57BL/6 mice. Spleens and cardiac grafts from the recipient animals were collected on the 3rd and 7th day after transplantation. (A) The percentages of Th17 cells in spleens on the 3rd (B) and 7th (C) day are shown. (D) (E) The percentages of Th17 cells in cardiac allografts are displayed. (F) The absolute numbers of Th17 cells in cardiac allografts were counted. (G) The percentages of Th1 cells in spleens on the 3rd (H) and 7th (I) day after allograft transplantation are shown. The splenocytes and infiltrating inflammatory cells from allografts were collected and activated with PMA (100 ng/ml) and ionomycin (1 μg/ml) in the presence of GolgiStop (1 μl/ml) for 4.5 h, followed by flow cytometry analysis. Data are shown as the mean ± SD (n = 3–5/group). Data from one representative out of three experiments are shown. $^{*}p < 0.05$; $^{**}p < 0.01$.

Th17 cells.

2.8. Western blot

Spleens and cardiac allografts removed on 3rd day after transplantation were homogenized, and the supernatant containing proteins was collected. Rabbit monoclonal anti-Signal transducer and activator of transcription 3 (STAT3) (1:2000, Cell Signaling) and anti-phospho-STAT3 (p-STAT3) (1:2000, Cell Signaling) were used in the experiments. After incubation with horseradish peroxidase (HRP)-conjugated secondary antibodies (1:5000, Servicebio, Wuhan, China), the stained blots were visualized by an enhanced chemiluminescent (ECL) system (Servicebio). The blot density was quantified by ImageJ.

2.9. Adoptive transfer experiments

$CD11c^{+}$ dendritic cells were labeled with PE-conjugated anti-CD11c antibody (BD) and then isolated from spleen cells of C57BL/6 wt or $TLR2^{-/-}$ mice by using the Anti-PE MicroBead Kit for mice (Miltenyi Biotec) as per the kit's instruction. The $TLR2^{-/-}$ $CD11c^{+}$ cells (6×10^6) and WT $CD11c^{+}$ cells (6×10^6) were then adoptively transferred into C57BL/6 wt mice, respectively. One day after the adoptive transfer, the C57BL/6 wt mice were used as recipients and transplanted with cardiac allograft from BALB/c mice. On the 3rd day after transplantation, Th17 cells in the spleens were analysed by flow cytometry.

2.10. Statistical analysis

Experimental data are presented as the mean ± SD. Student's *t*-test was used to analyse the differences between groups. Two-sided probability (p) values < 0.05 were considered significant. Allograft survival comparisons were analysed using the log-rank test.

3. Results

3.1. Neutrophils and Th17 cells were increased at the early stage of acute allograft rejection

Acute cardiac allograft rejection is characterized by inflammatory cell infiltration in the grafts and myocardial necrosis. Immunohistochemical staining was performed to detect the neutrophils

