Loss of Myeloid Related Protein-8/14 Exacerbates Cardiac Allograft Rejection

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Background—The calcium-binding proteins myeloid-related protein (MRP)-8 (S100A8) and MRP-14 (S100A9) form MRP-8/14 heterodimers (S100A8/A9, calprotectin) that regulate myeloid cell function and inflammatory responses and serve as early serum markers for monitoring acute allograft rejection. Despite functioning as a proinflammatory mediator, the pathophysiological role of MRP-8/14 complexes in cardiovascular disease is incompletely defined. This study investigated the role of MRP-8/14 in cardiac allograft rejection using MRP-14<sup>-/-</sup> mice that lack MRP-8/14 complexes.

Methods and Results—We examined parenchymal rejection after major histocompatibility complex class II allomismatched cardiac transplantation (bm12 donor heart and B6 recipients) in wild-type (WT) and MRP-14<sup>-/-</sup> recipients. Allograft survival averaged 5.9±2.9 weeks (n=10) in MRP-14<sup>-/-</sup> recipients compared with >12 weeks (n=15; P<0.0001) in WT recipients. Two weeks after transplantation, allografts in MRP-14<sup>-/-</sup> recipients had significantly higher parenchymal rejection scores (2.8±0.8; n=8) than did WT recipients (0.8±0.8; n=12; P<0.0001). Compared with WT recipients, allografts in MRP-14<sup>-/-</sup> recipients had significantly increased T-cell and macrophage infiltration and increased mRNA levels of interferon- γ and interferon-γ-associated chemokines (CXCL9, CXCL10, and CXCL11), interleukin-6, and interleukin-17 with significantly higher levels of Th17 cells. MRP-14<sup>-/-</sup> recipients also had significantly more lymphocytes in the adjacent para-aortic lymph nodes than did WT recipients (cells per lymph node: 23.7±0.7×10<sup>5</sup> for MRP-14<sup>-/-</sup> versus 6.0±0.2×10<sup>5</sup> for WT; P<0.0001). The dendritic cells (DCs) of the MRP-14<sup>-/-</sup> recipients of bm12 hearts expressed significantly higher levels of the costimulatory molecules CD80 and CD86 than did those of WT recipients 2 weeks after transplantation. Mixed leukocyte reactions with allo–endothelial cell–primed MRP-14<sup>-/-</sup> DCs resulted in significantly higher antigen-presenting function than reactions using WT DCs. Ovalbumin-primed MRP-14<sup>-/-</sup> DCs augmented proliferation of OT-II (ovalbumin-specific T cell receptor transgenic) CD4<sup>+</sup> T cells with increased interleukin-2 and interferon-γ production. Cardiac allografts of B6 major histocompatibility complex class II<sup>-/-</sup> hosts and of B6 WT hosts receiving MRP-14<sup>-/-</sup> DCs had significantly augmented inflammatory cell infiltration and accelerated allograft rejection compared with WT DCs from transferred recipient allografts. Bone marrow–derived MRP-14<sup>-/-</sup> DCs infected with MRP-8 and MRP-14 retroviral vectors showed significantly decreased CD80 and CD86 expression compared with controls, indicating that MRP-8/14 regulates B7-costimulatory molecule expression.

Conclusions—Our results indicate that MRP-14 regulates B7 molecule expression and reduces antigen presentation by DCs and subsequent T-cell priming. The absence of MRP-14 markedly increased T-cell activation and exacerbated allograft rejection, indicating a previously unrecognized role for MRP-14 in immune cell biology. (Circulation. 2011;124:2920-2932.)

Key Words: heart transplantation ■ immune system ■ antigen-presenting cells ■ dendritic cells

The calcium-binding proteins myeloid-related protein (MRP)-8 (S100A8, calgranulin A) and MRP-14 (S100A9, calgranulin B) belong to the S100 protein family. MRP-14 and MRP-8 form homodimers, heterodimers, and higher-order complexes, although the MRP-8/14 heterodimer (S100A8/A9, calprotectin) is the dominant extracellular form in humans. MRP-8/14 heterodimers constitute ~45% of human neutrophil, ~1% of human monocyte, and 10% to

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≈20% of murine neutrophil cytosolic proteins.1,3–5 Myeloid cells such as neutrophils, monocytes, and dendritic cells (DCs), along with activated macrophages, platelets, and megakaryocytes, express MRP-8 and MRP-14, whereas most nonactivated macrophages, T cells, and B cells do not express them.3,6–8

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MRP-8/14 heterodimers translocate from cytoplasm to the cytoskeleton and membranes of phagocytes on elevation of intracellular calcium concentration,9 and secreted extracellular MRP-8/14 enhances CD11b/CD18 integrin-binding activity on phagocytes,10,11 promoting transendothelial migration of phagocytes. Vascular endothelium expresses several classes of MRP-8/14 receptors, including toll-like receptor-4,12 receptor for advanced glycation end products,13 CD36,14 special carboxylated N-glycans,15 and heparin-like glycoaminoglycans.16 MRP-8/14 complexes also contribute to wound repair,17 have antiproliferative effects on monocytes/macrophages and lymphocytes,18,19 and inhibit the growth of fibroblasts.20 MRP-8/14 complexes may participate in the pathogenesis of cardiovascular disease and allograft rejection.8,21 MRP-8/14–expressing macrophages appear during the early phase of cardiac allograft rejection.22 MRP-8/14 is a very early serum marker of acute rejection, with high sensitivity (67%) and specificity (100%).23 In a study of 56 patients with acute renal allograft rejection, elevated MRP-8/14 serum levels preceded acute rejection episodes by a median of 5 days, and a 3-day course of intravenous methylprednisolone therapy significantly reduced MRP-8/14 serum levels.

Conversely, previous work in transplantation showed that a subpopulation of monocytes lacking MRP-8/14 expression associate with chronic allograft rejection.24 Moreover, human renal allograft recipients without allograft vascular disease had significantly higher MRP-8/14 levels shortly after transplantation compared with lower levels in those recipients that developed vascular disease. Thus, MRP-8/14 has uncertain roles in transplantation biology.

