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ORIGINAL RESEARCH

IL-6/IL-6R Signaling Blockade Alleviates Chronic Allograft Rejection by Modulating Germinal Center B Cells and Allograft Inflammation in Murine Cardiac Transplantation

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Introduction: The activation of B cells to produce donor-specific antibody (DSA) and the infiltration of T and macrophages in the allografts are important factors leading to chronic rejection (CR). Interleukin-6 (IL-6) is particularly important in immune responses, playing a crucial role in the activation of B, T, and macrophages. In this study, we investigate the preventive efficacy and underlying mechanism of IL-6/IL-6R signaling blockade.

Methods: The CR model in mice was constructed using allogeneic cardiac transplantation with CTLA4-Ig injection. We used anti-IL-6R monoclonal antibody tocilizumab and IL-6 knockout mice to block IL-6/IL-6R signaling, observed its preventive effects on CR, and explored the mechanism from its effects on B cell activation, DSA production, and inflammatory cell infiltration in the allografts.

Results: IL-6/IL-6R signaling ablation significantly prolonged allograft survival and alleviated key pathological features of CR, including interstitial fibrosis, C4d deposition, inflammatory cell infiltration, myocardial ischemic necrosis, and neointimal hyperplasia. Mechanistically, blocking IL-6/IL-6R signaling reduced serum DSA-IgG levels, suppressed B cell response and germinal center B formation, and decreased inflammatory cell infiltration in allografts. Moreover, IL-6 knockout demonstrated superior efficacy compared to tocilizumab, suggesting that complete IL-6 signaling ablation offers greater protection against CR. This study also provides the first comprehensive assessment of IL-6/IL-6R blockade on germinal center B cells and graft-infiltrating immune cells, highlighting its dual role in attenuating humoral and cellular immune responses.

Discussion: IL-6/IL-6R signaling represents a pivotal therapeutic target for CR, and its blockade offers a promising strategy for improving long-term allograft outcomes.

Keywords: IL-6, IL-6R, chronic rejection, tocilizumab, cardiac transplantation

Introduction

Chronic rejection (CR) remains the leading cause of long-term allograft failure, characterized by interstitial fibrosis, inflammatory cell infiltration, myocardial ischemic necrosis, and neointimal hyperplasia.[1–6] The pathogenesis of CR is multifactorial, involving the interplay of multiple inflammatory cells, including macrophages, T cells, and B cells. A key contributor to CR is the production of donor-specific antibody (DSA) following B cell activation and differentiation. These DSAs bind to the endothelial cells in the allografts, activating the complement cascade through classical pathways and mediating immune injury.[7,8] In addition, antigen exposure and chemokines recruit macrophages and T cells to allografts further exacerbating inflammation.[9,10] Notably, macrophages can transition into fibroblasts, directly

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contributing to interstitial fibrosis.[11] Thus, the intricate involvement of macrophages, T cells, and B cells underscores the complexity of CR pathogenesis.

Interleukin-6 (IL-6) plays a crucial role as a cytokine that primarily functions by binding to its receptor IL-6R to activate downstream signaling, driving immune cell activation and proliferation.[12] In humoral immunity, IL-6 plays a central role by promoting T-follicular helper cell activation, germinal center formation, B cell differentiation into plasma cells, and production of high-affinity antibodies.[13–16] Furthermore, IL-6 enhances IL-2 expression, supporting the expansion of $CD8^+$ T cells and influences $CD4^+$ T cell differentiation by favoring T-helper 17 polarization while inhibiting regulatory T cell development.[17–19] IL-6 activates macrophages, inducing their differentiation into M2 phenotype,[20] which is associated with tissue remodeling and fibrosis.

Considering its role in orchestrating immune responses across multiple cell types, IL-6/IL-6R signaling has emerged as a promising therapeutic target for CR. In 2009, a CR model using allogeneic mouse cardiac transplantation with $CD4^+$ T cell depletion demonstrated that anti-IL-6 mAb could effectively prevent CR.[21] More recent studies with clazakizumab (anti-IL-6 mAb) and tocilizumab (humanized anti-IL-6R mAb) have yielded encouraging preliminary results, suggesting their potential as novel strategies for the prevention and treatment of CR in clinic.[22,23] However, critical gaps remain. Specifically, the role of IL-6 signaling in germinal center B cell responses within the transplantation setting is poorly understood, and comprehensive assessments of its effects on inflammatory cell infiltration into allografts are lacking. Therefore, a more comprehensive elaboration of the preventive effects of blocking IL-6/IL-6R signaling on CR, as well as a preliminary exploration of its mechanisms, provides a stronger theoretical basis and reference for the clinical use of clazakizumab and tocilizumab in preventing and treating CR.

Here, we addressed these knowledge gaps by constructing a robust CR model using allogeneic cardiac transplantation combined with CTLA4-Ig treatment, which mimics clinically relevant immune dynamics. Using this model, we systematically evaluated the impact of IL-6/IL-6R signaling blockade on CR, employing IL-6 knockout recipients and tocilizumab-treated mice. Our study provides a detailed assessment of germinal center B cell dynamics and inflammatory cell infiltration in the graft microenvironment, offering novel insights into the mechanisms by which IL-6/IL-6R blockade mitigates CR.

Materials and Methods

Experimental Groups

To establish CR model, abdominal cardiac transplantation surgeries were performed using wild-type male BALB/c and C57BL/6 mice as donors and recipients, respectively. In the CTLA4-Ig treatment group, recipient mice were administered CTLA4-Ig (BioXCell, USA) 0.25 mg via intraperitoneal injection within the first 2 d post-surgery, while the control group was injected with an equal amount of saline at the same time points. In order to explore the preventive effect of blocking IL-6/IL-6R signaling on CR, three experimental groups were designed: control, tocilizumab, and IL-$6^{-/-}$ groups. The control group, cardiac transplantation (wild-type BALB/c mice as donors and wild-type C57BL/6 mice as recipients) with CTLA4-Ig treatment. In the tocilizumab group, recipient mice received intraperitoneal injections of tocilizumab 0.25 mg twice a week from the day of surgery until graft failure or specimen acquisition, this usage and dosage was determined based on our previously published article.[24] In the IL-$6^{-/-}$ group, IL-$6^{-/-}$ mice were used as recipients.

Antibodies and Mice

Immunohistochemical staining was performed using anti-mouse antibodies targeting CD68 (GB113109, 1:400), CD3 (ab16669, 1:200), α-SMA (14–9760-82, 1:200), Foxp3 (12653T, 1:500), C4d (HP8033, 1:200), CD8 (ab217344, 1:400), CD4 (D7D2Z, 1:100), CD138 (ab128936, 1:500), and CD19 (ab245235, 1:1000). Flow cytometry antibodies were acquired from BioLegend (USA), including PE-Dazzle-CD19, PE-CD138, FITC-GL7, APC-Fas, PerCP-Cy5.5-CD4, APC-Cy7-CD45, APC-CD138, PE-Cy7-CD11b, FITC-F4/80, APC-CD206, and PE-CD8.

