Reduced intragraft mRNA expression of matrix metalloproteinases Mmp3, Mmp12, Mmp13 and Adam8, and diminished transplant arteriosclerosis in Ccr5-deficient mice

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Experimental and human organ transplant studies suggest an important role for chemokine (C-C-motif) receptor-5 (CCR5) in the development of acute and chronic allograft rejection. Because early transplant damage can predispose allografts to chronic dysfunction, we sought to identify potential pathophysiologic mechanisms leading to allograft damage by using wild-type and Ccr5-deficient mice as recipients of fully MHC-mismatched heart and carotid-artery allografts. Gene expression in rejecting heart allografts was analyzed 2 and 6 days after transplantation using Affymetrix GeneChips. Microarray analysis led to identification of four metalloproteinase genes [matrix metalloproteinase (Mmp)3, Mmp12, Mmp13 and a disintegrin and metalloprotease domain (Adam)8] with significantly diminished intragraft mRNA expression in Ccr5-deficient mice at day 6. Accordingly, allografts from Ccr5-deficient mice showed less tissue remodeling and hence better preservation of the myocardial architecture compared with allografts from wild-type recipients. Moreover, survival of cardiac allografts was significantly increased in Ccr5-deficient mice. Carotid artery allografts from Ccr5-deficient recipients showed better tissue preservation, and significant reduction of neointima formation and $CD3^+$ T cell infiltration. Ccr5 appears to play an important role in transplant-associated arteriosclerosis that may involve metalloproteinase-mediated vessel wall remodeling. We conclude that early tissue remodeling may be a critical feature in the predisposition of allografts to the development of chronic dysfunction.

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1 Introduction

Acute vascular damage within allografts is a critical component of transplant dysfunction. This early damage can predispose the graft to develop chronic allograft rejection, the leading cause of late graft failure [1, 2]. Chemokine (C-C-motif) receptor-5 (CCR5) plays an important role in acute as well as chronic allograft rejection [3]. CCR5 binds the chemokines CCL3/MIP-1α, CCL4/MIP-1β and CCL5/RANTES and is mainly expressed by macrophages, activated/memory T cells, dendritic cells, NK cells and microglia [4]. CCR5 also represents the principal co-receptor for macrophage-tropic strains of HIV [4]. In human populations of European origin, a nonfunctional allele of the CCR5 gene with a 32-bp deletion (CCR5Δ32) is frequently found [4].

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Abbreviations: ADAM: A disintegrin and metalloprotease domain **CCR5:** Chemokine (C-C-motif) receptor-5 **CsA:** Cyclosporine A **HPF:** High-powered field **MMP:** Matrix metalloproteinase **MST:** Median survival time **RPA:** RNase-protection assay **wt:** Wild-type

Homozygous carriers of the CCR5Δ32 allele can be regarded as human "knockouts" for CCR5.

A role for CCR5 in allograft rejection was first demonstrated in a human kidney transplantation study which showed that long-term graft survival was significantly increased in patients homozygous for the CCR5Δ32 allele [5]. Animal models of CCR5 deficiency have been experimentally generated by targeted disruption of the murine Ccr5 gene [6–8]. The phenotype of Ccr5-deficient mice has been analyzed in several disease models including heart and islet allograft rejection [9, 10]. Experiments performed by Gao et al. using a model of heterotopic cardiac allograft rejection showed a prolongation of graft survival in Ccr5-deficient mice [9].

The mechanisms underlying CCR5-mediated allograft damage are not well understood. To better characterize these mechanisms, we generated Ccr5-deficient mice and used them as recipients of fully MHC-mismatched cardiac and carotid-artery allografts. Microarray analysis was used to compare gene expression in heterotopic heart allografts in wild-type (wt) and Ccr5-deficient recipients. These experiments led to the identification of selected metalloproteinase genes that showed reduced intragraft mRNA expression in Ccr5-deficient recipients. The importance of Ccr5 for vascular remodeling was assessed in carotid artery transplantation experiments, which revealed a significant reduction of graft T cell infiltration and vascular remodeling in Ccr5-deficient recipients. Our findings may be relevant for understanding the mechanisms involved in tissue remodeling during transplant rejection.

2 Results

2.1 Generation of Ccr5-deficient mice

Ccr5-deficient mice were generated using the targeting strategy outlined in Fig. 1A. Deletion of the Ccr5 gene was verified by Southern-blot analysis (Fig. 1B, C). The