This study investigated the role of MRP-14 in cardiac allograft rejection using MRP-14−/− mice lacking MRP-8/14 complexes.25,26 The results show that host MRP-14 deficiency augmented antigen presentation by DCs, markedly increased T-cell activation, and exacerbated allograft rejection.

Methods

Animals

C57BL/6 (B6; H-2b, I-Ak), B6.C-H2<bm12>KhEg (bm12; H-2b<bm12>, I-Ak), B6.129S7-Rag2<tm1Mom>tg(scid) transgenic (TcraTcrb) 425Cbn (Rag1 knockout/OT-II (ovalbumin-specific) T-cell receptor transgenic, H-2b), and BALB/c (B/c, H-2d, I-Ad) mice were obtained from Taconic Farm (Hudson, NY) or The Jackson Laboratory (Bar Harbor, ME). MRP-14−/− mice were generated with GK129 embryonic stem cells and backcrossed 12 times on the B6 background as described previously.25 Mice were maintained on acidified water in a pathogen-free facility. The National Institutes of Health, and the American Association for Accreditation of Laboratory Animal Care and performed in accordance with the guidelines of the American Association for Accreditation of Laboratory Animal Care and the National Institutes of Health.

Vascularized Heterotopic Cardiac Transplantation

B/c (total allomismatch) or bm12 (major histocompatibility complex [MHC] class II mismatch) donor hearts were transplanted heterotopically into B6 recipients without immunosuppression, as shown previously (details in the online-only Data Supplement).27 Because minor histoincompatibility can significantly influence alloimmune responses, we examined inflammatory responses in B6 wild-type (WT) cardiac allografts in MRP-14−/− recipients. We did not detect any inflammatory cell accumulation in the B6 WT cardiac allografts in MRP-14−/− recipients 4 weeks after transplantation (Figure 1 in the online-only Data Supplement), indicating that variation in genetic background between WT and MRP-14−/− mice does not account for differences in the cardiac transplantation assays.

Graft Harvest

Harvested allografts were sectioned transversely into 3 parts. In sectioned hearts, the most basal part was used for routine hematoxylin and eosin morphological examination. A second midtransverse section was frozen for immunohistochemical staining, and the apical portion was used for total RNA extraction for measuring mRNA levels of cytokines and chemokines by quantitative real-time polymerase chain reaction (PCR).28 For cellular extraction, hearts were digested at 37°C in 2 mg/mL collagenase (Sigma-Aldrich, St. Louis, MO) and 2% bovine serum albumin in buffered saline, followed by straining and Ficoll density gradient centrifugation (Organon Teknika, Durham, NC).27

Quantification of mRNA by Real-Time, Quantitative Reverse-Transcription PCR

mRNA levels of cytokines and chemokines were quantified from cardiac allografts harvested 2 weeks after transplantation or from in vitro cultured marrow-derived DCs with a LightCycler-based real-time PCR. Quantitative reverse-transcription PCR protocols used the LightCycler-DNA Master SYBR Green I Kit as described previously.29 Total RNA was extracted from cardiac allografts with Trizol (Invitrogen, Carlsbad, CA) and purified with the RNeasy kit (Qiagen, Valencia, CA); CDNA was synthesized with a First-Strand cDNA Synthesis Kit, followed by DNase treatment (Invitrogen). The TagStart antibody (CLONTECH, Palo Alto, CA) was used to prevent generation of nonspecific amplification products. Quantification was performed with primers designed by the Primer3 program (www.genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi). The mRNA levels of the various genes tested were normalized to GAPDH as an internal control. Data represent the mean±SEM of 6 to 7 determinations of the rates of mRNA expression relative to the control WT recipient allografts, which were set to 1.

Mixed Lymphocyte Reaction, T-Cell Proliferation Assay, and Cytokine ELISA

One-way mixed lymphocyte reaction (MLR) was performed as described previously with irradiated BALB/c splenocytes as stimulators and B6 splenocytes as responders. T-cell or B-cell proliferation assays were performed with anti-CD3 antibody–coated 96-well plates or by adding anti-CD40 monoclonal antibodies (3/23, BD Pharmingen), respectively.20 We performed primed MLR by coculturing B6 WT or B6 MRP-14−/− marrow-derived DCs with bm12 endothelial cells (ECs) for 72 hours. ECs were isolated from the bm12 murine hearts as described previously.29,30 These DCs were then used as stimulators for naïve B6 T-cell responders. T cells were extracted from naïve B6 WT splenocytes with MACS beads (Miltenyi Biotec Inc, Auburn, CA) with negative selection according to the manufacturer’s instructions. We cocultured irradiated allo-EC–primed DCs (50,000 cells per well) and naïve B6 T cells (500,000 cells per well, stimulator:responder=1:10) in 96-well plates and measured T-cell proliferation by use of [3H]-thymidine incorporation.29 Nonprimed DCs were used as negative controls.

Bone Marrow–Derived DC Preparation for Allo-EC–Primed MLR and/or Flow Cytometry

Bone marrow from femurs and tibias from 8- to 12-week-old male B6 WT or B6 MRP-14−/− mice was flushed with RPMI-1640 with a
syringe with a 27G needle. Clusters within the marrow suspension were dissociated by vigorous pipetting and filtered through a 70-μm cell strainer. Erythrocytes were lysed with ACK lysis buffer (0.15 mol/L NH₄Cl, 1.0 mmol/L KHCO₃, 0.1 mmol/L EDTA). Approximately 1 to 2×10⁵ marrow cells were obtained per mouse. Bone marrow cells were suspended in complete media (C10, RPMI-1640) supplemented with 10% FBS, 2 mmol/L L-glutamine, 1% nonsessential amino acids, 100 U/mL penicillin, and 100 μg/mL streptomycin. All culture reagents were purchased from Life Technologies (Rockville, MD). Ten million cells in 5 mL C10 were cultured in 6-cm dishes for up to 7 days in the presence of 1000 U/mL of granulocyte macrophage colony-stimulating factor and interleukin (IL)-4 (R&D Systems, Minneapolis, MN) at 37°C and 5% CO₂. On days 2 and 4 of culture, floating and loosely adherent cells were discarded, and 50% of the media was replaced with fresh media containing the same amount of cytokines. Monocytes cultured in this cytokine combination for this duration developed into immature DCs that characteristically bear high endocytic and macropinocytic activity and low expression of costimulatory signals. On day 7, these DCs were cocultured with allo-ECs for 72 hours to allow the DCs to process antigen and develop into mature DCs; DCs cultured without allo-ECs were used as negative controls. On day 10, CD11c⁺ DCs were recovered with anti-mouse CD11c antibody-bound magnetic beads (Miltenyi Biotec Inc) according to the manufacturer’s instructions. DCs were irradiated (3000 rad) and cultured at 5×10⁵ cells per well in 96-well dishes with naïve B6 T cells at 5×10⁵ cells per well. For flow cytometry analysis, WT and MRP-14⁺⁻ DCs were first stimulated with 500 U/mL interferon-γ (IFN-γ) for 18 hours.