Charles River Laboratories (Beijing, China) provided the IL-$6^{-/-}$ and wild-type C57BL/6 and BALB/c mice. Adult male mice (20–25 g) were used for transplantation and bred in the environment of pathogen-free at Guangzhou Jennio

Biotech Co., Ltd. (Guangzhou, China). All animal experiments were conducted by the Ethics of Guangdong experimental animal management regulations. The experimental protocol was approved by the Committee of Guangzhou Jennio Biotech Co., Ltd. (approval number JENNIO-IACUC-2023-A065).

Murine Heterotopic Cardiac Transplantation

The surgical procedure for murine heterotopic cardiac transplantation was performed as previously described.[24–26] Briefly, the donor cardiac allograft's ascending aorta was anastomosed end-to-side to the recipient's abdominal aorta, while the pulmonary artery was similarly anastomosed to the recipient's inferior vena cava. Postoperatively, graft viability was assessed daily by palpating the heartbeat through the abdominal wall, with cessation of contractility serving as the definitive indicator of allograft failure.

Detection of Serum DSA

Serum samples from recipient mice were collected at predetermined time points to assess DSA levels. In brief, splenocytes harvested from BALB/c donor mice were incubated with serum samples for 30 mins. Following incubation, the cells were washed thoroughly and stained with fluorochrome-conjugated anti-mouse antibodies specific for IgM and IgG (BioLegend, USA) for 1 hour at 4 °C. DSA binding was quantified by measuring the mean fluorescence intensity (MFI) of IgM and IgG on the surface of splenocytes by flow cytometry.

Histology

Cardiac allografts were collected at specific time points, formalin-fixed and paraffin-preserved. Allograft samples were sliced into 4-μm sections, which were subsequently deparaffinized and rehydrated. Pathological characteristics in allografts were assessed through staining with Masson's trichrome, hematoxylin and eosin (HE), α-SMA, C4d, CD3, CD4, Foxp3, CD68, CD8, CD19, and CD138. The scoring criteria for C4d deposition followed the guidelines set by the International Society of Heart and Lung Transplantation: grade 0 represented less than 10% of all capillaries; grade 1 represented between 10% and 50% of all capillaries; and grade 2 represented more than 50% of all capillaries.[27] The α-SMA deposition was analyzed using ImageJ software and quantified by indicator integrated optical density (IOD).

Spleen of recipient mice from the control, tocilizumab and IL-6$^{-/-}$ groups were obtained. For immunofluorescence procedures, tissue specimens embedded in O.C.T. compound (SkuraFinetek) were sectioned into 6-μm slices. Following air-drying, the slides underwent fixation through immersion in chilled 100% acetone (4 °C, 10 minutes), followed by a 30-minute blocking step with 10% bovine serum albumin (BSA) under ambient conditions. Subsequent immunolabeling involved overnight incubation with primary antibodies at 4 °C. The primary antibodies were as follows: anti-IgD (Biolegend, clone 11–26c.2a, Cat#405734), anti-GL7 Antigen (Biolegend, clone GL7, Cat#144614), anti-CD4 antibody (Abcam, clone: EPR19514, Cat#ab183685).

Flow Cytometry

The cardiac allografts, blood, bone marrow, lymph nodes, and spleen were collected at 3 weeks post-transplantation. The lymph node, spleen, and cardiac grafts were processed by milling and filtering using nylon screen (200-mesh). Peripheral blood samples were collected, and red blood cells were lysed to isolate leukocytes. Bone marrow cells were obtained by flushing both femurs with sterile buffer after sectioning. Single-cell suspensions prepared from the lymph nodes, bone marrow, blood, and spleen were stained using antibodies with fluorochrome-conjugated targeting CD138, CD19, GL-7, and Fas. Cells isolated from cardiac allografts were stained with antibodies specific for CD45, CD4, CD8, CD11b, F4/80, CD206, CD19, and CD138. Flow cytometric analysis was performed using a CytExpert flow cytometer (Beckman Coulter).

Statistics

Data were analyzed by IBM SPSS Statistics software (version 20.0) and presented as the mean ± standard deviation (SD). The Kaplan–Meier curves were generated, and Log rank tests were used to compare survival rates between groups. For two group comparisons, Student's *t*-test was applied to analyze normally distributed data, while the Mann–Whitney

U-test was used for non-normally distributed data. For three group comparisons, one-way ANOVA followed by Tukey's test was used to evaluate differences. $P < 0.05$ was considered statistically significant.

Results

CTLA4-Ig Treatment Establishes a Progressive Chronic Rejection Model

Based on our previous studies and those of other groups,[25,26,28,29] we established a robust CR model using allogeneic cardiac transplantation combined with CTLA4-Ig treatment. CTLA4-Ig was shown to effectively suppress acute rejection, resulting in a significant extension of cardiac allograft survival (26.0 ± 3.8 vs 7.0 ± 0.6 d, $P < 0.001$) (Figure 1A). To characterize the progression of CR in this model, serum DSA levels and the pathological features of the allografts were assessed weekly after transplantation.

The analysis revealed that DSA-IgG levels began to elevate markedly from 2 weeks post-surgery and continued to increase over time in the CTLA4-Ig-treated group (Figure 1B). However, DSA-IgM levels remained unchanged within 4 weeks after surgery (Figure 1C), suggesting that IgG is the predominant DSA subtype implicated in the pathogenesis of CR in this model. Histopathological evaluation demonstrated early signs of inflammatory cell infiltration and mild interstitial fibrosis as early as 1 week post-surgery. These pathological changes progressively worsened, with the development of myocardial ischemic necrosis and neointimal hyperplasia observed at 3 and 4 weeks post-transplant. Immunohistochemical analysis further confirmed mild C4d (antibody-mediated injury marker) deposition and a progressive increase in α-SMA staining, beginning at 1 week and intensifying over time (Figure 1D–F). These findings are consistent with the characteristics pathology of CR, validating the relevance and reproducibility of this model.

To further delineate the cellular dynamics of CR, we performed immunohistochemical staining to quantify infiltering T cells (CD3, CD4, and CD8), B cells (CD19), plasma cells (CD138), and macrophages (CD68) in allografts. The results indicated a significant increase in the infiltration of these cell populations starting at 1 week post-surgery and continued to increase over time (Figure 2). The pathological characteristics and quantitative data of inflammatory cell infiltration at weekly time points post-surgery in this CR model are summarized in Table 1.

IL-6/IL-6R Signaling Ablation Attenuates CR and Promotes Acceptance

Based on establishing the CR model, we investigated the effects of IL-6/IL-6R signaling ablation on CR using IL-6 knockout (IL-$6^{-/-}$) mice as recipients or the anti-IL6R monoclonal antibody tocilizumab. Both approaches dramatically prolonged allograft survival and promote immune tolerance. All allografts in the IL-$6^{-/-}$ group and approximately 67% of those in the tocilizumab-treated group achieved long-term survival (up to 100 days) (Figure 3A). Pathological evaluation at 3 weeks post-transplantation demonstrated that IL-6 knockout and tocilizumab treatment markedly alleviated the hallmarks of CR, including inflammatory cell infiltration, interstitial fibrosis, myocardial ischemic necrosis, and neointimal hyperplasia. Further analysis of surviving allografts at 60 and 100 days post-surgery revealed significantly milder pathological changes in the IL-$6^{-/-}$ and tocilizumab groups compared to the control group at 3 weeks (Figure 3B). These results confirmed the potent preventive effects of IL-6/IL-6R signaling blockade on CR, suggesting its potential to induce long-term graft acceptance and mitigate pathological progression in this model.