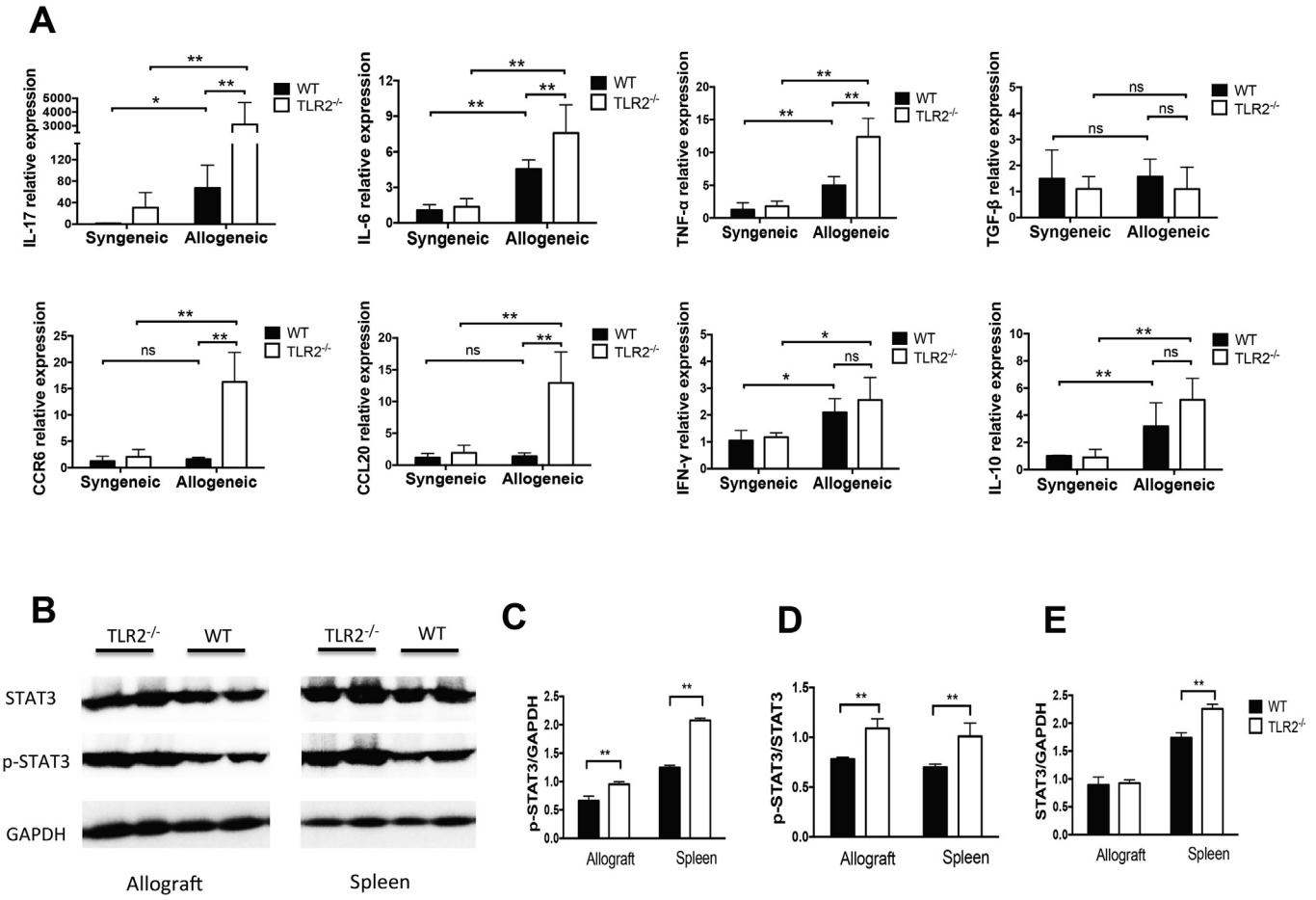

Fig. 5. The expression of IL-17, IL-6, TNF-α, CCR6, CCL20 and p-STAT3 was enhanced in TLR2-deficient mice. (A) Real-time PCR analysis of IL-17 and related cytokines in the cardiac grafts. Cardiac grafts from BALB/c mice (allogeneic) or C57BL/6 (syngeneic) mice were transplanted into ($TLR2^{-/-}$ or WT) C57BL/6 mice. Cardiac grafts were collected on the 3rd day after transplantation. Total RNA of the cardiac grafts was prepared for real-time PCR analysis of each indicated cytokine as shown. Data are shown as the mean ± SD (n = 5/group). Data from one representative out of three experiments are shown. $^{*}p < 0.05$; $^{**}p < 0.01$. (B) The expression of STAT3 and p-STAT3 in spleens and allografts was examined by Western blot. Spleens and allografts were collected on the 3rd day after allograft transplantation and the blots were quantified by ImageJ. (C) The relative expression of p-STAT3/GAPDH. (D) The relative expression of p-STAT3/STAT3. (E) The relative expression of STAT3/GAPDH. These data are shown as the mean ± SD (n = 4/group). Data from one representative out of three experiments are shown. $^{**}p < 0.01$.

and macrophages infiltration in the grafts on the 3rd and 7th day after transplantation. The neutrophils were the major infiltrates in the early stage (day 3 after transplantation) of allograft rejection, whereas macrophages were increased in the late stage (day 7 after transplantation) of allograft rejection (Fig. 1A). To investigate the roles of Th17 cells and Th1 cells in acute allograft rejection, the kinetics of Th17 cell and Th1 cells in recipient mice after cardiac transplantation were examined. Flow cytometry revealed that the strongest Th17 alloreactive response was noted in the early stage of allograft rejection (Fig. 1B and D). However, in the late stage of allograft rejection, Th17 alloreactive response became weakened and Th1 alloreactive response was increased (Fig. 1C and D). In syngeneic groups, the production of both Th17 cells and Th1 cells were much lower than those in allogeneic groups.

3.2. TLR2 deficiency aggravated acute allograft rejection

To investigate the effects of TLR2 signalling on acute allograft rejection, we first performed cardiac allograft transplantation in mice. Pathological analysis revealed that the inflammatory leukocytes infiltrate in the cardiac allograft of $TLR2^{-/-}$ recipients was apparently more severe than in the wild-type control animals, especially on the 3rd day after cardiac transplantation (Fig. 2A). The ISHLT scores of heart rejection of the $TLR2^{-/-}$ mice were 2R (moderate rejection), while the scores of the WT mice were 1R (mild rejection) on the 3rd day after cardiac transplantation (Fig. 2B). The cardiac allograft mean survival time (MST) in the $TLR2^{-/-}$ recipient mice (5.75 ± 0.10 days) was shorter than that of WT control animals (6.70 ± 0.21 days) (Fig. 2C). These data indicate that TLR2 deficiency led to more severe inflammation in the early stage of acute cardiac allograft rejection.

3.3. Neutrophil, macrophage and T cell infiltration in allografts was enhanced in TLR2-deficient mice

Next, we examined the types of infiltrating inflammatory leukocytes in the cardiac allografts. Immunohistochemical staining was performed to detect the neutrophils and macrophages infiltration in the grafts on the 3rd and 7th day after transplantation. Significantly increased neutrophils were observed in the cardiac allografts on the 3rd day for the $TLR2^{-/-}$ mice compared with those in the WT mice (Fig. 3A and C). On both the 3rd and 7th day after cardiac allograft transplantation, macrophages infiltration in the allografts was increased in the $TLR2^{-/-}$ mice compared to that in the WT mice (Fig. 3B and C). Moreover, the numbers of $CD3^+$ T cells, $CD4^+$ T cells and $CD8^+$ T cells in the cardiac allografts were significantly increased in the $TLR2^{-/-}$ mice compared to that in the WT mice (Fig. 3D).