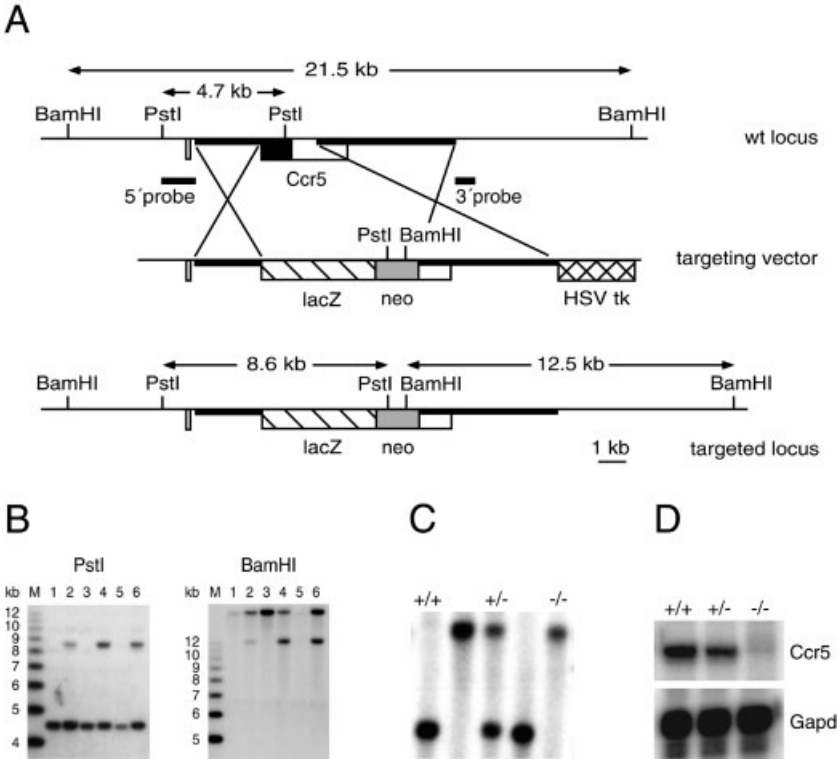

Fig. 1. Generation and identification of Ccr5-deficient mice. (A) Schematic representation of the targeting strategy. The murine Ccr5 wt locus, the targeting vector and the targeted locus are shown together with restriction sites and probes relevant for Southern blot analysis. Black box: coding region of the Ccr5 gene. Open boxes: 5′ and 3′ untranslated regions. Thick lines: homology arms. Gray box: neomycin resistance gene. Hatched box: lacZ reporter gene. Double-hatched box: HSV tk gene. (B) Southern blot analysis of different ES cell clones using the probes depicted in (A). (C) Southern blot analysis of mouse littermates using the 5′ probe. (D) RPA for Ccr5 and Gapd using spleen RNA from $Ccr5^{+/+}$, $Ccr5^{+/-}$ and $Ccr5^{-/-}$ mice.

absence of Ccr5-specific mRNA was demonstrated by RNase-protection assay (RPA) (Fig. 1D). Ccr5-deficient mice were viable, fertile and showed neither gross changes in morphology nor developmental abnormalities in comparison to their wt littermates (not shown).

2.2 Heart transplantation in Ccr5-deficient mice

We tested the influence of Ccr5 on the survival of heterotopically transplanted cardiac allografts using the MHC class I and class II mismatched mouse strain combination BALB/c (H-2^d) donors to C57BL/6 (H-2^b) recipients. C57BL/6 $Ccr5^{+/+}$ mice rejected hearts from BALB/c donors within 7–8 days with a median survival time (MST) of 7 days (n=9), whereas $Ccr5^{-/-}$ mice rejected BALB/c hearts between 10 and 13 days (MST 11 days; n=10) (Fig. 2A) ($p=6.9\times10^{-6}$). C57BL/6 isografts survived >28 days (the time when the experiment was terminated) and showed no signs of rejection (n=5) (Fig. 2A). It has been reported that cyclosporine A (CsA) given at a low dose (10 mg/kg) leads to permanent engraftment of cardiac allografts in $Ccr5^{-/-}$ recipients [9]. We repeated these experiments and administered CsA at a daily dose of 10 mg/kg using osmotic minipumps implanted subcutaneously on the back of the recipients. In $Ccr5^{+/+}$ mice the transplant was rejected between 8 and 12 days (MST 10 days; n=7), and in $Ccr5^{-/-}$ recipients between 10 and 15 days (MST 12 days; n=9) (Fig. 2B) (p=0.028).

Histological analysis was performed on additional grafts on post-transplant day 6 (n=5/group), i.e. prior to cessation of ventricular contractions (Fig. 3). Grafts from $Ccr5^{-/-}$ recipients showed reduced leukocyte infiltration and better preservation of the myocardial architecture. Quantitative assessment of the infiltrate on post-transplant day 6 indicated a trend towards a reduction in $CD3^+$ T cells in $Ccr5^{-/-}$ mice [19.8±7.4 cells / high-powered field (HPF) *vs.* 11.7±4.8 cells/HPF]. The number of $F4/80^+$ macrophages was also lower (10.6±7.5 cells/HPF *vs.* 6.7±4.0 cells/HPF), but neither difference reached statistical significance. Quantitative analysis of rejection on day 6 showed no differences in the interstitial rejection score (171.0±38.6 in wt *vs.* 165.0±38.9 in $Ccr5^{-/-}$ recipients), but a significant reduction of vascular rejection in grafts from $Ccr5^{-/-}$ mice (vascular rejection score 58.8±14.1 *vs.* 33.8±14.3; p=0.035).

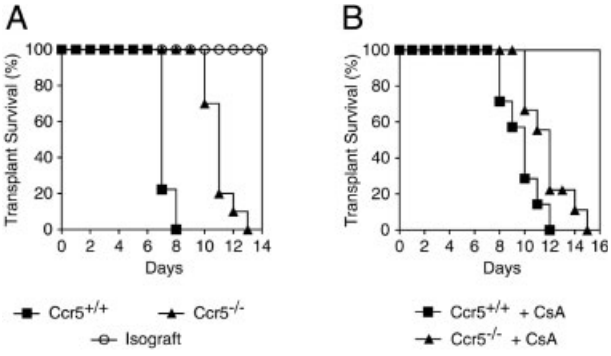

Fig. 2. Cardiac allograft survival in wt and Ccr5-deficient recipients. (A) Survival of BALB/c hearts in C57BL/6 wt mice (n=9) or Ccr5-deficient mice (n=10) and survival of C57BL/6 isografts (n=5) in C57BL/6 wt mice. (B) Influence of CsA on the survival of BALB/c hearts in C57BL/6 wt mice (n=7) or Ccr5-deficient mice (n=9).