**Cellular Surface Staining and Flow Cytometric Analysis**

IFN-γ-stimulated DCs were analyzed by flow cytometry after surface staining with the use of described previously methods. Antibodies included anti–MHC II-phycocyanin (PE) (I-A/I-E, M5/114.15.2); anti–CD40-PE, CD86-PE, or inducible T-cell co-stimulator ligand-PE antibodies; PE-conjugated rat IgG2b (negative control, Pharmingen); or anti–CD80-PE and PE-conjugated hamster IgG (negative control). Allophycocyanin (APC)–conjugated anti–CD11c antibody was used for DC staining.

**Intracellular Cytokine Staining for Flow Cytometry**

We performed intracellular cytokine staining and flow cytometry as described previously (details in the online-only Data Supplement). We isolated CD4⁺ T cells from the spleen of OT-II T-cell receptor transgenic CD4⁺ T cells and coculture of OT-II CD4⁺ T Cells and Ovalbumin-Primed DCs

We isolated CD4⁺ T cells from the spleen of OT-II T-cell receptor transgenic mice using the magnetic cell-sorting (MACS) system (Miltenyi Biotec Inc), following the manufacturer’s instructions. We then labeled CD4⁺ T cells with the carboxyfluorescein succinimidyl ester (CFSE) labeling kit (Invitrogen). Five million CFSE-labeled OT-II CD4⁺ T cells per well were cocultured with bone marrow-derived B6 WT or MRP-14⁻⁻ DCs in the presence of 10 μmol/L ovalbumin in a 6-well plate. We collected supernatants for cytokine analysis by ELISA 2 to 4 days after T-cell plating and harvested T cells and DCs for flow cytometry 4 days after T-cell plating. We also performed flow cytometry and defined T-cell proliferation by calculating the proliferation index, ie, the percentage of the sum of the cells in all generations divided by the calculated number of original parent cells, as described previously.

**Isolation of Lymphocytes From Spleen and Cardiac Allografts**

We isolated lymphocytes from spleen and cardiac allografts as described previously (details in the online-only Data Supplement).

**Construction of MRP-8/14 Expression Vectors and DC Retrovirus Infection**

MRP-8 and MRP-14 cDNAs were cloned by PCR amplification and inserted into the mouse stem cell retrovirus-based internal ribosomal entry site–enhanced green fluorescent protein vectors, which use the viral endogenous promoter to direct the expression of the cloned cDNAs. The expression plasmids were transfected into the Phoenix packaging cell line with TransIT293 transfection reagent (Mirus Bio Corp, Madison, WI) according to the manufacturer’s instructions, and the supernatants were collected, filtered, and stored at −80°C. Typically, retrovirus titers after packaging were ~1×10⁶/mL. The mixture of viral supernatants was placed on the cultured DCs and centrifuged for 3 hours at 700g at room temperature. This step was repeated 3 times, and then the media was replaced with 10% FCS Dulbecco modified Eagle medium and cultured for 24 hours before costimulatory molecule analysis. MRP-14⁻⁻ DCs infected with MRP-8 and MRP-14 retroviral vectors expressed MRP-8 and MRP-14 proteins, and MRP-14⁻⁻ DCs infected with control, MRP-8, and MRP-14 vectors showed enhanced green fluorescent protein.

**Statistical Analysis of Graft Survival and Cell Number, Cell Proliferation, and Cytokine Production**

Graft survival curves were estimated by the Kaplan-Meier method and compared between the 2 groups by the log-rank test. Comparisons between treatment groups on the frequency of graft-infiltrating cells, cell proliferation, ELISA, parenchymal rejection, and graft arteriolar disease (GAD) scores used t tests accounting for unequal variances when heterogeneity was present (when F test for equal variances yielded P<0.05). We used 2-way ANOVA and the Bonferroni method for statistical analysis of the lymphocyte count in lymph nodes before and after transplantation in a comparison of WT and MRP-14⁻⁻ recipients and cytokine production levels in the supernatant of OT-II CD4⁺ T cells cocultured with WT or MRP-14⁻⁻ DCs in the presence or absence of ovalbumin. Two-tailed tests were used, with values of P<0.05 considered statistically significant. We used GraphPad Prism 4 or Statview software for Macintosh for statistical analysis.

**Results**

**Deficiency ofRecipient MRP-14 Accelerates Parenchymal Rejection and Exacerbates Graft Survival in MHC Class II–Allomismatched Allografts**

Survival of totally allomismatched murine cardiac allografts (BALB/c donor hearts and B6 recipients) was comparable between B6 WT and MRP-14⁻⁻ recipients; allograft survival averaged 8.3±1.3 days (mean±SD; n=18) in WT recipients and 8.3±0.8 (n=7; P=0.8565) in MRP-14⁻⁻ recipients (Figure 1A). In contrast, in MHC class II–allomismatched murine cardiac allografts (bm12 donor hearts and B6 recipients), MRP-14⁻⁻ recipients had significantly reduced allograft survival compared with WT recipients; survival averaged 5.9±2.6 weeks (mean±SD; n=10) for allografts in MRP-14⁻⁻ recipients compared with >12 weeks (n=15; P<0.0001) in WT recipients (Figure 1B). Failing grafts in MRP-14⁻⁻ recipients showed severe coronary arteritis with perivascular edema and confluent areas of myocardial necrosis at 2 and 4 weeks after transplantation (Figure 2A). At the same time point, grafts in WT recipients exhibited virtually no arterial inflammation, edema, or coagulative necrosis despite the presence of multifocal parenchymal inflammatory infiltrates (Figure 2A). Reflecting these differences, allografts
MRP-14 Deficiency Increases Intragraft Accumulation of CD4+ and CD8+ T Lymphocytes and Macrophages