IL-6/IL-6R Signaling Ablation Reduces C4d Deposition and Impacts Germinal Center B Cell Response

To evaluate the effect of IL-6/IL-6R signaling ablation on humoral immune responses, we performed both immunohistochemical and flow cytometric analyses. At 2 and 3 weeks post-surgery, IL-6 knockout (IL-$6^{-/-}$) and tocilizumab treatment significantly reduced serum DSA-IgG levels (Figure 4A), as well as decreased C4d deposition, B ($CD19^{+}$) and plasma cells ($CD138^{+}$) infiltration in the cardiac allografts (Figure 4B).

Flow cytometric analysis further revealed that IL-$6^{-/-}$ and tocilizumab-treated recipients exhibited significantly decreased frequencies of circulating B cells ($CD19^{+}$) and plasma cells ($CD138^{+}$) across multiple compartments, including

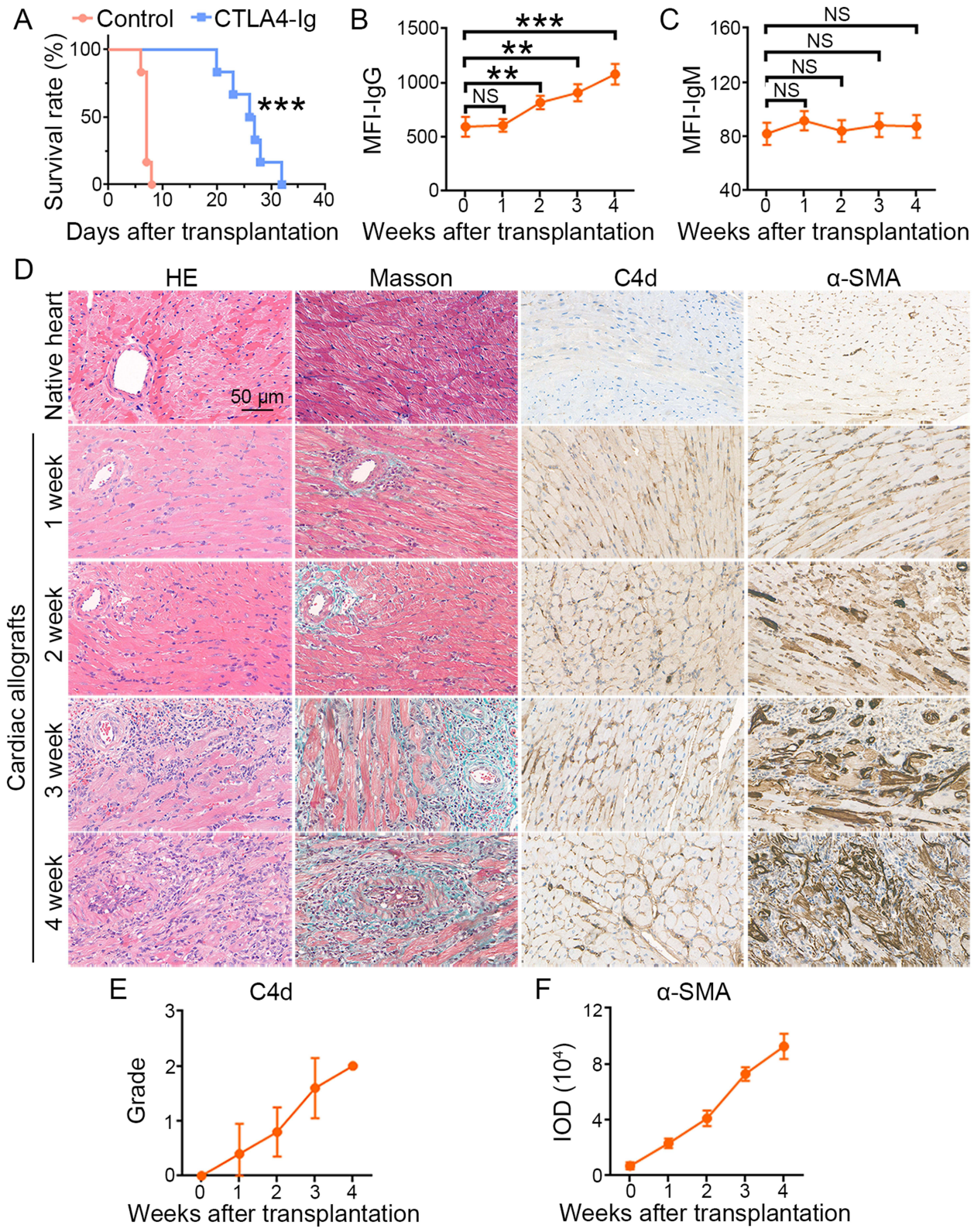

Figure 1 Establishment of chronic cardiac allograft rejection in mice via allogeneic transplantation with CTLA4-Ig treatment. (**A**) Survival rates of cardiac allografts in the control and CTLA4-Ig treatment groups (n = 6/group). (**B** and **C**) Sequential analysis of serum DSA (IgG and IgM) in the CTLA4-Ig treatment group (n = 4/group/time point). (**D**) Representative native heart and cardiac allograft images in the CTLA4-Ig treatment group stained with HE, Masson's trichrome, C4d, and α-SMA at the indicated time points post-transplantation. (**E** and **F**) Sequentially quantitative analysis of C4d and α-SMA staining in the CTLA4-Ig treatment group (n = 5/time point). **P < 0.01; ***P < 0.001.

Abbreviations: MFI, mean fluorescence intensity; DSA, donor specific antibody; IOD, integrated optical density; NS, not significant.

