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Retraction

At the request of the authors and in agreement with the Editor-in-Chief and Wiley-Blackwell, the following article from *Transplant International*, 'Chronic cardiac allograft rejection in mice is alleviated by inhibition of CCR5 in combination with cyclosporine A' by Jun Li, Kailun Zhang and Jiahong Xia, published online on 27 October 2008 in Wiley InterScience (http://www.interscience.wiley.com), has been retracted. The retraction has been requested and agreed due to unintentional errors in the analysis of the data presented.

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ORIGINAL ARTICLE

Chronic cardiac allograft rejection in mice is alleviated by inhibition of CCR5 in combination with cyclosporine A

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Keywords
anti-CCR5 mAb, cardiac transplantation, chronic rejection, cyclosporine A, immune system, pathology.

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Summary

The chemokine receptor CCR5 plays important roles in acute allograft rejection. In this study, we examined the inhibition of CCR5 in combination with the treatment with cyclosporine A (CsA) in chronic rejection in cardiac transplantation. Forty-five transplant recipients were randomized to three groups. Recipients in group A were treated with anti-CCR5 mAb and CsA, mice in group B were given anti-CCR5 mAb alone, and animals in group C were administered with only CsA. On day 45 after transplantation, the allografts were harvested and examined by immunohistologic technique and PT-PCR methods. Allografts treated with anti-CCR5 mAb and CsA showed significantly prolonged survival (44.73 ± 0.258 days, $P < 0.01$) as compared with CsA-treated group (37.00 ± 2.04 days). Treatment with anti-CCR5 mAb plus CsA significantly inhibited the progression of cardiac allograft vasculopathy. Our findings demonstrated that anti-CCR5 mAb in combination with CsA can prolong the survival of allograft through their cardio-protective and immunomodulative properties. Thus, combined administration of anti-CCR5 mAb and CsA may become a new therapeutic approach for the prevention of cardiac graft failure that has not been obviated by conventional immunosuppressive agents.

Introduction

Cardiac transplantation is the last resort for patients with end-stage heart failure. Short-term patient survival of acute rejection has been substantially improved over the past years thanks to better immunosuppressive management, but long-term survival has not been dramatically raised so far. The predominant obstacle has been cardiac allograft vasculopathy (CAV) [1], which refers to a concentric thickening of the blood vessel wall caused by proliferation of smooth muscle cells (SMCs) in the intima (neointima) of the coronary arteries. Hence, research effort has been directed at exploring strategies that can overcome the shortcoming of conventional immunosuppressive to effectively inhibit the development of CAV.

Chemokines are small cytokines that mediate cell chemotaxis and activation [2,3]. Many studies with animal models demonstrated that the graft survival was prolonged in recipients with knockout of genes encoding specific chemokines or receptors, or in recipients treated with antibodies against these molecules [4–7]. But reports about their effect in chronic rejection have been scanty. Increasing evidence suggested that specific chemokines play significant roles in the recruitment of mononuclear cells during chronic rejection [8,9] and chemokines form a complex network, which is involved in vascular remodeling [10]. Moreover, CCR5 also plays significant parts in the development of CAV [11–13]. Until now, the researchers have not agreed about the roles of T- and B-lymphocyte pathways in chronic vascular injury. Some studies indicated that CAV mainly involved T lymphocytes [14–16] and others demonstrated an association of humoral immunity with CAV [17,18]. On the basis of these studies, we were led to hypothesize that anti-CCR5 mAb in combination with CsA might regulate chronic rejection in cardiac transplantation. In this study, we administered anti-CCR5 mAb and CsA into model mice to see whether they could significantly prolong the survival of cardiac allografts, and inhibit the progression of CAV.

Materials and methods

Animals

Adult male BALB/c (H2d) mice, and C57Bl/10 (H2b) mice aged 6 and 8 weeks were from the Center of Experimental Animals, Tongji Medical College of HUST, China. Effort was made to make sure the investigation conformed to the guidelines for the handling of experimental animals formulated by the Research Committees of HUST University, Wuhan, China.

Antibodies

Biotinylated and PE-conjugated rat anti-mouse CCR5 mAb, rat anti-mouse CD3 mAb, rat anti-mouse interleukin (IL)-2 mAb, rat anti-mouse IL-10 mAb were purchased from Mebtech Corporation (Stockholm, Sweden). Anti-α-smooth muscle actin (α-SMA) antibody (E2464) was bought from Spring Bioscience (Fremont, USA). Anti-nonmuscle myosin heavy-chain B (MHC-B) antibody (PRB-445P) was product of Covance Research (Virginia, USA). Rat anti-mouse CD4, CD8, MOMA-2, CD68 were procured from Pharmingen and Serotec USA (San Diego, USA). Rat anti-mouse C4 mAb was from Novus Biologicals (Colorado, USA).

Heterotopic cardiac transplant

Donor hearts were heterotopically transplanted into recipient mice [19]. The mice were anesthetized by a single intraperitoneal injection of ketamine/xylazine (100:10 μg/kg). BALB/c hearts were transplanted into C57 recipients as allografts, and C57 hearts were transplanted into the same strain as isografts. The strength and quality of cardiac impulses were graded by palpation on daily basis, as previously described [20]. For each graft, rejection of cardiac grafts was considered to be complete by the cessation of impulse and was confirmed visually for each graft by laparotomy.

