Leukocyte Integrin Mac-1 Promotes Acute Cardiac Allograft Rejection

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Background—In allograft rejection, recipient leukocytes and alloantibodies first target donor endothelial cells. Although the leukocyte integrin Mac-1 (αMβ2, CD11b/CD18) facilitates cell–cell interactions among leukocytes and interactions between leukocytes and endothelial cells or platelets, its role in allograft survival and vasculopathy is incompletely defined.

Methods and Results—This study examined parenchymal rejection and graft arterial disease after total allomismatched cardiac transplantation (BALB/c donor heart and B6 recipients) in wild-type (WT) and Mac-1-deficient (Mac-1−/−) recipients. Recipient Mac-1 deficiency attenuated parenchymal rejection and significantly prolonged cardiac allograft survival from 8.3±1.3 days in WT recipient allografts (n=18) to 13.8±2.3 days in Mac-1−/− recipient allografts (n=6; P<0.0001). Accumulation of neutrophils and macrophages significantly decreased in Mac-1−/− compared with WT recipients. Adoptive transfer of WT but not Mac-1−/− macrophages to Mac-1−/− recipients exacerbated parenchymal rejection and reduced allograft survival; in contrast, adoptive transfer of WT neutrophils did not affect graft survival. Mac-1−/− macrophages expressed significantly lower levels of costimulatory molecules both in vivo and in vitro, and mixed lymphocyte reaction using alloantigen-primed Mac-1−/− macrophages resulted in significantly lower antigen-presenting function than for WT macrophages. Tumor necrosis factor-α production also fell in cultures with Mac-1−/− macrophages. Despite attenuation of acute rejection, recipient Mac-1-deficiency did not prevent late graft arterial disease.

Conclusions—These studies demonstrate critical participation of Mac-1 in alloresponses during cellular allograft rejection. These observations establish a molecular target for modulating recipient responses to prolong graft survival. (Circulation. 2008;117:1997-2008.)

Key Words: antigen-presenting cells • immunology • macrophage-1 antigen • pathogenesis • rejection • transplantation

In allografts, recipient leukocytes and alloantibodies attack donor endothelial cells (ECs) early in acute rejection. Cell–cell interactions among leukocytes and interactions between leukocytes and ECs or platelets critically influence immune responses. These adhesive interactions require multistep adhesive and signaling events, including selectin-mediated attachment and rolling, leukocyte activation, and integrin-mediated firm adhesion and diapedesis, that result in the infiltration and accumulation of inflammatory cells into tissues.1 Firm attachment depends on members of the β2-integrin family, LFA-1 (αLβ2, CD11a/CD18), Mac-1 (αMβ2, CD11b/CD18), p150,95 (αMβ2, CD11c/CD18), and CD11d/CD18, expressed exclusively by hematopoietic cells.

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Formation of the immunological synapse requires direct interaction between T-cell integrins and cognate ligands on antigen-presenting cells (APCs).2 LFA-1 binding to intracellular adhesion molecule-1 (ICAM-1) reduces the level of antigen required to form the immune synapse, thereby lowering B-cell activation thresholds.3 Engagement of ICAM-1 on target cells leads to major histocompatibility complex class I (MHC-I) recruitment to contact areas and enhances presentation of cognate peptide/MHC-I complexes to cytotoxic T cells.4

Mac-1 resides on neutrophils, monocytes, macrophages, dendritic cells, CD8+ T cells, and natural killer cells.5–7
Endothelial ligands for Mac-1 include ICAM-1,8–10 endothe-
lum-associated extracellular matrix proteins (eg, fibrino-
gen),11–13 CD154,14 and glycosaminoglycans.15 These adhe-
sive interactions facilitate firm attachment and transmigration
into interstitial tissue.16,17

Numerous studies have shown that the donor ECs in
rejecting cardiac allografts robustly express ICAM-1 and that
augmented expression of adhesion molecules precedes leuk-
ocyte accumulation within vessels.18–20 These results sug-
gest that ICAM-1 induction during early rejection contributes
to mononuclear cell recruitment and renders ECs more
susceptible to cell-mediated injury, including graft arterial
disease (GAD).20,21 Nevertheless, it remains unclear whether
donor adhesion molecules contribute critically to allograft
rejection. Treatment with monoclonal antibodies against
ICAM-1 and LFA-1 resulted in indefinite cardiac allograft
survival between fully histoincompatible mice strains22 but had
no effect on allograft survival in fully incompatible rat
strains.23 In 1 study, ICAM-1–deficient donor hearts did not
exhibit prolonged graft survival or attenuated GAD24; in
another report, ICAM-1–deficient donor hearts had modest (2
to 2.5 days), albeit significantly prolonged, graft survival.25