We performed immunohistochemical analysis of immune cell infiltrates in transplanted hearts in WT and MRP-14−/− recipients 2 weeks after engraftment. Both the number of CD4+ and CD8+ T cells and the number of macrophages increased significantly in MRP-14−/− recipients compared with WT recipients as assessed by staining with anti-CD4, anti-CD8, or anti-CD11b antibodies, respectively (Figure 2C and 2D). To verify that differences in leukocyte accumulation in MRP-14−/− mice did not result from differences in peripheral blood leukocyte counts, we performed complete blood count analysis in WT and MRP-14−/− mice. WT recipients (n=4) and MRP-14−/− recipients (n=4) showed no statistically significant differences in peripheral blood neutrophils (2.12±0.67 versus 1.95±0.60×10^3/μL, mean±SD; p=0.71), monocytes (0.11±0.06 versus 0.09±0.06×10^3/μL; p=0.60), or lymphocytes (6.85±0.67 versus 6.95±0.78; p=0.85), respectively.

**Elevated Cytokine and Chemokine mRNA Expression in Allografts of MRP-14−/− Recipients**

The augmented graft immune cell infiltration in MRP-14−/− recipients could result from changes in local chemokine and cytokine expression. To test this possibility, we performed quantitative real-time PCR to measure chemokine and cytokine mRNA expression from allografts harvested 2 weeks after transplantation. MRP-14−/− recipients had significantly increased allograft expression of IFN-γ, IL-6, IL-17, monocyte chemotactic protein-1 (CCL2), IFN-γ-inducible protein-10 (CXCL10), monokine induced by IFN-γ (CXCL9) mRNA, and IFN-inducible T-cell chemoattractant (CXCL11) compared with WT recipients. MRP-14−/− and WT recipients had comparable levels of tumor necrosis factor-α, transforming growth factor-β, Foxp3, and regulated on activation normal T-cell expressed and secreted (CCL5) mRNA expression (Figure 3A–3C).

IFN-γ, IL-17, T-Bet, and Retinoic Acid Receptor-γt Levels Increased in CD4+ T Cells in Allografts of MRP-14−/− Recipients

Flow cytometry analysis of graft-infiltrating cells revealed significantly higher levels of IFN-γ and IL-17 in CD4+ T cells in MRP-14−/− host allografts compared with WT host allografts (Figure 3D–3G). Levels of CD4+ CD25+ Foxp3+ T cells of WT and MRP-14−/− host allografts did not differ (Figure II in the online-only Data Supplement).

**Increased Size and Cell Number of Adjacent Para-Aortic Lymph Nodes in MRP-14−/− Hosts**

MRP-14−/− recipients showed a prominent lymphadenopathy of the adjacent para-aortic lymph nodes 2 weeks after transplantation (Figure 4A). Although WT lymph node size increased 1.2 times after transplantation compared with nontransplanted lymph nodes, lymph node size increased ≈5-fold in MRP-14−/− transplant recipients. Flow cytometry of adjacent lymph nodes showed that WT and MRP-14−/− recipients had comparable cellular compositions, but the numbers of CD4+ and CD8+ T cells, B220+ B cells, and CD11b+ macrophages were relatively increased in MRP-14−/− recipients (Figure 4B).
MRP-14−/− Splenocytes Have Increased MLRs but Normal Responses to Anti-CD3 and Anti-CD40 Antibodies

Enlargement of local adjacent lymph nodes suggests augmented interaction of APCs and T cells with subsequent enhanced alloresponse and is consistent with the increased allograft inflammatory cell infiltration in MRP-14−/− recipients.35 MRP-14 deficiency could also directly modulate T-cell, B-cell, and/or monocyte/macrophage/DC function and may alter the local cytokine milieu.36 We therefore examined whether MRP-14 deficiency modulates MLR using WT or MRP-14−/− splenocytes as responders and irradiated bm12 splenocytes as stimulator cells. In this assay, MRP-14−/− splenocytes showed a significantly higher proliferation rate compared with WT splenocytes after 3 days of culture (Figure 5A). To dissect the mechanisms by which MRP-14−/− splenocytes showed augmented proliferative responses, we stimulated splenocytes directly with anti-CD3 monoclonal antibodies (T-cell stimulation) or anti-CD40 (3/23) monoclonal antibodies (B-cell stimulation). Interestingly, WT and MRP-14−/− splenocytes proliferated comparably under these conditions (Figure 5B and 5C), suggesting that MRP-14−/− T cells and B cells have normal intrinsic proliferative capability.

MRP-14−/− DCs Promote Greater CD4+ T Cell Proliferation and Higher IFN-γ Production

MLR using bm12 stimulator splenocytes enhanced proliferation of MRP-14−/− splenocyte responders compared with WT responders, whereas direct stimulation of T cells and B cells showed comparable responses. We therefore hypothesized that MRP-14 deficiency increases the immu-
**Figure 3.** Cytokine and chemokine mRNA expression and TH17 cells in transplanted hearts. mRNA expression of cytokines (A; interferon-γ [IFN-γ], tumor necrosis factor-α [TNF-α], interleukin [IL]-6, IL-10, and transforming growth factor-β [TGF-β]) and chemokines (B; monocyte chemotactic protein-1 [MCP-1], regulated on activation normal T cell expressed and secreted [RANTES], IFN-γ–inducible protein-10 [IP-10], monokine induced by IFN-γ [Mig], and IFN-inducible T-cell chemoattractant [I-TAC]) and Foxp3 and IL-17 (C) in bm12 allografts was examined by quantitative real-time polymerase chain reaction 2 weeks after transplantation into WT (open bars) or MRP-14 /– (solid bars) recipients. Data represent mean ± SEM (n = 6 per group). D through G, Flow cytometric analysis of IFN-γ (D), T-bet (E), IL-17 (F), and retinoic acid receptor-γ (RORγt; G) in CD4+ T cells of graft-infiltrating cells in WT and MRP-14 /– recipient allografts 2 weeks after transplantation. Shaded gray curve shows negative control using isotype-matched immunoglobulin; blue lines show cytokine expression levels in the merged histogram (D, c and d; E, c and d; F, c and d).
MRP-14−/− DCs Express Higher Levels of Costimulatory Molecules and Have Augmented Alloantigen-Presenting Function