Post-transplant therapies

Allograft recipients of group A were treated with rat anti-mCCR5 mAb, at 1 μg/g/day, i.p. [21] and cyclosporine A (CsA) (Sigma, St Louis, MO, USA), dissolved in olive oil and administered daily (10 mg/kg i.p.), for 14 days post-transplant. Allograft recipients of the group B were treated with rat anti-mouse CCR5 mAb, at 1 μg/g/day, i.p. for 14 days post-transplant. Group C were treated with CsA daily (10 mg/kg i.p.) for 14 days post-transplant. The donor hearts were harvested on day 45 after transplantation (n = 14, in group treated with anti-CCR5 mAb plus CsA; n = 9, in anti-CCR5 mAb-treated group; n = 6, in CsA-treated group).

Histopathologic and immunohistochemical study

Frozen sections (4 μm) of cardiac allografts obtained 45 days post-transplant were immunostained for CD4, CD8, MOMA-2, α-SMA and MHC-B. For evaluation of cell infiltration, five fields (1.6 × 10−1 mm2 for each field) were randomly selected from one section, and the number of nuclei in each field was counted. Paraffin-embedded sections were stained with hematoxylin–eosin, elastica-van Gieson's and Masson's trichrome (MT) stains for regular histopathologic analysis. To evaluate CAV, three different sections were observed and only vessels exceeding 80 μm in diameter were included [22], the area encompassed by the lumen and internal elastic lamina was analyzed with computer-based software (Optimas, Houston, USA); The luminal occlusion rate was calculated by the following formula: Luminal occlusion rate = (internal elastic lamina area − luminal area)/internal elastic lamina area. Data were analyzed for the severity of CAV (Table 1), as recently described in detail [23,24]. CAV was defined as grade 1 or greater, significant CAV was defined as grade 3 changes. To evaluate the proportion of interstitial fibrosis, the whole area of the coronal section of the upper ventricle and the area of fibrosis (green area with MT staining) of the same section in each specimen were measured, and then the fibrosis rate was calculated. In addition, C4d deposition was localized by immunoperoxidase staining by using affinity-purified polyclonal rat antibody to mouse C4d [25,26], and macrophages were visualized by immunoperoxidase staining for CD68.

ELISPOT assay

Priming of alloantigen-specific T cells from heart allograft recipients was investigated by enumerating IL-2- and IL-10-producing T cells by using ELISPOT assay, as previously described [27,28]. Briefly, ELISPOT plates (Unifilter 350; Polyfiltronics) were coated with 2 μg/ml IL-2 or IL-10-specific mAb and incubated overnight at 4 °C. The plates were blocked with 1% bovine serum albumin/phosphate-buffered saline (PBS) and then washed four times

Table 1. Histologic grading of severity of transplant vasculopathy.

Grade	Vasculopathy
0	Vessel unaffected
1	Accumulation of inflammatory cells along intimal surfaces but with <10% occlusion of the lumen
2	More advanced 1esion including definite intimal proliferation and thickening with <50% occlusion of the lumen
3	High-grade occlusion of the vessel with >50% occlusion of its lumen

with PBS. Spleen cell suspensions from graft recipients were prepared on day 45 post-transplant and used as responder cells. Spleen cells from BALB/c, C57Bl/6, and DBA mice were prepared and treated with mitomycin C to be used as stimulator cells in the assay, as described above. Responder and stimulator cells (1:2) were cultured in serum-free HL-1 medium (BioWhittaker, Basel, Switzerland) supplemented with 1 mM L-glutamine. After 24 h of cell culture at 37 °C in 5% CO_2, cells were removed from the plate by extensive washing with PBS. Biotinylated anti-IL-2mAb (2 μg/ml) or anti-IL-10 (4 μg/ml) was added, and the plate was incubated for 6 h at room temperature. The plate was washed three times with PBS/0.05% Tween 20, and streptavidin-conjugated alkaline phosphatase was added to each well. After 2 h at room temperature, the plates were washed with PBS, and NBT-5-bromo-4-cloro-3-indolyl substrate (Kirkegaard & Perry Laboratories, Maryland, USA) was added for the detection of IL-2- or IL-10-producing cells. The resulting spots were counted with an ImmunoSpot Series I analyzer (Cellular Technology, Cleveland, USA) that was designed to detect ELISPOT spots with predetermined criteria for spot size, shape, and colorimetric density.

RT-PCR

Relative levels of mRNAs for the genes of interest were assessed by real-time reverse transcription-polymerase chain reaction (RT-PCR) with use of the ABI Prism 5700 system (PE Applied Biosystems, Inc, Beijing, China). CCR5: upward: 5-CTGAAGAGCGTGACTGAT-3, downward: 5-AGGACAATGTTGTAGGGA-3. IL-2: upward: 5-CACCCTTGCTATCACTCCT-3, downward: 5-TCTCCTC AGAAAGTCCACCA-3. IL-10: upward: 5-CTACCAAAGC CACAAAGCAG-3, downward: 5-CATGGCCTTGTAGA CACCTT-3. The expression levels of each targeted gene were normalized by subtracting the corresponding glyceraldehyde 3-phosphate dehydrogenase threshold cycle (C_T) values by using the $\Delta\Delta C_T$ comparative method [29].

