

Inhibition of C-X-C motif chemokine 10 reduces graft loss mediated by memory CD8⁺ T cells in a rat cardiac re-transplant model

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Abstract. The interaction of chemokine (C-X-C motif) ligand 10 (CXCL10) with its receptor (CXCR3) is a critical process in recruiting donor reactive T cells to a graft and alloantigen-specific memory T (T_m) cells exert a principal function in promoting graft dysfunction during accelerated cardiac rejection. However, whether CXCL10 chemokine exerts any effects on acute accelerated rejection mediated by CD8⁺ T_m cells in a re-transplant model has remained elusive. The present study established a cardiac transplant model by advanced microsurgery technology and improved organ storage. A novel rat model of cardiac re-transplantation was established at 40 days following primary heart transplant. The experiment included two parts, and when models were established, the rats were divided into two groups: Primary cardiac transplant (HTx) and re-transplantation without treatment (HRTx). In part 1, recipients from part 2, including re-transplantation without treatment (HRTx+NS) and re-transplantation treated with anti-CXCL10 antibodies (500 µg every other day by intraperitoneal injection; HRTx+CXCL10 Abs group). The graft survival time was observed and graft infiltration by inflammatory cells was assessed via histology of cardiac graft sections; in addition, the gene expression and the serum concentration of CXCL10 in each group was assessed. Indexes such as rejection-associated cytokines were assayed by reverse-transcription quantitative PCR and ELISA kits, and flow cytometry of splenocytes was used to detect T_m cells in the re-transplantation groups. The results demonstrated that

level of CXCL10 was significantly increased and the graft mean survival time was shortened accompanied with aggravated lymphocyte cell infiltration in the HRTx group when compared that in the HTx group; in addition, the serum levels and mRNA expression of interleukin (IL)-2 and interferon (IFN)-γ were increased, while transforming growth factor (TGF)-β was decreased in the HRTx group. Furthermore, neutralization of CXCL10 prolonged the graft mean survival time and delayed accelerated rejection. Compared with that in the HRTx+NS group, serum levels and graft tissue mRNA expression of IFN-γ and IL-2 were decreased in the HRTx+CXCL10 Abs group, while TGF-β mRNA was significantly increased but the serum concentration was not significantly affected. In addition, there was no difference in IL-10 between the two groups, while delayed accelerated rejection paralleled with inflammatory cell infiltration decreased and the proliferation and differentiation of CD8⁺ T_m cells in secondary lymphoid organs were reduced in the HRTx+CXCL10 Abs group vs. those in the HRTx+NS group. The present study demonstrated that CXCL10 had a crucial role in cardiac transplantation and re-transplantation, and that treatment with CXCL10 antibodies delays accelerated acute rejection mediated by T_m cells in a rat model of cardiac re-transplantation.

Introduction

A multidisciplinary program has been initiated to better establish transplantation for prolonging survival time of cardiac transplantation recipients; however, 45% succumbed to primary or secondary graft failure with the underlying pathogenesis including coronary vasculopathy and acute rejection. Previous studies have demonstrated that outcomes of cardiac re-transplantation are worse compared with those of the first transplant (1-3), while cardiac re-transplantation is thought to be a sole definitive treatment strategy for cardiac allograft failure (4).

A second graft is associated with more complications and a poorer microenvironment than the first transplantation. Following exposure to antigens, infective agents and other factors, the organism generates memory T (T_m) cells, which provide long-term protective immunity. A fundamental

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feature of Tm lymphocytes is enhanced responsiveness to a secondary challenge with the cognate antigen (5), which may be ascribed to lower activation thresholds and vigorous proliferation compared with those of naïve T cells (6). Furthermore, Tm cells generated by homeostatic proliferation vigorously respond to transplanted allografts and mediate accelerated rejection that is highly resistant to toleration (7). Hence, acute accelerated rejection episodes mediated by alloreactive Tm cells have posed an obstacle to tolerance induction and survival time in the clinic. An increasing number of studies have focused their attention onto chemokines, a family of powerful chemotactic small peptides (8-10 kDa), which have a pivotal role in the control of leukocyte trafficking during inflammatory conditions and also in allograft rejection (8). Chemokine (C-X-C motif) ligand 10 (CXCL10) as a major chemokine, as well as the absence of acid-leucine-arginine peptide chemokine, detected in an allograft and isograft model, was found to accelerate acute allograft rejection after organ transplantation (9-11); this occurred via binding to the G-protein-coupled receptor CXCL receptor 3 (CXCR3), which was expressed in multiple cells such as primed effector T cells producing interferon (IFN)- γ and Tm cells (11,12). CXCL10 has been demonstrated to have an important role in the generation of T lymphocyte effector functions in infection and first transplants (13); however, few, if any, experimental and clinical studies on the role of CXCL10 in accelerated rejection mediated by Tm cells are available.

