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The role of recipient mast cells in acute and chronic cardiac allograft rejection in C57BL/6-*Kit*$^{W\text{-}sh/W\text{-}sh}$ mice

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KEYWORDS:
mast cells;
acute rejection;
chronic rejection

BACKGROUND: Mast cells are hypothesized to promote rejection and adverse remodeling in cardiac allografts. In contrast, it has been reported that mast cells may enhance cardiac allograft survival in rats. We used C57BL/6-*Kit*$^{W\text{-}sh/W\text{-}sh}$ mast cell-deficient and corresponding wild-type mice to investigate possible contributions of recipient mast cells to acute or chronic cardiac allograft rejection.
METHODS: FVB (H-2^{q}; acute rejection), or C-H-2^{bm12}KhEg (H-2^{bm12}; chronic rejection) donor hearts were heterotopically transplanted into C57BL/6-*Kit*$^{W\text{-}sh/W\text{-}sh}$ (H-2^{b}) or C57BL/6-*Kit*$^{+/+}$ (H-2^{b}) mice. The degree of acute rejection was assessed at 5 days and chronic rejection, at 52 days.
RESULTS: In the acute rejection model, donor heart vascular cell adhesion molecule-1 (VCAM-1) expression was significantly lower in C57BL/6-*Kit*$^{W\text{-}sh/W\text{-}sh}$ than in wild-type recipients; however, acute rejection scores, graft survival, inflammatory cells, or cytokine expression did not differ significantly. In the chronic rejection model, the number of mast cells/mm^2 of allograft tissue was significantly increased 52 days after transplantation in allografts transplanted into C57BL/6-*Kit*$^{+/+}$ but not C57BL/6-*Kit*$^{W\text{-}sh/W\text{-}sh}$ mice; however, no substantial differences were noted in graft coronary artery disease, graft inflammatory cells, or levels of graft tissue expression of cytokines or adhesion molecules.
CONCLUSIONS: Cardiac allografts undergoing chronic rejection in wild-type C57BL/6-*Kit*$^{+/+}$ mice exhibit increased numbers of mast cells, but acute or chronic cardiac allograft rejection can develop in C57BL/6-*Kit*$^{W\text{-}sh/W\text{-}sh}$ mice even though these recipients virtually lack mast cells. These findings indicate that recipient mast cells are not required for acute or chronic cardiac allograft rejection in the models examined.
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Congestive heart failure has become a worldwide public health problem. Although medical and non-transplant surgical options are considered first-line therapies for patients with end-stage heart disease, cardiac transplantation remains the gold standard treatment. Despite advances in surgical technique, donor organ preservation, and immunosuppressive agents, allograft rejection still represents the major cause of graft failure after cardiac transplantation.[1]

Substantial advances have been made in our understanding of transplant immunology, but the cellular and molecular mechanisms that underlie acute and chronic rejection have not been fully delineated. Acute rejection is typically a cell-mediated immune response, with clinical consequences varying from asymptomatic to circulatory collapse.

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Chronic rejection, or graft coronary artery disease (GCAD), is characterized by a diffuse intimal proliferation in the coronary arteries that leads to luminal obliteration and, eventually, graft failure.[2]

There currently is great interest in understanding the potential role of mast cells in transplant rejection. Because mast cells are present in the human heart, investigators have focused on the possible functions of mast cells in several cardiovascular disorders,[3] including atherogenesis,[4–6] aortic aneurysm formation,[7] and cardiac allograft rejection.[8–10] The participation of mast cells in cardiac transplant rejection remains controversial. For example, studies in out-bred genetically mast cell-deficient or corresponding wild-type rats suggest that mast cells may contribute to the survival of heterotopic cardiac allografts.[10] By contrast, morphologic findings in post-transplant endomyocardial biopsy specimens from human heart transplant recipients have been interpreted to indicate that mast cells can promote fibrosis and chronic rejection of cardiac allografts.[8]

Accordingly in the present study, we used genetically mast cell-deficient C57BL/6-*Kit*$^{W\text{-}sh/W\text{-}sh}$ mice and the corresponding wild-type mice to investigate the biologic importance of mast cells in acute and chronic allograft rejection after cardiac transplantation in mice.

Materials and Methods

The mice used in this study were maintained in the animal care facility at Stanford University Medical Center (Stanford, CA) and were treated in compliance with the *Guide for the Care and Use of Laboratory Animals* and published by the National Academy Press (revised 1996).

Animals

Six- to 10-week-old male FVB (H-2^{q}) and B6.C-H-2^{bm12}KhEg (Bm12, H-2^{bm12}) mice were obtained from Jackson Laboratory (Bar Harbor, ME). Mast cell-deficient C57BL/6-*Kit*$^{W\text{-}sh/W\text{-}sh}$ (H-2^{b}) mice and the corresponding C57BL/6-*Kit*$^{+/+}$ (H-2^{b}) wild-type mice were generously provided by Dr Peter Besmer (Memorial Sloan-Kettering, New York, NY). C57BL/6-*Kit*$^{W\text{-}sh/W\text{-}sh}$ mice are profoundly mast cell-deficient; in 12-week-old mice, virtually no mature mast cells are detectable in the heart, lung, or in many other sites.[11] The *W-sh* mutation is an ~3 Mb inversion upstream of *Kit,*[12] which includes a region (~150 kb upstream of *Kit*) that regulates KIT expression in mast cells,[13] and that disrupts corin,[14] which encodes a cardiac serine protease that converts the proform of atrial natriuretic peptide to the active peptide. *Kit*$^{W\text{-}sh/W\text{-}sh}$ mice are not anemic or sterile,[15] but have cardiomegaly and increased numbers of neutrophils and platelets.[14]

Heterotopic cardiac transplantation

Heterotopic abdominal cardiac transplantations were performed according to the method of Corry et al.[16] Donor hearts from (A) FVB mice (total allograft mismatch) for acute rejection or (B) Bm12 mice (major histocompatibility complex class II-mismatch) for chronic rejection (GCAD) were transplanted into *Kit*$^{W\text{-}sh/W\text{-}sh}$ and *Kit*$^{+/+}$ mice. Allografts were harvested on Post-operative Day (POD) 5 or at the time of graft failure in the acute rejection model and at POD 52 for the chronic rejection model. To study the effect of resident donor mast cells on acute rejection, donor hearts from (A) *Kit*$^{W\text{-}sh/W\text{-}sh}$ or (B) wild type *Kit*$^{+/+}$ mice were transplanted into mast cell-competent FVB (H-2^{q}) recipients.