Statistics

Kaplan–Meier curve was used to estimate graft-survival time, other results were analyzed by ANOVA followed by Bonferroni correction. A value of $P < 0.05$ was considered to be statistically significant. Data were expressed as mean ± SD.

Results

Effect of anti-CCR5 mAb plus CsA on cardiac allograft survival

The survival of allografts treated with both anti-CCR5 mAb and CsA was significantly prolonged (anti-CCR5 mAb combining CsA treated, 44.73 ± 0.258 days; anti-CCR5 mAb treated, 41.07 ± 1.53 days; CsA treated, 37.00 ± 2.04 days). 14 allografts treated with anti-CCR5 mAb plus CsA, nine allografts treated with anti-CCR5 mAb and six allografts treated with CsA showed prolonged survival longer than 45 days (Fig. 1).

The inhibitory effect of anti-CCR5 mAb plus CsA on the expression of CCR5

To evaluate whether CCR5 expression was regulated after transplantation, the levels of CCR5 in the allografts were measured by immunohistochemistry and RT-PCR. In allografts treated with both anti-CCR5 mAb and CsA, CCR5 expression was much weaker as compared with the anti-CCR5 mAb-treated allografts and CsA-treated allografts. CsA-treated allografts have the highest expression of CCR5 (Fig. 2).

Effects of anti-CCR5 mAb plus CsA on allografts in the chronic phase

In allografts receiving CsA alone, 24% (34 of 145 arteries) of the intramyocardial and epicardial arteries were significantly occluded by thickened intima and only 36% (75 of 210 arteries) had grade-2 lesions in the allografts that received anti-CCR5 mAb. In contrast, 36% (86 of 238 arteries) were intact and 42% (101 of 238 arteries) had

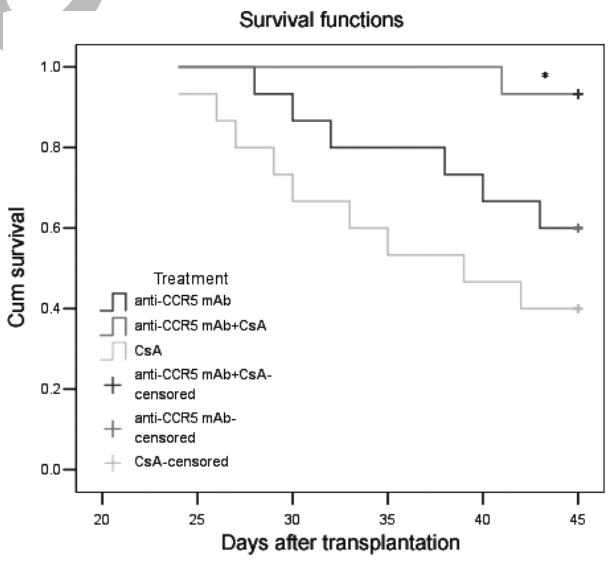

Figure 1 Effects of anti-CCR5 mAb plus CsA on graft survival. Recipients in group A were treated with anti-CCR5 mAb plus CsA, mice in group B were given anti-CCR5 mAb alone and only CsA were administrated in group C. $n = 15$ in each group. Recipient treatment with both anti-CCR5 mAb and CsA significantly prolonged heart allograft survival versus CsA treatment $^*P < 0.01$ vs CsA.

grade-1 lesions in allografts treated with both anti-CCR5 mAb and CsA. The overall incidence of intimal thickening of arteries in allografts administrated with anti-CCR5 mAb in combination with CsA was significantly lower than in those from CsA-treated and anti-CCR5 mAb-treated groups. Similarly, the severity of intimal thickening of arteries was significantly lower than the other two groups (Table 2 and Fig. 3).

Immunohistochemically, α-SMA was expressed in the thickened intima and medial layer of the allografts treated with CsA alone. And MHC-B, which reflects phenotypic change of vascular SMCs, was also expressed in the thickened intima and medial layer of allografts treated with CsA alone (Fig. 3c2 and c3). In contrast, intima was almost negative for α-SMA after the treatment with anti-CCR5 mAb regardless of CsA administration (Fig. 3a2 and b2). Moreover, MHC-B expression was almost negative in both the intima and media in anti-CCR5 mAb-treated allografts (Fig. 3a3 and b3). In allografts treated with CsA alone, three out of six allografts showed severe fibrosis in both the epicardium and intramyocardium, without a reduction in contractility, and the others exhibited mild fibrosis (Fig. 3c4 and 3.3). In the long-surviving allografts treated with