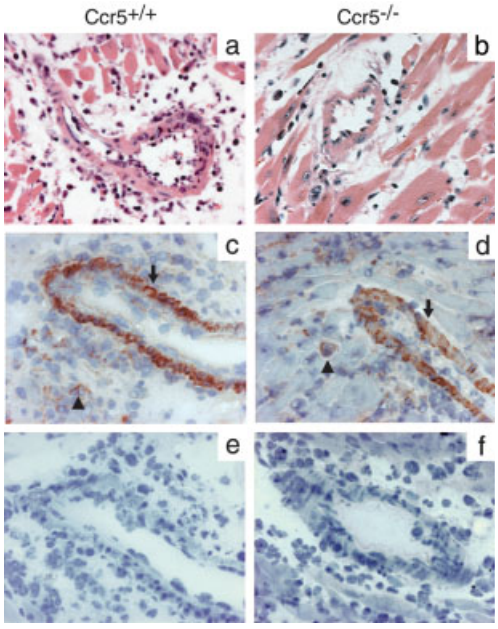

Fig. 3. Histological and immunohistochemical analysis of cardiac allografts from wt and Ccr5-deficient recipients 6 days post-transplantation. (a, b) HE-stained sections. (a) Intramural artery of a transplant from a wt recipient showing signs of acute vascular rejection with infiltration of the subendothelial space and the vessel wall by mononuclear cells. A prominent perivascular edema and a moderate inflammatory infiltrate of the myocardium are visible. (b) Intramural artery of a transplant from a Ccr5-deficient recipient. The artery is free of mononuclear cells. The periadventitial area is slightly edematous. The interstitial mononuclear infiltrate is slightly reduced to that found in wt recipients. (c–f) Frozen sections were stained with a goat anti-murine-Mmp12 antiserum (c, d) or without first antibody as control (e, f). A positive label was found on inflammatory cells in the myocardium (arrowheads). Additionally smooth-muscle cells of intramural arteries (arrows) stained positive in wt (c) and Ccr5-deficient recipients (d). Magnification 400×.

2.3 DNA microarray analysis of cardiac allografts from Ccr5-deficient mice

To characterize potential mechanism(s) underlying Ccr5-mediated transplant damage, allograft RNA from wt or $Ccr5^{-/-}$ recipients was subjected to microarray analysis. Cardiac isograft RNA from wt recipients was analyzed in parallel. Grafts were removed on days 2 or 6 after transplantation, *i.e.* in the early and late phases of rejection. Microarray analysis of all six experimental groups was performed using Affymetrix GeneChips containing ≈6000 functionally characterized sequences and ≈6000 EST. Identification of differentially expressed genes was performed as described previously [11]. Of all 12488 genes analyzed, 3162 genes were considered to be differentially expressed (>2-fold) between at least two of the six groups.

Cluster analysis was used to arrange all differentially expressed genes according to similarity in their pattern of gene expression [11]. In a two-way cluster analysis (Fig. 4C, left), the six experimental groups could be distributed to the following three clusters: allografts from wt or $Ccr5^{-/-}$ recipients at day 6 (groups 1+2), isografts from wt recipients at day 2 or day 6 (groups 3+4), and allografts from wt or $Ccr5^{-/-}$ recipients at day 2 (groups 5+6). Groups 3–6 formed together a new cluster, which differed considerably from the cluster formed by groups 1 and 2.

A one-way cluster analysis was performed to obtain a more intuitive time-based arrangement of the groups (Fig. 4C, middle). At day 2, no major differences in the gene expression pattern are evident, neither between isografts and allografts, nor between allografts from wt or