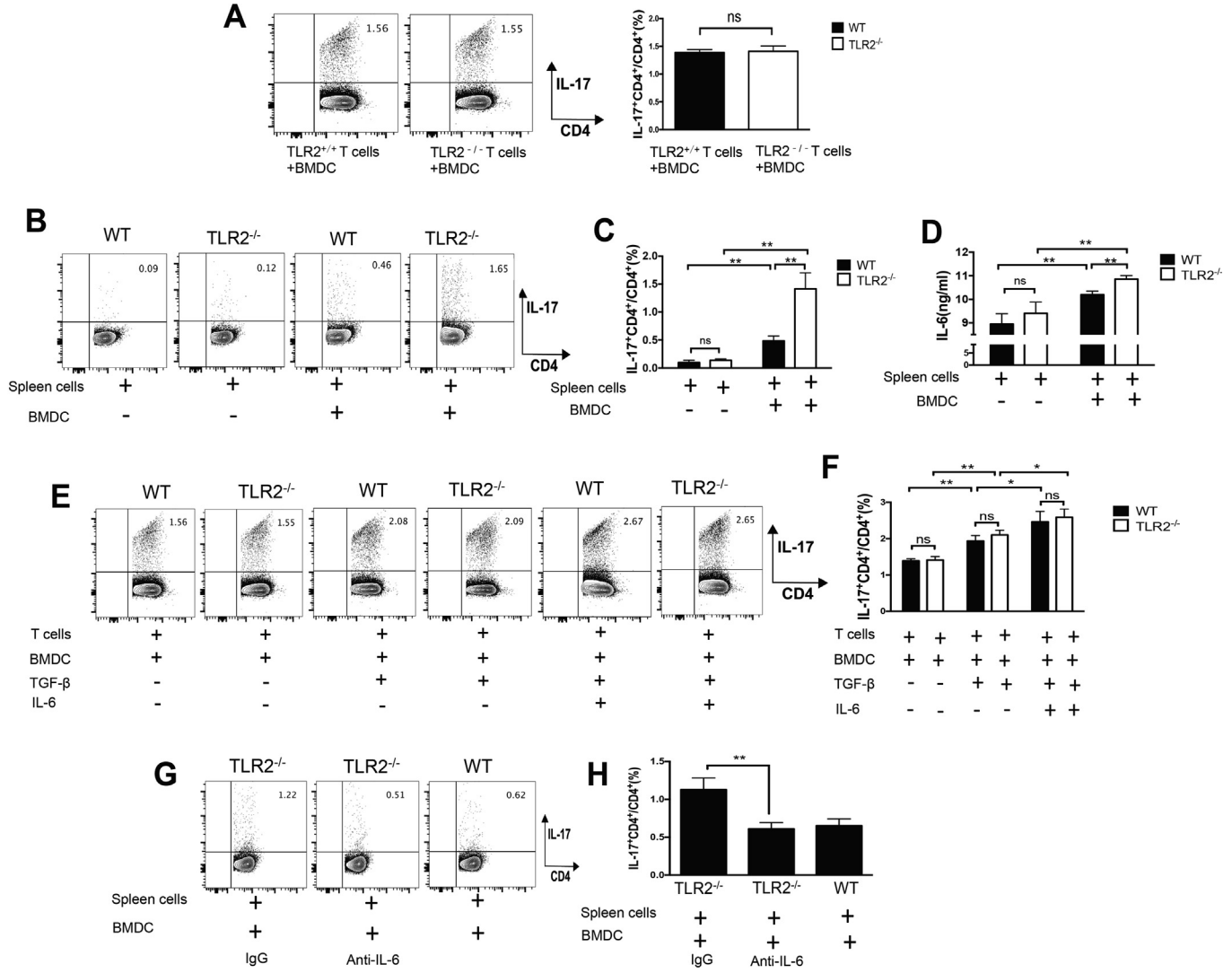

Fig. 6. The increase in Th17 cells of TLR2$^{-/-}$ mice was related to the increased production of IL-6 from spleen cells. Bone marrow-derived dendritic cells (BMDCs) from BALB/c mice bone marrow were stimulated with LPS (1 μg/ml) for 48 h and inactivated by mitomycin C (40 μg/ml) before the co-culture experiments. (A) The production of Th17 cells showed no difference between the TLR2$^{-/-}$ and WT groups in the mixed lymphocyte culture (MLC) of T cells and BMDCs. Approximately 2×10^6 of CD3$^+$ T cells isolated from spleen cells of recipient mice (TLR2$^{-/-}$ or WT) were co-cultured with 2.5×10^5 of BMDCs from BALB/c mice for 4 days. The percentages of Th17 in the co-culture system were analysed by flow cytometry. (B) The production of Th17 cells was increased in TLR2$^{-/-}$ group in the MLC of spleen cells and BMDCs. Approximately 2.5×10^6 of spleen cells from TLR2$^{-/-}$ or WT mice were co-cultured with 2.5×10^5 of BMDCs from BALB/c mice for 4 days. (C) The percentages of Th17 in the co-culture system were analysed by flow cytometry. (D) The concentration of IL-6 in the supernatant were analysed by ELISA. (E) (F) Approximately 2×10^6 of CD3$^+$ T cells isolated from spleen cells of recipient mice (TLR2$^{-/-}$ or WT) were co-cultured with 2.5×10^5 of BMDCs from BALB/c mice for 4 days in the presence of TGF-β and IL-6. The percentages of Th17 cells in the co-culture system were analysed by flow cytometry. (G)(H) Blockade of IL-6 suppressed Th17 cells. To examine the effect of IL-6 on the generation of Th17 cells, anti-IL-6 or IgG was added into the co-culture system. Approximately 2.5×10^6 of spleen cells from TLR2$^{-/-}$ or WT mice were co-cultured with 2.5×10^5 of BMDCs from BALB/c mice for 4 days followed by flow cytometry analysis. These data are shown as the mean ± SD (n = 3/group). Data from one representative out of three experiments are shown. $^*p < 0.05$; $^{**}p < 0.01$.