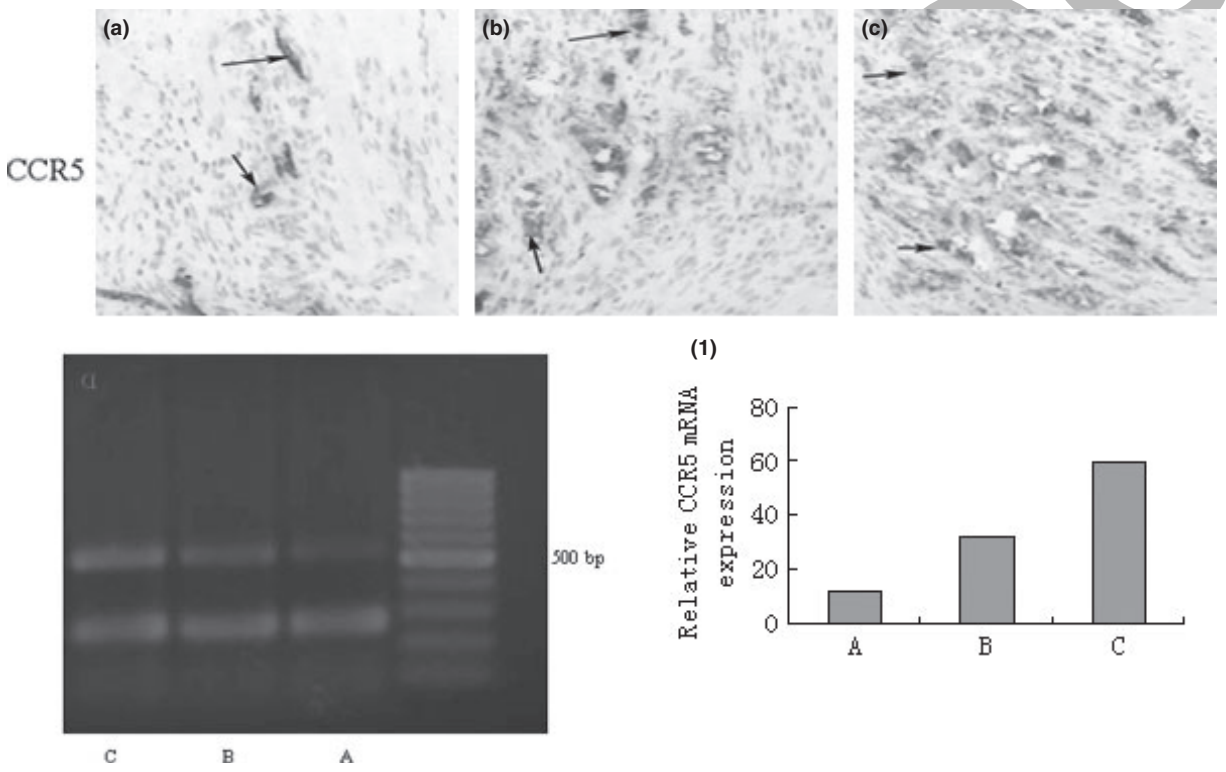

Figure 2 Effect of anti-CCR5 mAb plus CsA on expression of CCR5. (a–c) CCR5 immunostaining (×200); α: RT-PCR for CCR5. Figure 2.1 Relative level of CCR5 mRNA. Significant reduction in level of CCR5 mRNA is observed in allografts treated with anti-CCR5 mAb plus CsA versus CsA-treated allografts ($P < 0.01$). A indicates anti-CCR5 mAb plus CsA-treated allografts; B, allografts that received anti-CCR5 mAb alone; C, allografts that received CsA alone. All other abbreviations are defined in text.

Table 2. Grading of transplant vasculopathy.

	Group A (n = 14)	Group B (n = 9)	Group C (n = 6)	P-value (group A versus group B)	P-value (group A versus group C)
Vessels graded, n	238	210	145		
CAV vessels ± SD, %	60 ± 13	78 ± 11	87 ± 8	<0.05	<0.01
Mean CAVgrade ± SD	0.85 ± 0.74	1.25 ± 0.83	175 ± 093	<0.05	<0.01
CAV grade 0,1 2, 3, % (n)	36 (86), 42 (101), 21 (51), 0 (0).	20 (43), 39 (82), 36 (75), 5 (10)	11 (16), 26 (38), 39 (57), 24 (34)		

CAV, cardiac allograft vasculopathy.