The present study investigated the potential role for CXCL10 in cardiac allograft rejection mediated by Tm cells. Although previous research by our group demonstrated increased levels of chemokine (C-C motif) ligand 5 and CXCL9 expression as well as secretion in skin-allograft and Tm cell transfer models compared to those in the control groups, those two models are not able to closely mimic the scenario encountered in the clinic due to the absence of blood supply in the skin-allograft model and unsteady factors in the Tm-cell transfer model (14). Therefore, the present study established a new model of two consecutive heterotopic heart transplantations in rats, for which advanced microsurgical technology was used. This procedure included cervical and abdominal heterotopic cardiac transplantation. The recipients were then treated with anti-CXCL10 antibodies to further verify whether CXCL10 directly affected the Tm cell subset in this cardiac re-transplantation model.

Materials and methods

Animals. Adult female *Rattus norvegicus* (age, 5-6 weeks; weight, 160-180 g; n=6 per group) and Lewis rats (age, 5-6 weeks; weight, 100-110 g; n=6 per group) were purchased from the Beijing Vital River Laboratory Animal Central (Beijing, China) and used as recipients and donors, respectively. Animals were housed in a room with the humidity at 45-60% and temperature at 22-27°C; they were given access to standard diet purified water *ad libitum*. The study was approved by the ethics committee of the First Affiliated Hospital of Xiamen University (Xiamen, China).

Cardiac primary transplantation and re-transplantation. The primary cardiac+transplantation was performed using

standard methods of heterotopic cervical heart transplantation as outlined by Wang *et al* (15). The vessels of Lewis rat donor hearts were anastomosed to the recipients' neck aorta and vena using a non-suture cuff technique and the graft was considered a success when the survival time was >6 days as determined by abdominal palpation at 9:00 a.m. and 9:00 p.m. every day. All procedures were performed under anesthetic treatment in order to minimize suffering of the rats. Rats with efficient first transplant were selected by observing the survival time, which were then selected to undergo cardiac re-transplantation, performed at 40 days following the first transplantation. A modified version of the method by Plenter and Grazia (16) was performed for intra-abdominal heterotopic repeat cardiac graft and the operative process was performed using microsurgical techniques. The strength and quality of heartbeats were monitored by abdominal palpation twice daily.

Experimental groups. The present study consisted of two parts. In part 1, *Rattus norvegicus* were divided into two groups without treatment, namely the primary heart transplant group (HTx; n=6) and the cardiac re-transplantation group (HRTx; n=6). Subsequently, re-transplantation rats were divided into the HRTx+NS group and the HRTx+CXCL10 Abs group according to whether recipient rats were treated with anti-CXCL10 antibodies. Rats in the HRTx+CXCL10 Abs were treated with 500 μ g anti-CXCL10 antibodies [intra-peritoneal (i.p.); Biosynthesis Biotechnology Co., Ltd., Beijing, China] on at 0, 1 and 3 days following re-transplantation. Rats in the HRTx+NS group were injected with normal saline at the same time-points. The association between CXCL10 levels and rejection mediated by CD8⁺ Tm cells in cardiac re-transplantation was evaluated.

Histology. Donor hearts were harvested 4 days after transplantation. Portions of ventricles were placed in 10% neutral buffered formalin (Elabscience Biotechnology Co., Ltd., Wuhan, China) and then embedded in paraffin (Elabscience Biotechnology Co., Ltd.). Sections (4 μ m thick) of ventricles were deparaffinized with xylene I for 20 min, xylene II for 20 min, 100% ethanol II for 1 min, 80% ethanol for 1 min, 70% ethanol for 1 min, then stained with hematoxylin for 5 min and eosin for 8 min at room temperature (H&E; Elabscience Biotechnology Co., Ltd.). The grade of rejection, which included the extent of inflammatory cell infiltration and myocyte necrosis, was confirmed according to the International Society for Heart and Lung Transplantation (ISHLT) criteria (17).

