Anti-CCL25 Antibody Prolongs Skin Allograft Survival by Blocking CCR9 Expression and Impairing Splenic T-Cell Function

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Abstract Chemokines, by virtue of their ability to recruit immune cells into allografts, play critical roles in acute transplantation rejection. CCR9 and its ligand, CCL25, is one of the key regulators of thymocyte migration and maturation in normal and inflammatory conditions. Moreover, several studies have revealed that high expression of CCR9 and CCL25 participated in many kinds of diseases. However, the role of CCR9 in allograft rejection is still unclear. In this study, we established a murine skin transplantation model of acute rejection. Our findings showed that the proportion of CCR9-expressing T cells was significantly increased in the spleen of allotransplanted mice compared with syngeneic transplantation. Furthermore, expression of CCL25 in allograft was similarly increased. Neutralization of CCL25 by intravenous injection of anti-CCL25 monoclonal antibody significantly prolonged skin allograft survival, decreased the number of infiltrating cells, and simultaneously suppressed the chemotactic ability and the proliferation of the splenic T cells in response to allogeneic antigens. Finally, blockade of CCL25 also diminished the secretion of IFN- γ by splenic

T cells. These studies indicated that CCR9/CCL25 was involved in acute transplantation rejection and anti-CCL25 strategies might be useful in preventing acute rejection.

Keywords CCR9 · CCL25 · Transplantation · Rejection

Introduction

Organ transplantation is a commonly used method to treat end-stage organ failure. However, acute or chronic rejection after transplantation often leads to loss of graft function (Creput et al. 2007; Hall and Dorsch 1984; Sayegh and Carpenter 2004). Immunosuppressive agents are used to suppress rejection effectively, but it often carries the burden of risk for infection, cancer, and many other secondary diseases (Ajithkumar et al. 2007; Fishman 2007; Tantravahi et al. 2007). Thus, there is a continuing need to find effective strategies to prevent acute allograft rejection.

Chemokine and its receptor play a key role in acute transplantation rejection (Fischereder and Schroppel 2009; Hancock et al. 2001; Nelson and Krensky 2001; Yopp et al. 2004). Therapeutics targeted at chemokine/chemokine receptor axis blockade as a pharmacological mechanism to prevent rejection have been shown to be effective (Liu et al. 2011). Recent studies reported that a hamster anti-mouse CXCR3 monoclonal antibody (mAb) could significantly prolong cardiac and islet allograft survival when combined with a subtherapeutic regimen of rapamycin (Uppaluri et al. 2008). In addition, combined CXCR3 and CCR5 blockade attenuated acute and chronic rejection in a robust murine model (Schnickel et al. 2008).

CCR9 is a representative receptor that is expressed both constitutively and in inflammation, which has only one

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identified ligand, thymus-expressed chemokine/CCL25 (Zaballos et al. 1999). Originally, the CCR9–CCL25 interaction was found to be involved in T cell development in the thymus (Vicari et al. 1997) and T-cell homing to the small intestine (Kunkel et al. 2000; Wurbel et al. 2000). However, accumulating evidences showed that the CCR9/CCL25 axis participated in a variety of disease processes (Johnson-Holiday et al. 2011; Nakamoto et al. 2012; Schmutz et al. 2010). In Crohn's disease patients (M et al. 2007) and in a murine model (Apostolaki et al. 2008; Rivera-Nieves et al. 2006), CCL25 was up-regulated following accumulation of CCR9⁺ CD4⁺ T and CD8⁺ T cells. Moreover, interfering with the CCR9/CCL25 axis using neutralizing antibody has been proved to be effective in the animal model. CCX282-B, an antagonist of CCR9, has also been tested in inflammatory bowel disease in clinical trials (Walters et al. 2010). However, little information is available about the role of CCR9 in allograft rejection.

In the current study, we used a murine skin transplantation model to investigate the role of CCR9 in acute allograft rejection. We found that proportion of CCR9-expressing T cells significantly increased with rejection progression, and injection of anti-CCL25 mAb prolonged skin allograft survival.