3.4. TLR2 deficiency led to a sustained increase in Th17 cells

Previously, we reported that IL-17-producing T cells play a critical role at the early stage of acute allograft rejection through inducing the recruitment of neutrophils into allografts, and cardiac allograft survival became longer after the blockade of IL-17A in recipient mice [23]. We analysed whether the exacerbation of early acute allograft rejection was correlated with the increase of Th17 cells in TLR2$^{-/-}$ mice. For this purpose, the percentage of Th17 cells in spleens and allografts was analysed by flow cytometry. TLR2 deficiency led to a significant increase in Th17 cells in the spleens on the 3rd and 7th day after cardiac allograft transplantation compared to WT mice (Fig. 4A–C). Higher percentages and absolute numbers of Th17 cells were also detected in cardiac allografts of recipient TLR2$^{-/-}$ mice compared to the wild-type control animals (Fig. 4D–F). Meanwhile, Th1 cells in their spleens were also examined, and no significant difference was observed between recipient TLR2$^{-/-}$ and WT mice (Fig. 4G–I).

3.5. TLR2 deficiency led to increased expression of IL-17, IL-6, TNF-α, CCR6, CCL20 and p-STAT3

Cytokines are important inflammatory mediators in cardiac allograft acute rejection. TGF-β and IL-6 are important cytokines that trigger the production of IL-17 from CD4$^+$ T cells [30]. CCR6 and CCL20 induce the recruitment of Th17 cells to inflammatory sites [31]. TNF-α a is an important pro-inflammatory cytokine that can be produced by Th17 in allograft acute rejection [32]. IL-10 is a pleiotropic anti-inflammation cytokine and attenuates posttransplant immune