Analyses of graft survival and allograft function

Graft viability was assessed by daily direct abdominal palpation of the transplanted heart. Cardiac graft function was expressed as the beating score, assessed by the Stanford cardiac surgery laboratory graft scoring system as 0 (no contraction), 1 (barely palpable), 2 (obvious decrease in contraction strength), 3 (strong, coordinated beat but noticeable decrease in strength), or 4 (strong contraction of both ventricles, regular rate).

Histologic evaluation

Grafts and arteries were analyzed after Mallory, hematoxylin and eosin, or elastica van Gieson staining. The areas within the internal elastic lamina (IEL), the external elastic lamina (EEL), and the lumen were carefully traced, and planimetric areas were calculated with SPOT Advanced 3.4.2 software (Diagnostic Instruments, Inc, Sterling Heights, MI). The cross-sectional area of luminal stenosis was calculated as follows: luminal narrowing = [(IEL area − luminal area)/IEL area] × 100 (%). The intima-to-media (I/M) ratio was calculated as follows: I/M = (IEL area − lumen area)/(EEL area − IEL area). The percentage of vessels affected by GCAD was quantified by calculating the number of arterial segments in a given heart section with intimal proliferation > 10% and dividing this number by the total number of arterial segments in that section.[17]

Parenchymal rejection was assessed in allografts on POD 5 for acute rejection and on POD 52 for chronic rejection. The severity of parenchymal rejection was graded with a modified scale of the International Society of Heart and Lung Transplantation (ISHLT) classification.[18] The fibrotic areas in allografts on POD 52 were measured with an image analysis system and scored as follows: 0 (no fibrotic areas), 1 (increased number of interstitial collagen fibers), 2 (0%–10% fibrosis), 3 (10%–50% fibrosis), or 4 (50%–100% fibrosis).

Quantification of mast cells

Mast cell numbers were counted in sections stained with 0.1% toluidine blue (pH 1) and expressed as total number of mast cells/cross sectional area of tissue analyzed (mm^2).

Immunohistochemistry

Frozen sections (5 μm) were fixed in acetone for 10 minutes at 4°C. After washing, sections were incubated with primary antibodies overnight (4°C), biotinylated secondary antibodies at room temperature for 30 minutes, and detected with an avidin-biotin-horseradish peroxidase complex (Nichirei, Tokyo, Japan). AEC (3-amino-9-ethylcarbazole) was used as the chromogen, and counterstaining was with hematoxylin.

Cytokine antibody arrays

Cytokine antibody arrays (Raybiotech, Norcross, GA) were used to identify intragraft expression of the cytokines interferon-γ (IFN-γ), tumor necrosis factor-α (TNF-α), interleukin-2 (IL-2), IL-4, IL-6, IL-10, and IL-17; intracellular (ICAM-1) and vascular cell adhesion molecule (VCAM-1), and the chemokine monocyte chemotactic protein-1 (MCP-1). Membranes were covered with 250 μg of protein from tissue lysates. Integrated densities were calculated using ImageJ 1.38 software (National Institutes of Health, Bethesda, MD).

Statistical analysis

Data are expressed as mean ± standard error of the mean. The Kaplan–Meier analysis and the Mann–Whitney *U*-test were used for statistical evaluation of graft survival and survival differences between 2 groups, respectively. A 2-tailed *t*-test was used for statistical evaluation of parenchymal rejection scores, infiltrating cell number, luminal narrowing, I/M ratio, and fibrotic areas. One-way analysis of variance was used for comparisons between groups for cytokine array data. A value of $p < 0.05$ was considered statistically significant.