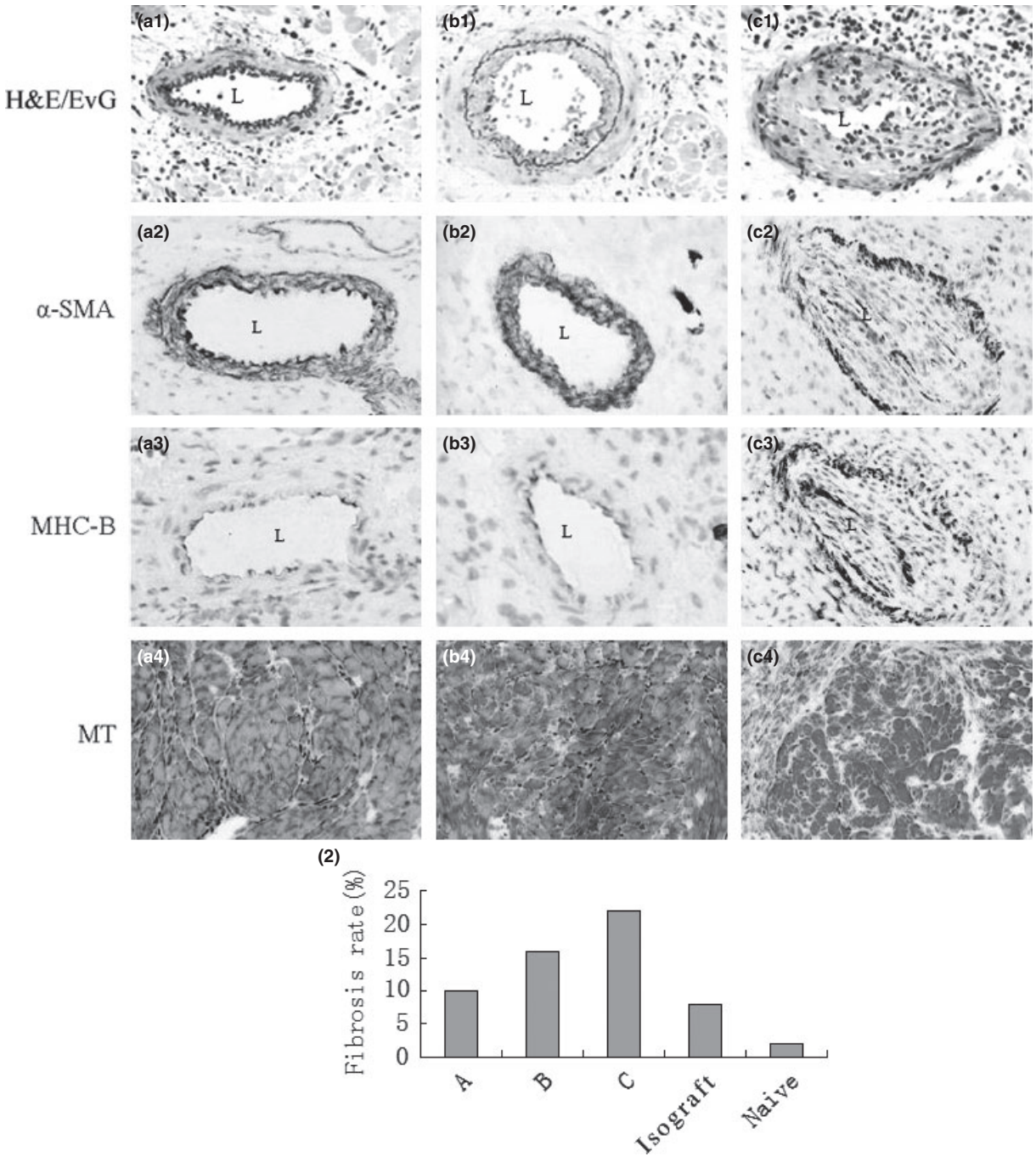

Figure 3 Representative sections of donor hearts from recipients harvested on day 45 after transplantation. (a1–c1) HE/Elastica-van Gieson's (EvG) staining (×100); (a2–c2) α-SMA immunostaining (×100); (a3–c3) MHC-B immunostaining (×100); (a4–c4) MT staining (×100). Figure 3.2: Fibrosis rate of allograft. Significant decrease in fibrosis rate is observed in allografts treated with anti-CCR5 mAb plus CsA versus CsA-treated allografts ($P < 0.05$). All other abbreviations are defined in text.

anti-CCR5 mAb plus CsA, interstitial fibrosis was significantly milder than that in the allografts treated with CsA (Fig. 3a4).

In addition, immunohistochemical staining confirmed that treatment with anti-CCR5 mAb significantly decreased the recruitment of CD4+, CD8+ T lymphocytes and MOMA-2+ cells (Fig. 4). In addition, C4d deposits and macrophage infiltrates were decreased in allografts from both the recipients treated with anti-CCR5 mAb plus CsA and the recipients with anti-CCR5 mAb alone.