Cytokine ELISA. Peripheral serum of recipients was prepared for ELISA determination of the concentrations of CXCL10 (cat. no. EL-R0546c; Elabscience Biotechnology Co., Ltd.), IFN- γ (cat. no. ERC101g.96), transforming growth factor (TGF)- β (cat. no. ERC107b.96), interleukin (IL)-2 (cat. no. ERC001.96) and IL-10 (cat. no. ERC004.96) (all from NeoBioscience Technology Co., Ltd., Shenzhen, China) according to the manufacturer's instructions for the respective kits. The concentration of each cytokine was calculated by generating a standard curve with determination using ELISA analyzer (iMark™; Bio-Rad Laboratories, Inc., Hercules, CA, USA).

RNA extraction from graft tissue and reverse-transcription quantitative polymerase chain reaction (RT-qPCR). Total RNA was extracted from graft apical tissues of recipients with TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) according to the manufacturer's instructions. Total RNA samples (2 µg) were used to synthesize complementary DNA using RevertAid First Strand cDNA Synthesis kit (cat. no. K1622; Thermo Fisher Scientific, Inc.). RT products were then subjected to qPCR analysis (ABI StepOne system; Applied Biosystems; Thermo Fisher Scientific, Inc.) using the SYBR-Green system (SYBR™ Select Master mix; cat. no. 4472908; Thermo Fisher Scientific, Inc.). β-actin was used as an internal control and PCR primer sequences were as follows: CXCL10 forward, 5'-TCGTGCTGC TAGTCTGAGT-3' and reverse, 5'-CAACATGCGGACAGG ATAGA-3'; IL-2 forward, 5'-TCAACAGCGCATCCACTT CAAG-3' and reverse, 5'-GTTGAGATGATGCTTTGACAG A-3'; β-actin forward, 5'-TACTGCCCTGGCTCCTAGCA-3' and reverse, 5'-TGGACAGTGAGGCCAGGATAG-3'; IL-10 forward, 5'-ATTACCTCTGATACCTCAGT-3' and reverse, 5'-AGCCTTAGGATCGAAGT-3'; TGF-β forward, 5'-CAA GGGCTACCATGCCAACT-3' and reverse, 5'-CCGGGTTGT GTTGTTGTAGA-3'; IFN-γ forward, 5'-AGGACGGTAACA CGAAA-3' and reverse, 5'-CTGTGGGTTGTTCACCTC-3'. The thermocycling conditions were as follows: 10 min denaturation at 95°C, followed by a total of 40 cycles (annealing at 50°C for 2 min, denaturation at 95°C for 2 min and 15 sec, and then extension at 60°C for 60 sec) and a final elongation step of 72°C. Relative mRNA expression was calculated using the $2^{-\Delta\Delta C_q}$ method as previously described (18).

Flow cytometry and fluorescent antibodies. Spleen cells from recipients of part 2 of the experiment were prepared for flow cytometric analysis on day 4 post-transplantation. A total of 1×10^6 cells in a cell suspension in a tube were incubated with fluorescently labeled antibodies in the dark for 30 min at 4°C. Then the cell suspension underwent centrifugation at $300 \times g$ for 5 min at 4°C; the supernatant was discarded. Fragment crystallizable (Fc) region block antibodies (PerCP-Cyanine5.5-labeled CD16/CD32; cat. no. 45-0161-80; dilution, 1:160) were added to each tube to block non-specific sites and incubated in the dark at 4°C for 15 min. Data were from the spleen cells were collected and analyzed by the Coulter® Epics® XL™ using Kaluza software version 1.3 (both from Beckman Coulter, Inc., Brea, CA, USA). All antibodies incubated in the dark for 30 min at 4°C, including fluorescein isothiocyanate-labeled anti-CD8 (cat. no. 11-0084-80; dilution, 1:200) and anti-CD4 (cat. no. 11-0040-81; dilution, 1:200), phycoerythrin-labeled anti-CD44 (cat. no. 12-0444-80, dilution, 1:300) and Fc block antibodies were purchased from eBioscience, Inc. (San Diego, CA, USA).

Statistical analysis. Values are expressed as the mean \pm standard deviation. Differences between groups were analyzed by Student's t-test, and survival curves were plotted using the Kaplan-Meier method. GraphPad prism software (version 5; GraphPad, Inc., La Jolla, CA, USA) was used for statistical analysis. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Increased CXCL10 levels are associated with poor graft function and outcome of heart re-transplantation. To test whether increased CXCL10 levels are associated with poor survival, the present study assessed graft survival time by Kaplan-Meier analysis, as well as the levels of CXCL10 in the serum and grafts of rats in part 1 of the experiment (Fig. 1). The HRTx group had a median cardiac allograft survival time (MST) of 3.50 days, which was significantly shorter than that in the HTx group (MST=7.16 days; $P < 0.01$; Fig. 1A). Furthermore, the serum levels of CXCL10 in the HTx group were higher than those in the HRTx group (Fig. 2A), and the mRNA expression of CXCL10 in the graft tissue was significantly increased (1.5-fold) in the HRTx group ($P < 0.001$; Fig. 2B). In each graft type group, 6 rats were assessed.