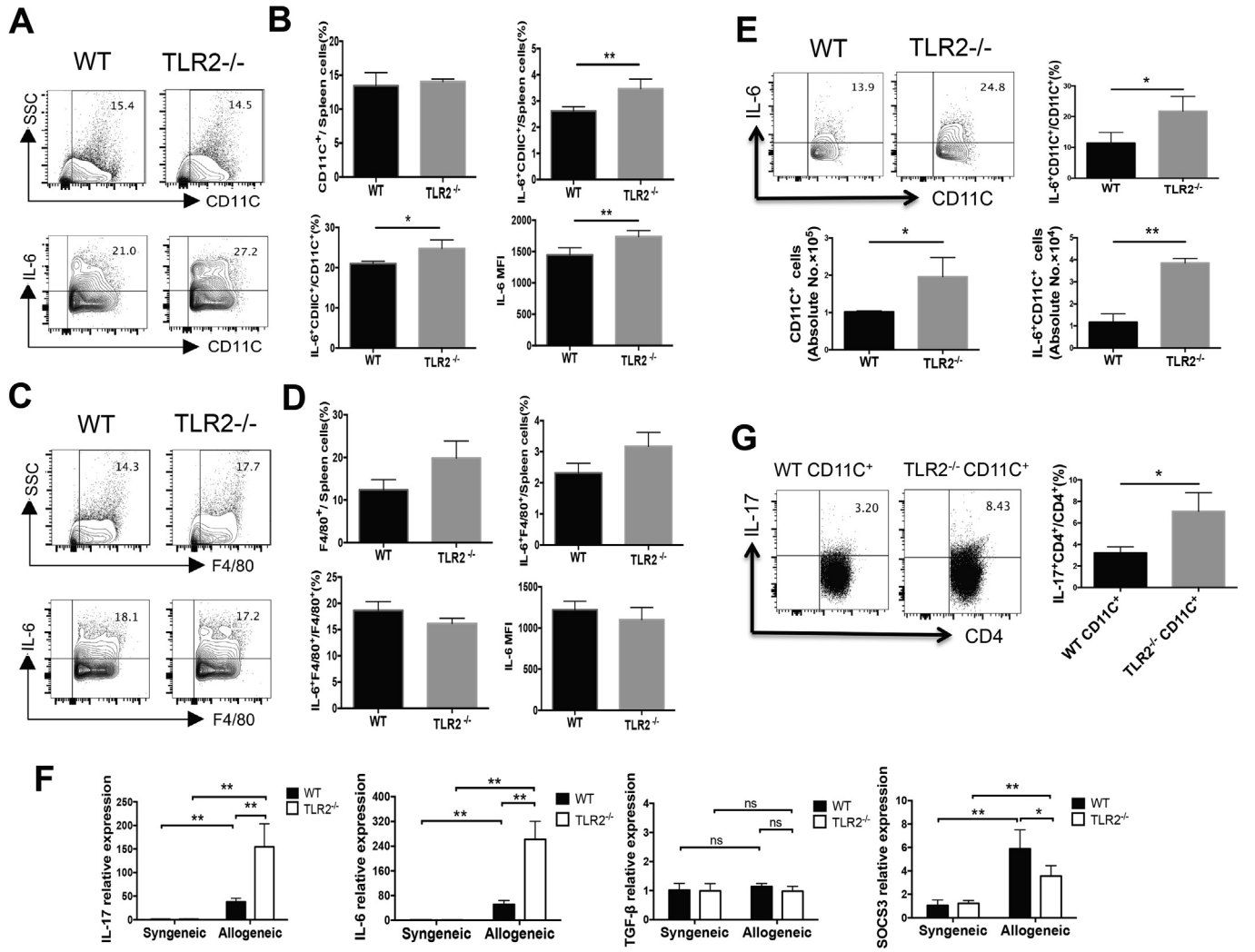

Fig. 7. The production of IL-6 in dendritic cells was increased in TLR2-deficient mice after allograft transplantation. Cardiac grafts from BALB/c mice were transplanted into ($TLR2^{-/-}$ or WT) C57BL/6 mice. Spleens were collected on the 3rd day after allograft transplantation and the production of IL-6 in dendritic cells (A) and macrophages (C) was detected by flow cytometry analysis. (B) The percentage of $CD11C^+$ cells and $IL\text{-}6^+CD11C^+$ cells in total spleen cells, the percentage of $IL\text{-}6^+CD11C^+$ cells in $CD11C^+$ cells, as well as the MFI of IL-6 in DCs. (D) The percentage of $F4/80^+$ cells and $IL\text{-}6^+F4/80^+$ cells in total spleen cells, the percentage of $IL\text{-}6^+F4/80^+$ cells in $F4/80^+$ cells, as well as the MFI of IL-6 in macrophages. These data are shown as the mean ± SD (n = 3/group). Data from one representative out of three experiments are shown. $^*p < 0.05$; $^{**}p < 0.01$. (E) Cardiac allografts were collected on the 3rd day after allograft transplantation. The percentage of $IL\text{-}6^+CD11C^+$ cells in $CD11C^+$ cells, the absolute numbers of $CD11C^+$ cells and $IL\text{-}6^+CD11C^+$ cells in cardiac allografts are displayed. These data are shown as the mean ± SD (n = 3/group). Data from one representative out of three experiments are shown. $^*p < 0.05$; $^{**}p < 0.01$. (F) Real-time PCR analysis of IL-17, IL-6, TGF-β and SOCS3 in the spleens. Spleens were collected on the 3rd day after transplantation. Total RNA of the spleens was prepared for real-time PCR analysis of each indicated cytokine as shown. Data are shown as the mean ± SD (n = 4/group). Data from one representative out of two experiments are shown. $^*p < 0.05$; $^{**}p < 0.01$. (G) The $TLR2^{-/-}$ $CD11c^+$ cells (6×10^6) and WT $CD11c^+$ cells (6×10^6) were adoptively transferred into C57BL/6 wt mice, respectively. One day after the adoptive transfer, the C57BL/6 wt mice were used as recipients and transplanted with cardiac allograft from BALB/c mice. The percentages of Th17 cells in spleens on the 3rd day after transplantation were analysed by flow cytometry. Data are shown as the mean ± SD (n = 3/group). Data from one representative out of two experiments are shown. $^*p < 0.05$.

responses [33]. Thus, the expression of IL-17 and related cytokines in allografts on the 3rd day after transplantation were analysed by using quantitative real-time PCR (Fig. 5A). mRNA expression of IL-17, IL-6 and TNF-α within the allografts markedly increased in recipient $TLR2^{-/-}$ mice compared to WT mice. Moreover, the expression of CCR6 and CCL20 within the allografts was also significantly increased in the recipient $TLR2^{-/-}$ mice compared to the WT mice. Meanwhile, no significant difference was observed in mRNA levels of TGF-β, IL-10 and IFN-γ between the recipient $TLR2^{-/-}$ and WT mice. These results suggest that TLR2 deficiency in the recipient can promote the expression of IL-17, IL-6, TNF-α, CCR6 and CCL20 in cardiac allografts at the early stage of rejection. The significant increase in CCR6 and CCL20 indicates that Th17 cells were recruited into the allograft in the recipient $TLR2^{-/-}$ mice. The increase in IL-6 but not TGF-β suggests that IL-6 might play an important role in the promotion of IL-17-producing alloreactive T cells after cardiac allograft transplantation in the recipient $TLR2^{-/-}$ mice.

The generation and survival of IL-17-expressing T cells is related to the STAT3 signalling pathway, which could be activated by IL-6 [34]. Western blots were used to investigate whether the expression of STAT3 and p-STAT3 was enhanced in $TLR2^{-/-}$ mice after cardiac allograft transplantation. As shown in Fig. 5B, lack of TLR2 signalling resulted in a remarkable increase of p-STAT3 both in spleens and allografts. These results suggest that TLR2 deficiency leads to upregulation of STAT3 phosphorylation, which could promote the expansion of IL-17-producing alloreactive T cells (Fig. 5B–E).