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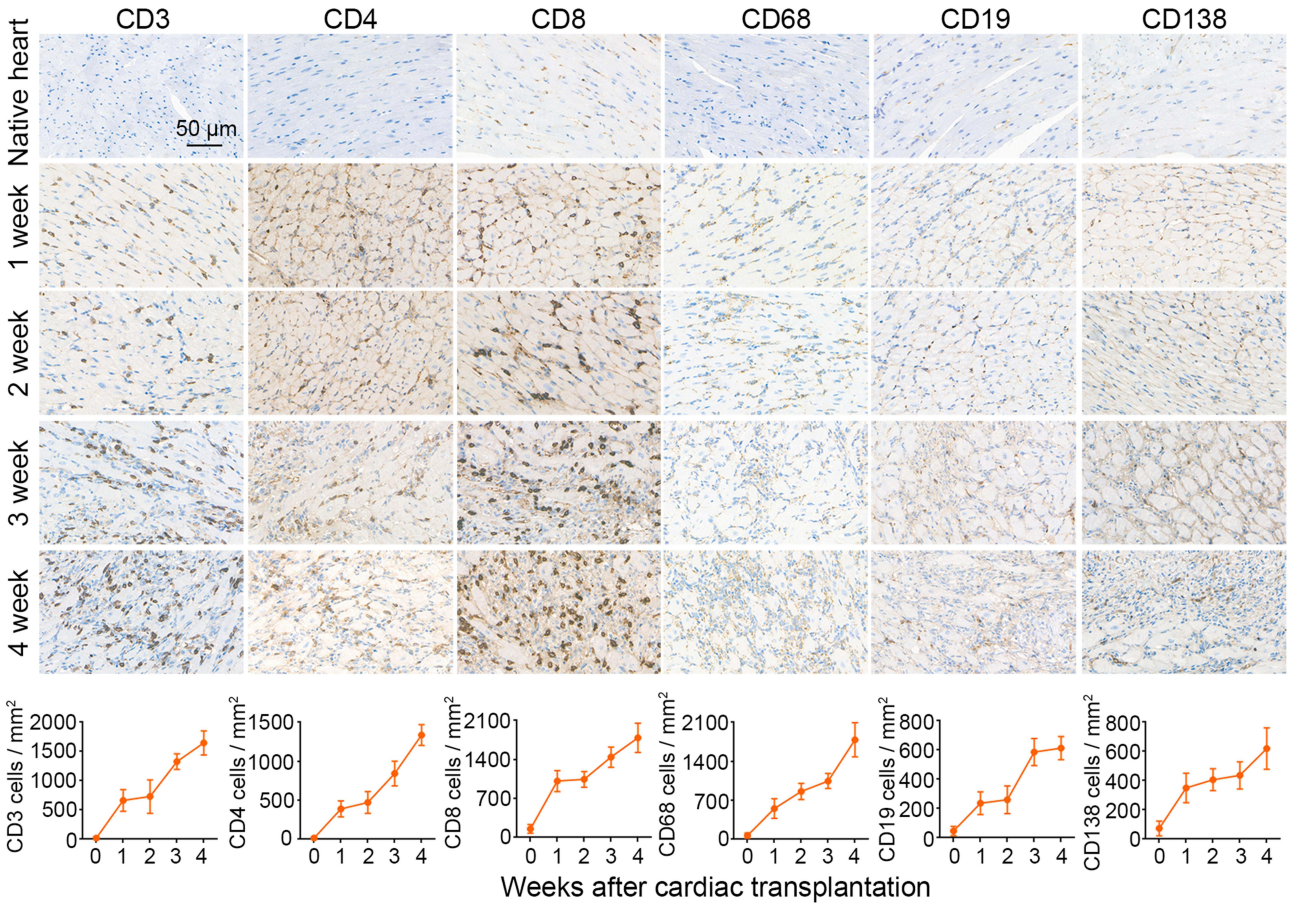

Figure 2 Sequential analysis of inflammatory cell infiltration in allografts with CR. Representative images and quantitative analysis of total T (CD3), $CD4^+$ T (CD4), $CD8^+$ T (CD8), macrophage (CD68), B (CD19), and plasma (CD138) cell infiltration in allografts of the CTLA4-Ig treatment group at the indicated time points post-transplantation (n = 5/group).

blood, bone marrow, lymph nodes, and spleen. There was no statistically significant difference in the frequency of B cells and plasma cells between the tocilizumab-treated group and the IL-$6^{-/-}$ group in these immune organs (Figure 5A and B). Additionally, germinal center B cells ($CD19^+GL7^+Fas^+$), critical for DSA generation,[30,31] were significantly reduced in the lymph nodes and spleen of both treatment groups. Interestingly, the frequency of lymph node germinal center B cells was higher in the tocilizumab group compared to the IL-$6^{-/-}$ group (Figure 5C). Furthermore, the immunofluorescence staining results indicated that tocilizumab treatment and IL-6 knockout significantly reduce the formation of germinal centers ($CD4^+GL7^+IgD^-$) and the level of germinal center B cells ($GL7^+$) in the recipient spleen (Figure 5D). These findings highlight the pivotal role of IL-6/IL-6R signaling in regulating B cell responses and reducing antibody-mediated injury in CR.

IL-6/IL-6R Signaling Ablation Reduces Inflammatory Cell Infiltration in Cardiac Allografts

To assess the impact of IL-6/IL-6R signaling ablation on the recruitment of inflammatory cells into the graft, we performed immunohistochemistry and flow cytometry analyses on cardiac allografts. IL-6 knockout and tocilizumab treatment significantly reduced α-SMA expression, indicative of attenuated fibrotic processes, and markedly suppressed the infiltration of macrophages ($CD68^+$), and T cells ($CD3^+$, $CD4^+$, and $CD8^+$) within the allografts. Furthermore, IL-6 knockout and tocilizumab treatment did not decrease Treg cell infiltration in allografts (Figure 6).

Table 1 Sequential Analysis of Histological Characteristics in a Mouse Chronic Allograft Rejection Established by Allogeneic Transplantation with CTLA4-Ig Treatment

Histology	Week After Cardiac Transplantation				
	0	1	2	3	4
Myocardial ischemic necrosis	–	–	–	+	+
Neointimal hyperplasia	–	–	–	+	+
Inflammatory cell infiltration	–	Mild	Moderate	Severe	Severe
Interstitial fibrosis	–	Mild	Mild	Moderate	Severe
Capillary C4d distribution (grade)	0	0.4 ± 0.5	0.8 ± 0.4	1.6 ± 0.5	2
α-SMA staining (IOD, 10^4)	0.7 ± 0.2	2.3 ± 0.3	4.1 ± 0.5	7.3 ± 0.4	9.3 ± 0.8
$CD3^+$ cell infiltration (cells / mm^2)	19.8 ± 19.4	663.0 ± 165.0	729.8 ± 255.6	1326.6 ± 120.5	1646.6 ± 184.5
$CD4^+$ cell infiltration (cells / mm^2)	16.4 ± 14.8	389.8 ± 92.3	472.8 ± 124.1	843.0 ± 141.9	1336.4 ± 119.5
$CD8^+$ cell infiltration (cells / mm^2)	153.0 ± 71.0	1013.0 ± 168.0	1043.2 ± 126.0	1443.0 ± 161.3	1789.6 ± 234.4
$CD68^+$ cell infiltration (cells / mm^2)	66.4 ± 43.3	553.2 ± 156.9	859.6 ± 131.8	1046.4 ± 120.3	1786.4 ± 274.2
$CD19^+$ cell infiltration (cells / mm^2)	46.4 ± 28.5	236.4 ± 69.4	259.8 ± 84.6	586.4 ± 82.5	613.0 ± 71.0
$CD138^+$ cell infiltration (cells / mm^2)	73.2 ± 45.4	349.8 ± 89.9	406.2 ± 67.2	436.2 ± 83.2	619.6 ± 127.3

Notes: The scoring criteria of C4d deposition was according to the International Society of Heart and Lung Transplantation: grade 0, <10% of all capillaries; grade 1, 10–50% of all capillaries; grade 2, >50% of all capillaries. Data are mean±standard deviation. Histological evaluations were performed using light microscopy by an independent observer, who was blinded to the experimental conditions.
Abbreviations: IOD, Integrated Optical Density; –, negative; +, positive.