A definitive role for Mac-1 in allograft rejection is uncer-
tain. Antibody targeting of Mac-1 did not affect graft survival
time but decreased the accumulation of graft macrophages.26
However, antibody experiments have several important limi-
tations: (1) Complete blockade is difficult, particularly for
those integrins with mobilizable intracellular storage pools
such as Mac-1;8–10; (2) antibodies binding to the surface of
leukocytes can stimulate changes in cellular functions and
behavior (eg, through activation of Fc receptors when immu-
noglobulin G [IgG] antibodies are used); and (3) because
Mac-1 associates closely with and influences other cell
surface receptors such as the urokinase receptor,28 antibody
binding may cause steric interference with the associated
receptor. The present study avoided these limitations of
antibody administration by using a genetic approach, exami-
nining parenchymal rejection (PR) and GAD after cardiac
transplantation in Mac-1−/− recipients.

Methods

Animals

C57BL/6 (B6; H-2b), B6.C-H2<bm12>KhEg (bm12; H-2bm12), and
BALB/c (B/c) mice were from Taconic Farms (Germantown, NY) or
The Jackson Laboratory (Bar Harbor, Me). Mac-1−/− C57BL/6 mice
were kindly provided by Dr Christie M. Ballantyne (Baylor College
of Medicine, Houston, Tex).29 Mice were maintained on acidified
water in barrier animal facilities. Animal care and procedures were
reviewed and approved by the Harvard Medical School Standing
Committee on Animals and performed in accordance with the
guidelines of the American Association for Accreditation of Labo-
ralory Animal Care and the National Institutes of Health.

Vascularized Heterotopic Cardiac Transplantation

B/c (total allomismatch to assess effects on acute rejection) or bm12
(MHC-II mismatch to assess effects on GAD) donor hearts were
transplanted heterotopically into B6 recipients without immunosup-
pression.30 We assessed graft function by palpation twice daily. In
total allomismatched experiments, 2 cohorts of wild-type (WT) and
Mac-1−/− recipients were used. The first group of animals served to
determine graft survival, defined as the day of cessation of beating or
when the strength of beating declined to the point that failure would
occur (by prior experience) within the next 12 hours. In the second
group of animals, beating cardiac allografts were harvested at the
prespecified time point of 7 days after transplantation. For single
MHC-II–mismatched transplantation, grafts were harvested at 4 and
12 weeks.

PR and the severity and extent of GAD were quantified with a
4-point scoring system by blinded observers as described previ-
ously.30 PR was scored on a scale of 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). GAD was scored on
a scale of 0 to 4 on the basis of the extent of luminal stenoses averaged
over >10 arteries (0, <10%; 1, 10% to 25%; 2, 25% to 50%; 3, 50%
to 75%; and 4, >75% stenosis).

Graft Harvest

Harvested allografts were transversely sectioned into 3 parts. In
sectioned hearts, the most basal part was used for routine hematox-
ylin and eosin morphological examination. A second midtransverse
section was frozen for immunohistochemical staining, and the apical
portion was used for total RNA extraction for RNase protection
assay.30 For cellular extraction, hearts were digested at 37°C in 2
mg/mL collagenase (Sigma, St Louis, Mo) and 2% BSA in buffered
saline, followed by straining and Ficoll density gradient centrifuga-
tion (Organon Teknika, West Chester, Pa).30

Immunohistochemistry

Cryosections (5 μm) were fixed in acetone before incubation with
0.5% H2O2. Slides were incubated with 10% normal goat serum and
stained with purified monoclonal anti–CD4 (RM4-5), anti–CD8
(53-6.7), anti–Mac-3 (macrophage), or anti–Gr-1 (neutrophil, RB6-
8C5) antibodies. Sections were incubated with biotinylated goat
anti-rat IgG antibodies (1 μg/mL, Southern Biotechnology Associ-
ates, Inc, Birmingham, Ala), followed by streptavidin-peroxidase
(Dako, Glostrup, Denmark). Antibody binding was visualized with 3-ami-
no-9-ethyl carbazole (Dako). Nuclei were counterstained with
Gill’s hematoxylin (Sigma).