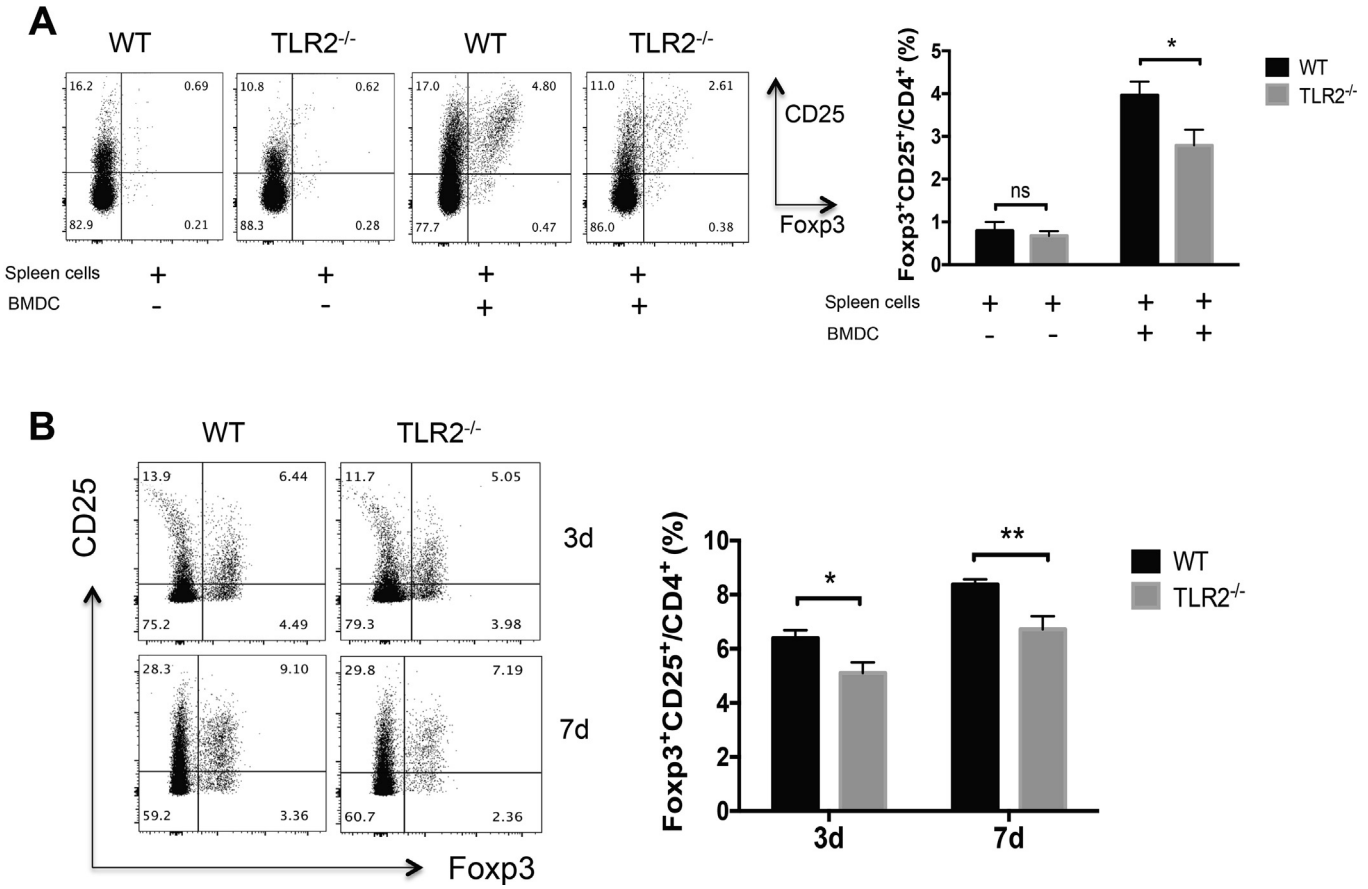

Fig. 8. TLR2 deficiency led to a significant decrease in the generation of regulatory T cells. (A) The percentages of $CD4^+CD25^+Foxp3^+$ T cells in the MLC. Approximately 2.5×10^6 of spleen cells from $TLR2^{-/-}$ or WT mice were co-cultured with 2.5×10^5 of BMDCs from BALB/c mice for 4 days, followed by flow cytometry analysis. (B) The percentages of $CD4^+CD25^+Foxp3^+$ T cells in spleens collected on the 3rd and 7th day after allograft transplantation. These data are shown as the mean ± SD (n = 3/group). Data from one representative out of three experiments are shown. $^*p < 0.05$; $^{**}p < 0.01$.

3.6. The increase in Th17 cells of $TLR2^{-/-}$ mice was related to the increased production of IL-6 from DCs

Our results showed that T cells from mice express TLR2 (data not shown). Therefore, increased generation of IL-17-producing alloreactive T cells could be due to the absence of TLR2 signalling of these $TLR2^{-/-}$ T cells. To analyse whether the skewing towards Th17 cells during acute allograft rejection in the recipient $TLR2^{-/-}$ mice was due to direct effects on the T cells themselves, T cells from recipient mice ($TLR2^{-/-}$ and WT) were co-cultured with BMDCs from donor mice for 4 days, then the percentages of Th17 cells were analysed by flow cytometry. Interestingly, no difference in the percentage of Th17 was noted between the $TLR2^{-/-}$ and WT groups (Fig. 6A). These data indicate that deficiency of TLR2 signaling in T cells does not affect the alloreactive Th17 cell responses.

Next, spleen cells from recipient mice ($TLR2^{-/-}$ and WT) were co-cultured with BMDCs from donor mice, and the percentages of Th17 cells were analysed by flow cytometry on the 4th day. In agreement with the in vivo results, TLR2 deficiency led to a marked increase in the production of Th17 cells in the mixed lymphocyte culture compared with the WT groups (Fig. 6B and C). These results suggest that skewing towards Th17 cells during acute allografts rejection in $TLR2^{-/-}$ mice was related to the effects from other cells in the spleen. To define the factors contributing to the promotion of this IL-17-producing alloreactive T cell development, the concentration of IL-6 in the supernatant was detected by ELISA. Interestingly, TLR2 deficiency led to a higher production of IL-6 in the mixed lymphocyte culture compared with the WT group (Fig. 6D).

To investigate whether TLR2 deficiency of T cells influences the Th17 skewing under the stimulation of TGF-β and IL-6, $CD3^+$ T cells from recipient mice ($TLR2^{-/-}$ and WT) were co-cultured with BMDCs from donor mice for 4 days in the presence of TGF-β and IL-6. As shown in Fig. 6E and F, there was no difference in the production of Th17 cells between the $TLR2^{-/-}$ and WT groups under the same conditions of TGF-β and IL-6 induction. Furthermore, TGF-β and IL-6 enhanced the production of Th17 in both the $TLR2^{-/-}$ and WT groups (Fig. 6E and F). To confirm the role of IL-6 in driving Th17 skewing in $TLR2^{-/-}$ mice, an anti-IL-6 antibody was used in the mixed lymphocyte culture, and the percentages of Th17 cells were analysed by flow cytometry on the 4th day. It is noteworthy that blockade of IL-6 suppressed Th17 cell production in $TLR2^{-/-}$ group (Fig. 6G and H).