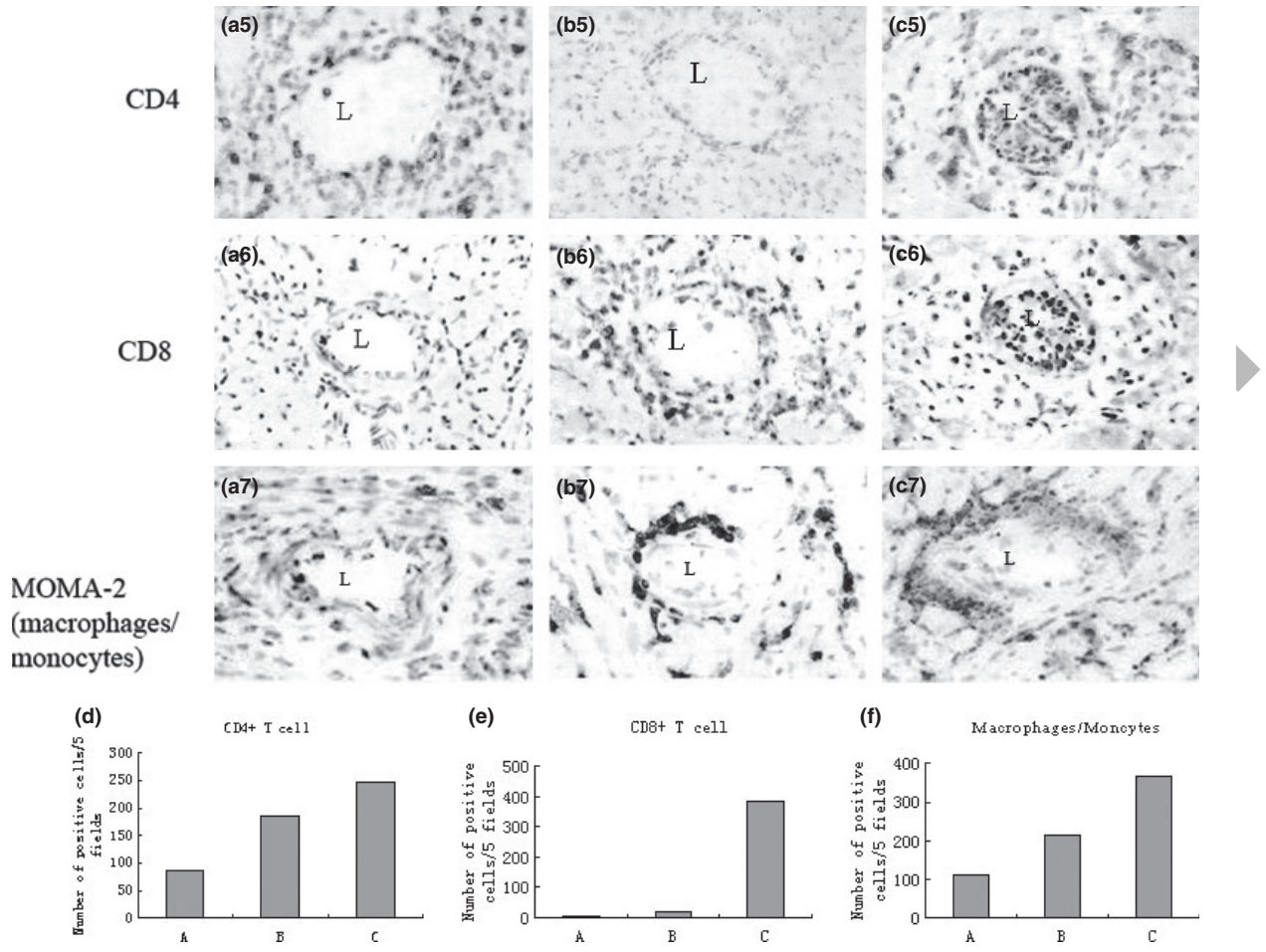

Figure 4 Representative sections of donor hearts from recipients harvested on day 45 after transplantation. (a5–c5) CD4 immunostaining (×100); (a6–c6) CD8 immunostaining (×100); (a7–c7) MOMA2+ immunostaining (×100); d: Number of CD4+ T cells; e: Number of CD8+ T cells; f: Number of macrophage/monocytes; Decrease of CD4+ T cells,CD8+ T cells and macrophage/monocytes are observed in allografts treated with anti-CCR5 mAb plus CsA versus CsA-treated allografts ($P < 0.01$). All other abbreviations are as defined in text.

In contrast, heart allografts from CsA-treated recipients displayed intense staining for C4d and CD68 (Fig. 5a8–c9).

Effect of anti-CCR5 mAb plus CsA on cytokine expression

Expression of IL-2 mRNA was significantly higher in the allografts than in the isografts, regardless of the treatment with anti-CCR5 mAb (Fig. 6a). The expression of IL-10 mRNA was significantly higher in both the anti-CCR5 mAb- treated allografts and allografts treated with both CsA and anti-CCR5 mAb as compared with the isografts, whereas the allografts that had been treated with CsA alone had lower expression than their anti-CCR5 mAb-treated counterparts (Fig. 6b). And the results were further confirmed by ELISPOT (Fig. 7).

Discussion

In the present study, we demonstrated that the administration of anti-CCR5 mAb in combination with CsA significantly prolonged survival in cardiac allografts in the murine model and hindered the progression of CAV and interstitial fibrosis.

Our results showed that CCR5 expression was down-regulated in the myocardium of cardiac allografts after the administration of anti-CCR5 mAb, independent of the treatment with CsA. The study indicated that anti-CCR5 mAb exerted a cardio-protective effect on the heart transplant and CsA worked synergistically with anti-CCR5 mAb.

The immune reaction after transplantation can be successfully suppressed by the continuous administration of calcineurin inhibitors [30,31]. These agents, however, can