Mixed Lymphocyte Reaction, T-Cell Proliferation

Assay, and Cytokine ELISA

One-way mixed lymphocyte reaction (MLR) was performed as
described previously30 with irradiated B/c splenocytes as stimulators
and B6 splenocytes as responders. T-cell or B-cell proliferation assay
was performed with anti–CD3 antibody-coated 96-well plates or by
adding anti–CD40 monoclonal antibodies (3/23, BD PharMingen,
San Diego, Calif).

We also performed MLR using allograft-derived macrophages as
stimulators and naive B6 T cells as responders. We isolated macro-
phages from the allografts of B6 WT or B6 Mac-1−/− recipients 7
days after transplantation as described previously.30 We extracted T
cells from naive B6 WT splenocytes using MACS beads (Miltenyi
Biotec Inc, Auburn, Calif) with negative selection according to the
manufacturer’s methods. We plated irradiated allograft-derived mac-
rophages (50 000 cells per well) and naive B6 T cells (500 000 cells
per well, S:R = 1:10) in the 96-well plates and measured T-cell
proliferation using 3H-thymidine incorporation.30 We also measured
tumor necrosis factor-α (TNF-α) levels in the supernatant of the
coculture by ELISA as described previously30 using purified anti–
TNF-α and biotinylated anti–TNF-α monoclonal antibodies (BD
PharMingen).

Total RNA Extraction and RNase

Protection Assay

Total RNA was isolated from allografts with the TRIzol reagent
(Invitrogen, Carlsbad, Calif). RNase protection assay was performed
(RiboQuant, PharMingen) with 10 μg RNA from each heart accord-
ing to the manufacturer’s recommendations.30
Murine Peritoneal Macrophage and Bone Marrow Neutrophil Isolation
Peritoneal cells were collected via peritoneal lavage using 10 mL ice-cold RPMI 1640. Lavage cells were pelleted, resuspended in 5 mL hypertonic RBC lysis buffer (ACK lysis buffer, Invitrogen) at 37°C for 5 minutes, washed with RPMI 1640 twice, and resuspended in 5 mL RPMI 1640. Cells were centrifuged in Percoll gradient solution for 30 minutes. Cells in the upper layer were resuspended in 10% FCS RPMI 1640, plated on plastic dishes for 60 minutes, and washed twice to remove nonadherent cells, resulting in >95% pure macrophages, confirmed by Mac-3 staining and flow cytometry. To isolate neutrophils from the bone marrow, we removed the femur and tibia from both hind legs and cut off the extreme distal tip of each extremity. RPMI 1640 medium was forced through the bone with a syringe. After dispersing cell clumps and erythrolysis, the cell suspension was centrifuged (400g for 10 minutes at 4°C) and resuspended in 1 mL RPMI 1640. Cells were then treated on a 3-layer Percoll gradient exactly as described above for peritoneal cells. Flow cytometry using anti–Ly-6G and Gr-1 antibodies showed >95% purity of neutrophils.

Murine EC Isolation and Coculture With Macrophages
ECs were isolated from the B/c murine hearts as described previously and incubated for 72 hours with the peritoneal macrophages extracted from naive B6 WT or Mac-1−/− mice. We performed flow cytometry analysis for constitutual molecule expression of the B/c EC–primed macrophages as described below.

Cellular Surface Staining and Flow Cytometric Analysis
Graft-infiltrating cells and B/c–EC–primed macrophages were analyzed by flow cytometry after surface staining using methods described previously. Antibodies included anti–MHC-II phycoerythrin (PE) (I-A/I-E, M5/114.15.2), anti–CD40-PE, anti–CD86-PE, or anti–ICOSL-PE antibodies; PE–conjugated rat IgG2b (negative control, PharMingen); or anti–CD80-PE and PE–conjugated hamster IgG (negative control). Anti–MAC-3 antibody was used for macrophage staining.

Statistical Analysis of the Graft Survival and Cell Number, Proliferation, and Cytokine Production
Comparisons between the 2 groups for graft survival used Kaplan–Meier and log-rank tests; frequency of graft-infiltrating cells, cell proliferation, and ELISA used 1-way ANOVA. We described PR and GAD scores as mean±SD and analyzed them by Fisher exact test using the GraphPad Prism 4 software for Macintosh (GraphPad Software, San Diego, Calif).