The first encounter of recipient leukocytes with donor cells in an allograft is through ECs; DCs serve as the most potent APCs and play a central role in the initiation of immunity or tolerance.37 To assess whether DCs (B6) can process and present EC alloantigens in vitro, we precultured B6 WT or MRP-14−/− DCs with bm12 ECs for 72 hours to allow DCs to capture and process alloantigens. We then cocultured irradiated allo-EC–primed DCs as stimulators and naïve B6 T cells as responders, measuring T-cell proliferation by 3H-thymidine incorporation (with nonprimed DCs serving as a negative control). Allo-EC–primed MRP-14−/− DCs induced significantly higher T-cell proliferation compared with allo-EC–primed WT DCs (Figure 6A). These results suggest that MRP-14−/− DCs provide more effective T-cell priming.

We then tested the hypothesis that enhanced MRP-14−/− DC APC function is associated with increased costimulatory molecule expression. We stimulated WT or MRP-14−/− DCs with IFN-γ for 18 hours and assessed DC MHC class II and costimulatory molecule expression by flow cytometry. IFN-γ-treated MRP-14−/− DCs showed significantly higher expression of CD80 (mean fluorescence intensity, 162.5 ± 15.5), CD86 (130.9 ± 26.7), and ICOSL (35.1 ± 1.5; graph not shown) compared with IFN-γ–treated WT DCs (CD80, 69.9 ± 9.3, P<0.0001; CD86, 62.2 ± 11.5, P<0.0001; n=4; ICOSL, 30.6 ± 1.8, P=0.029; n=4; Figure 6B and 6C). The percentages of CD80+ and/or CD86+ cells were also greater in MRP-14−/− DCs than in WT DCs (Figure 6C). MHC class II expression showed identical levels (data not shown). The MLR and DC costimulatory molecule expression data demonstrate that MRP-14 regulates DC antigen-presenting capacity and DC-mediated T-cell activation.

Retroviral Infection With MRP-8 and MRP-14 Retroviral Vectors Reduced CD80 and CD86 Expression in MRP-14−/− DCs

A previous study indicated that MRP-14−/− animals lack both MRP-8 and MRP-14 protein expression, even though they express MRP-8 mRNA.25 Moreover, DCs overexpressing MRP-8/14 exhibit DC maturation.38 We therefore tested whether MRP-14−/− DCs reconstituted with MRP-8/14 show reduced expression of costimulatory molecules. We reconstituted MRP-14−/− DCs with MRP-8 and MRP-14 retroviral vector infection and performed flow cytometry to assess costimulatory molecule expression 18 hours after IFN-γ stimulation. Reconstituted MRP-14−/− DCs with MRP-8/14 retroviral vector showed significantly lower levels of CD80 (mean fluorescence intensity, 117.2 ± 9.1) and CD86 (161.3 ± 30.2) compared with control vector-infected MRP-14−/− DCs (CD80, 175.5 ± 25.4, P<0.0001; CD86, 278.4 ± 32.9, P<0.0001; n=4; Figure 7B and 7C). The percentages of CD80+ and/or CD86+ cells were also comparable, as shown by mean fluorescence intensity. These findings indicate that MRP-8/14 inhibits costimulatory molecule expression on the surface of DCs.

nogenic and proinflammatory antigen-presenting capacity of DCs. To test this hypothesis, we cultured OT-II T cells and bone marrow–derived WT or MRP-14−/− DCs in the presence of the nominal antigen of the OT-II cell, ovalbumin. We incubated CFSE-labeled OT-II splenocytes at 5×10⁶ cells per 1 mL in 6-well plates with B6 WT or MRP-14−/− DCs with 10 nmol/L ovalbumin peptide (Sigma) for 4 days and then harvested the supernatant for IL-2 and IFN-γ measurement by ELISA and CFSE-labeled OT-II cell proliferation by flow cytometry. This experiment demonstrated that, compared with WT, MRP-14−/− DCs induced significantly more IL-2 and IFN-γ and promoted higher degrees of CD4+ T cell proliferation, indicating that MRP-14 deficiency increases the immunogenic APC function of DCs (Figure 5D and 5E). In contrast, IL-10 production did not differ between WT and MRP-14−/− DCs, and flow cytometry showed no difference in the expression of the inhibitory costimulatory molecules PDL1 and PDL2 (data not shown).
Cardiac Allografts of B6 MHC Class II−/− Hosts Receiving MRP-14−/− DCs Show Augmented Inflammatory Cell Content

To test whether MRP-8/14 in recipient DCs regulates antigen-presenting function and affects allograft rejection, we performed adoptive transfer experiments using MHC class II−/− animals. Bm12 cardiac allografts in MHC class II−/− hosts do not develop acute rejection because host APCs lack MHC class II and do not recognize bm12 antigen. We specifically tested whether MRP-14−/− DCs in such MHC class II−/− hosts could augment APC function and drive acute rejection. DCs from B6 MHC class II−/−, B6 WT, or MRP-14−/− mice were transferred into B6 MHC class II−/− mice; 1 week later, bm12 hearts were transplanted into these hosts. We examined graft-infiltrating inflammatory cells 2 weeks after cardiac transplantation in each host. Allografts in animals receiving MRP-14−/− DCs showed significantly higher levels of inflammatory cell accumulation compared with allografts in hosts receiving MHC class II−/− DCs or WT DCs (Figure 8B and 8C).