Neutralization of CXCL10 prolongs cardiac allograft survival time after re-transplantation. In order to further explore the role of CXCL10 in the re-transplantation model, recipients in the HRTx+CXCL10 Abs group were treated with CXCL10 antibodies every other day following re-transplantation. Treatment with CXCL10 antibodies obviously prolonged re-allograft survival time (MST=11.33 days; $P < 0.001$ vs. HRTx+NS group; Fig. 1B). In parallel with the prolonged survival time, the CXCL10 serum concentration and the mRNA expression in the HRTx+CXCL10 Abs group was significantly declined ($P < 0.001$; Fig. 2C and D, respectively).

Allograft injury is increased in secondary transplants and attenuated by CXCL10 inhibition. To assess the degree of rejection, grafts were retrieved on day 4 post-transplantation and subjected to pathological examination by H&E staining. As demonstrated in Fig. 3, the re-transplant graft (ISHLT grade 4) had a significantly higher level of inflammatory cell infiltration, which caused more severe acute cellular rejection accompanied with myocyte necrosis and edema compared with that in the first transplant (ISHLT grade 3; $P < 0.05$). Treatment with anti-CXCL10 antibodies (ISHLT grade 2) significantly reduced allograft rejection compared to the re-transplant graft, resulting in multi-focal infiltrates with myocyte damage but without edema and hemorrhage ($P < 0.001$; Fig. 4). These results suggested that increases in CXCL10 may be associated with allograft injury and that treatment with anti-CXCL10 antibodies reduced graft damage.

CXCL10 antibodies regulate rejection-associated cytokine levels. The present study evaluated inflammatory cytokine levels, which reflect the intensity of rejection episodes, in grafts and serum obtained at 4 days after re-transplantation. The cytokine serum concentrations determined by ELISA kits are presented in Fig. 5A. IL-2 was significantly downregulated in the group treated with CXCL10 antibodies ($P < 0.001$). Furthermore, IFN-γ was reduced, but IL-10 was not affected by CXCL10 antibodies. In addition, TGF-β was obviously increased in the CXCL10Ab group. The relative mRNA expression of these inflammatory factors in re-allograft tissues was also assessed by RT-qPCR (Fig. 5B). The mRNA expression of IL-2 and IFN-γ was significantly downregulated by

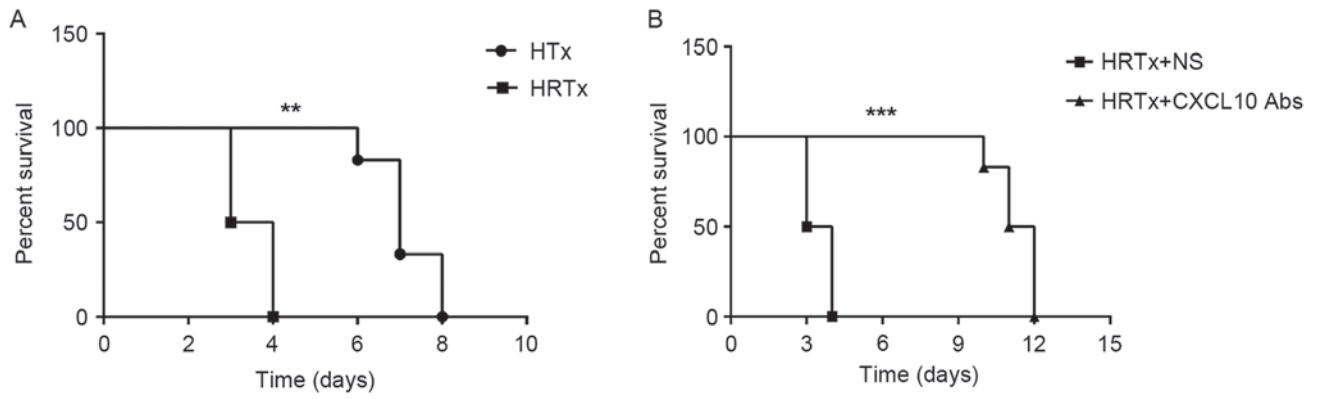


Figure 1. Kaplan-Meier survival curves of Brown Norway rats receiving donor hearts from Lewis rats, with secondary transplantation at 40 days after primary transplantation (n=6 in each group). (A) The mean survival time in the HRTx group was 3.5 days and that in the HTx group was 7.16 days (**P<0.01). (B) Rats subjected to re-transplantation were divided into two groups according to treatment with or without anti-CXCL10 antibodies. The mean survival time in the HRTx+NS group was 3.5 days, and that in the HRTx+CXCL10 Abs group was 11.33 days (***P<0.001). HTx, primary transplantation; HRTx, re-transplantation; HRTx+CXCL10 Abs, re-transplantation group treated with antibodies to chemokine (C-X-C motif) ligand; HRTx+NS, re-transplantation group with normal saline treatment.