Results

Deficiency of Recipient Mac-1 Attenuates PR and Prolongs Graft Survival in Total Allomismatched Allografts
In total allogeneic murine cardiac allografts (B/c donor hearts and B6 recipients), survival averaged 13.8±2.3 days (mean±SD; n=6) for allografts in Mac-1−/− recipients compared with 8.2±1.3 days (n=18) in WT recipients (Figure 1A). Failing grafts in WT recipients showed severe coronary arteritis with perivascular edema and confluent areas of myocardial necrosis 7 days after transplantation. In comparison, at the same time point, grafts in Mac-1−/− recipients exhibited virtually no arterial inflammation, edema, or coagulative necrosis, despite the presence of multifocal parenchymal inflammatory infiltrates (Figure 1B). Reflecting these differences, the PR scores were significantly lower for allografts in Mac-1−/− recipients 7 days after transplantation (2.3±0.4, mean±SD; n=6) compared with WT recipients (3.0±0.4; n=13; P=0.0408). At this 7-day time point, inflammatory cells and thrombi populated vessel luminal areas in allografts of WT recipients. These lesions differ from chronic GAD lesions that consist predominantly of smooth muscle–like cells. Nevertheless, we scored the extent of early luminal occlusion using the GAD scoring system. Scores were significantly lower for grafts in Mac-1−/− (0.01±0.02, mean±SD; n=6) compared with WT (0.63±0.44; n=13; P=0.0108) recipients (Figure 1C). Thus, deficiency of recipient Mac-1 prolongs allograft survival and is associated with diminished inflammatory cell accumulation, including reduced luminal inflammatory cells. At the time of eventual graft failure in Mac-1−/− recipients (13.8±2.3 days), PR scores were 3.2±0.9, with luminal stenosis graded at 1.3±1.1.

Mac-1 Deficiency Reduces the Intragraft Accumulation of Neutrophils, CD8+ T Lymphocytes, and Macrophages
Immunohistochemical analysis examined the characteristics of the immune cell infiltration in transplanted hearts in WT and Mac-1−/− recipients 7 days after allotraging. Anti–Gr-1 identified neutrophils, anti–CD4 or anti–CD8 for T cells, and anti–Mac-3 for macrophages. Neutrophil (Gr-1–positive cells), CD8+ T cell, and macrophage (Mac–3–positive cells) accumulation all decreased significantly in Mac-1−/− compared with WT recipients. The transplanted hearts of both Mac-1−/− and WT recipients had comparable numbers of CD4+ T cells (Figure 2A and 2B). To verify that differences in leukocyte recruitment in Mac-1−/− mice were not secondary to differences in peripheral blood leukocyte counts, we performed complete blood count analysis in WT and Mac-1−/− mice. No statistical differences were found in the number of peripheral blood neutrophils (2.12±0.67 versus 2.10±0.53×106/μL; mean±SD; P=0.96), monocytes (0.11±0.06 versus 0.09±0.04×106/μL; P=0.53), and lymphocytes (6.85±0.67 versus 6.52±0.28; P=0.40) between WT (n=4) and Mac-1−/− (n=4), respectively.

WT Macrophage but Not Neutrophil Adoptive Transfer Reduces Allograft Survival in Mac-1−/− Recipients
Both neutrophils and monocytes/macrophages can participate in acute graft failure. Deficiency of Mac-1 reduces the recruitment of both neutrophils and monocytes/macrophages at sites of vascular injury. To determine the cell type involved in acute graft dysfunction, we performed an adoptive transfer experiment using neutrophils and macrophages from WT donors. Mac-1−/− recipients of B/c heart allografts were injected intravenously with either neutrophils (5×10⁶) or macrophages (5×10⁵) extracted from WT or Mac-1−/− mice 1 day after transplantation. Neither WT nor Mac-1−/− neutrophil adoptive transfer affected graft survival in Mac-1−/− recipients (Figure 3A). However, adoptive transfer of WT but not Mac-1−/− macrophages significantly reduced...
graft survival of Mac-1−/− recipients (Figure 3B). PR scores of allografts also increased significantly in Mac-1−/− recipients receiving WT (2.3±0.4; mean±SD; n=6) versus Mac-1−/− (1.5±0.5; n=5; P=0.024) macrophages (Figure 3C). These observations indicate that macrophages expressing Mac-1 participate in graft inflammatory cell accumulation and influence graft survival in total allomismatched allografts.