To investigate whether dendritic cells (DCs) and macrophages enhanced the production of IL-6 in $TLR2^{-/-}$ mice, $IL\text{-}6^+CD11C^+$ cells and $IL\text{-}6^+F4/80^+$ cells in spleens on the 3rd day after cardiac allograft transplantation were analysed by flow cytometry. Production of IL-6 from DCs was significantly increased in $TLR2^{-/-}$ mice (Fig. 7A and B), whereas no significant difference in the IL-6 expression in macrophages was observed between $TLR2^{-/-}$ and WT mice (Fig. 7C and D). Higher percentages and absolute numbers of $IL\text{-}6^+CD11C^+$ cells were also detected in cardiac allografts of recipient $TLR2^{-/-}$ mice compared to the wild-type control animals (Fig. 7E).

At the same time, the mRNA expression of IL-17, IL-6, TGF-β and SOCS3 in the spleens on the 3rd day after transplantation were also analysed (Fig. 7F). TGF-β can induce the development of both Th17 and Treg; however, IL-6 polarizes Th17 response together with TGF-β and inhibits Treg differentiation.[35] SOCS3 is the primary inhibitor of IL-6

and JAK-STAT3 pathway.[36] In present study, the expression of IL-17 and IL-6 within the spleens markedly increased in recipient TLR2$^{-/-}$ mice compared to WT mice. Whereas the expression of SOCS3 in the spleens decreased in recipient TLR2$^{-/-}$ mice compared to WT mice.

To confirm the role of DCs in driving Th17 skewing in TLR2$^{-/-}$ mice, TLR2-deficient DCs (TLR2$^{-/-}$ CD11C^{+}) were isolated from spleens of TLR2$^{-/-}$ mice and adoptive transferred to the C57BL/6 wild-type mice. DCs isolated from spleens of WT mice (WT CD11C^{+}) were conducted as control group. Then the mice were transplanted with cardiac allograft and the percentages of Th17 cells in spleens were analysed by flow cytometry on the 3rd day after transplantation. The adoptive transfer study showed that TLR2-deficient DCs are responsible for the induction of increased Th17 cells (Fig. 7G).

These data suggest that TLR2 deficiency promotes the production of IL-6 in DCs, which is a positive regulator of Th17 cells.

3.7. The expansion of regulatory T cells was reduced in TLR2-dedicient mice

Tregs and Th17 cells differentiation is closely related, and IL-6 may serve as a switch between Tregs and Th17 cells differentiation [35]. Tregs in spleens were also examined, and flow cytometric analysis revealed that the Tregs percentages in spleens on the 3rd and 7th day after allograft transplantation were significantly decreased in recipient TLR2$^{-/-}$ mice compared to wild-type mice (Fig. 8B). Next, spleen cells from recipient mice (TLR2$^{-/-}$ and WT) were co-cultured with BMDCs from donor mice, and the percentages of Tregs were analysed by flow cytometry on the 4th day. In agreement with the in vivo results, TLR2 deficiency led to a marked decrease in the production of Tregs in the mixed lymphocyte culture compared with the WT groups (Fig. 8A).

4. Discussion

In the present study, we investigated the effects and mechanisms of TLR2 deficiency on the generation of IL-17^{+} alloreactive T cells in a mouse cardiac allograft model. We demonstrated that deficiency of TLR2 signalling in the recipient resulted in an increased population of alloreactive Th17 cells during allograft cardiac transplantation. This increase of alloreactive Th17 cells in TLR2-deficient recipients was caused by increased IL-6 secretion by TLR2-deficient dendritic cells.

Previous studies have found that the expression of TLR2 on peripheral mononuclear cells and in allografts is increased in organ transplant rejection, suggesting a relationship between TLR2 and allograft rejection [19–21,37]. TLR2/4-, MyD88- and TRIF-deficient mice have markedly improved excretory function and ameliorate the pathological damage of chronically rejecting kidney grafts [1]. This study suggests that TLR2 and TLR4 may play a pro-inflammatory role in transplant rejection by MyD88- and TRIF-dependent signaling [1], but the effect of TLR2 alone was not investigated. On the other hand, Li et al. have found that trichosanthin and its derived peptide tetramer induces tolerance of cardiac allografts by instigating type 2 responses via the TLR2-promoted activation of MCP-1, suggesting a protective role of TLR2 signaling in cardiac transplantation [38]. In our study, we found that TLR2 deficiency in the recipient can promote inflammatory cell infiltration and mRNA expression of IL-17, IL-6, TNF-α, CCR6 and CCL20 in cardiac allografts at the early stage of rejection. Our finding suggests a regulatory effect of TLR2 on acute rejection after cardiac transplantation. It is worth noting that our study is focused on the effects of TLR2 from the recipient; however, the TLR2 expressed in an allograft may play a different role. For example, corneal epithelial and stromal cells transfected with lentiviral vector (LV)-mediated TLR2 small interference RNA (siRNA) demonstrate less edema, opacity and neovascularization after corneal allograft transplantation, and LV-mediated TLR2 siRNA significantly prolongs the survival of transfected allografts compared to nontransfected allografts [39]. What is more, different mice strains have shown remarkable immunological differences [40]. More studies are required to confirm the effects of TLR2 using different mice as donors and recipients, such as using C57BL/6 mice as donors and Balb/c mice as recipients.