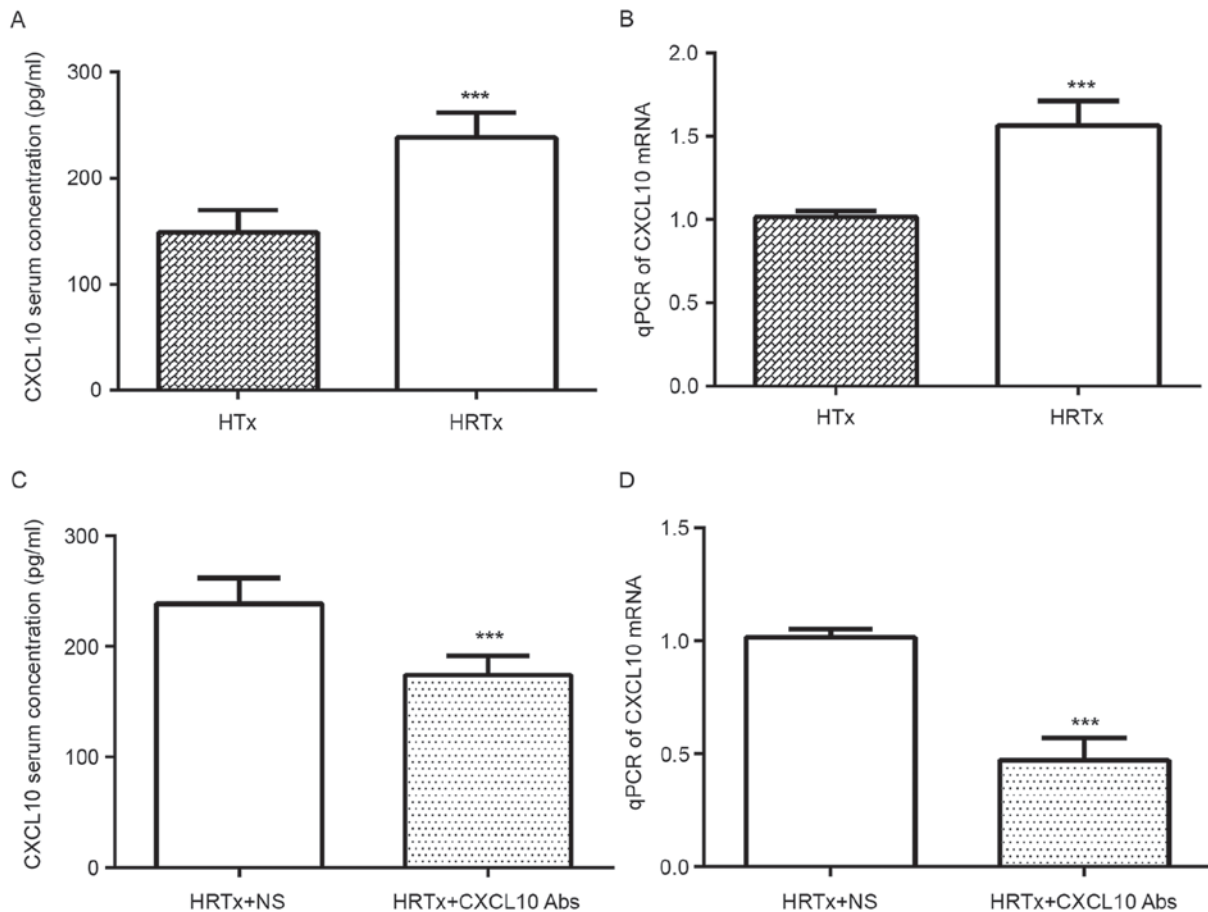


Figure 2. Expression of CXCL10 in HTx, HRTx, HRTx+NS and HRTx+CXCL10 Abs groups. (A) Serum levels of CXCL10 and (B) mRNA expression of CXCL10 in the graft of the HTx group were higher than those in the HRTx group. The mRNA expression of CXCL10 in the graft of the HRTx group was 1.5-fold increased (P<0.001). (C) Serum levels of CXCL10 and (D) mRNA expression of CXCL10 in the graft of the HRTx+CXCL10 Abs group was significantly declined compared with that in the HRTx+NS group. ***P<0.001 vs. HTx or HRTx+NS group. HTx, primary transplantation; HRTx, re-transplantation; HRTx+CXCL10 Abs, re-transplantation group treated with antibodies to CXCL10; HRTx+NS, re-transplantation group with normal saline treatment; CXCL10, chemokine (C-X-C motif) ligand.

treatment with CXCL10 antibodies (P<0.001). By contrast, TGF- β expression was significantly elevated by one fold in the antibody-treated group (P<0.001), while IL-10 was not significantly affected (P>0.05).