Materials and Methods

Animals

Female C57BL/6(H-2^b) or BALB/c(H-2^d) mice (6–8 weeks old) were obtained from the animal facilities at Tongji Medical College (Wuhan, China). Mice were maintained under specific pathogen-free conditions and the experiments were carried out in compliance with institutional animal care and use guidelines.

Skin Transplantation

C57BL/6(H-2^b) mice, as recipients, and BALB/c(H-2^d) mice, as donors. Full-thickness trunk skin allograft was placed using standard technique (Rosenberg 1991). Isograft transplantation was used as a control. The transplanted graft was scored visually daily and considered fully rejected when >80 % of it was necrotic.

Flow Cytometry

Splenic or peripheral lymph node (pLN) single-cell suspensions from recipient mice were prepared and co-stained with CD3 (clone 145-2C11), CD8 (clone 53-6.7), and CCR9 (clone CW-1.2; Biolegend, Inc) for 30 min at 4 °C.

Surface marker expression levels were analyzed using a FACSCalibur flow cytometer (Becton–Dickinson, San Jose, CA). Collected data were analyzed using the Cell Quest software (BD Biosciences, Mountain View, CA).

Western Blot Assays

Proteins (80 µg) extracted from skin-graft were separated by 12 % SDS-PAGE, transferred onto polyvinylidene difluoride membranes, and incubated with the CCR9 (ab1662, Abcam), CCL25 (89818, R&D Systems), or GAPDH (FL-335, Santa Cruz) antibodies, after which signals were visualized using enhanced chemiluminescence (Amersham).

Histological Evaluation of Skin Grafts

Skin grafts were either stained with H&E (hematoxylin and eosin) or immunostained. For immunohistochemistry, the primary antibodies were goat anti-mouse CCR9 antibody and rat anti-mouse CCL25 mAb. Immunohistochemistry was performed using the ABC immunoperoxidase technique. For immunofluorescence detection, frozen sections were incubated with the primary antibodies goat anti-mouse CCR9 and rabbit anti-mouse CD3 (3256-1, Epitomics, Inc., California) and secondary antibodies (FITC-conjugated anti-goat and TRITC-conjugated anti-rabbit). Then, the preparations were observed using a laser scanning confocal microscopy (FV500, Olympus Inc., Japan).

Animal Treatment with Anti-CCL25 Antibody

C57BL/6 recipients received rat anti-mouse CCL25 mAb (89818, R&D Systems; 2 µg) or rat IgG2a (bs-0293P, Beijing Biosynthesis Biotechnology Co, Ltd) isotype control every other day via tail vein. The treatments were started after the transplants were in place. All treatments lasted until animals were killed.

Chemotaxis Assay

Migration assays were performed in 24-well Transwell plates (Corning Costar, Cambridge, MA) with 5-µm pore polycarbonate filters. 100 µL of purified splenic T cells isolated from iso- or allo-transplanted C57BL/6 mice (3 × 10⁷ cells/mL) was placed in the upper chamber, and 600 µL of medium or a given dilution of recombinant mouse CCL25 (R&D Systems, Minneapolis, MN) was placed in the lower chamber. After incubation for 2 h at 37 °C, the cells in the lower chamber were collected and counted. Results were expressed as the chemotactic index (CI); that is, the ratio between the numbers of migrating cells in the sample and in the control medium.

Mixed Lymphocyte Reaction, Cell Proliferation Responses, and Cytokine Quantification

Alloantigen-specific proliferation of splenic T cells from recipient mice was tested by performing one-way mixed lymphocyte reaction (MLR). Splenic T cells (purified using nylon wool columns) isolated from recipient mice, serving as responders (2×10^6 /well), were treated with $4.5 \mu\text{M}$ CFSE dye (molecular probes). Dendritic cells (DCs) from BALB/c mice without transplantation as stimulators were treated with mitomycin C ($50 \mu\text{g/mL}$, Sigma-Aldrich), then incubated with responder T cells for 7 days. T-cell proliferation (proliferation index: PI) was assessed by serial dilution of the intracellular CFSE dye as described previously (Lyons 2000). Culture supernatants from one-way MLR were recovered at day 7 for determination of interferon (IFN)- γ , interleukin (IL)-12, IL-10, and IL-4 production using mouse IFN- γ ELISA kit (NeoBioscience Technology Co, Ltd), mouse IL-10 ELISA Max Deluxe (Biolegend, San Diego, CA), mouse IL-4 and IL-12p70 ELISA kit (eBioscience, San Diego, CA).