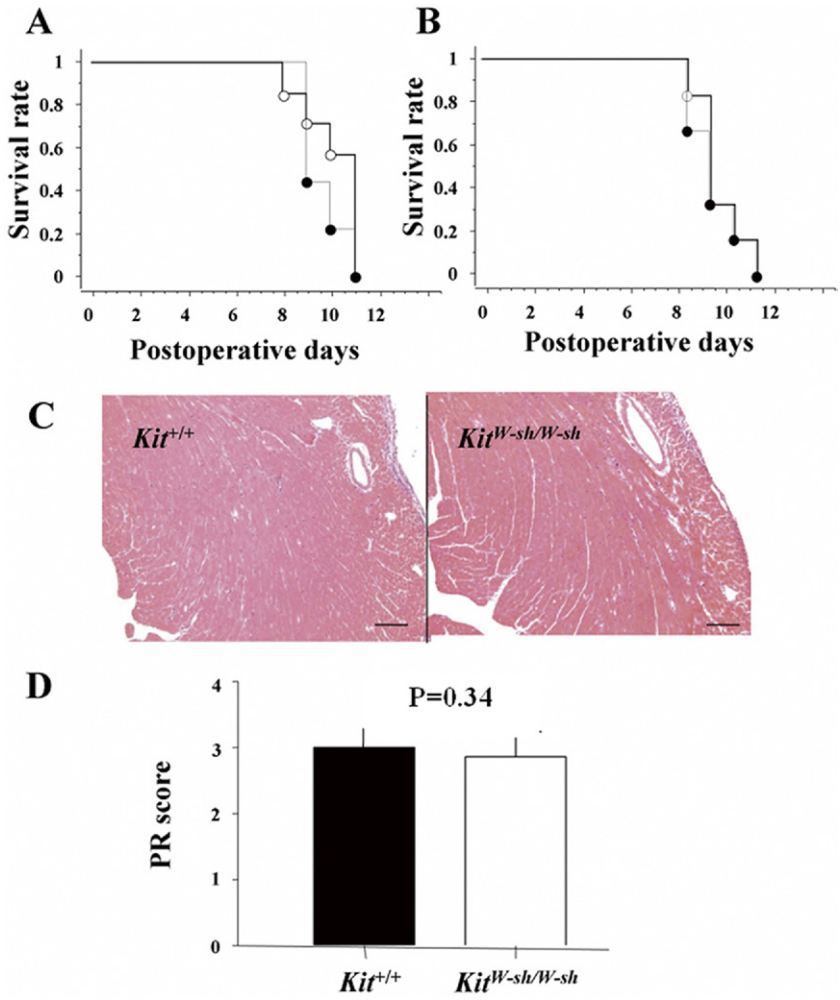

Figure 1 (A) Survival of cardiac allografts in wild-type ($Kit^{+/+}$) mice (filled circles; $n = 8$) or genetically mast cell-deficient ($Kit^{W\text{-}sh/W\text{-}sh}$) mice (open circles; $n = 8$) during acute rejection. (B) Survival of cardiac allografts from donor $Kit^{+/+}$ mice (filled circles; $n = 6$) or mast cell-deficient $Kit^{W\text{-}sh/W\text{-}sh}$ mice (open circles; $n = 6$) transplanted into FVB recipient mice during acute rejection. (C) Histology of cardiac allografts transplanted into $Kit^{+/+}$ or mast cell-deficient $Kit^{W\text{-}sh/W\text{-}sh}$ mice at 5 days after transplantation. The bar = 100 μm; hematoxylin and eosin stain; original magnification × 100. (D) Parenchymal rejection (PR) scores at 5 days after transplantation. Data are the mean and standard error of the mean for 8 mice per group. NS, not significant ($p > 0.05$).

Results

Recipient mast cells are not required for acute rejection of heart transplants

To test whether recipient mast cells can influence the development of acute rejection, donor hearts from FVB mice were transplanted into mast cell-deficient C57BL/6-*Kit*$^{W\text{-}sh/W\text{-}sh}$ mice and the corresponding wild-type control C57BL/6-*Kit*$^{+/+}$ mice. There was no significant difference in acute rejection between donor hearts transplanted into C57BL/6-*Kit*$^{W\text{-}sh/W\text{-}sh}$ or C57BL/6-*Kit*$^{+/+}$ recipients (10.1 ± 1.2 vs 9.7 ± 0.9 days, $p = 0.56$; Figure 1A). Parenchymal rejection scores were also very similar between the 2 groups 5 days after transplantation (Figure 1C). Histologically, the numbers of graft-infiltrating CD4$^+$ lymphocytes, CD8$^+$ lymphocytes, and CD11b$^+$ cells (macrophages) in allografts at 5 days were somewhat higher in C57BL/6-*Kit*$^{+/+}$ than in C57BL/6-*Kit*$^{W\text{-}sh/W\text{-}sh}$ mice, but these differences did not achieve statistical significance (Figure 2). Similarly, although levels of immunoreactivity for certain cytokines, MCP-1, ICAM-1, and VCAM-1 were higher in donor hearts transplanted into wild-type mice than in those transplanted into C57BL/6-*Kit*$^{W\text{-}sh/W\text{-}sh}$ mice, only the difference in VCAM-1 levels achieved statistical significance (Figure 3).

These experiments indicate that in our acute model of cardiac allograft rejection, the allografts can be acutely rejected by mast cell-deficient C57BL/6-*Kit*$^{W\text{-}sh/W\text{-}sh}$ mice as rapidly as by wild-type mice. Although cardiac allografts undergoing acute rejection in C57BL/6-*Kit*$^{W\text{-}sh/W\text{-}sh}$ mice developed slightly reduced levels of graft-infiltrating lymphocytes and macrophages, cytokines, and adhesion molecules than did the corresponding wild-type mice, except for levels of VCAM-1, such differences did not achieve statistical significance. Thus, even if mast cells in recipient mice might have slightly enhanced inflammation in the allografts during acute rejection, these effects did not significantly influence the ultimate outcome of the process: acute graft rejection.

Mast cells in recipient mice are not required for the development of features of chronic rejection

The number of donor heart mast cells has been reported to correlate positively with the severity of GCAD in human hearts.[8] To test whether mast cells can influence the development of chronic cardiac allograft rejection, donor hearts from Bm12 mice were transplanted into mast cell-deficient C57BL/6-*Kit*$^{W\text{-}sh/W\text{-}sh}$ mice and the corresponding wild-type control C57BL/6-*Kit*$^{+/+}$ mice. On POD 52, mast cell numbers in allografts from C57BL/6-*Kit*$^{+/+}$ recipients ($2.5 \pm 0.4/mm^2$) were significantly higher than the