Flow cytometric analysis further confirmed a notable reduction in both the frequency and total quantity of leukocytes ($CD45^+$) in the treated groups (Figure 7A). While the frequencies of $CD4^+$ and $CD8^+$ T cells remained unchanged, their absolute quantities were significantly reduced in both IL-$6^{-/-}$ and tocilizumab groups (Figure 7B). Additionally, macrophages ($CD11b^+F4/80^+$), M2 macrophages ($CD11b^+F4/80^+CD206^+$), B cells ($CD19^+$), and plasma cells ($CD138^+$) were significantly reduced in treated allografts compared to the control group. Interestingly, the frequency of B cells was higher in the tocilizumab-treated group compared to the IL-$6^{-/-}$ group (Figure 7C–F). These results demonstrate that IL-6/IL-6R signaling ablation effectively diminishes inflammatory cell infiltration, alleviating graft inflammation and fibrosis.

Discussion

CR is a major cause of long-term allograft failure and has garnered increasing attention because of its complex pathogenesis involving multiple inflammatory cell types, including macrophages, T cells, and B cells.[32–34] These immune cells collectively contribute to processes such as interstitial fibrosis, neointimal hyperplasia, and DSA production. Consequently, therapies targeting a single cell type have been largely inadequate in effectively mitigating CR. Our study addresses this challenge by establishing a robust and clinically relevant CR mouse model and investigating the therapeutic potential of IL-6/IL-6R signaling blockade. Mechanistically, IL-6/IL-6R signaling ablation suppressed B cell activation and germinal center formation, reducing DSA production, and subsequently attenuated antibody-mediated injury. Furthermore, it markedly reduced macrophage and T cell infiltration into the graft, thereby mitigating inflammation and fibrosis. Notably, the IL-$6^{-/-}$ group exhibited more pronounced effects compared to the tocilizumab group, suggesting the potential involvement of non-IL-6R-dependent pathways in CR progression.

To construct the the CR model, we performed allogeneic cardiac transplantation with CTLA4-Ig treatment, which mimics the clinical scenario of insufficient immunosuppression leading to CR. This approach effectively suppressed acute rejection while inducing features characteristic of CR, including the progressive elevation of DSA-IgG levels, inflammatory cell infiltration, and pathological changes in the allografts. Unlike previously reported CR models relying on MHC-II-mismatched strains (cardiac transplantation from BM12 mice to B6 mice)[35–37] or $CD4^+$ T cells depletion,[21,38] which deviate from clinical setting, the CTLA4-Ig model closely recapitulates the immune dynamics observed in patients receiving immunosuppressants. By sequentially

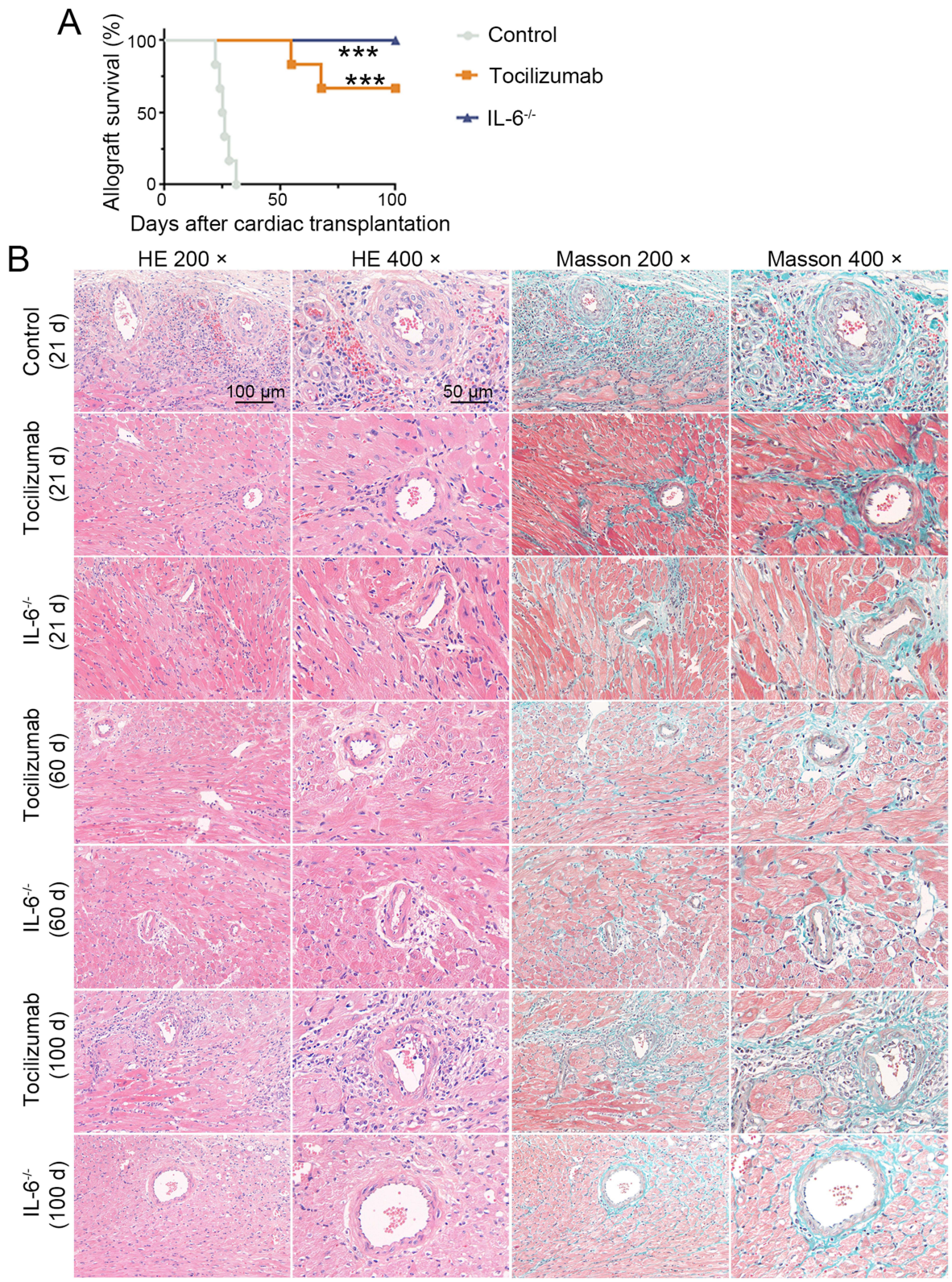

Figure 3 IL-6/IL-6R signaling ablation attenuates CR and promotes acceptance. (**A**) Survival rates of cardiac allografts in the control, Tocilizumab and IL-6$^{-/-}$ groups (n = 6/group) (**B**) Representative cardiac allograft images of HE and Masson's trichrome staining at the indicated time points post-transplantation in each group. ***P < 0.001.