**Lack of Mac-1 Reduced Early PR and GAD but Not Chronic GAD in MHC-II–Mismatched Cardiac Transplantations**

We examined PR and GAD after MHC-II–mismatched murine heart transplantation using bm12 donor hearts and WT or Mac-1−/− B6 recipients without immunosuppression. Single MHC class mismatch permits graft survival for the assessment of GAD. Grafts were harvested at 4 and 12 weeks after transplantation. The 12-week time point is commonly used to evaluate arterial lesions at a more chronic stage when GAD lesions typically are well developed.32,35 Four weeks after transplantation, both PR (Mac-1−/−, 1.9±0.5 [n=6] versus WT, 2.8±0.8 [n=8]; P=0.026) and GAD (Mac-1−/−, 0.0±0.0 [n=6] versus WT, 1.2±1.1 [n=8]; P=0.031) scores were reduced in Mac-1−/− compared with WT recipients. However, at 12 weeks, GAD lesions were comparable in Mac-1−/− (1.7±1.1; n=8) and WT (2.2±1.5; n=11; P=0.65) recipients (Figure 4). PR at 12 weeks was reduced compared with 4 weeks but was similar in Mac-1−/− (1.5±1.1) and WT (2.0±1.0; P=0.63) recipients.
Chemokine mRNA Expression Is Comparable in Allografts From WT and Mac-1<sup>−/−</sup> Recipients

The reduction in graft immune cell infiltration in Mac-1<sup>−/−</sup> recipients could result from diminished EC adhesion via leukocyte Mac-1 or from changes in local chemokine and cytokine expression. To test the latter possibility, we performed RNase protection assay to measure chemokine and cytokine mRNA expression from allografts harvested 7 days after transplantation. Allograft expression of regulated on activation, normal T cell expressed and secreted (RANTES), monocyte chemotactic protein-1, macrophage inflammatory protein (MIP)-1α, MIP-1β, MIP-2, and IFN-inducible protein 10 mRNA was similar in WT and Mac-1<sup>−/−</sup> recipients. Among cytokine mRNA expression profiles, only TNF-α decreased significantly in Mac-1<sup>−/−</sup> compared with WT recipients (Figure 5).

Mac-1<sup>−/−</sup> Splenocytes Have Attenuated MLR but Normal Responses to Anti-CD3 and Anti-CD40 Antibodies

Interaction of APCs and T cells, as well as the subsequent strength of the alloresponse, may affect cellular recruitment. Mac-1 also can directly limit T-cell and/or dendritic cell function and may alter the local cytokine milieu. Therefore, we examined whether Mac-1 deficiency modulates MLR using WT or Mac-1<sup>−/−</sup> splenocytes as responders and irradiated B/c splenocytes as stimulator cells. Mac-1<sup>−/−</sup> splenocytes showed significantly less proliferation compared with WT splenocytes after 3 to 5 days of culture (Figure 6A). To dissect the mechanisms by which Mac-1<sup>−/−</sup> splenocytes show defective proliferative responses, we stimulated splenocytes with anti-CD3 monoclonal antibodies (ie, direct T-cell stimulation) or anti-CD40 (3/23) monoclonal antibodies (direct B-cell stimulation). Interestingly, both WT and Mac-1<sup>−/−</sup>
splenocytes proliferated comparably under these conditions (Figure 6B and 6C), suggesting that Mac-1–deficient T and B cells have normal intrinsic proliferative capability. The results indicate that Mac-1–/– splenocytes have attenuated antigen-presenting functions.

To examine whether Mac-1–deficient splenocytes ineffectively prime T cells, we performed B/c-primed MLR. B/c-primed splenocytes from WT or Mac-1–/– recipients of B/c cardiac allografts were recovered 6 days after transplantation and were stimulated with or without (control) irradiated naive B/c splenocytes. Interestingly, splenocytes harvested from Mac-1–/– transplant recipients showed significantly less proliferation compared with splenocytes recovered from WT recipients (Figure 6D). The results suggest that Mac-1–/– splenocytes lead to ineffective T-cell priming in vivo.