Statistical Analysis

For pair-wise comparisons, the groups were analyzed using a Student's *t* test, and for multiple comparisons, a one-way ANOVA with Dunnett's method was used. In both cases, $p < 0.05$ were considered to indicate statistical significance.

Results

CCR9 is Markedly Up-Regulated in Splenic T Cells from Recipient Mice after Allograft Transplantation, Primarily in CD8⁺ T Cells

The migration of T cells in secondary lymphoid organs to allografts plays an important role in transplantation rejection (Trivedi 2007). Thus, we first analyzed CCR9 expression in splenic and pLN T cells from recipient mice at different time intervals during the course of rejection. Compared with that in isogeneic recipient mice spleen, there was an obvious increased expression of CCR9 after allogeneic transplantation (Fig. 1a, b). Moreover, flow cytometry analysis showed that CCR9 was mainly expressed in CD3⁺ cells. Along with rejection after allograft transplantation, the predominant CCR9-expressing population was identified within CD8⁺ T cells, and the ratio of CD8⁺ T to CD4⁺ T in alltransplantation was almost double than that in isograft transplantation on day 7 after transplantation (Fig. 1c). Whereas there is no significant difference of the expression of CCR9 in T cells between the two groups in pLN (Fig. 1d).

Fig. 1 Analysis of the expression and distribution of CCR9 in recipient's secondary lymphoid organs and skin grafts. **a** Western blot of skin grafts obtained from isogeneic transplantation recipients or allotransplantation recipients at day 7 for protein detection of CCR9 and CCL25 (GAPDH as control). **b** Splenic T cells were isolated from iso- or allo-transplantation recipients for 1, 3, 5, or 7 days. Procedure for cell staining is described in the "Materials and Methods". The graph shows expression of CCR9 after gating within CD3⁺ splenic cells. **c** The graph shows the ratio of CD8⁺/CD4⁺ after gating within CD3⁺CCR9⁺ cells. **d** pLN T cells from iso- or allotransplantation recipients were analyzed. Assays were carried out in triplicate and a representative result is shown (* $p < 0.05$, ** $p < 0.01$)

Next, expression of CCL25 in skin grafts was examined by Western blotting at day 7 post-transplantation. Compared with isogeneic recipient mice, CCL25 was also significantly upregulated in the allograft (Fig. 1a).

In addition, we examined inflammatory cell infiltration in the skin and detected the expression of CCR9 and CCL25 using H&E and immunohistochemical staining. There were only a few lymphocytes residing in the isogeneic skin on day 7 after transplantation, while extensive immune infiltration into the allograft was observed in the allograft (Fig. 2a). Immunohistochemical staining revealed that CCR9 was highly expressed in infiltrating cells (Fig. 2a). To determine whether the cells that stained positive with anti-CCR9 antibody were T cells, immunofluorescence experiments were performed using anti-CD3 antibody and sections were analyzed by confocal microscopy. The results showed that CCR9 was highly expressed in CD3⁺ cells and the

Fig. 2 Histological analysis of skin grafts. Skin allografts were obtained from isogenic transplant recipient mice or allotransplanted mice on day 7. Paraffin sections were prepared and stained with H&E (**a**, *left*). For immunohistochemical staining, sections were stained with goat anti-mouse CCR9 (**a**, *middle*) or rat anti-mouse CCL25 mAb (**a**, *right*). Arrows indicated CCR9⁺ or CCL25⁺ cells. **b** CD3 and CCR9 colocalization. CD3 protein was detected by rabbit anti-mouse CD3 antibody (*red*), CCR9 was detected by goat anti-mouse antibody (*green*), and the nuclei were stained with DAPI (*blue*). The right bottom is an enlarged picture of the area within the square. Five different tissue sections were examined, and a representative histological result is shown. Magnification: HE $\times 100$; immunohistochemistry $\times 200$. Laser scanning confocal microscopy: $\times 400$; magnified region: $\times 800$

co-localization of CCR9 and CD3 was more significant in allogeneic transplanted mice than in isograft group (Fig. 2b). Moreover, CCL25 was markedly elevated in allograft compared with the isogenic skin graft (Fig. 2a).