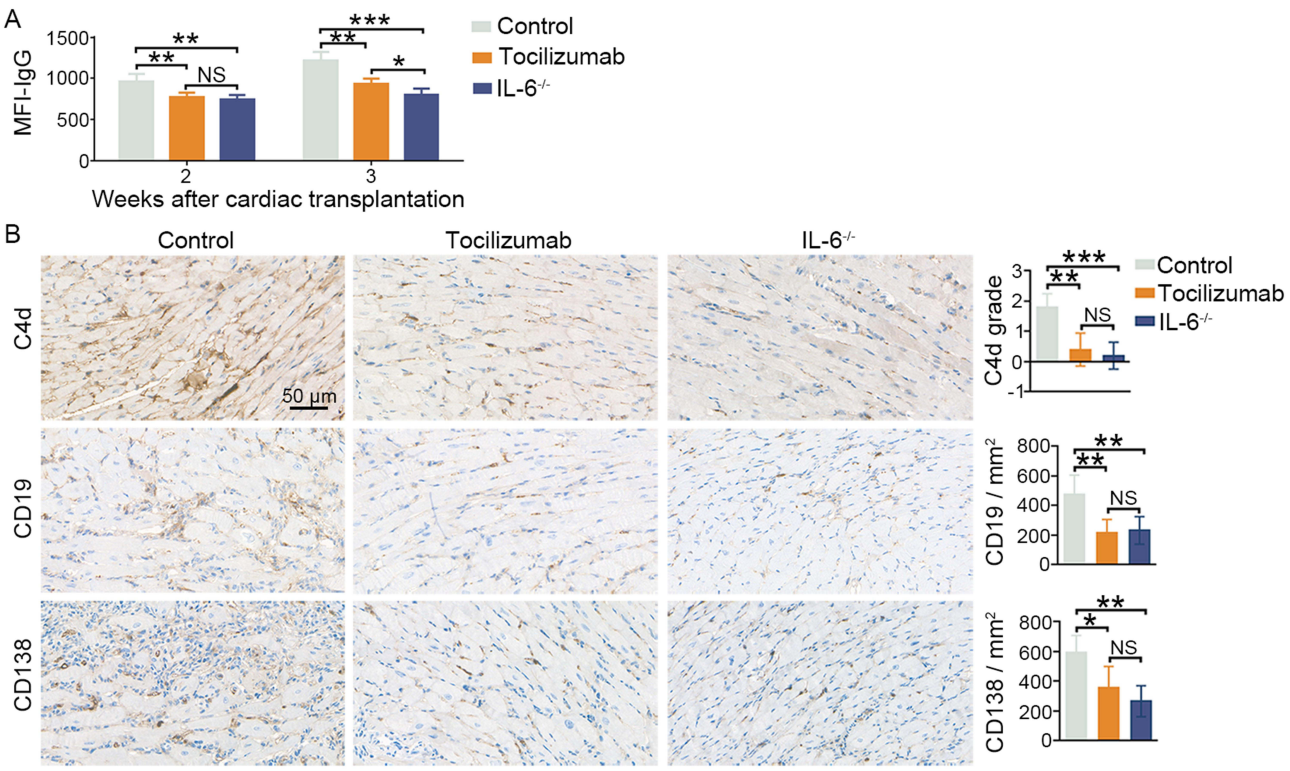

Figure 4 IL-6/IL-6R signaling ablation reduces serum DSA-IgG levels, as well as decreases C4d deposition, B and plasma cells infiltration in the cardiac allografts. (**A**) Serum DSA-IgG levels at 2 and 3 weeks post-transplantation in the control, tocilizumab, and IL-6$^{-/-}$ groups (n = 4/group/time point). (**B**) Representative images and quantitative analysis of C4d, CD19, and CD138 staining in each group at 3 weeks post-transplantation (n = 5/group). *P < 0.05; **P < 0.01; ***P < 0.001.
Abbreviations: MFI, mean fluorescence intensity; DSA, donor-specific antibody; NS, not significant.

analyzing DSA levels and pathological changes, we established a temporal understanding of CR progression and its immune mediators.

IL-6, a cytokine with redundant pleiotropic activity, is crucial in the activation and differentiation of inflammatory cells including macrophages, T cells, and B cells.[39] Current immunosuppressive regimens predominantly target T cells, with limited effects on B cells and macrophages, which may explain the persistent high incidence of CR in clinical practice. Our findings provide compelling evidence that blocking IL-6/IL-6R signaling alleviates CR by targeting both humoral and cellular immune mechanisms. Specifically, IL-6 knockout and tocilizumab treatment significantly reduced DSA-IgG levels, suppressed germinal center B cell formation, and attenuated antibody-mediated injury. These effects were accompanied by marked reductions in inflammatory cell infiltration, including macrophages and T cells, within the allografts.

In fact, the critical involvement of IL-6/IL-6R signaling in transplant rejection has been documented in multiple preclinical studies. Pioneering work in cardiac allograft models demonstrated markedly elevated IL-6 expression in CR lesions characterized by progressive fibrosis, with subsequent anti-IL-6 mAb intervention effectively reversing these pathological changes. This seminal finding established the therapeutic potential of IL-6/IL-6R axis blockade for CR management.[21] Subsequent investigations by Zhao et al extended these observations, revealing that combinatorial therapy targeting both IL-6 signaling and Th1 immune responses produces synergistic effects in enhancing allograft survival.[40] Our prior research further substantiated this therapeutic strategy by demonstrating that IL-6/IL-6R blockade significantly attenuates acute antibody-mediated rejection via suppression of pathogenic B-cell activation.[24] Our current investigation advances this field through three principal findings: First, longitudinal analysis up to 100 days post-transplantation revealed that prophylactic IL-6/IL-6R blockade confers durable protection against CR development, as evidenced by histopathological evaluation. Second, through multi-color immunofluorescence and flow cytometric analysis, we elucidated the