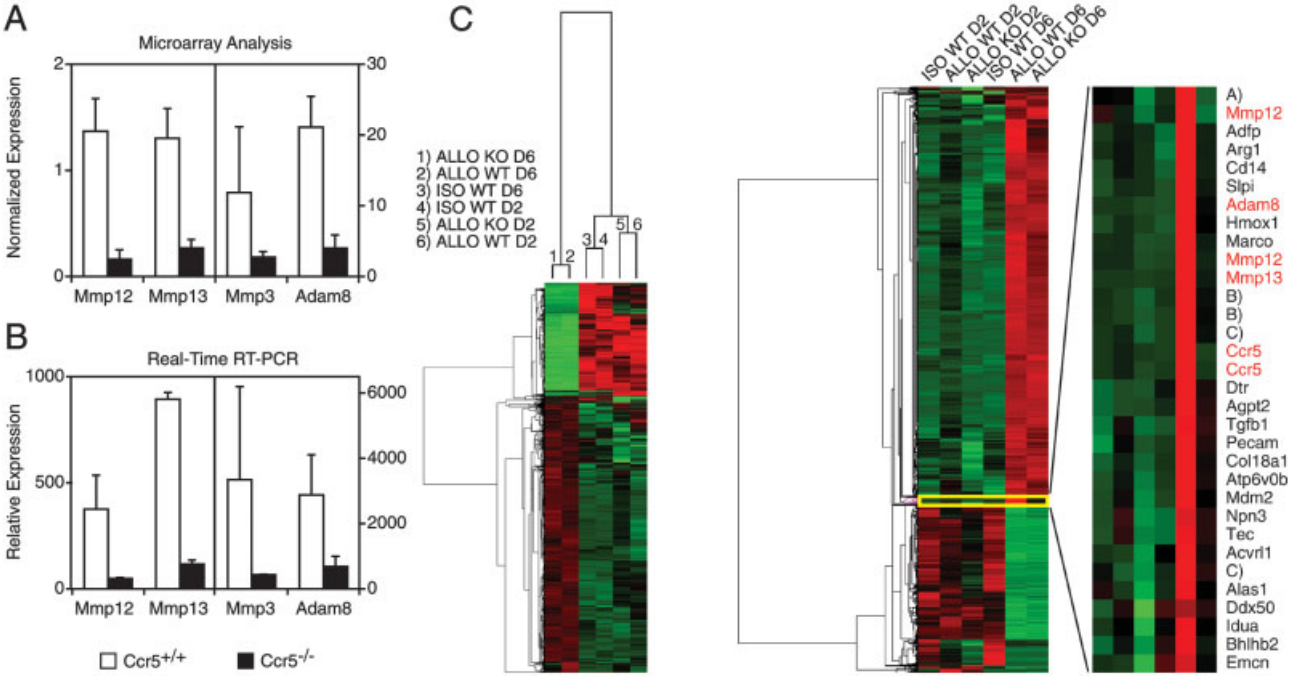

Fig. 4. Microarray analysis of cardiac allografts from wt and Ccr5-deficient recipients. (A) Cardiac allograft RNA from three wt and three Ccr5-deficient recipients were subjected to Affymetrix GeneChip analysis. Relative mRNA expression levels of four metalloproteinase genes with significant differences between wt and Ccr5-deficient recipients at post-transplant day 6 are shown (mean±SD, $p<1\times10^{-5}$ for Mmp12, Mmp13 and Adam8 and $p<0.02$ for Mmp3; Student's *t*-test). (B) Confirmation of microarray results shown in (A) by quantitative real-time RT-PCR (mean±SD). (C) Cluster analysis of gene expression on post-transplant day 2 (D2) or day 6 (D6) in cardiac isografts (ISO) and allografts (ALLO) from WT and Ccr5-deficient (KO) recipient mice. In each group, cardiac graft RNA from three different animals was analyzed. On the left, a two-way cluster analysis is shown for all genes that were considered to be differentially expressed. ALLO at D6, ALLO at D2 and ISO at D2 and D6 cluster together. In the middle, a one-way cluster analysis of the same genes is shown and the six groups are arranged in a more intuitive order. The part of the cluster picture showing the strongest differences between ALLO from WT and KO recipients at D6 is marked by a yellow box. A blow-up of this region is shown on the right and the genes are indicated by their official gene symbols. Some of the genes are represented by two probe sets on the Affymetrix U74Av2 chip and are therefore listed twice. For the three genes designated A), B) and C) no official gene symbol is available.

$Ccr5^{-/-}$ recipients. At day 6, however, massive changes in gene expression are visible between isografts and allografts from wt or $Ccr5^{-/-}$ recipients, and >2000 genes are up-regulated. For the majority of these genes no differences are visible between allografts from wt or $Ccr5^{-/-}$ recipients. A small group of ≈30 genes, however, was induced in allografts from wt recipients, and repressed in allografts from $Ccr5^{-/-}$ recipients. The cluster region containing these interesting genes is marked by a yellow box (Fig. 4C, middle) and a blow-up of this region together with a list of the corresponding genes is shown next to it (Fig. 4C, right). As expected, Ccr5 belonged to this group of genes. The most interesting finding was the presence of several metalloproteinase genes: Two members of the matrix metalloproteinase (MMP) family, Mmp12 and Mmp13, and a member of the ADAM (a disintegrin and metalloprotease domain) gene family, Adam8, clustered together with Ccr5 (Fig.4C, right).

Each day-6 allograft RNA sample was analyzed with two technical replicates and the resulting microarray data were re-evaluated by employing the Affymetrix Microarray Suite v4 algorithm and standard *t*-test statistics. In a 2D hierarchical clustering analysis of the experiments, the technical replicates paired up well. In general, the expression data obtained from all six animals showed a very high correlation. About 5% of the genes were differentially expressed (>2-fold) between allografts from wt and $Ccr5^{-/-}$ recipients. The majority of differentially expressed genes showed lower expression levels in $Ccr5^{-/-}$ mice. Amongst the 15 different chemokine receptors present on the chip, a ≈3-fold lower expression level was observed for Ccr1 in allografts from $Ccr5^{-/-}$ mice.

Analysis of chemokines, cytokines, cytokine receptors, growth factors, adhesion molecules, Fc receptors, NK cell and macrophage markers, various CD antigens, and selected transcription factors revealed only minor differences in mRNA levels. When the genes were ranked based on fold expression levels, Mmp12, Mmp13 and Adam8 showed up in the top 10 differentially expressed genes. All other MMP and ADAM family members present on the chip were individually checked for differences in gene expression. These analyses revealed that Mmp3 expression was also significantly reduced in allografts from $Ccr5^{-/-}$ recipients. The relative mRNA expression levels of all four affected metalloproteinase genes at day 6 are shown in Fig. 4A. The reduced intragraft mRNA expression of Mmp3, Mmp12, Mmp13 and Adam8 in allografts from $Ccr5^{-/-}$ mice was verified by quantitative real-time RT-PCR (Fig. 4B).

We wanted to know whether the differences observed at the RNA level can be detected at the protein level as well. The lack of commercially available antibodies directed against murine MMP represented a major obstacle. Only immunostaining for Mmp12 could be performed on frozen sections from cardiac allografts explanted on post-transplant day 6 from wt or $Ccr5^{-/-}$ recipients (Fig. 3c–f). The infiltrating inflammatory cells within the myocardium stained positive for Mmp12, and, in addition, a strong positive staining was observed on the smooth-muscle cell layer of intramural arteries in both wt (Fig. 3c) and $Ccr5^{-/-}$ recipients (Fig. 3d). Fig. 3c and 3d suggest a lower Mmp12 expression in allografts from $Ccr5^{-/-}$ recipients. Attempts to quantify allograft Mmp12 protein levels were unsuccessful because the antibody did not work in Western blot experiments.

2.4 Verification of cytokine, chemokine and chemokine receptor expression in cardiac allografts

As the cytokine milieu of the graft will play an important role in the development of allograft injury, the expression of selected cytokines, chemokines and chemokine receptors was re-analyzed in cardiac allografts from wt and $Ccr5^{-/-}$ recipients and compared with the expression pattern of native hearts from the same animals. For this purpose, grafts and native hearts were explanted on day 6, total RNA was extracted from two or three samples per group and analyzed by multi-probe RPA. All cytokines analyzed were up-regulated in the allografts and no significant differences between wt and $Ccr5^{-/-}$ recipients were detected (Fig. 5A). It is noteworthy that at this time point the signal corresponding to IFN-γ was similar in wt and $Ccr5^{-/-}$ animals. Expression of 19 different chemokines including the three major ligands for CCR5, *i.e.* CCL3/MIP-1α, CCL4/MIP-1β and CCL5/RANTES, was analyzed using different multi-probe template sets. There was no major difference (>3-fold) in expression of these chemokines between allografts from wt and $Ccr5^{-/-}$ recipients (not shown).