Anti-CXCL10 antibodies reduce CD8⁺ Tm cells in splenocytes. In order to further test whether anti-CXCL10 antibodies affect Tm cells *in vivo*, spleens were obtained from recipients at 4 days post-re-transplantation and analyzed by flow cytometry. As

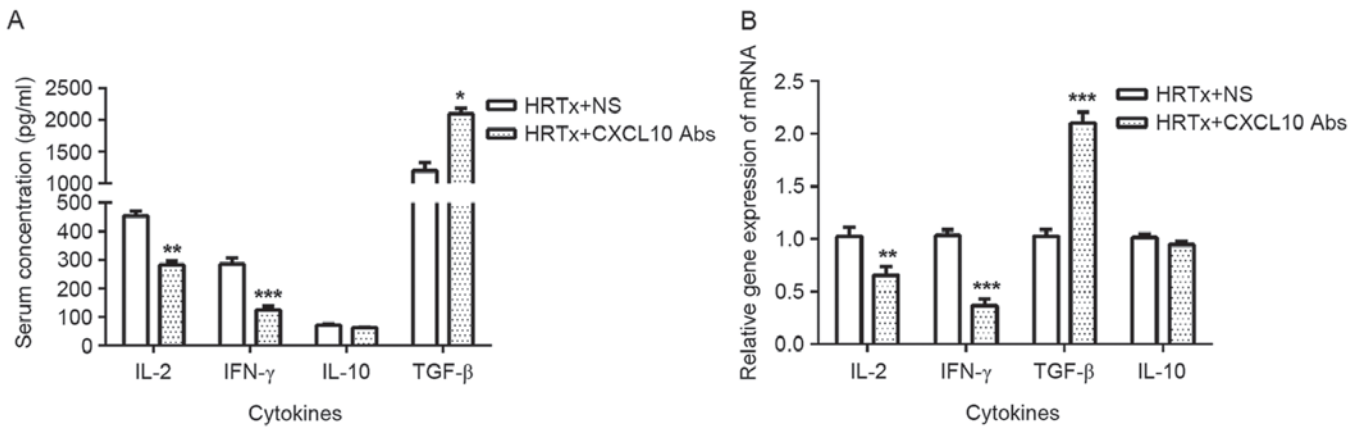


Figure 5. CXCL10 antibodies regulate rejection-associated cytokine levels. Allograft and serum was collected at 4 days following re-transplantation to evaluate relative cytokines that imply a rejection episode. (A) IL-2, IFN- γ , IL-10 and TGF- β in serum of recipients. (B) mRNA expression of IL-2, IFN- γ , IL-10 and TGF- β in allografts determined by reverse-transcription quantitative polymerase chain reaction. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$ vs. HRTx+NS group. HRTx+CXCL10 Abs, re-transplantation group treated with antibodies to CXCL10; HRTx+NS, re-transplantation group with normal saline treatment; IL, interleukin; IFN, interferon; TGF, transforming growth factor; CXCL10, chemokine (C-X-C motif) ligand.