Cardiac Allografts of B6 WT Hosts Receiving MRP-14−/− DCs Also Show Augmented Inflammatory Cell Content

We also performed adoptive transfer experiments using B6 WT animals. DCs from B6 WT or MRP-14−/− mice were transferred into B6 WT mice; 1 week later, bm12 hearts were transplanted into these hosts. We examined graft-infiltrating inflammatory cells 2 weeks after cardiac transplantation in each host. Allografts in animals receiving MRP-14−/− DCs showed significantly higher levels of inflammatory cell accumulation compared with allografts in hosts receiving WT DCs (Figure 8C and 8D). The results support the conclusions...
that MRP-14 mutes APC function and that the absence of MRP-14 leads to augmented allograft rejection.

**Discussion**

This study found that MRP-14−/− recipients have accelerated failure of MHC class II–mismatched cardiac allografts, associated with increased intragraft accumulation of inflammatory cells and Th-17 cells, and elevated mRNA expression of proinflammatory cytokines (IFN-γ, IL-6), IL-17, and IFN-γ–associated chemokines (CCL2, CXCL9, CXCL10, and CXCL11). We also found significant enlargement of adjacent para-aortic lymph nodes in MRP-14−/− recipients with increased expression of costimulatory molecules (CD80, CD86, ICOSL) in IFN-γ–stimulated MRP-14−/− DCs and augmented APC function. Reconstitution of MRP-14−/− DCs with MRP-8 and MRP-14 retroviral vectors suppressed APC function.

MRP-14−/− mice have normal organ development and live a normal lifespan despite lacking both MRP-14 and MRP-8 proteins.25 The lack of MRP proteins did not affect baseline lymph node size or the number of circulating monocytes, neutrophils, lymphocytes, or natural killer cells; myeloid cell numbers in lymphoid organs such as the spleen and bone marrow were also normal. Several lines of evidence suggest that the maturity of myeloid cells from MRP-14−/− mice is similar to that in WT mice; MRP-14−/− and WT neutrophils have similar expression of adhesion receptors (e.g., L-selectin and the integrins Mac-1, lymphocyte function-associated antigen-1, and α4β1), either constitutively or after activation with macrophage inflammatory protein-2, tumor necrosis factor-α, or phorbol ester.25

Murine MRP-14 protein constitutes 10% to 20% of neutrophil soluble protein.5 Murine leukocytes also express other S100 proteins such as S100A1 and A4.39,40 Consequently, an increase in other S100 proteins might compensate for the loss of MRP-14 and MRP-8, but the expression of both S100A1 and A4 remained unaltered in MRP-14−/− bone marrow cells.25 Studies of mice deficient in other S100 protein family members indicate a similar lack of compensatory increases in associated S100 family members.25 Thus, myeloid and other innate inflammatory cell types apparently develop and function reasonably normally in the absence of their distinctive S100 proteins.
We recently demonstrated that MRP-8/14 broadly regulates vascular inflammation and contributes to the biological response to vascular injury in experimental atherosclerosis, after arterial injury, or in small-vessel vasculitis by promoting leukocyte recruitment. Nevertheless, the response of neutrophils, monocytes, and other leukocytes to thioglycolate-induced peritonitis was comparable to that in WT mice, indicating that the effect of MRP-14 likely depends on the precise inflammatory stimulus. MRP-14 inhibits a number of macrophage-activating activities, including proliferation and phagocytic activity and respiratory burst of O$_2^-$ or H$_2$O$_2$. Release from activated macrophages. MRP-14 also inhibits immunoglobulin synthesis by B cells in vitro. Some inhibitory effects may arise from deficient actin polymerization and phagolysosome maturation.

CD4$^+$ T cells regulate alloimmune response. After transplantation, the local cytokine milieu produced by innate cells activates naïve CD4$^+$ T cells and promotes them to differentiate into Th1, Th2, and Th17 effector subsets, as well as regulatory T cells. The expression of specific transcription factors (eg, T-bet for Th1 cells, GATA-3 for Th2, retinoic acid receptor-γ for Th17, and Foxp3 for regulatory T cells) identifies each Th subset. Both Th1 and Th2 effector cells cause acute rejection of bm12 allografts in B6 hosts. Th17 cells may contribute to the immune response during allograft rejection. IL-12 and IL-23 promote Th1 and Th17 differentiation, respectively; they also share the same p40 subunit, paired with the p35 subunit to form IL-12. IL12/23/p40 blockade prolongs cardiac allograft survival, with a decrease in both T-bet and retinoic acid receptor-γ expression in the allografts, indicating that both Th1 and Th17 cells cause allograft rejection. Regulatory T cells can inhibit IL-2, IFN-γ, and IL-13 by CD4$^+$ T cells; suppress both types of responses; and attenuate skin allograft rejection. In contrast, regulatory T cells enhance IL-17 production in an MHC II–mismatched MLR and promote the Th17-mediated pathway of allograft rejection.

The costimulatory molecules CD80 (B7–1) and CD86 (B7–2), expressed on APCs, interact with the receptors CD28 and CD152 (CTLA-4) on T cells. Costimulation through the B7/CD28 pathway is an important mechanism for the activation of lymphocytes by alloantigens; blockade of the B7/CD28 costimulatory pathway attenuates acute rejection of cardiac allografts. This study showed augmented B7 molecule expression of MRP-14$^{-/-}$ DCs and reduced B7 molecule expression of MRP-8/14 gene-transferred MRP-14$^{-/-}$ DCs, indicating that MRP-14 inhibits CD80 and CD86 expression on DCs. Emerging evidence demonstrates that DCs have distinct inflammatory phenotypes, denoted as DC1 or DC2. DC1s serve as immunogenic or proinflammatory APCs; they produce proinflammatory cytokines such as IL-12, activate Th1 cells, initiate inflammation, orchestrate the defense against infectious agents or alloantigens, and promote allograft rejection. In contrast, DC2s serve as tolerogenic or inhibitory APCs; they produce IL-10, induce Th2 cells and/or regulatory T cells, and promote transplant tolerance.
the basis of the finding that MRP-14−/− DCs accelerate cardiac allograft rejection and increase IFN-γ production and T-cell proliferation compared with WT DCs in coculture with OT-II T cells in the presence of their nominal antigen, ovalbumin, it appears that MRP-14−/− DCs function as DC1s. The recent report that MRP-14−/− DCs have increased toll-like receptor–mediated cytokine expression further supports a role for MRP-14 in the control of DC-mediated T-cell activation and inflammation.56

S100 proteins regulate cathepsins and MMPs.57–59 In particular, the transcriptional activation of MMP-2 requires MRP-8/14,60 and antigen presentation via MHC class II involves proteolytic degradation of both the internalized protein and invariant chains.61 We performed quantitative real-time PCR for cathepsins B, L, S, and K in DCs but found no differences between WT and MRP-14−/− DCs (data not shown).