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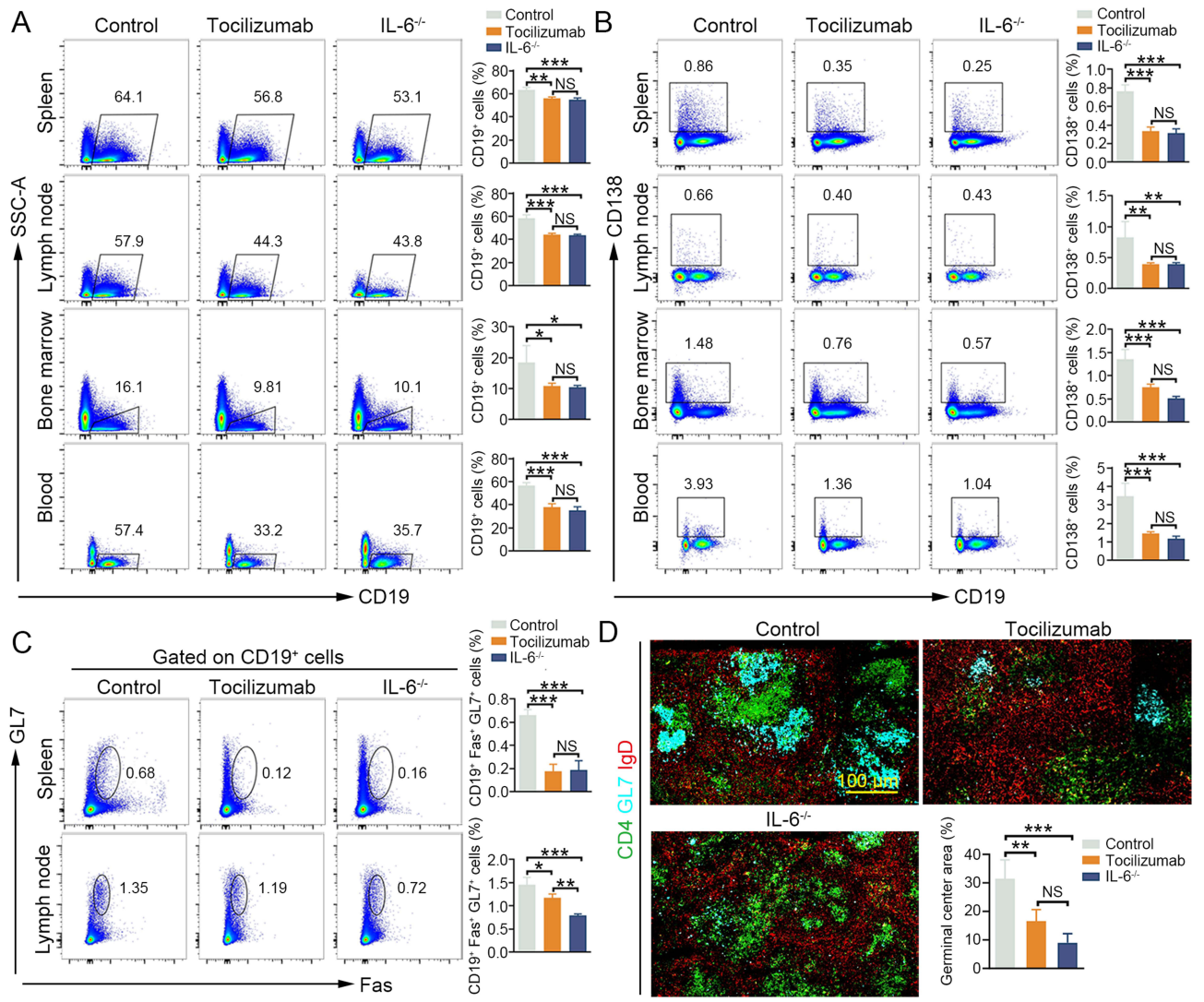

Figure 5 IL-6/IL-6R signaling ablation inhibited B cell response in recipients. (**A** and **B**) Representative dot plots for the frequencies and quantitative analysis of B cells ($CD19^+$) and plasma cells ($CD138^+$) in the spleen, lymph nodes, bone marrow, and blood in the control, tocilizumab, and IL-$6^{-/-}$ groups at 3 weeks post-transplantation (n = 4/group). (**C**) Representative dot plots for the frequencies and quantitative analysis of germinal center B cells ($CD19^+Fas^+GL7^+$) in the spleen and lymph nodes of each group (n = 4/group). (**D**) CD4, GL7, and IgD immunofluorescence staining revealed the levels of germinal center formation and germinal center B cell levels in the spleens of each group (n = 4/group). *P < 0.05; **P < 0.01; ***P < 0.001.

Abbreviation: NS, not significant.

specific inhibitory effects of this intervention on germinal center B-cell maturation - a critical process in DSA production. Finally, systematic quantification of inflammatory infiltrates demonstrated significant reductions in multiple leukocyte subsets (including T cells, B cells, macrophages, and plasma cells) within CR-affected allografts following treatment.

Interestingly, our results showed that IL-6 knockout demonstrated greater efficacy in preventing CR compared to tocilizumab. This discrepancy may be attributed to some reasons: First, the intraperitoneal administration of tocilizumab necessitates an absorption period to reach therapeutic concentrations, with potential fluctuations in blood drug levels over time. Second, gene knockout completely blocks IL-6 signaling, whereas tocilizumab only partially blocks IL-6R signaling within safe therapeutic doses. Additionally, the possibility of IL-6 acting through non-IL-6R pathways requires further exploration. These observations underscore the complexity of IL-6 signaling in CR and highlight the need for a deeper mechanistic understanding.

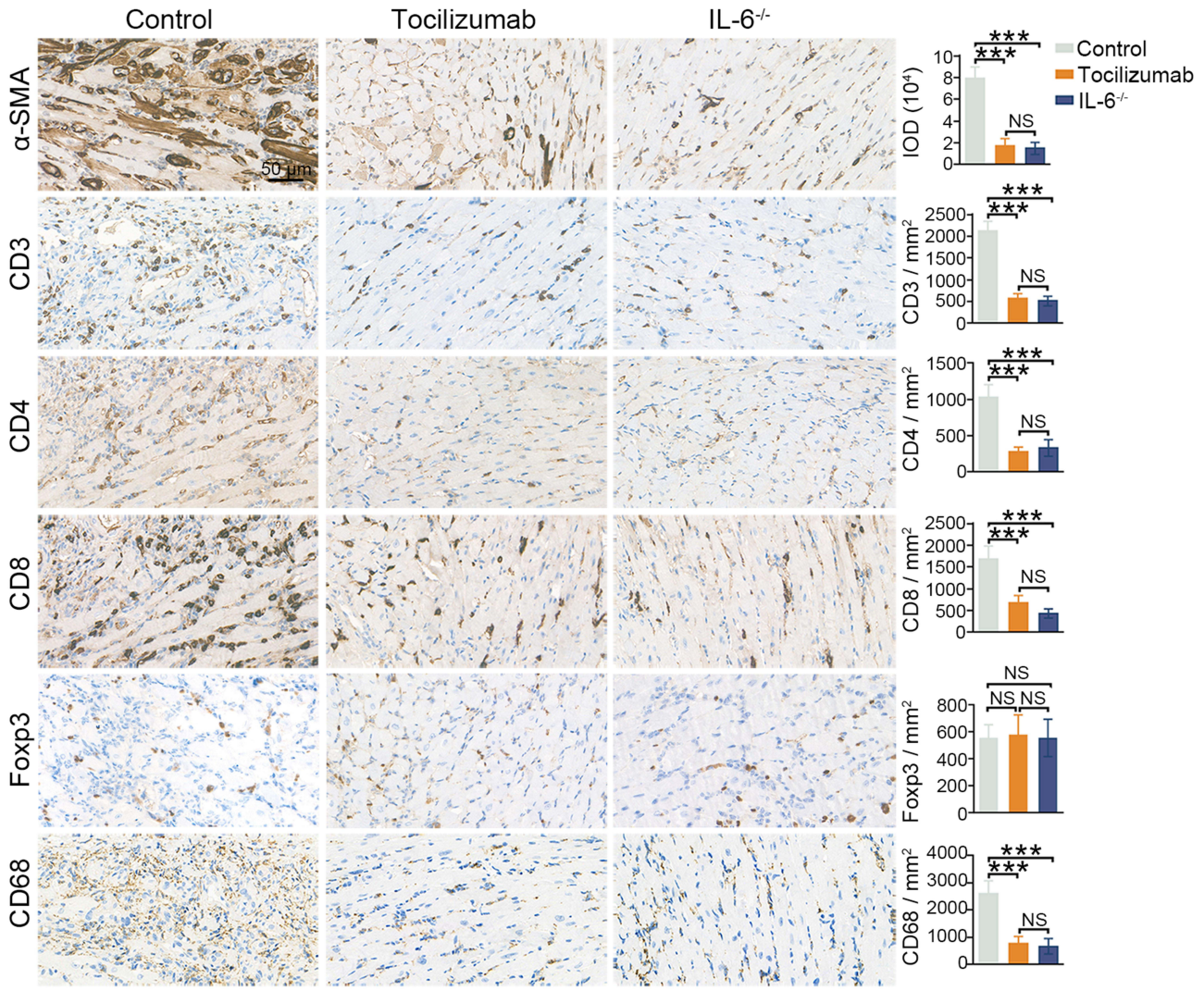

Figure 6 Reduced α-SMA expression, and T cells and macrophages infiltration in allografts following IL-6/IL-6R signaling ablation. Representative images and quantitative analysis of α-SMA, CD3, CD4, CD8, Foxp3 and CD68 staining in the control, tocilizumab, and IL-6$^{-/-}$ groups at 3 weeks post-transplantation (n = 5/group). ***P < 0.001. **Abbreviation**: NS, not significant.