The chemokine receptors Ccr1, Ccr2 and Ccr5 were strongly up-regulated in allografts from wt mice (Fig. 5B, lane 3). In allografts from $Ccr5^{-/-}$ recipients, Ccr5 was undetectable and a clear reduction of Ccr1 and Ccr2 expression was observed by RPA (Fig. 5B, lane 4). The chemokine receptor Cxcr3 plays a pivotal role in the initiation of acute allograft rejection [12]. In our microarray analyses, we observed a slightly higher expression for Cxcr3 in allografts from $Ccr5^{-/-}$ recipients (1.5-fold). Due to the lack of a suitable RPA probe, the intragraft expression pattern of Cxcr3 was verified by real-time RT-PCR. Cxcr3 was strongly expressed in allografts from both $Ccr5^{+/+}$ (n=3) and $Ccr5^{-/-}$ (n=3) recipients (relative expression 1839±601 *vs.* 2630±159). In summary, all

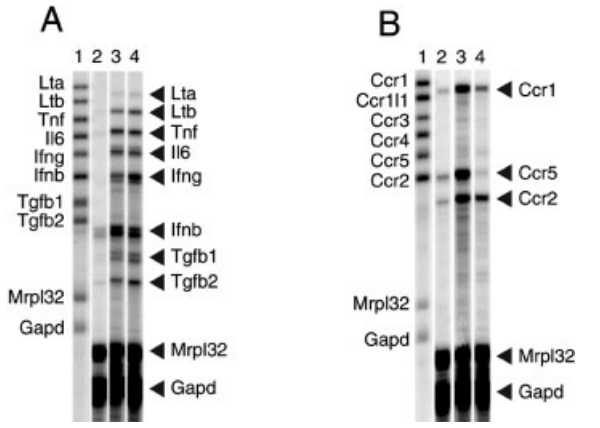

Fig. 5. Verification of cytokine and chemokine receptor expression in cardiac allografts from wt and Ccr5-deficient recipients 6 days post-transplantation. RNA from cardiac allografts or native hearts was analyzed by multi-probe RPA using various template sets. (A) Cytokines. (B) Chemokine receptors. Lane 1, undigested probes; lane 2, native hearts; lane 3 allografts of wt recipients; and lane 4, allografts of Ccr5-deficient recipients. Analyses were performed with two or three different RNA samples from each group.

RPA or quantitative real-time RT-PCR experiments confirmed the corresponding microarray data for cytokines, chemokines and chemokine receptors.

2.5 Carotid artery transplantation experiments in Ccr5-deficient mice

Transplant vasculopathy is a hallmark of chronic allograft failure [2]. To further investigate the role of Ccr5 in chronic rejection, we used murine carotid artery transplantation as a model of accelerated transplant arteriosclerosis [13]. Carotid artery grafts from BALB/c donors were transplanted into C57BL/6 wt mice (n=6) or C57BL/6 Ccr5$^{-/-}$ mice (n=8). The mice received no treatment and grafts were harvested 35 days after transplantation. Fig. 6a and b show representative histological pictures of Giemsa-stained sections.

Changes in vessel wall architecture were quantified by morphometric analysis of transverse sections of grafts (Fig. 7A). The mean intimal cross-section area was significantly reduced (by ≈50%) in Ccr5$^{-/-}$ recipients ($p<0.005$, repeated-measures ANOVA) (Fig. 6a, 6b and 7A), whereas lumen cross-sectional area and media thickness were not different. The intima-to-media ratio, a measure that is used to quantify vascular remodeling, was also significantly reduced in Ccr5$^{-/-}$ recipients (0.71±0.09 *vs.* 0.37±0.05; $p<0.005$). The media displayed a more intense staining with an antibody to α

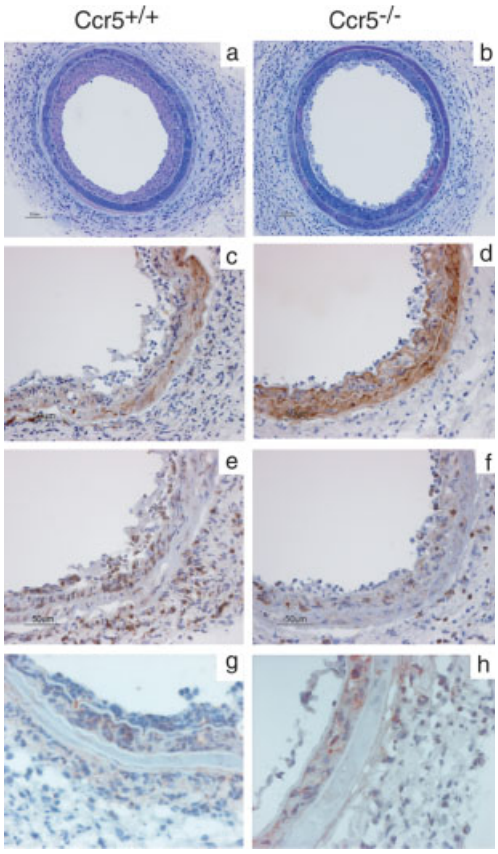

Fig. 6. Histological and immunohistochemical analysis of carotid-artery allografts from wt and Ccr5-deficient recipients. BALB/c carotid arteries grafted into C57BL/6 wt mice (n=6) or Ccr5-deficient mice (n=8) were harvested 35 days post-transplantation and analyzed by histology and immunohistochemistry. (a, b) Giemsa-stained sections of methacrylate-embedded carotid allografts. Frozen sections from wt (c, e, g) and Ccr5-deficient mice (d, f, h) were stained with antibodies against αSMA (c, d), CD3 (e, f), and Mmp12 (g, h). Magnification 200× (a, b) or 400× (c–h).

smooth muscle actin (αSMA) in grafts from Ccr5$^{-/-}$ recipients than in grafts from wt recipients (Fig. 6c, d), indicating a better media preservation in Ccr5$^{-/-}$ mice. Furthermore, grafts from Ccr5$^{-/-}$ recipients contained a lower number of infiltrating CD3$^+$ T cells in comparison with grafts from wt recipients (Fig. 6e, f). Quantitative assessment of the cellular infiltrate showed a significant reduction of the total number of cells forming the neointima (p=0.01) and of the number of CD3$^+$ T cells (p=0.007) in Ccr5$^{-/-}$ mice (Fig. 7B). Although apparently lower in Ccr5$^{-/-}$ recipients, the number of F4/80$^+$ macrophages did not differ significantly between the two groups (p=0.12) (Fig. 7B). Cells in the neointima and adventitia stained positive for Mmp12 both in wt and Ccr5$^{-/-}$ recipients (Fig. 6g, h).