MRP-14 also serves as a key molecule in regulating tolerogenic DCs rather than immunogenic DCs. This conjecture is supported by the observation that IL-10 and transforming growth factor-β induce a tolerogenic DC phenotype53; IL-10 raises MRP-8/14 levels in DCs,36 and MRP-14 overexpression inhibits DC differentiation,38 suggesting that tolerogenic DCs may associate with increased MRP-8/14 expression. MRP-14 deficiency, as in these experiments, would therefore show overall reduced tolerance and increased allograft responses.

Conclusions

These results indicate that MRP-14 regulates B7 molecule expression and reduces antigen presentation by DCs and subsequent T-cell priming. The absence of MRP-14 markedly increased T-cell activation and exacerbated allograft rejection, indicating a previously unrecognized role for MRP-14 in immune cell biology.

Figure 8. Cardiac allografts in B6 major histocompatibility complex (MHC) class II−/− hosts or B6WT receiving myeloid-related protein-14 (MRP-14)− deficient MHC II−/− dendritic cells (DCs) showed augmented inflammatory cell infiltration. A, Immunohistochemistry examined inflammatory cell infiltration into transplanted hearts in MHC class II−/− hosts receiving MHC class II−/− DCs (a, d, g), B6 WT MHC II−/− DCs (b, e, h), and MHC II−/− MRP-14−/− DCs (c, f, i) 2 weeks after transplantation. Anti-CD4 (a, b, c), anti-CD8 (d, e, f), and anti-CD11b (g, h, i for macrophages) staining was performed. B, Inflammatory cell accumulation was quantified by determining the average number of cells per high-power field (×100) in MHC class II−/− hosts receiving MHC class II−/− DCs (open bar), B6 WT MHC II−/− DCs (open bar), and MRP-14−/− MHC II−/− DCs (solid bar). Data represent mean±SD; n=6 per group. C, Immunohistochemistry examined inflammatory cell infiltration into transplanted hearts in B6WT hosts receiving B6WT DCs (a, c, e) and MRP-14−/− DCs (b, d, f) 4 weeks after transplantation. Anti-CD4 (a, b), anti-CD8 (c, d), and anti-CD11b (e, f for macrophages) staining was performed. D, Inflammatory cell accumulation was quantified by determining the average number of cells per high-power field (×100) in B6WT hosts receiving B6WT DCs (open bar) and MRP-14−/− DCs (solid bar). Data represent mean±SD; n=6 per group.
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Disclosures

None.

References


**CLINICAL PERSPECTIVE**

Graft arterial disease (GAD) limits long-term survival in cardiac transplant recipients. GAD shares some pathophysiological features with conventional atherosclerosis; APCs present alloantigens (eg, antigens on donor ECs) or autoantigens (eg, oxidized low-density lipoprotein cholesterol) to T cells, initiating differentiation and activation of T cells, B cells, and inflammatory cell responses with costimulatory signaling. Nevertheless, there are important differences in the pathology and distribution of these diseases. Although hyperlipidemia, a well-established risk factor for conventional atherosclerosis, is common after transplantation, GAD lesions tend to be lipid poor. GAD involves large and medium-sized vessels and the microvasculature and affects the media, adventitia, and intima. MRP-14 deficiency can attenuate wire injury–induced vascular lesions and atherosclerotic lesions in atherogenic animals. In contrast, the present study demonstrates that recipient MRP-14 deficiency exacerbates allograft vasculopathy. This study examined the effect of MRP-14 deficiency on parenchymal rejection and GAD after MHC class II–mismatched murine heart transplantation without immunosuppression. Clinical application requires future studies evaluating the effects of MRP-14 expression in the setting of immunosuppressive therapy.
Loss of Myeloid Related Protein-8/14 Exacerbates Cardiac Allograft Rejection

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SUPPLEMENTAL MATERIAL

Supplemental Methods:

Vascularized heterotopic cardiac transplantation

B/c (total allo-mismatch) or bm12 (MHC class II-mismatch) donor hearts were transplanted heterotopically into B6 recipients without immunosuppression, as shown previously. We assessed graft function by palpation twice daily. In MHC class II-mismatched experiments, two cohorts of WT and MRP-14−/− recipients were used. The first group of animals served to determine graft survival — defined as the day of cessation of heartbeat, or when the strength of the heartbeat declined to the point that failure would occur (by prior experience). WT grafts typically survive more than 12 weeks. In the second group of animals, beating cardiac allografts were harvested 2 weeks after transplantation.

Parenchymal rejection (PR) and the severity and extent of vascular involvement were quantified using a four-point scoring system by blinded observers, as described previously. PR was scored on a scale from 0 to 4 (0: no rejection, 1: focal mononuclear cell infiltrates, 2: focal mononuclear infiltrates with necrosis, 3: multifocal infiltrates with necrosis, and 4: widespread infiltrates with hemorrhage and/or vasculitis), and vascular stenosis was scored on a scale from 0 to 4 based on the extent of luminal stenoses averaged over ≥ 10 arteries (0: <10%, 1: < 10-25%, 2: 25-50%, 3: 50-75%, and 4: >75% stenosis).