Neutralization of CCL25 with Anti-CCL25 mAb Significantly Prolonged Allograft Survival

The findings mentioned above suggested that CCR9 might be involved in the progression of acute skin allograft rejection in mice. To determine whether blocking the interaction between CCR9 and CCL25 could prolong graft survival, recipient mice were injected with anti-CCL25 mAb every other day. We observed an obvious prolongation of skin allograft survival in anti-CCL25 mAb

treatment group, compared with the IgG2a treatment (Fig. 3a, b). Isografts were maintained on all recipients for longer than 20 days. The skin grafts were subjected to H&E staining at day 7 post-transplantation. Widespread inflammatory cell infiltration in IgG2a isotype control group was observed and markedly less immune cells were seen in the anti-CCL25 treated group; indeed, the latter was similar to the isograft (Fig. 3c).

Anti-CCL25 mAb Treatment Decreased the Expression of CCR9 in Allogeneic Recipient Mice

To explore the mechanism(s) underlying prolonged allograft survival time in recipient mice treated with anti-CCL25 mAb, we analyzed expression of CCR9 on splenic

Fig. 3 Blockade of CCR9 with an anti-CCL25 mAb prolonged skin allograft survival. Groups of C57BL/6 recipients accepted skin allografts from BALB/c mice, and then were injected with an anti-CCL25 mAb ($n = 10$) or rat IgG2a isotype control ($n = 10$) via the tail vein every other day from day 1 until complete rejection. Isogeneic skin transplantation was used as control ($n = 8$). Survival time of the iso- or allograft (**a** and **b**). Injection of anti-CCL25 mAb extended skin allograft survival significantly ($p = 0.03$). **c** Histological examination of skin grafts at day 7 after transplantation. Five different sections were examined, and a representative histological result is shown. Magnification: $\times 100$

T cells from mice that received different treatments at day 7 after transplantation. Interestingly, a higher proportion of CCR9⁺ T cells in splenic cells was observed in IgG2a-treated mice compared with isogeneic transplant recipient mice ($9.63 \pm 0.85\%$ vs. $5.89 \pm 1.21\%$), whereas infiltrated T cells were significantly reduced in anti-CCL25 mAb treatment group (Fig. 4a). Consistent with above results, compared with IgG2a, a large decline in the ratio of CD8⁺ to CD4⁺ cells in CCR9-expressing splenic T cell was observed in the group receiving anti-CCL25 mAb (ratio: 0.72 ± 0.36 vs. 2.12 ± 0.43) (Fig. 4b). The Western blotting results also indicated that injection of anti-CCL25 significantly decreased CCR9 protein expression in skin allografts (Fig. 4c). Our data indicated that anti-CCL25 mAb reduced the expression of CCR9 in spleen and skin allografts of recipient mice.

Anti-CCL25 mAb Suppressed the Chemotactic and Proliferative Capacity of Splenic T Cells

We then evaluated the functionality of CCR9 on CCL25 stimulation in splenic T cells using chemotaxis assays. First, CCL25 induced significant migration of recipient mice splenic T cells in a dose-dependent manner, and the optimal response was obtained at 500 ng/mL (Fig. 5a). Using this optimal concentration, we further tested the chemotactic

Fig. 4 Anti-CCL25 mAb administration decreased the expression of CCR9 in splenic T cells and skin grafts. Splenic T cells were obtained from the anti-CCL25 mAb- or control IgG2a isotype-treated group at day 7 after allotransplantation. Splenic cells from isogeneic transplantation mice were used as a control. **a** Graph shows expression of CCR9 after gating within CD3⁺ splenic cells. **b** The ratio of CD8⁺/CD4⁺ after gating within CD3⁺CCR9⁺ cells. **c** Expression of CCR9 protein in the skin allograft tissue. Assays were carried out in triplicate and a single representative experiment is shown. $*p < 0.05$

ability of splenic T cells in the different treatment groups. CCL25 induced very weak chemotactic migration in freshly isolated isogeneic transplant recipient splenic T cells (CI: 2.48 ± 1.04 ; Fig. 5b), while chemotaxis towards CCL25