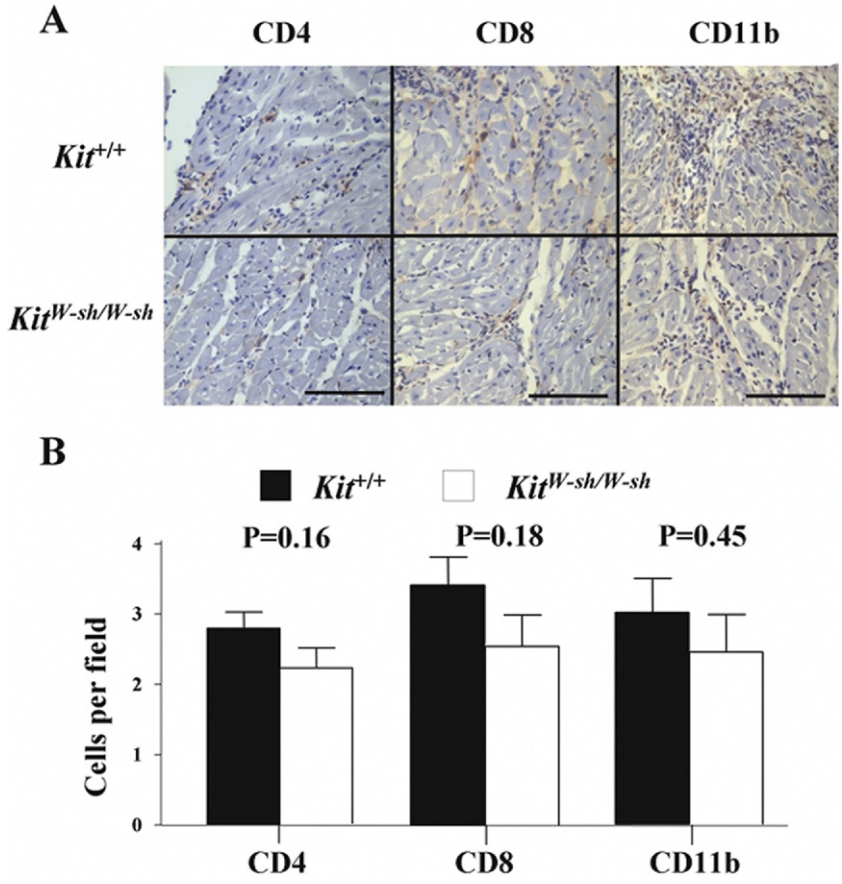

Figure 2 (A) Immunohistochemical staining of CD4$^+$, CD8$^+$, and CD11b$^+$ cells in allografts at 5 days after transplantation during acute rejection. The bar = 100 μm; original magnification $\times$400. (B) Quantitative analysis of CD4$^+$, CD8$^+$, and CD11b$^+$ cells. Data are the mean and standard error of the mean of values derived from an analysis of 20 fields.

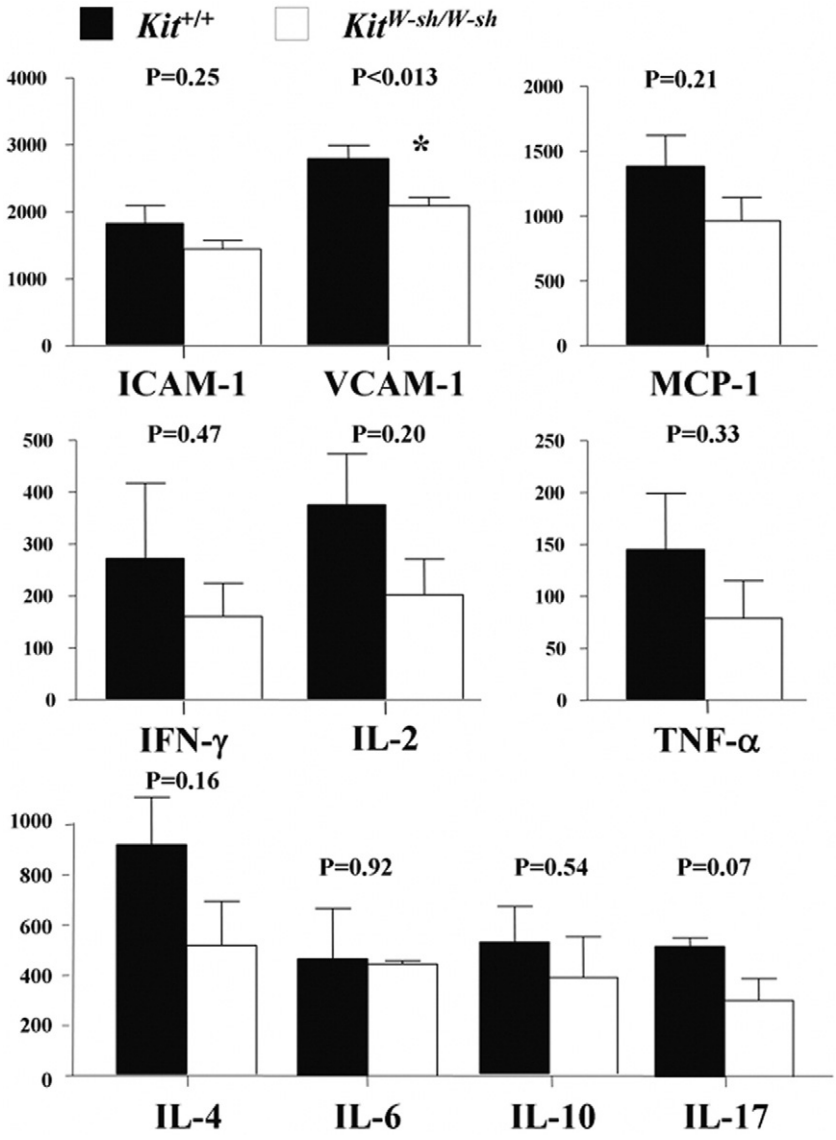

Figure 3 Expression of adhesion molecules, cytokines, and the chemokine monocyte chemotactic protein-1 (MCP-1) is shown during acute rejection in allografts transplanted into $Kit^{+/+}$ mice (filled bars; $n = 6$) or $Kit^{W\text{-}sh/W\text{-}sh}$ mice (open bars; $n = 6$). *$p < 0.05$ vs values for $Kit^{+/+}$ mice. Data show the mean and standard error of the mean in each group. ICAM, intracellular cell adhesion molecule; IFN-γ, interferon-γ; IL, interleukin; VCAM, vascular cell adhesion molecule.

corresponding values in non-transplanted donor hearts ($1.2 \pm 0.1/mm^2$), isografts transplanted into syngeneic Bm12 mice ($1.1 \pm 0.1/mm^2$), or allografts transplanted into C57BL/6-$Kit^{W\text{-}sh/W\text{-}sh}$ mice ($0.9 \pm 0.1/mm^2$; Figure 4).

After 52 days, there was no significant difference in the beating scores for cardiac allografts in wild-type (C57BL/6-$Kit^{+/+}$) vs mast cell-deficient (C57BL/6-$Kit^{W\text{-}sh/W\text{-}sh}$) recipient mice (Figure 5A). At POD 52, neointimal thickening and luminal narrowing were 42.0% ± 9.3% in C57BL/6-$Kit^{W\text{-}sh/W\text{-}sh}$ mice vs 43.8% ± 13.5% in C57BL/6-$Kit^{+/+}$ recipients ($p = 0.91$; Figure 5B). GCAD severity, assessed by the mean percentage of luminal narrowing, the I/M ratio, and the percentage of diseased vessels, was also virtually identical between the 2 groups (Figure 5C).