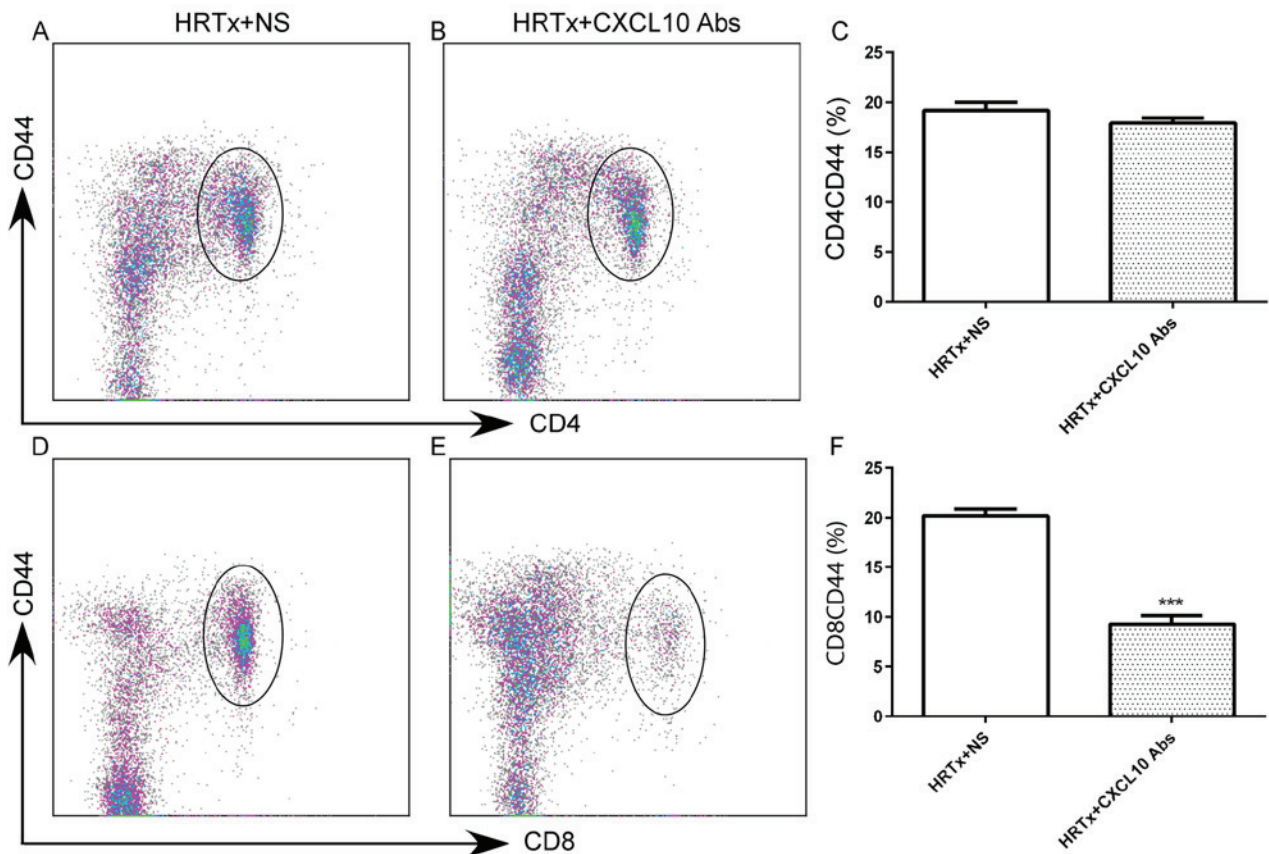


Figure 6. Spleens were obtained from recipients at 4 days post-re-transplantation and analyzed by flow cytometry. (A and B) Dot plots for the quantification of CD4⁺ Tm cells in (A) the HRTx+NS and (B) the HRTx+CXCL10 Abs group. (C) Percentages of CD4⁺ Tm cells in the two groups. (D and E) Dot plots for the quantification of CD8⁺ Tm cells in (D) the HRTx+NS and (E) the HRTx+CXCL10 Abs group. (F) Percentages of CD8⁺ Tm cells in the two groups. *** $P < 0.001$ vs. HRTx+NS. HRTx+CXCL10 Abs, re-transplantation group treated with antibodies to chemokine (C-X-C motif) ligand; HRTx+NS, re-transplantation group with normal saline treatment; Tm cell, T memory cell.

and CXCL11 (19). CXCL10, a factor determined to have the highest predictive value for early cardiac acute rejection (20), was initially reported to be induced by IFN- γ and secreted by various cell types, including monocytes, neutrophils, endothelial cells, keratinocytes and fibroblasts (21,22). After the first transplantation, CXCL10 was released into the

circulation and recognized by CXCR3, which was expressed on the surface of Th1 cells. This interaction of CXCL10 and CXCR3 augmented mononuclear cells producing IFN- γ . This cytokine strongly stimulated lymphocyte cell proliferation and induced Th1 cells to produce CXCL10. Therefore, CXCL10 and IFN- γ -producing Th1 cells formed a self-promoting loop,

which initiated and amplified the response to the allograft. The present study investigated whether CXCL10 facilitates acute accelerated rejection and examined the possible mechanism in a novel rat model.