Mac-1–/– Macrophages Express Reduced Levels of Costimulatory Molecules and Have Diminished Alloantigen-Presenting Function

Macrophages make up most of inflammatory cells at the later phases of acute rejection.30,38 Macrophages can exhibit direct cytotoxicity against certain cells39 and can function in allograft rejection as APCs.40 We tested the hypothesis that intragraft Mac-1–/– macrophages have impaired activation and exhibit reduced antigen-presenting function compared with WT macrophages. To do so, immune cells were extracted from cardiac allografts harvested from WT and Mac-1–/– recipients 6 days after transplantation; flow cytometry was performed to assess macrophage MHC-II and costimulatory molecule expression. Graft macrophages in Mac-1–/– recipients showed significantly lower expression of CD40, CD80, CD86, ICOSL, and MHC-II compared with those in WT recipients (Figure 7A and 7B).

We also used the macrophages extracted from B/c allografts of B6 WT or Mac-1–/– recipients as a stimulator of B6 naive T cells and performed primed MLR (primed macrophages as stimulator and naive T cells as responder). The results demonstrated that naive T cells stimulated by the primed macrophages extracted from the allografts of Mac-1–/– recipients had a lower proliferative capacity (Figure 7C) and secreted less TNF-α (Figure 7D) compared with those stimulated by the primed macrophages extracted from the allografts of WT recipients. These results suggested that Mac-1–/– macrophages have lower antigen-presenting capacity.

Because recipient leukocytes first encounter donor ECs after transplantation, the ability of donor ECs to facilitate antigen presentation probably is important. Thus, to assess
whether macrophages (B6) can process and present a foreign antigen of the ECs (B/c) in vitro, we performed B/c-EC and B6-macrophage coculture and examined the antigen-presenting capacity of the macrophages to B6 T cells. Naive B6 peritoneal macrophages also were extracted from WT and Mac-1/H11002/H11002/B6 animals and cocultured with B/c ECs. After 3 days, macrophages were isolated by gradient centrifugation and used for flow cytometry analysis. Mac-1/H11002/H11002/H11002/macrophages expressed significantly lower levels of CD40 (mean fluorescence intensity, 22.7 ± 4.5) and CD86 (71.7 ± 9.5) compared with WT macrophages (CD40, 87.7 ± 21.3, P = 0.0016, n = 4; CD86, 150 ± 18.2, P = 0.0089, n = 4) (Figure 7E). Of note, no significant differences occurred in the expression (mean ± SD fluorescence intensity) of CD40 (Mac-1/H11002/H11002/H11002, 19.8 ± 5.9 versus WT, 20.0 ± 7.0; P = 0.96), CD80 (20.8 ± 10.4 versus 22.8 ± 7.5; P = 0.77), CD86 (48.3 ± 14.9 versus 50.5 ± 24.0; P = 0.77), and ICOSL (15.0 ± 6.5 versus 13.5 ± 5.1; P = 0.73) on naive peritoneal macrophages isolated from Mac-1/H11002/H11002/H11002, compared with WT mice. These findings indicate that Mac-1/H11002/H11002/H11002/macrophages cocultured with B/c ECs have weaker antigen-presenting capacity than WT macrophages.

**Discussion**

The present study provides in vivo evidence that Mac-1 promotes acute allograft rejection. Recipient Mac-1 deficiency reduced allograft accumulation of immune cells, prolonged allograft survival, but did not prevent GAD. The
results demonstrated an important role for Mac-1 in macrophage antigen presentation by showing that Mac-1−/− macrophages expressed significantly lower levels of the costimulatory molecules both in vivo and in vitro and that MLR using alloprimed Mac-1−/− B6 splenocytes resulted in significantly lower antigen-presenting function than WT macrophages. Furthermore, WT but not Mac-1−/− macrophage adoptive transfer to Mac-1−/− recipients exacerbated PR and reduced allograft survival. In comparison, adoptive transfer of WT neutrophils did not affect graft survival. We also have observed that blockade of neutrophil recruitment with anti-Gr1 antibody (RB6-8C5) treatment does not prevent acute rejection (K.S., unpublished data, 2004).