We also detected no differences between wild-type C57BL/6-$Kit^{+/+}$ or mast cell-deficient C57BL/6-$Kit^{W\text{-}sh/W\text{-}sh}$ recipient mice in the histologic appearance of the allografts on POD 52 (Figure 6A) or in the parenchymal rejection or fibrotic scores (Figure 6B). The numbers of infiltrating $CD4^+$ lymphocytes, $CD8^+$ lymphocytes, and $CD11b^+$ cells did not differ significantly between wild-type C57BL/6-$Kit^{+/+}$ and C57BL/6-$Kit^{W\text{-}sh/W\text{-}sh}$ recipient mice (Figure 6C). Finally, the expression of levels of allograft immunoreactivity for the adhesion molecules ICAM-1 and VCAM-1, the cytokines IFN-γ, IL-2, IL-4, IL-6, IL-10, IL-17, and TNF-α, and the chemokine MCP-1, were also very similar in wild-type (C57BL/6-$Kit^{+/+}$) and mast cell-deficient (C57BL/6-$Kit^{W\text{-}sh/W\text{-}sh}$) recipient mice (data not shown).

Taken together, these findings do not support the hypothesis that mast cells in the tissues of recipient C57BL/6 mice contribute significantly to cardiac allograft fibrosis or chronic rejection in this model.

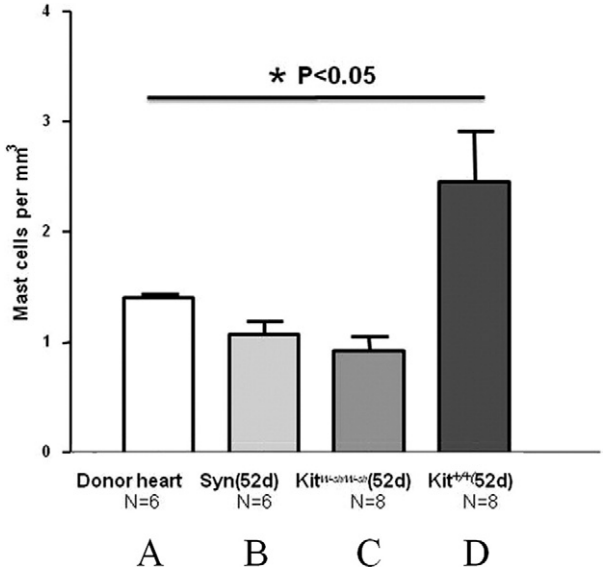

Figure 4 (A) Baseline mast cell numbers in non-transplanted donor hearts. (B) Mast cell numbers/mm^2 detected in Bm12 allografts transplanted into Bm12 mice (isograft controls). (C) Bm12 allografts transplanted into C57BL/6-*Kit*$^{W\text{-}sh/W\text{-}sh}$ mice. (D) Mast cell numbers/mm^2 in allografts from C57BL/6-*Kit*$^{+/+}$ recipients 52 days after transplantation. The error bar shows the standard error of the mean.

Discussion

It is now appreciated that mast cells can have either positive or negative roles in innate or adaptive immune responses.[3,19] This new understanding permits the reconsideration of hypotheses about mast cell function that was based on morphologic observations. For example, because rejection severity seemed to correlate with mast cell numbers in human post-transplant heart biopsy specimens,[8] several laboratories have focused on mechanisms by which mast cells might promote transplant rejection.

By contrast, 2 recent reports have presented evidence that mast cells can enhance allograft survival. Experiments in genetically mast cell-deficient C57BL/6-*Kit*$^{W\text{-}sh/W\text{-}sh}$ mice indicate that mast cells can promote peripheral tolerance of skin allografts, which is a process dependent on Treg.[20] The conclusion that mast cells can have effects that promote allograft survival is also supported by the findings of Boerma et al,[10] who investigated heterotopic cardiac transplantation using genetically mast cell-deficient out-bred *Ws/Ws* rats. *Ws/Ws* rats are profoundly deficient in mast cells because of a 12-base deletion in the tyrosine kinase domain of c-*kit* that results in marked loss of function in KIT.[21,22] At 12 weeks after heterotopic transplantation, the survival of *Ws/Ws* hearts transplanted into *Ws/Ws* rats (3 of 7 hearts) was significantly reduced compared with that of the corresponding wild-type hearts transplanted into wild-type rats (8 of 8, $p = 0.015$).

As acknowledged by the authors, however, these findings[10] must be interpreted cautiously, because the *Ws/Ws* and corresponding wild-type rats used were the F2 generation of hybrids of BN/*f*Mai-*Ws*/+ and wild-type Donryu strain animals. In addition, the rats in that study were receiving treatment with cyclosporine to induce immunosuppression.

The mast cell-deficient mouse model used in this study (C57BL/6-*Kit*$^{W\text{-}sh/W\text{-}sh}$) permits the analysis of allograft rejection in mast cell-deficient and wild-type mice on the same genetic background. Moreover, the analysis was performed without treating the mice with immunosuppressive agents, which might influence the function of mast cells as well as T cells.[23]

The central finding of this study was that recipient mast cells do not appear to have essential positive or negative involvement in acute or chronic cardiac allograft rejection in our model. These findings are of interest, because mast cells can have many effects with the potential to influence several aspects of inflammation, tissue remodeling, and T lymphocyte migration and activation.[3,24] However, even though numbers of graft-infiltrating CD4$^+$ or CD8$^+$ lymphocytes and CD11b$^+$ cells in allografts at POD 5 in the acute rejection model were somewhat higher in C57BL/6-*Kit*$^{+/+}$ than in C57BL/6-*Kit*$^{W\text{-}sh/W\text{-}sh}$ mice, these differences did not achieve statistical significance. Similarly, although levels of immunoreactivity for certain cytokines, MCP-1, ICAM-1, and VCAM-1, were higher in donor hearts transplanted into wild-type than C57BL/6-*Kit*$^{W\text{-}sh/W\text{-}sh}$ mice, only the difference in VCAM-1 levels achieved statistical significance. These results are consistent with the possibility that mast cells contribute to leukocyte recruitment and survival and to cytokine and chemokine production in the acute rejection model; however, cardiac allografts transplanted into wild type or C57BL/6-*Kit*$^{W\text{-}sh/W\text{-}sh}$ mice had virtually identical parenchymal rejection scores and, importantly, survival curves.