Alloimmune T cells are important mediators of solid organ rejection and graft-versus-host disease [41]. Although Th1 cells are thought to be major effector cells in adaptive alloimmune responses, recent studies have shown a relationship between IL-17 and allograft rejection. In a fully MHC mismatched heterotopic mouse heart transplantation model, T cells deficient for *T*-bet versus RORγt rejected allografts at a more accelerated rate, indicating a predominance of Th17-driven over Th1-driven alloimmunity [42]. Recently, it has been reported that hyperlipidemic mice had an increased number of Th17 cells in their periphery, and rejected allografts from hyperlipidemic mice contained significant numbers of IL-17 producing T cells compared to controls. Blockade or genetic knockout of IL-17 prolonged the survival of the cardiac allografts transplanted into hyperlipidemic recipients compared to control animals, indicating that IL-17 production promotes accelerated rejection [43]. In addition, Th17 has been implicated in the pathogenesis and prognosis of acute T cell-mediated rejection in recipients of kidney allografts [26]. We have previously demonstrated that Th17 and γδ T cells are the dominant IL-17^{+} cells in the setting of cardiac allografts and contribute to the early phase of acute allograft rejection. Moreover, IL-17 induces early graft damage through neutrophil recruitment into the grafts [23,25]. In this study, we found that the development of IL-17^{+} alloreactive Th17 cells was significantly increased in the recipient animals in the absence of TLR2 signaling compared to the wild-type control mice, whereas the generation of IFN-γ^{+} Th1 cells was comparable between the TLR2-deficient and wild-type recipients. A lack of TLR2 signalling results in a remarkable increase in phosphorylation of STAT3, which enhances the inflammatory responses evoked by alloantigen stimulus, including the development of the alloreactive Th17 cells. Moreover, during the early period of acute cardiac allograft rejection, it was found that an infiltrate of inflammatory neutrophils in the cardiac allografts was apparently severe in the recipients of TLR2 deficient mice compared with those of wild-type control recipient mice. In humans, neutrophil infiltrates are the main inflammatory cells in biopsies of rejected allografts, and a link has been established between the degree of neutrophil infiltration and the severity of cardiac allograft rejection [44]. In line with our study, previously studies have demonstrated that TLR2 is a negative regulator of Th17 cells in several disease models, such as experimental brain abscesses [45] and an experimental pulmonary infection model [17]. Notably, previous research has shown that TLR2 signaling in CD4^{+}T lymphocytes promotes Th17 responses [46,47]. However, these studies are focused on the effects of TLR2 signaling in T cells. TLR2 is widely expressed on a variety of cells such as DCs, macrophages, lymphocytes and epithelial cells. We speculate that TLR2 signaling in different cells can have a pro-inflammatory or anti-inflammatory effect, depending on the regulation of inflammation in the body. For example, Konowich et al. reported distinct roles of TLR2 signaling on hematopoietic versus non-hematopoietic cells. In a mouse model of chronic *Mycobacterium tuberculosis* infection, TLR2 on hematopoietic cells contributes to the protective function in a non-redundant manner, whereas TLR2 on non-hematopoietic cells contributes to the pulmonary inflammation and bacterial dissemination [8]. Our present study suggests that deficiency of TLR2 in T cells does not affect the alloreactive T cell response. However, the deficiency of TLR2 in mice can promote alloreactive Th17 response by producing more IL-6 in DCs.

What is the mechanism by which TLR2 deficiency promotes the development of alloreactive IL-17-producing T cells? To answer this question, a mixed lymphocyte culture was performed, and intracellular cytokines cytometric analysis revealed that IL-6, an important cytokine for inducing the development of Th17 cells, significantly increased in the TLR2 deficiency of dendritic cells relative to that of the wild-type dendritic cells in response to alloantigen stimulus. In support of our observation, previous studies have suggested that activation of TLR2

signaling with or without HIV infection results in a reduction of IL-6 production by dendritic cells [48,49]. What is more, the activation of TLR2 signaling can suppress pro-inflammatory cytokines in DCs by induction of SOCS3, which is a negative regulator of IL-6 signal transduction [50]. In present study, we also found that the expression of SOCS3 in spleen was decreased in TLR2$^{-/-}$ mice. Of note, we observed that blockade of IL-6 resulted in a marked reduction of alloreactive Th17 cells in the mixed lymphocyte culture, which could be because IL-6 is an important cytokine that drives the development of these alloreactive Th17 cells. DCs are the most important cells connecting the innate and adaptive immune responses [51]. Further studies are required to define the signal transduction of molecules responsible for TLR2 signalling and regulating the production of IL-6 by dendritic cells. What is more, TLR2 deficiency led to a significant decrease in the generation of regulatory T cells, which could be another mechanism by which it enhances the development of inflammatory alloreactive Th17 cells in the setting of cardiac allograft transplantation. There is evidence that TLR2 is involved in modulating Tregs functions both directly or indirectly [52]. There is also research showing that IL-6 induces the development of Th17 cells and inhibits Tregs differentiation [35].

In conclusion, we have presented strong evidence to support that TLR2 signaling plays a crucial role in regulating the development of IL-17-producing alloreactive T cells in the context of cardiac allograft transplantation. This is a new finding that demonstrates a regulatory role of TLR2 in allograft transplantation, providing a reference value for the basic research and clinical treatment of organ transplantation.

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Conflict of interest

The authors have no conflicts of interest to disclose.

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