Fig. 5 Migration of CD3⁺ splenic T cells towards CCL25 and T cell proliferation responses. Freshly isolated splenic T cells from injection of anti-CCL25 or IgG recipients C57BL/6 mice at day 7 after allograft transplantation were prepared. **a** Migration of splenic T cells towards recombinant mouse CCL25 (rmCCL25) at the indicated concentrations or culture medium as negative control. **b** Migration of recipient mice splenic T cells in different treatment groups towards rmCCL25 at 500 ng/mL. **c** Proliferation of splenic T cells. T cells in spleen

originating from mice in the different treatment groups were used as responder cells for the MLR assay with donor-derived DCs (DC:T ratio of 1:5). The bar graph is a summary of proliferation index data of T cells isolated from different recipients. **d** Histograms of proliferation of splenic T cells are shown. The data shown are representative of five independent experiments and each assay was repeated three times. * $p < 0.05$

was greatly increased in IgG2a isotype control and returned to isogeneic transplantation levels in the group injected with anti-CCL25 mAb (CI: 4.36 ± 0.85 vs. 3.02 ± 0.68).

Next, we examined the proliferation of splenic T cells isolated from recipient mice that received different treatments after graft transplantation. The T cells from anti-CCL25-treated mice exhibited a low proliferative response (PI: 1.25 ± 0.25) when compared with T cells isolated from IgG2a-treated mice (PI: 2.56 ± 0.29 ; Fig. 5c, d).

Th1-Type Cytokine Secretion by Splenic T Cells Was Reduced with Anti-CCL25 mAb Administration

Th1-type immune response plays a dominant role in acute rejection (Mariotti et al. 2008). Finally, we investigated the effects of the injection of the anti-CCL25 mAb on Th1 and Th2 cytokine production in splenic T cells by ELISA. The level of IFN- γ increased progressively in the isotype-treated mice but decreased markedly in the anti-CCL25 mAb-treated mice (Fig. 6). IL-12 production was also reduced after anti-CCL25 mAb administration, while no difference was observed in the secretion of IL-4 or IL-10 (Fig. 6).

Discussion

In this report, we demonstrated that the expression of CCR9 in splenic T cells was upregulated markedly after allograft transplantation. In addition, we showed that anti-CCL25

mAb delayed skin allograft rejection in mice partly through reducing infiltrated cells and inhibiting splenic T lymphocyte proliferation.

Chemokine receptors transmit signals by interaction with their corresponding ligands, and eventually affect a variety of biological processes (Neel et al. 2005). CCR9, one of the CC subset chemokine receptors, was initially reported in thymus and small intestine (Vicari et al. 1997; Wurbel et al. 2000). More recent studies have shown that CCR9 was highly expressed in other tissues besides thymus and intestine, which were involved in many kinds of disease processes, including rheumatoid arthritis, inflammatory bowel disease and hepatitis (Apostolaki et al. 2008; Nakamoto et al. 2012; Schmutz et al. 2010), while the role of CCR9 in acute transplant rejection remains unclear.

Our results revealed that there was a significant difference expression of CCR9 in splenic T cells between isogeneic and allo-transplantation while there was not obvious as the one in pLN. However, the exact mechanism responsible for this observation is not clear. Previous study in a T-cell receptor transgenic transfer model has shown that CCR9 expression was functionally maintained on CD8 $\alpha\beta$ ⁺ lymphocytes following activation in mesenteric lymph nodes but downregulated on these cells in peripheral lymph nodes, indicating that select regulation of chemokine receptors was associated with the microenvironment of secondary lymphoid tissues (Svensson et al. 2002). Similarly, different structure and microenvironment between spleen and pLN generally have a complex interplay effect