Our prior chronic rejection studies showed that the development of GCAD is absolutely contingent on the presence of CD4$^+$ lymphocytes, and that CD8$^+$ lymphocytes augment GCAD severity.[25,26] Interestingly, although the allograft mast cell number was increased in the chronic rejection model in the wild-type but not mast cell-deficient recipients, this did not correlate with any substantial differences in donor heart mononuclear cell recruitment or subsequent allograft arteriosclerotic changes. As in the acute rejection model, numbers of CD4$^+$ lymphocytes in the allografts were somewhat higher in wild-type recipients than in C57BL/6-*Kit*$^{W\text{-}sh/W\text{-}sh}$ recipients. Although the importance of this finding is uncertain, it offers further support to the notion that mast cells in recipient mice may contribute marginally to lymphocyte recruitment and/or survival in allografts in this setting.

Notably, Boerma et al[10] also detected fewer T lymphocytes in mast cell-deficient donor hearts transplanted into mast cell-deficient rats than in the corresponding wild-type donor hearts transplanted into wild type rats; despite this finding, the mast cell-deficient allografts transplanted into mast cell-deficient rats had slightly (but not significantly) higher rejection scores.[10] They attributed these findings to reduced transforming growth factor-β (TGF-β) levels in the mast cell-deficient recipients, suggesting that TGF-β, which

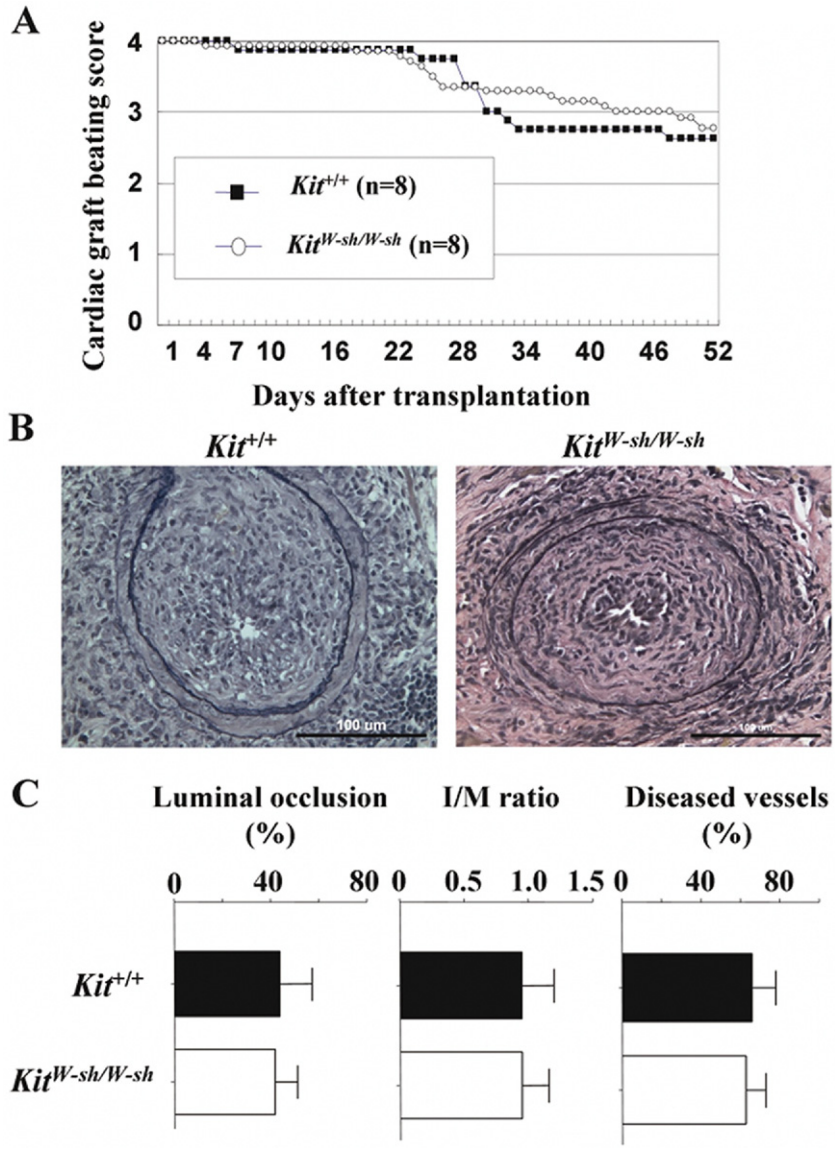

Figure 5 (A) The graft beating scores of allografts in $Kit^{+/+}$ mice (filled squares; $n = 8$) and $Kit^{W\text{-}sh/W\text{-}sh}$ mice (open circles; $n = 8$) after cardiac transplantation in the chronic rejection model, consisting of an major histocompatibility complex class II mismatch. (B) Elastic Van Gieson-stained representative sections of cardiac allografts undergoing chronic rejection 52 days after transplantation into $Kit^{+/+}$ or $Kit^{W\text{-}sh/W\text{-}sh}$ mice. Original magnification ×400. (C) Data from morphometric assessment of luminal occlusion, intima/media (I/M) ratio, and diseased vessels in sections like those shown in Panel A. Data are the mean and standard error of the mean for 8 mice per group.

can be secreted by mast cells,[27] may be anti-inflammatory and help to prevent rejection in this model.

Although they are known both to produce chemokines/cytokines and to influence chemokine/cytokine secretion in other cells types,[3] mast cells in recipient mice did not appear to alter substantially the levels of immunoreactive cytokines or of the chemokine MCP-1 in the cardiac allografts. The differences did not achieve statistical significance, but levels of most cytokines and MCP-1 at POD 5 in the acute rejection model were somewhat higher in the allografts transplanted into the wild-type mice than they were in the grafts from mast cell-deficient recipients. However, levels of cytokines and MCP-1 in allografts undergoing chronic rejection, as assessed at POD 52, were nearly identical in the 2 groups. The cytokines measured included IFN-γ, a cytokine that has an important effect in the development of GCAD in this strain combination. Indeed, Tellides et al[28] reported that IFN-γ alone induced intimal proliferation in human arterial loops that were transplanted into immunodeficient mice. Furthermore, we did not detect significant differences in IL-17 levels, even though mean levels of IL-17 immunoreactivity in allografts examined on POD 5 in the acute rejection model were about 74% higher in wild-type than in mast cell-deficient mice ($p = 0.07$).