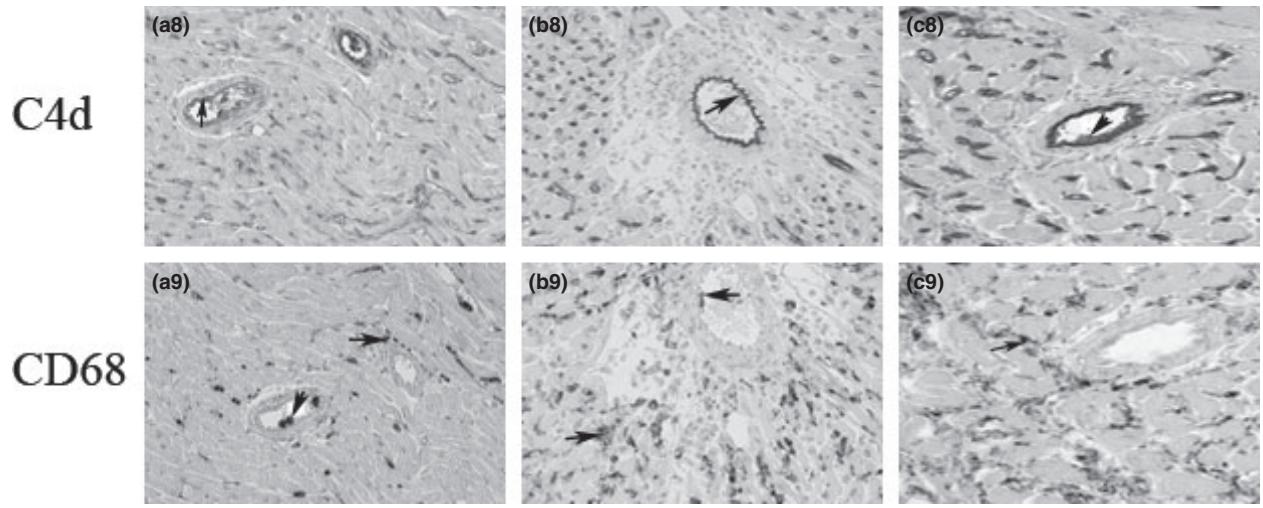

Figure 5 Representative sections of donor hearts from recipients harvested on day 45 after transplantation. (a8–c8) C4d immunostaining (×200); (a9–c9) CD68 immunostaining (×200). All other abbreviations are as defined in text.

not inhibit the development of vasculopathy and remodeling in the allografts. Although the pathogenesis of CAV development is not fully understood, previous studies suggested that CAV was primarily an immunity-mediated disease and IL-2 was involved in the triggering of the pathologic changes associated with CAV [32]. Therefore, suppressed cardiac expression of IL-2 by anti-CCR5 mAb seems to be a principal mechanism responsible for its preventive effect on the CAV. On the other hand, regulatory T (T_{Reg}) cells play a role in the immune responses of allograft rejection, and IL-10 modulates allograft rejection by regulating T_{Reg}-cell function [33,34]. So the increased expression of IL-10 may also contribute to the prevention of CAV. In addition, it is noteworthy that anti-CCR5 mAb facilitated re-endothelialization and inhibited neointimal formation in models of balloon injury. Together with the decreased expression of CCR5 in the chronic phase of cardiac transplantation in anti-CCR5 mAb-treated mice, the effects of anti-CCR5 mAb on vascular endothelial cells and the subsequent inhibition of neointimal formation by anti-CCR5 mAb may decrease the possibility of CAV development. Moreover, anti-CCR5 mAb used in combination with CsA strongly suppressed interstitial fibrosis.

Humoral rejection in heart allografts has been seen as an important clinicopathologic entity that is associated with accelerated graft arteriopathy, graft loss, and, in the case of cardiac allografts, death [35–38]. So far, no generally-accepted diagnostic criteria for humoral rejection are available. Nevertheless, in both renal and cardiac allografts, deposition of the complement fragment C4d in the microvasculature has been shown to be a marker of antibody-mediated graft injury and it was associated with poorer graft function or graft loss, independent of the presence of cellular rejection [39–42]. Other studies have looked at the correlation of C4d in tissue with circulating anti-donor reactive antibody [43,44] . What is more, some researches indicated that macrophages were the principal cells involved in humoral rejection-related myocyte injury and they were associated with C4d staining [45,46]. Accordingly, the decreased C4d deposits and CD68+ macrophage infiltrates in our experiment suggested that humoral rejection could be inhibited by anti-CCR5 mAb used in combination with CsA. Our results confirmed earlier findings that treatment with CCR5 antagonist plus CsA delayed alloantibody production, suppressed CAV, and thereby prolonged graft survival in primates [47].

Treatment with anti-CCR5 mAb plus CsA substantially reduced (by over 80%) the recruitment of CD4+ and CD8+ T lymphocytes and MOMA-2+ monocytes/macrophages to the donor heart. On the basis of previous findings that CAV in this model was a T-lymphocyte development process [14], our experiment further proved that inhibition of T-lymphocyte recruitment was involved in the mechanism by which anti-CCR5 mAb plus CsA reduced CAV. In addition, the rejection process, as characterized by C4d deposits on the arteries and capillary endothelium and infiltration of CD68+ macrophages, also suggested that antibody-mediated humoral rejection independently contribute to the reduction of CAV by CCR5 inhibitors in combination with CsA. Notably, anti-CCR5 mAb plus CsA did not completely prevent T lymphocyte/macrophage recruitment or C4d deposition or CAV development, suggesting that the interaction of other chemokines with their receptors may also be involved. It