While our study elucidates the pivotal role of IL-6/IL-6R signaling in CR, several limitations must be acknowledged. The JAK/STAT, MAPK/ERK, and PI3K/Akt signaling cascades represent principal intracellular pathways mediating IL-6/IL-6R biological effects, which play key roles in the activation of T cells, B cells, and macrophages.[41] In this study, we did not measure the levels of these pathways, therefore we did not fully elucidate the mechanisms by which blocking IL-6/IL-6R signaling prevents CR. Furthermore, our findings are based on murine models, necessitating validation in preclinical and clinical settings to assess the translatability of IL-6/IL-6R blockade as a therapeutic strategy. Moreover, some potential confounders cannot be ignored. For example, there are differences in the immune systems between mice and humans, and the clinical translation effect may not be as ideal as in mice. In clinical practice, patients also use other immunosuppressants, which may affect the effectiveness of blocking IL-6/IL-6R signaling in managing CR.

In conclusion, this study establishes a clinically relevant CR model and demonstrates that IL-6/IL-6R signaling blockade is a promising therapeutic approach for mitigating CR. By simultaneously targeting humoral and cellular immune responses, IL-6/IL-6R inhibition represents a comprehensive strategy for addressing the multifaceted pathogenesis of CR and improving long-term graft outcomes.

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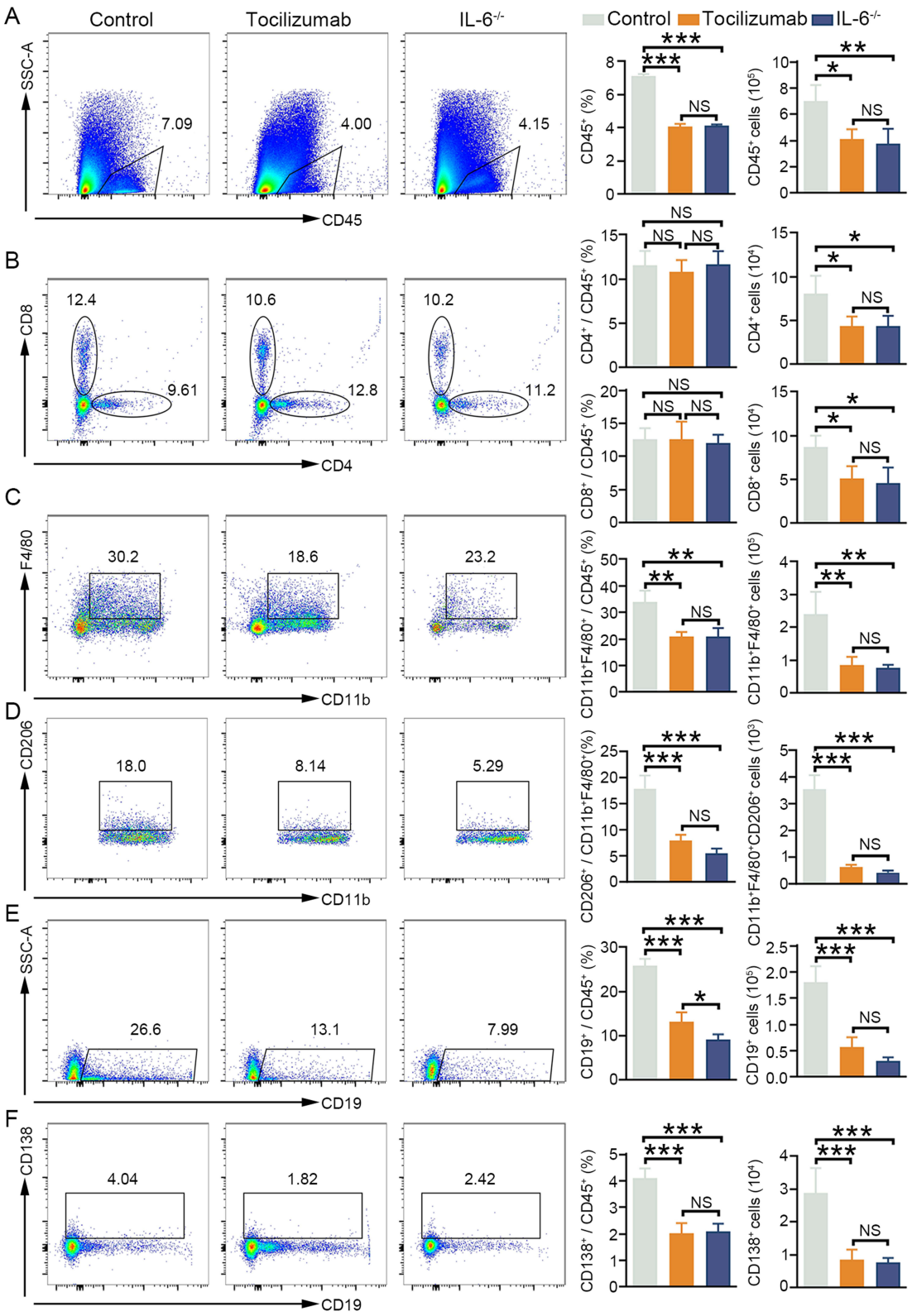

Figure 7 IL-6/IL-6R signaling ablation reduced inflammatory cell infiltration into cardiac allografts as shown by flow cytometry. Leukocytes ($CD45^+$), T cells ($CD4^+$ and $CD8^+$), macrophages ($CD11b^+F4/80^+$), M2 macrophages ($CD11b^+F4/80^+CD206^+$), B cells ($CD19^+$), and plasma cells ($CD138^+$) were detected using flow cytometry in the control, tocilizumab, and IL-$6^{-/-}$ groups at 3 weeks post-transplantation. Representative dot plots and quantitative analysis illustrate the frequencies and counts of leukocytes (**A**), T cells (**B**), macrophages (**C**), M2 macrophages (**D**), B cells (**E**), and plasma cells (**F**) in allografts from each group (n = 4/group). *P < 0.05; **P < 0.01; ***P < 0.001. **Abbreviation**: NS, not significant.

Abbreviations

CR, chronic rejection; DSA, donor-specific antibody; MFI, mean fluorescence intensity; HE, Hematoxylin and eosin; IOD, integrated optical density.

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Disclosure

The authors have no conflicts of interest to disclose for this work.

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