Because minor histoincompatibility can influence allo-immune responses significantly, we examined inflammatory responses in B6 WT cardiac allografts in MRP14−/− recipients. We did not detect any inflammatory cell accumulation in the B6 WT cardiac allografts in MRP14−/− recipients 4 weeks after transplantation (Supplemental Figure S1), indicating that variation in genetic background between WT and MRP14−/− mice does not account for differences in the
Immunohistochemistry

5-µm cryosections were fixed in 4% paraformaldehyde before incubation with 0.5% H2O2. Slides were incubated with 5% normal goat serum and stained with purified monoclonal rat anti-mouse antibodies (BD Pharmingen) to CD4 (RM4-5), CD8 (53-6.7), B220 (B cell), CD11b (macrophage), or CD11c (DCs); control sections were stained with non-specific isotype-matched antibodies. Sections were incubated with biotinylated goat anti-rat IgG (1 mg/ml, Southern Biotechnology Associates, Inc., Birmingham, AL), followed by streptavidin-peroxidase (DAKO, Carpinteria, CA). Antibody binding was visualized with 3-amino-9-ethyl carbazole (DAKO). Nuclei were counter-stained with Gill’s hematoxylin (Sigma).

Intracellular cytokine staining for flow cytometry

We performed intracellular cytokine staining and flow cytometry using methods described previously.27 Briefly, we stimulated isolated cells with 1 µg/ml ionomycin (Sigma) and 50 ng/ml PMA (Sigma), for 4 hours at 37°C under a 5% CO2 humidified atmosphere. We added monensin (5 µg/ml, Sigma) for the duration of the culture to block cytokine secretion and thereby improve detection. After stimulation, cells were centrifuged at 200 x g for 5 minutes, and washed in ice-cold PBS before being fixed at room temperature with 4% paraformaldehyde in PBS for 10 minutes. For intracellular staining, cells were permeabilized with saponin/PBS buffer (0.5% saponin [Sigma], 1% BSA, 0.1% NaN3 in PBS). After incubation with 0.25 µg Fc block
(PharMingen) for 5 minutes, cells were labeled with 10 µg/ml of a primary biotinylated anti-
cytokine Ab or biotinylated isotype-matched control Ab (PharMingen) for 30 minutes at room
temperature. After washing twice with saponin/PBS buffer, the cells were incubated with PE-
conjugated streptavidin (2 µg/ml) for 30 minutes at room temperature. For staining of
transcription factors (Foxp3, T-bet, or RORγt), the cells were incubated with PE-conjugated anti-
Foxp3, T-bet, RORγt, or isotype-matched Abs. The cells were washed twice with saponin/PBS
buffer, washed with PBS alone to seal the membranes, and stained with PE-conjugated or APC-
conjugated surface marker Abs (2.5 µg/ml) for 30 minutes at room temperature, followed by
washing in PBS.

Isolation of lymphocytes from spleen and cardiac allografts

We isolated lymphocytes from spleen and cardiac allografts as described previously. Briefly,
we removed spleens from the recipients of cardiac allografts and passed them through a
cytoscreen into RPMI 1640 (Life Technologies, Grand Island, NY). The cells and residue were
pelleted at 200 x g for 5 minutes, and resuspended in 5 ml Tris-ammonium chloride buffer
(0.83% NH₄Cl, 5 mM Tris buffer, pH 7.2) at 37°C for 5 minutes to lyse red blood cells.
Lymphocytes were washed twice more in PBS, and resuspended in RPMI with 10% FCS
supplemented with 100 U/ml penicillin, 100 µg/ml streptomycin sulfate, 2 mM L-glutamine, and
57 µM 2-ME (C/10 medium) at a concentration of 1 x 10⁷ cells/ml.

We minced portions of harvested cardiac allografts with a sterile blade and incubated the pieces
in 10 ml buffered saline with 2% BSA and 2 mg/ml collagenase at 37°C for 2 hours. The cells
were strained through a 70-µM nylon cell strainer (Becton Dickinson, Franklin Lakes, NJ). Lymphocytes were isolated from these cells by Ficoll (Organon Teknika, Durham, NC) density gradient centrifugation for 20 minutes at 800 rpm. After being washed twice in RPMI 1640, lymphocytes were resuspended in C/10.
Clinical Implications

Graft arterial disease (GAD) limits long-term survival in cardiac transplant recipients. GAD shares some pathophysiologic features with conventional atherosclerosis; APCs present alloantigens (e.g., antigens on donor ECs) or auto antigens (e.g., oxidized LDL cholesterol) to T cells, initiating differentiation and activation of T cells, B cells, and inflammatory cell responses with co-stimulatory signaling. Nevertheless, there are important differences in the pathology and distributions of these diseases. Although hyperlipidemia, a well-established risk factor for conventional atherosclerosis, is common after transplantation, GAD lesions tend to be lipid-poor. GAD involves large-sized and medium-sized vessels as well as the microvasculature, and affects the media and adventitia as well as the intima. MRP-14 deficiency can attenuate wire-injury–induced vascular lesions and atherosclerotic lesions in atherogenic animals. In contrast, the current study demonstrates that recipient MRP-14 deficiency exacerbates allograft vasculopathy. This study examined the effect of MRP-14 deficiency on PR and GAD after MHC class II-mismatched murine heart transplantation without immunosuppression. Clinical application will require future studies evaluating the effects of MRP-14 expression in the setting of immunosuppressive therapy.
Figure S1

Wild-type B6 cardiac allografts of MRP-14<sup>-/-</sup> recipients did not develop rejection and did not show inflammatory cell accumulation.

![B6WT → B6MRP14<sup>-/-</sup> (4w)](image)

**Figure S1.** To confirm whether MRP-14 deficient animals are on a pure C57BL/6 (B6) background, we performed cardiac transplantation of wild-type B6 hearts into MRP-14<sup>-/-</sup> recipients. We harvested cardiac allografts 4 weeks after transplantation and stained them with H&E (a), CD4 (b), CD8 (c), and CD11b (d).
Cardiac allografts of B6 WT and MRP14−/− recipients showed comparable levels of CD4+Foxp3+ cells.

Figure S2. Intracellular staining of the graft-infiltrated CD4+ T cells shows comparable expression levels of Foxp3 in WT (a, c) and MRP14−/− (b, d) recipient allografts. c and d, the histogram shows Foxp3 expression (bold lines) gated on CD4+ T cells obtained from WT (c) and MRP14−/− (d) recipient allografts 2 weeks after transplantation. Shaded gray curve shows isotype matched control. e, mean fluorescence intensity (MFI); f, percentages of Foxp3-positive cells gated on CD4+ cells, mean ±SD (n=4, each).