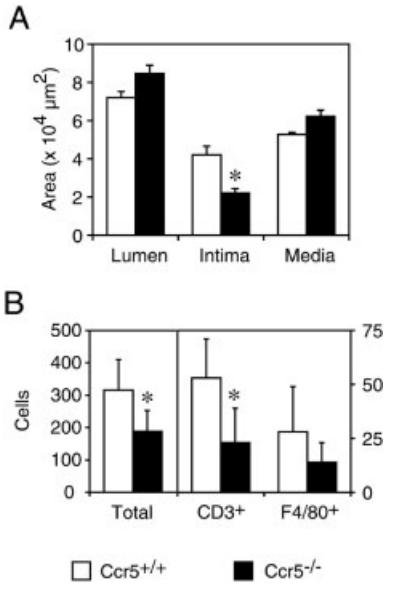

Fig. 7. Quantitative analysis of carotid-artery allografts from wt and Ccr5-deficient recipients. (A) Morphometric analysis of carotid allografts (mean±SEM; *$p<0.005$). (B) Quantitative assessment of cells involved in neointima formation (mean±SD; *$p\leq0.01$).

3 Discussion

Transplant arteriosclerosis and progressive fibrosis with increased extracellular matrix turnover are hallmarks of chronic rejection, which is a major problem in clinical transplantation [2]. Transplant rejection as seen in our mouse model of heterotopic heart transplantation across a full MHC barrier was less vigorous in the absence of Ccr5. This is documented by the significantly prolonged graft survival in $Ccr5^{-/-}$ recipients (Fig. 2A) and by a better tissue preservation not only at day 6 (Fig. 3), but also at the time of complete rejection (not shown).

Administration of a subtherapeutic dose of CsA by continuous s.c. infusion had only a modest effect on graft survival in wt and $Ccr5^{-/-}$ recipients (Fig. 2B). This finding is in apparent contradiction to data reported by Gao et al. [9], who observed permanent engraftment when $Ccr5^{-/-}$ mice were treated for 2 weeks with the same dose of CsA. One possible explanation for this discrepancy is the different mode of CsA administration. Our dosing regimen ensures constant CsA plasma levels over the entire duration of the experiment whereas Gao et al. administered the drug by a single i.p. injection every day. Some other differences observed (MST, mRNA levels for Cxcr3 and IFN-γ) might be explained by the use of different $Ccr5^{-/-}$ mouse lines with a different degree of backcrossing and therefore differences in genetic background. The $Ccr5^{-/-}$ mice used by Gao et al. were maintained on a mixed genetic background of C57BL/6 and 129 and F2 hybrids had been used as approximate controls.

CCR5 ligands are known to participate in T cell activation [14]. Therefore, the phenotype of $Ccr5^{-/-}$ mice in transplantation experiments might be explained by changes in the alloreactivity of lymphocytes. To exclude this possibility, we performed mixed lymphocyte reactions in the presence of antibodies neutralizing Ccr5 [15] (not shown). The results suggest that Ccr5 does not play a role in priming or proliferation of T cells.

To identify potential mechanism(s) underlying Ccr5-mediated transplant damage, microarray analysis was used. The most striking result from our microarray analyses of cardiac allografts was the decreased mRNA expression of Mmp3, Mmp12, Mmp13 and Adam8 in $Ccr5^{-/-}$ recipients on post-transplant day 6 (Fig. 4). The Affymetrix chip U74Av2 contains at least 19 more members of the MMP and the ADAM family. However, for these other family members either no signal was detected or the signals did not show significant differences between wt and $Ccr5^{-/-}$ mice. The same was true for the three tissue inhibitors of metalloproteinases (TIMP) present on the chip.

The MMP superfamily is represented to date by at least 26 metalloendopeptidases that degrade extracellular matrix. Collectively, these enzymes are responsible for the metabolism of extracellular matrix proteins. MMP are active during tissue development and differentiation, cellular infiltration and wound healing. These enzymes have also been implicated in chronic diseases such as multiple sclerosis and rheumatoid arthritis, as well as in tumor progression and metastasis (reviewed in [16]). MMP play key roles in leukocyte extravasation [17]. Furthermore, selected MMP have the ability to modulate inflammatory and immune responses by processing chemokines. MMP-mediated cleavage of chemokines can either generate a chemokine with increased potency or convert an agonist to a chemokine receptor antagonist [18, 19]. MMP activity is kept under tight biological control. Although MMP are regulated at the level of gene expression, they are also controlled at the level of protein synthesis, compartmentalization of enzyme activity and by the processing of MMP pro-enzymes to active enzymes. In addition, MMP activity is regulated by the expression of natural MMP inhibitors, *i.e.* TIMP.

It has been reported that stimulation of cells with a proinflammatory chemokine induces expression of specific MMP mRNA [20]. Locati et al. [20] treated human monocytes with either the CCR5 ligand CCL5/RANTES or lipopolysaccharide and compared the gene

expression profiles obtained by microarray analysis. Cells activated by CCL5 showed increased mRNA levels for MMP-9, MMP-19 and CCR1. The decreased Ccr1 expression levels that we observed in cardiac allografts from $Ccr5^{-/-}$ mice are in accordance with the findings in human monocytes. With respect to MMP-9 and MMP-19 expression, comparisons are not possible. For the murine ortholog Mmp9, which is present on the Affymetrix U74Av2 chip, no signal above background was detected and Mmp19 is not included in this GeneChip.