Given the complexity of attempting to understand how mast cells might influence cardiac allograft rejection, it is important to acknowledge the limitations of our study. First, we of course cannot rule out any contributions of resident donor organ mast cells (ie, passenger mast cells) to allograft rejection in our models. Mast cells normally are present in the healthy hearts of animals and humans. However, we found that when mast cell-deficient $Kit^{W\text{-}sh/W\text{-}sh}$ or wild-type

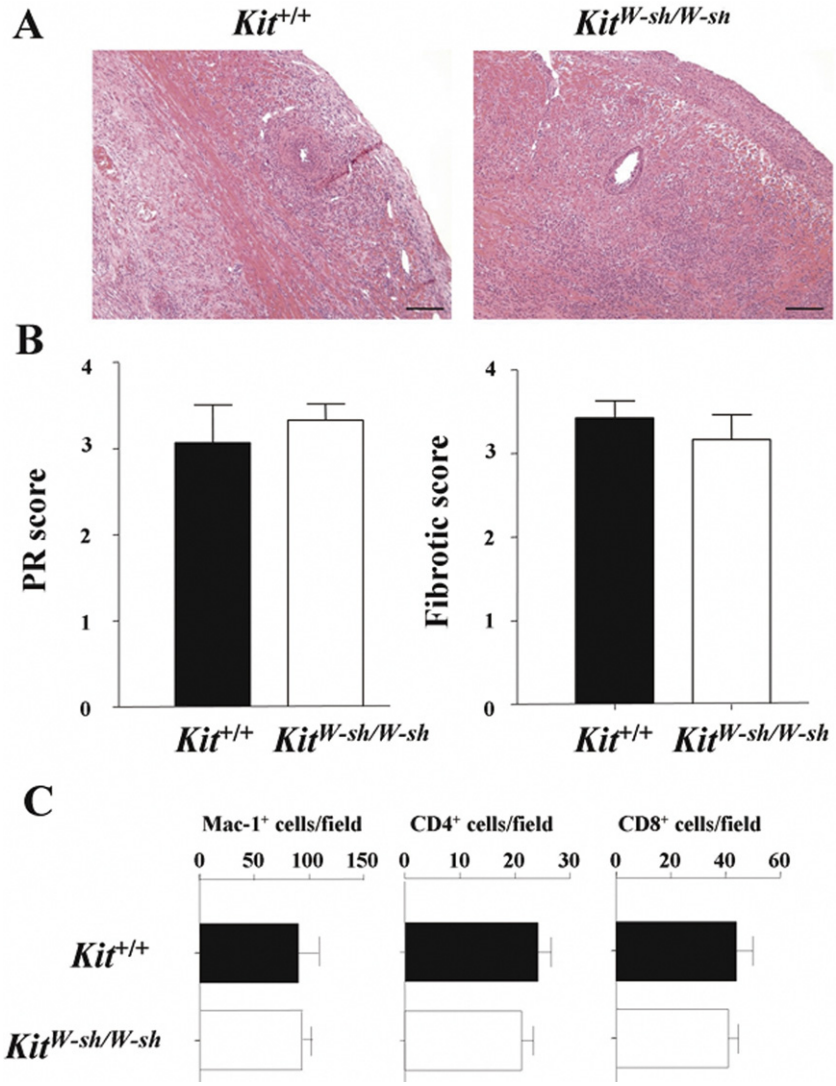

Figure 6 (A) Representative sections of cardiac allografts undergoing chronic rejection 52 days after transplantation into $Kit^{+/+}$ or $Kit^{W\text{-}sh/W\text{-}sh}$ mice. The bar = 100 μm; hematoxylin and eosin stain; original magnification ×100. (B) Parenchymal rejection (PR) and fibrotic scores in sections like those shown in Panel A. Data are the mean and standard error of the mean (SEM) from 8 mice per group. (C) Quantitative analysis of $CD4^+$, $CD8^+$, and $CD11b^+$ cells. Data are the mean and SEM of values derived from an analysis of 20 fields with 6 mice per group.

$Kit^{+/+}$ hearts were transplanted into mast cell-competent FVB ($H\text{-}2^q$) recipients, the differences in graft survival between donor $Kit^{W\text{-}sh/W\text{-}sh}$ and $Kit^{+/+}$ hearts were not significant (10.3 ± 0.5 vs 10.2 ± 0.5 days, $p = 0.56$; Figure 1B; preliminary data, $n = 3$). Moreover, Zweifel et al[29] have suggested that recipient mast cells, rather than donor mast cells, may be more important in cardiac allograft rejection in rats.[29]

Second, although $Kit^{W\text{-}sh/W\text{-}sh}$ mice are not anemic, they do exhibit cardiomegaly and increased numbers of blood neutrophils and platelets[14] and may have other abnormalities yet to be defined. The genetic inversion noted in $Kit^{W\text{-}sh/W\text{-}sh}$ mice results in the interruption of corin, a cardiac protease responsible for the activation of atrial natriuretic peptide and, consequently, mild hypertension and cardiomegaly. Some of the other phenotypic abnormalities in $Kit^{W\text{-}sh/W\text{-}sh}$ mice may possibly compensate in part for their lack of certain mast cell functions that may be important in acute or chronic cardiac allograft rejection. For example, it is possible that neutrophilia and thrombocytosis could influence the pathogenesis of GCAD in $Kit^{W\text{-}sh/W\text{-}sh}$ mice.

Third, mice in the chronic rejection model were sacrificed for examination of donor hearts at POD 52. It is possible that differences in numbers of infiltrating cells, levels of immunoreactive cytokines, or rejection scores might have been detected if the animals were sacrificed at earlier or later time points.

In conclusion, despite the limitations of our approach, we believe our data strongly support the hypothesis that recipient mast cells are not required for the development of either acute or chronic cardiac allograft rejection in C57BL/6 mice.