The present study adopted a novel transplant model to explore this topic. It was observed that the graft mean survival time after re-transplantation was significantly shortened when compared with that after primary transplantation, and that the ISHLT scores were higher, as determined through histological examination with H&E staining. The serum concentrations and mRNA expression in graft tissues of CXCL10 were also determined. In re-transplant recipients, the levels of CXCL10 had increased in the peripheral blood and allograft compared with those in the primary transplant model. In parallel with the increase in CXCL10, the proportion of CD8⁺ Tm cells in splenocytes was also elevated. These results provided evidence that accelerated rejection mediated by CD8⁺ Tm cells is a crucial factor in re-transplantation (23-25). Various studies have described the generation and function of CD4⁺ and CD8⁺ Tm cells (5,26-28). Tm cells may be divided into Tcm and Tem according to CD44 and CD62L expressed on the cell surface. Tcm, whose phenotype is CD44⁺CD62L⁺, refers to Tm cells with high proliferation potential, existing in the circulation and lymphoid organs, while Tem, whose phenotype is CD44⁺CD62L⁻, refers to Tm cells that display effector functions so that they provide rapid protection after antigen reencounter (29,30) as well as trafficking between spleen and non-lymphoid tissue. By definition, this increased propensity to activation, coupled with a shortened lag time to enter the cell cycle and acquire effector functions, allows Tm cells to quickly and robustly exert an effective recall response (31).

Various studies have indicated that Tm cells confer resistance to traditional immunosuppression, are difficult to eliminate by depletion therapies and have limited dependence on co-stimulatory blockage strategies (32,33). Hence, the present study adopted a strategy of applying anti-CXCL10 antibodies alone. The results indicated that neutralization of CXCL10 prolonged re-allograft mean survival time, reduced the proportion of CD8⁺ Tm cells and mitigated inflammatory cell infiltration. In addition, Tm cells also produce effector cytokines *in situ* to recruit additional immune cells that mediate early graft tissue damage (34,35); for instance, IL-2 secreted by tissue-residing Tm cells and IFN- γ secreted by effector Tm cells. In the present study, the serum and tissue expression levels of rejection-associated cytokines were assessed. IL-2 and IFN- γ are indicative of the degree of rejection, while IL-10 and TGF- β represent tolerance. IL-2 was decreased in the CXCL10 antibody-treated group in the serum concentration as well as at the mRNA level in the graft tissue. IFN- γ declined in the CXCL10 antibodies group, indicating that inhibition of CXCL10 reduces allograft rejection-associated cytokines by inhibiting effector T cells and Tm production. Statistical analysis of the tolerance-associated cytokines demonstrated that TGF- β mRNA was significantly upregulated in the CXCL10 antibody-treated group, while the serum concentration of TGF- β was not significantly affected. Compared with the HRTx+NS group, IL-10 in the serum and allograft tissues exhibited no marked variation in the antibody-treated group. From the above results, it may be deduced that the strategy to block CXCL10 is likely to be successful in prolonging survival

time, regulating rejection-associated cytokines and reducing the proportion of Tcm and Tem *in vivo*.

Although the accurate proportion of CXCR3 expression in Tcm or Tem has not been assessed in the present study, evidence suggested that CXCR3, expressed by CD4⁺ and CD8⁺ Tm cells, is a reliable marker for Tm cell responses (36,37). The above results suggested that CXCL10 exerted a crucial role in accelerating CD8⁺ Tm cell-mediated rejection of cardiac re-transplants.

As is known, there are direct and indirect paths for CD4⁺ and CD8⁺ T cells to mediate rejection, which lead to graft damage after the first organ transplantation, but Tm cells have a major role in re-transplantation. In the present study, CXCL10 antibodies prolonged graft survival time, at least in part through inhibiting Tm activation and reducing the expression and secretion of effector cytokines. Aside from its chemoattractant properties, additional pathways and mechanisms should be further investigated in order to elucidate the roles of CXCL10 to ultimately achieve the goal of attenuating or even eliminating accelerated rejection in re-transplantation.

In conclusion, re-transplantation still poses a marked limitation on survival time. It is clear that accelerated rejection mediated by Tm cells is the hallmark of re-transplantation. Additional fundamental studies regarding CXCL10 and other chemokines are required to be launched in order to facilitate experimental and clinical trials pursuing a strategy for reducing rejection in re-transplantation. In this light, the present study provided novel insight in inhibiting Tm cells to achieve immune tolerance of second cardiac transplants.

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