Mmp3 (also called stromelysin-1) is expressed by different cell types including stromal fibroblasts and degrades gelatin, fibronectin, laminins, collagens III, IV, IX, and X, tenascin C and vitronectin [21]. Mmp12 (also called macrophage metalloelastase) is produced by macrophages and degrades elastin [21]. Analysis of macrophages from $Mmp12^{-/-}$ mice has demonstrated that this metalloproteinase plays an important role in extracellular matrix proteolysis and tissue invasion [22]. Mmp13 (also called collagenase-3) is expressed by different cell types including stromal fibroblasts. Mmp13 degrades collagen I, II, III, gelatin, fibronectin, laminins, and tenascin [21]. Adam8, (also called MS2 or murine CD156), is a member of the ADAM family [23], which are membrane-bound proteins that can act as cell-to-cell and cell-to-matrix adhesion molecules, degrade the extracellular matrix, and play a role in tissue morphogenesis. Adam8 is highly expressed on cells of the monocytic lineage.

The reduced mRNA expression levels for Mmp3, Mmp12, Mmp13 and Adam8 in allografts from $Ccr5^{-/-}$ recipients may relate to the observed decrease in the number of infiltrating macrophages, but also to differences in the activation state of infiltrating cells or to secondary effects on resident cells. A direct effect of Ccr5 activation on Mmp12 mRNA expression was excluded in a control experiment. Peritoneal macrophages from $Ccr5^{+/+}$ and $Ccr5^{-/-}$ mice were stimulated with the Ccr5-specific ligand Ccl4/MIP-1β, and Mmp12 mRNA expression was measured by quantitative real-time RT-PCR. No significant differences were found (not shown). Therefore, the exact mechanism remains to be determined. In any case, the reduced mRNA expression of these proteases in the absence of Ccr5 nicely correlates with the reduced tissue remodeling and the better preservation of grafts observed in our experiments (Figs. 3, 6). It is tempting to speculate that there may be a functional link between MMP expression and tissue remodeling during allograft rejection.

On the basis of the results described in this report, we would like to conclude that the loss of Ccr5 has only a modest effect on transplant survival in acute transplant rejection as evidenced in the cardiac allograft model (Fig. 2A). However, in the absence of Ccr5 grafts are better preserved with less vasculopathy. This is true for grafts undergoing acute rejection, and also for long-term carotid-artery transplants.

We propose that the role of Ccr5 in acute and chronic transplant rejection may be mediated at least in part by selected MMP involved in leukocyte extravasation and tissue remodeling. The reduced mRNA expression of these proteases in the absence of Ccr5 may lead to a reduction in transplant-associated arteriosclerosis and an enhancement of long-term graft survival. The identification of the metalloproteinases as mediators of transplant remodeling may also be of interest for future therapeutic exploration.

4 Materials and methods

4.1 Animals

C57BL/6NCrl (C57BL/6) and BALB/cAnNCrl (BALB/c) mice were obtained from Charles River (Sulzfeld, Germany). Mice were housed in individually ventilated cages under SPF conditions. All animal experiments were performed in compliance with governmental and institutional guidelines.

4.2 Generation of Ccr5-deficient mice

A targeting vector was constructed based on the plasmid pHM2 [24]. A 2.5-kb *Xba*I/*Cla*I genomic fragment was inserted upstream of the lacZ reporter gene and a 5.2-kb *Bgl*II fragment followed by a HSV tk cassette was placed downstream of the *neo* cassette (Fig. 1A). A more detailed description is available upon request. The targeting vector was electroporated into E14-1 ES cells and clones resistant to both G418 and gancyclovir were picked and screened by Southern blot for homologous recombination using external probes (Fig. 1A, B). Three positive ES cell clones were aggregated with morulae from CD1 mice and the resulting chimeras were mated with CD1 wt mice for germline transmission. The resulting $Ccr5^{+/-}$ mice were backcrossed for five generations to C57BL/6 and then intercrossed to obtain the $Ccr5^{-/-}$ mice that were used for all experiments.

4.3 Heterotopic heart transplantation and CsA administration

BALB/c ($H\text{-}2^{d}$) donor hearts (or C57BL/6 hearts, for isograft controls) were heterotopically transplanted into C57BL/6 ($H\text{-}2^{b}$) wt or $Ccr5^{-/-}$ mice as described previously [25]. Grafts were monitored daily by palpation and were considered rejected when there were no palpable ventricular contrac-

tions. Grafts were explanted on post-transplant day 2 (for gene expression analysis), day 6 (for gene expression analysis and histology) or on the day when they had stopped beating (for histology). Grafts were cut transversally into several slices and mid-ventricular samples were immersion-fixed either in 4% formaldehyde or in methacarn, and embedded in paraffin or in OCT compound (Sakura Finetek) for histology/immunohistology or were snap-frozen in liquid nitrogen for RNA extraction. Allograft recipients were treated in some experiments with CsA (Novartis, Basel, Switzerland) at a daily dose of 10 mg/kg. CsA was infused continuously via subcutaneously implanted Alzet osmotic minipumps (model 2002) at a delivery rate of 0.5 μl/h. Graft survival was statistically analyzed using log-rank tests.