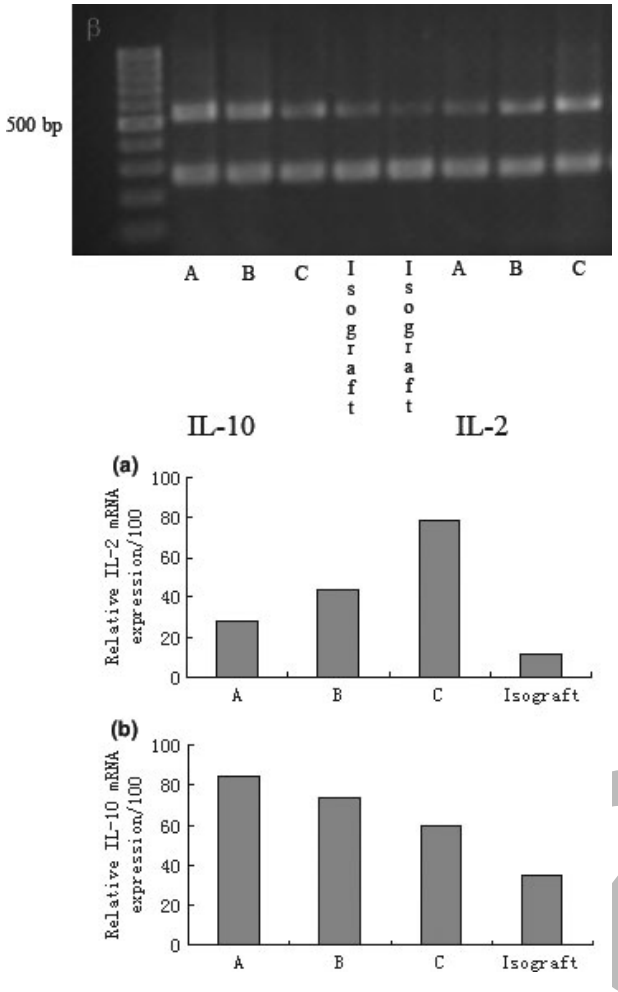

Figure 6 Effect of anti-CCR5 mAb plus CsA on cytokine mRNA expression in cardiac allografts. β: RT-PCR for IL-2 and IL-10. a: Relative level of IL-2 mRNA, significant reduction in level of CCR5 mRNA is observed in allografts treated with anti-CCR5 mAb plus CsA versus CsA-treated allografts ($P < 0.05$). b: Relative level of IL-10 mRNA, significant increase in level of CCR5 mRNA is observed in allografts treated with anti-CCR5 mAb plus CsA versus CsA-treated allografts ($P < 0.05$). All other abbreviations are defined in text.

should be noted, however, that CsA did not inhibit the development of both CAV and cardiac remodeling, even though it suppressed acute rejection, but early treatment with anti-CCR5 mAb plus CsA remarkably inhibited them. Still, the possibility that CAV might eventually develop (after 45 days) can not be ruled out, especially when the anti-CCR5 mAb plus CsA is not given on continuous basis.

In conclusion, our results provide strong evidence that anti-CCR5 used in combination with CsA plays important roles in preventing chronic rejection via its cardioprotective and immuno-regulatory effects in the murine cardiac transplantation model. In this model, anti-CCR5 plus CsA inhibited CAV by modulating cytokine expression, reducing recruitment of mononuclear cells and suppressing humoral rejection. Although further investigations are needed to fully clarify the precise molecular and cellular mechanism involved in the immuno-regulation, the administration of CCR5 blockader in combination with CsA may be of therapeutic benefit in controlling the development of chronic rejection in heart transplants.

Authorship

LJ: performed research/study, collected date, analyzed data, wrote the paper. ZK and XJ: designed research/study, reviewed the paper.

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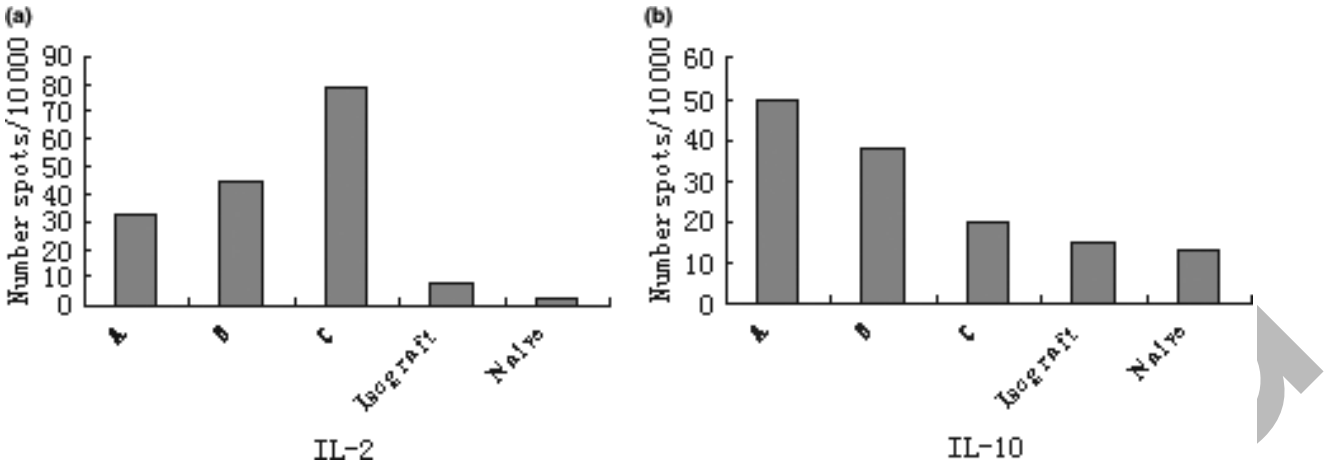

Figure 7 Frequency of IL-2 and IL-10-producing cells in the spleen of recipients of cardiac allografts. a: Number of T cells producing IL-2. Significant decrease in the number of T cells producing IL-2 is observed in allografts treated with anti-CCR5 mAb plus CsA versus CsA-treated allografts ($P < 0.05$). b: Number of T cells producing IL-10. Significant increase in the number of T cells producing IL-2 is observed in allografts treated with anti-CCR5 mAb plus CsA versus CsA-treated allografts ($P < 0.05$). All other abbreviations are defined in text.

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