**Possible Mechanism of Mac-1 Action in Acute Rejection**

Mac-1 localizes on neutrophils, monocytes, macrophages, natural killer cells, and CD8+ T cells. The functional relevance of β2-integrins such as Mac-1 (CD11b/CD18) on APCs remains unclear. Varga and coworkers reported recently that Mac-1 on APCs, including dendritic cells and macrophages, directly inhibited T-cell activation. In that study, CD18−/− macrophages stimulate allogeneic T cells more potently than their WT counterparts. However, Mac-1−/− (CD11b−/−) APCs were not examined, thereby precluding definitive conclusions on the precise role of Mac-1 given the possible contributions of other β2-integrins, including...
Figure 7. Mac-1⁻/⁻ macrophages have diminished APC function. A, Representative figure shows expression of costimulatory molecules and MHC-II (solid graph) on macrophages extracted from day 7 transplanted cardiac allografts in Mac-1⁻/⁻ (top) and WT (bottom) recipients assessed by flow cytometry and double staining with PE-conjugated anti-CD40, anti-CD80, anti-CD86, anti-ICOSL, or anti-MHC-II (IA/IE) and FITC-conjugated anti–Mac-3. Clear graph shows negative control using PE-conjugated rat immunoglobulin G2a (IgG2a; negative control for CD40, CD86, ICOSL, and MHC-II) or hamster IgG (negative control for CD80). B, Bar graph shows values representing mean±SEM fluorescent intensity (MFI) of the Mac-3⁻/⁻ macrophages extracted from WT (solid bars) and Mac-1⁻/⁻ (open bars) recipient allografts (n=4 per group). C and D, Proliferation of naive wild-type B6 T cells stimulated by the macrophages extracted from B/c allografts of B6 WT (solid bars) and Mac-1⁻/⁻ (open bars) recipients (C) and production of TNF-α from those T cells (D) were assessed as described in Methods. Macrophage antigen presentation after interaction with allogeneic ECs. E, Expression of costimulatory molecules on B/c EC-primed WT (solid) and Mac-1⁻/⁻ (open) macrophages was assessed by flow cytometry as described above. Data represent average values (mean±SEM) of 3 independent experiments (n=4 per group).
LFA-1 (CD11a/CD18), p150,95 (CD11c/CD18), and CD11d/CD18, to immune synapse function.

In contrast, the present work shows unambiguously that Mac-1\(^{-/-}\) macrophages have defective APC function. Direct T-cell receptor stimulation by anti-CD3 antibodies and direct B-cell stimulation with anti-CD40 antibodies induced comparable proliferation from WT and Mac-1\(^{-/-}\) splenocytes, indicating that Mac-1\(^{-/-}\) T and B cells have normal T- and B-cell receptor responses. At the same time, graft-infiltrating macrophages in Mac-1\(^{-/-}\) recipients and Mac-1\(^{-/-}\) macrophages cocultured with allo-ECs express significantly lower levels of costimulatory molecules and MHC-II compared with WT macrophages. Mac-1\(^{-/-}\) macrophages also stimulated T-cell proliferation more poorly than WT macrophages. Over all, our data suggest that the diminished APC function of Mac-1\(^{-/-}\) macrophages may prolong allograft survival by reducing T-cell priming but may do so by reducing TNF-\(\alpha\) expression.

**Mac-1 and GAD**

By virtue of binding diverse ligands such as fibrinogen, ICAM-1,44 factor X, C3bi, platelet glycoprotein Ib,14,15,42,43 CD154,44 and JAM-3, Mac-1 potentially regulates important leukocyte functions relevant to vascular injury, including adhesion, migration, coagulation, proteolysis, phagocytosis, oxidative burst, and signaling.29,31,40–52

Our prior report identified Mac-1 as a molecular determinant of neointimal thickening after endothelium-denuding injury; selective absence of Mac-1 impaired transplatelet leukocyte migration into the vessel wall, diminishing medial leukocyte accumulation and neointimal thickening after experimental angioplasty.54 In the present study, deficiency of Mac-1 attenuated allograft leukocyte accumulation and rejection but had no effect on neointimal thickening in GAD at 12 weeks. These discrepant effects on neointimal thickening likely reflect a distinct cell recruitment mechanism provoked by mechanical arterial injury (ie, platelet-dependent neutrophil recruitment mediated in part by integrin interactions between neutrophil Mac-1 and platelet glycoprotein Ib\(\alpha\)) compared with the pathways downstream of immunological injury involving intact, activated endothelium (ie, endothelium-dependent mononuclear cell recruitment mediated by mononuclear \(\beta_1\)- and \(\beta_2\)-integrins and endothelial ICAM-1 and vascular cell adhesion molecule-1). Similar to GAD, deficiency of Mac-1 has no effect on high-fat-diet–induced atherosclerotic lesion formation in low-density lipoprotein receptor–deficient mice.53