4.4 Heart histology and immunohistochemistry, and determination of rejection score

Light microscopy was performed on 3-μm sections stained by hematoxylin and eosin (HE). Rejection was quantified in two ways. Firstly, vascular and interstitial rejection scores were calculated as described previously [26] and analyzed with Student's *t*-test on at least four allografts from wt or $Ccr5^{-/-}$ recipients. Secondly, the number of infiltrating $CD3^+$ T cells and $F4/80^+$ macrophages were counted in at least 15 HPF (40×) per section and were recorded as mean per HPF±SD and analyzed with Student's *t*-test. Immunohistochemical staining was performed on 5-μm sections of frozen tissue, using rat anti-mouse monoclonal antibodies against CD3 (clone 17A2; Pharmingen, San Diego, USA) and F4/80 (clone CI:A3-1; Serotec, Oxford, GB). An alkaline phosphatase anti-alkaline phosphatase (APAAP) (for CD3) or an avidin–biotin enhanced horseradish peroxidase (HRP) (for F4/80) detection system was applied for visualization. Controls, omitting the first antibody or replacing the first antibody by a nonimmune IgG, were negative for each section tested. Mmp12 expression was demonstrated by immunohistochemistry using a goat anti-mouse-Mmp12 antiserum and an HRP-labeled donkey anti-goat conjugate (sc-8839, sc 2056; Santa Cruz, Heidelberg, Germany).

4.5 Carotid artery transplantation

Carotid artery segments (approximately 6 mm long) from BALB/c wt donors were interpositioned into the carotid artery of C57BL/6 wt mice (n=6) or $Ccr5^{-/-}$ mice (n=8) using end-to-end anastomoses. Animals were exsanguinated 35 days later and the vasculature was perfused with 5 ml ice-cold PBS through the left ventricle. Perfusion was continued with 10 ml of cold 4% formaldehyde and then the graft and contralateral carotid were removed and cut transversally in the middle under an operating microscope. One half of the graft was embedded in 2-hydroxyethyl methacrylate (HistoResin; Leica), the other half was embedded in OCT after cryoprotection in 25% sucrose. Methacrylate sections (3 μm) were taken at 0, 50, 200, 350 μm from the center of the graft and were stained with Giemsa. Morphometric analyses of the lumen, neointima and media were performed using a Zeiss Image Analysis system KS400 (Carl Zeiss, Oberkochen, Germany). Comparisons between groups were done using repeated-measures ANOVA. Immunohistochemical analysis of carotids was performed on 5-μm-thick frozen sections. Streptavidin/peroxidase reactions were performed using primary antibodies to SMA (clone 1A4; DAKO, Glostrup, Denmark), CD3 (clone CD3-12; Serotec) and F4/80 (clone CI:A3-1; Serotec). Semiquantitative analysis of smooth-muscle cells and quantitative analysis of the infiltrated $CD3^+$ and $F4/80^+$ cells was performed by counting all cells carrying the respective markers in the neointima. Mmp12 expression was demonstrated by immunohistochemistry as described for transplanted hearts.

4.6 Microarray analysis

BALB/c cardiac allografts from three wt and three $Ccr5^{-/-}$ C57BL/6 recipients were explanted either on post-transplant day 2 or 6. Three C57BL/6 cardiac isografts from C57BL/6 wt recipients were explanted at the same two time points and total RNA from all 18 samples was isolated and purified using the RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The integrity of purified RNA was assessed on an Agilent 2100 Bioanalyzer using a RNA 6000 Nano LabChip kit (Agilent Technologies, Waldbronn, Germany). Microarray analysis was performed using Affymetrix murine U74Av2 GeneChips (Affymetrix, Santa Clara, CA, USA). Each of the six allograft RNA samples from post-transplant day 6 was analyzed on two GeneChips, whereas all other RNA samples were analyzed on one GeneChip each. In total, 24 GeneChips were analyzed. Probe synthesis, hybridization, washing, staining and scanning of the microarrays was performed according to the manufacturer's instructions. Identification of differentially expressed genes and cluster analysis was performed according to Hoffmann et al. [11]. The raw data from the microarray experiments performed in duplicate were re-analyzed by a second method. Probe level fluorescence intensities were calculated with Microarray Suite v4 software (Affymetrix). Prenormalized data [trimmed mean (top and bottom 2%) set to a constant value] was imported into Expressionist (GeneData, Basel, Switzerland) from a generic chip database. Data were normalized with arithmetic mean (100) and parametric tests performed (standard Student's *t*-test as implemented in Expressionist). Genes with p-values <0.001 were selected for further analysis. Quality was controlled by analyzing Affymetrix quality-control features (noise, background, spikes, etc.) as well as 2D hierarchical clustering.

4.7 RPA

For RPA, the commercial template sets mCK-3 (cytokines) and mCR-5 (chemokine receptors) (Pharmingen, Heidelberg, Germany) were used according to the manufacturer's instructions. RPA were performed with 3 μg (cytokines) or 10 μg (chemokine receptors) total graft RNA.

4.8 Real-time RT-PCR

Intragraft mRNA expression of selected genes was quantified by real-time RT-PCR using an Applied Biosystems 7700 Sequence Detection system (PE Biosystems, Weiterstadt, Germany) and normalized to 18S RNA [27]. The resulting values were multiplied by a factor of 10^8 and expressed as mean±SD. The following primer pairs and probes were used (F=forward primer, R=reverse primer, P=probe):

Mmp3:
F5′-CCTTTTGATGGGCCTGGAA-3′,
R5′-CAACCAGGAATAGGTTGGTACCA-3′,
P5′-TTGGCTCATGCCTATGCACCTGGAC-3′

Mmp12:
F5′-GACTGGTTCTTCTGGTGGAAGCT-3′,
R5′-TGGGATGCTTGGCCATATG-3′,
P5′-TGGGAGTCCAGCCACCAACATTACTTCT-3′

Mmp13:
F5′-TTGTGTTTGCAGAGCACTACTTGA-3′,
R5′-AACTGTGGAGGTCACTGTAGACTTCTT-3′,
P5′-CTGCGACTCTTGCGGGAATCCTGA-3′

Adam8:
F5′-GCCCCTTGAACGCTCCTT-3′,
R5′-TTCCATCCATGCAAACCTTTC-3′,
P5′-TATTGCAGGGCACCAAGTGCGAGG-3′

Cxcr3:
F5′-CCTGCTCCACCTGGCTGTAG-3′,
R5′-CCCCTGCATAGAAGTTGATGTTG-3′,
P5′-CCCTGGCCTCTGCAAAGTGGCA-3′

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