Fig. 6 Cytokine secretion by splenic T cells was examined in splenic T/DC cell supernatants. Splenic T cells from anti-CCL25-treated mice or IgG-treated mice that received allotransplantations were cultured with DCs from BALB/c mice (DC:T ratio of 1:5). The supernatants were harvested on days 1, 3, 5, and 7 and cytokine levels (IFN- γ , IL-12, IL-4, and IL-10) were quantified by ELISA. Splenic cells from isogenic transplantation mice were used as a control. The data are from the six mice examined in each study group (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$)

on the expression of chemokine/chemokine receptor. Our results may also due to an organ-specific expression of chemokine receptor.

Moreover, the findings based on flow cytometry, immunohistochemical staining and western blotting, revealed that CCR9 and CCL25 were significantly increased in recipient mice after allotransplantation. In addition, chemotaxis and proliferation of splenic T cells were significantly enhanced compared with isogenic transplantation. Taken together, our data suggested for the first time that CCR9/CCL25 might play a dominant role in acute transplant rejection in the skin.

Furthermore, neutralization of CCL25 using anti-CCL25 mAb after allotransplantation could prolong skin allograft survival and greatly reduce the infiltration of lymphocytes into allografts. Also, it showed that the proportion of CCR9⁺ splenic T cells in allotransplant recipient mice was significantly decreased, and their proliferation and IFN- γ secretion were markedly suppressed. The results mentioned above might be one possible explanation for the prolongation of skin allograft by CCL25 neutralization.

Our findings demonstrated that the proliferation of splenic T cells was obviously increased after allogeneic transplantation and then markedly decreased by neutralization of CCL25. This result was consistent with previous

research studies that demonstrated that CCR9 binding to its ligand could promote cell proliferation Johnson-Holiday et al. (2011) and up-regulate anti-apoptotic signaling (Datta et al. 1999; Sharma et al. 2010; Youn et al. 2001). However, the underlying molecular mechanism(s) remain incompletely characterized.

IFN- γ is shown to be produced mostly by splenic effector and graft-infiltrating T cells, and it is commonly used as a marker of effector priming to alloantigens (Hidalgo and Halloran 2002). Recent researches have demonstrated that CCR9⁺ T cells from inflamed small bowel have a more activated phenotype and significantly produced more IFN- γ (Papadakis et al. 2003; Saruta et al. 2007). Similar to these investigations, our experiment also found that IFN- γ was fiercely secreted by splenic T cells in allotransplant recipient mice. In addition, IFN- γ level was significantly decreased after neutralization of CCL25, and this inhibitory effect was consistent with previous research that CXCR3 antagonism could depress the generation of IFN- γ -producing CD8⁺ T cells in the spleen, prolonging cardiac allograft survival (Rosenblum et al. 2009).

Based on these results, we concluded that the inhibitory effect of the neutralizing antibody reflected its ability not only to block chemokine binding but also to depress chemotactic ability and proliferation. We also found that blockade of the CCR9/CCL25 axis could suppress cytokine secretion. Taken together, our data suggest that CCR9 plays a dominant role in acute transplant rejection process in the skin.

In summary, the present data demonstrated that interfering with CCR9/CCL25 using anti-CCL25 mAb prolonged mouse skin allograft survival, indicating the therapeutic potential of anti-CCL25 mAb in preventing acute transplant rejection. We plan to further investigate the precise molecular and cellular mechanism(s) involved in the regulation of immune response after administration of CCR9 neutralizing antibody.

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Conflict of interest All the authors who have taken part in this study declared they have nothing to disclose regarding competing interests or funding from industry with respect to this manuscript. Li Jie participated in this work, including study design, analysis and interpretation, manuscript preparation, data collection and statistical analysis. Tan Jinquan participated in the study design and manuscript preparation. Xiong Tao, Xiong Ali and Chen Jie participated in statistical analysis and manuscript preparation, Zheng Yingcheng and He Yuling participated in sample collection and processing. Xiao Ruijing, Ehtisham Altaf and Zhu Guoguo participated in manuscript revision.

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