**Clinical Implications**

Allograft rejection, both cellular and antibody mediated, remains a clinically important limitation to human cardiac transplantation. More than 40% of patients will experience an episode of rejection in the first year after transplant that requires therapy; the average patient will experience close to 2 episodes of rejection in the first year.54 Severe rejection episodes can cause heart failure, malignant arrhythmias, and sudden cardiac death. Higher grades of rejection (International Society for Heart and Lung Transplantation grade 3 or 4) portend poor outcomes.54 Current treatment strategies often resolve the acute episode effectively and typically include the use of augmented doses of glucocorticoids or calcineurin inhibitors, cytolytic agents such as OKT3, and plasmapheresis. However, they are limited by the inherent toxicities of immunosuppression, including fatal infections, aggressive malignancies, and renal failure. Furthermore, antibody-mediated rejection, in particular, has no consensus treatment to date.55 Novel therapies are greatly needed not only to decrease the morbidity of conventional immunosuppression but also to improve the outcomes of patients with specific forms of allograft rejection, especially acute antibody-mediated rejection. Although deficiency of Mac-1 did not prevent chronic rejection in our study, targeting Mac-1 is an attractive strategy to modulate acute rejection after cardiac transplantation. Indeed, previous clinical trials demonstrated improved renal allograft survival in high-risk patients (either highly immunized or retransplant recipients) who received a monoclonal antibody to one of the Mac-1 cognate ligands, ICAM-1.56

**Study Limitations**

We examined the effect of Mac-1 deficiency on PR and GAD after MHC-II–mismatched murine heart transplantation without background immunosuppression. Clinical application requires future studies evaluating the effect of Mac-1 deficiency and immunosuppressive therapy, alone or in combination, on rejection scores and GAD.

Mac-1 deficiency may affect the development and function of immune cells. The present experiments using adoptive transfer of normal cells provide compelling evidence for the importance of macrophage Mac-1 in acute rejection. Nonetheless, we cannot rule out an additional role of Mac-1 in the development and function of immune cells.

**Conclusion**

This study demonstrates that recipient Mac-1 deficiency reduces accumulation of graft-infiltrating cells, macrophage APC function, T-cell proliferation, and TNF-\(\alpha\) production and attenuates acute allograft rejection.

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**Disclosures**

None.

**References**

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**CLINICAL PERSPECTIVE**

Allograft rejection remains a clinically important limitation to human cardiac transplantation. More than 40% of patients will experience an episode of rejection that requires therapy in the first year after transplantation. Severe rejection episodes can cause heart failure, malignant arrhythmias, and sudden cardiac death. Higher grades of rejection (International Society for Heart and Lung Transplantation grade 3 or 4) portend poor outcomes. Current treatment strategies often resolve the acute episode effectively and typically include the use of augmented doses of glucocorticoids or calcineurin inhibitors, cytolytic agents such as OKT3, and plasmapheresis. However, they are limited by the inherent toxicities of immunosuppression, including fatal infections, aggressive malignancies, and renal failure. Furthermore, antibody-mediated rejection, in particular, has no consensus treatment to date. Novel therapies are greatly needed not only to decrease the morbidity of conventional immunosuppression but also to improve the outcomes of patients with specific forms of allograft rejection, especially acute antibody-mediated rejection. Although deficiency of Mac-1 did not prevent chronic rejection in our study, targeting Mac-1 is an attractive strategy to modulate acute rejection after cardiac transplantation. Indeed, previous clinical trials demonstrated improved renal allograft survival in high-risk patients (either highly immunized or retransplant recipients) who received a monoclonal antibody to one of the Mac-1 cognate ligands, intracellular adhesion molecule-1. This study demonstrates that recipient Mac-1 deficiency reduces accumulation of graft-infiltrating cells, macrophage antigen-presenting cell function, T-cell proliferation, and tumor necrosis factor-alpha production and attenuates acute allograft rejection. Clinical application requires future studies evaluating the effect of Mac-1 deficiency and immunosuppressive therapy, alone or in combination, on allograft rejection.
Leukocyte Integrin Mac-1 Promotes Acute Cardiac Allograft Rejection
Koichi Shimizu, Peter Libby, Rica Shubiki, Masashi Sakuma, Yunmei Wang, Kenichi Asano,
Richard N. Mitchell and Daniel I. Simon

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