Disclosure statement

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References

1. Taylor DO, Edwards LB, Boucek MM, et al. Registry of the International Society for Heart and Lung Transplantation: twenty-fourth official adult heart transplant report—2007. J Heart Lung Transplant 2007;26:769-81.
2. Weis M, von Scheidt W. Cardiac allograft vasculopathy: a review. Circulation 1997;96:2069-77.
3. Galli SJ, Grimbaldeston M, Tsai M. Immunomodulatory mast cells: negative, as well as positive, regulators of immunity. Nature reviews. Immunology 2008;8:478-86.
4. Sun J, Sukhova GK, Wolters PJ, et al. Mast cells promote atherosclerosis by releasing proinflammatory cytokines. Nat Med 2007;13:719-24.
5. Kovanen PT. Mast cells and degradation of pericellular and extracellular matrices: potential contributions to erosion, rupture and intraplaque hemorrhage of atherosclerotic plaques. Biochem Soc Trans 2007;35:857-61.
6. Bot I, de Jager SC, Zernecke A, et al. Perivascular mast cells promote atherogenesis and induce plaque destabilization in apolipoprotein E-deficient mice. Circulation 2007;115:2516-25.
7. Sun J, Sukhova GK, Yang M, et al. Mast cells modulate the pathogenesis of elastase-induced abdominal aortic aneurysms in mice. J Clin Invest 2007;117:3359-68.
8. Li QY, Raza-Ahmad A, MacAulay MA, et al. The relationship of mast cells and their secreted products to the volume of fibrosis in posttransplant hearts. Transplantation 1992;53:1047-51.
9. Koskinen PK, Kovanen PT, Lindstedt KA, Lemstrom KB. Mast cells in acute and chronic rejection of rat cardiac allografts—a major source of basic fibroblast growth factor. Transplantation 2001;71:1741-47.
10. Boerma M, Fiser WP, Hoyt G, et al. Influence of mast cells on outcome after heterotopic cardiac transplantation in rats. Transpl. Int 2007;20:256-65.
11. Grimbaldeston MA, Chen CC, Piliponsky AM, Tsai M, Tam SY, Galli SJ. Mast cell-deficient W-sash c-kit mutant $Kit^{W\text{-}sh/W\text{-}sh}$ mice as a model for investigating mast cell biology in vivo. Am J Pathol 2005;167:835-48.
12. Nagle DL, Kozak CA, Mano H, Chapman VM, Bucan M. Physical mapping of the Tec and Gabrb1 loci reveals that the Wsh mutation on mouse chromosome 5 is associated with an inversion. Hum Mol Genet 1995;4:2073-79.
13. Berrozpe G, Agosti V, Tucker C, Blanpain C, Manova K, Besmer P. A distant upstream locus control region is critical for expression of the Kit receptor gene in mast cells. Mol Cell Biol 2006;26:5850-60.
14. Nigrovic PA, Gray DH, Jones T, et al. Genetic inversion in mast cell-deficient W^{sh} mice interrupts Corin and manifests as hematopoietic and cardiac aberrancy. Am J Pathol 2008;173:1693-701.
15. Duttlinger R, Manova K, Chu TY, et al. W-sash affects positive and negative elements controlling c-kit expression: ectopic c-kit expression at sites of kit-ligand expression affects melanogenesis. Development 1993;118:705-17.
16. Corry RJ, Winn HJ, Russell PS. Primarily vascularized allografts of hearts in mice. The role of H-2D, H-2K, and non-H-2 antigens in rejection. Transplantation 1973;16:343-50.
17. Armstrong AT, Strauch AR, Starling RC, Sedmak DD, Orosz CG. Morphometric analysis of neointimal formation in murine cardiac allografts. Transplantation 1997;63:941-47.
18. Billingham ME, Cary NR, Hammond ME, et al. A working formulation for the standardization of nomenclature in the diagnosis of heart and lung rejection: Heart Rejection Study Group. The International Society for Heart Transplantation. J. Heart Lung Transplant 1990;9:587-93.
19. Metz M, Grimbaldeston MA, Nakae S, Piliponsky AM, Tsai M, Galli SJ. Mast cells in the promotion and limitation of chronic inflammation. ImmunolRev 2007;217:304-28.
20. Lu LF, Lind EF, Gondek DC, et al. Mast cells are essential intermediaries in regulatory T-cell tolerance. Nature 2006;442:997-1002.
21. Niwa Y, Kasugai T, Ohno K, et al. Anemia and mast cell depletion in mutant rats that are homozygous at "white spotting (Ws)" locus. Blood 1991;78:1936-41.
22. Tsujimura T, Hirota S, Nomura S, et al. Characterization of Ws mutant allele of rats: a 12-base deletion in tyrosine kinase domain of c-kit gene. Blood 1991;78:1942-46.
23. Wershil BK, Furuta GT, Lavigne JA, Choudhury AR, Wang ZS, Galli SJ. Dexamethasone or cyclosporin A suppress mast cell-leukocyte cytokine cascades. Multiple mechanisms of inhibition of IgE- and mast cell-dependent cutaneous inflammation in the mouse. J Immunol 1995;154:1391-98.
24. Mekori YA, Metcalfe DD. Mast cell-T cell interactions. J Allergy Clin Immunol 1999;104:517-23.
25. Fischbein MP, Yun J, Laks H, et al. CD8+ lymphocytes augment chronic rejection in a MHC class II mismatched model. Transplantation 2001;71:1146-53.
26. Fischbein MP, Yun J, Laks H, et al. Regulated interleukin-10 expression prevents chronic rejection of transplanted hearts. J Thorac Cardiovasc Surg 2003;126:216-23.
27. Gordon JR, Galli SJ. Promotion of mouse fibroblast collagen gene expression by mast cells stimulated via the Fc epsilon RI. Role for mast cell-derived transforming growth factor beta and tumor necrosis factor alpha. J Exp Med 1994;180:2027-37.
28. Tellides G, Tereb DA, Kirkiles-Smith NC, et al. Interferon-gamma elicits arteriosclerosis in the absence of leukocytes. Nature 2000;403:207-11.
29. Zweifel M, Hirsiger H, Matozan K, Welle M, Schaffner T, Mohacsi P. Mast cells in ongoing acute rejection: increase in number and expression of a different phenotype in rat heart transplants. Transplantation 2002;73:1707-16.