Importance of Donor- and Recipient-Derived Selectins in Cardiac Allograft Rejection

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ABSTRACT

The selectins expressed on activated endothelial cells (E- and P-selectin), leukocytes (L-selectin), and platelets (P-selectin) play crucial roles in the rolling and tethering of leukocytes. We explored the importance of donor and recipient selectins in acute and chronic cardiac allograft rejection using mice deficient in all three selectins ($ELP^{-/-}$). In BALB/c recipients, survival of fully allomismatched hearts from $ELP^{-/-}$ C57BL/6 donors was almost double that of wild-type grafts. In $ELP^{-/-}$ cardiac allografts, mononuclear cell infiltration and vasculitis of intramyocardial coronary arteries were significantly reduced. Interestingly, $ELP^{-/-}$ grafts were rejected similarly in both the presence and the absence of recipient selectins, and both wild-type and $ELP^{-/-}$ recipients promptly rejected wild-type hearts. Alternative adhesive molecules such as $\alpha4\beta7$ integrin may compensate for the lack of selectins and may mediate rejection in $ELP^{-/-}$ recipients. Chronic rejection was evaluated in a major histocompatibility complex (MHC) class II mismatch model using C57BL/6.C-H2[bm12] mice. While lack of selectins in recipients did not offer protection against chronic rejection, luminal stenosis of coronary arteries in $ELP^{-/-}$ grafts was markedly diminished. In conclusion, donor-derived selectins contribute to the development of both acute and chronic cardiac allograft rejection, and targeting donor selectins may open novel therapeutic approaches in clinical transplantation.

Selectins play critical roles in inflammatory responses by mediating transient adhesion of leukocytes to the endothelium during the process of rolling. The binding affinity between selectins and selectin-ligands is relatively weak on the resting endothelium; however, once endothelial cells and leukocytes are activated in response to inflammatory stimuli, chemokines and integrins assist to form potent adhesive bridges between leukocytes and the endothelium.[1] Selectins are three structurally related proteins, which were named after the tissue in which they were first identified.[2–4] L-selectin is expressed by leukocytes and mediates the attachment of lymphocytes to high endothelial venules of peripheral lymph nodes, thereby promoting naïve T cell homing.[5] L-selectin also mediates leukocyte recruitment to the site of inflammation.[6] E-selectin is found on endothelial cells, and P-selectin is stored in α-granules of platelets and Weibel-Palade bodies

of endothelial cells. E- and P-selectins also play a role in leukocyte rolling.[7]

Previous reports demonstrated that selectins are critically involved in the development of acute rejection *via* mediation of T cell recruitment into allografts. Critical roles of P- and E-selectin expressed on postcapillary venules, as well as the interaction between T cell L-selectin and its ligands in allografts, have recently been demonstrated in a SCID reconstitution model of skin graft rejection.[8] In a murine model of cardiac transplantation, allograft survival was significantly prolonged in recipients who were treated with either anti–E- or anti–P-selectin mAb.[9,10] Efomycine M, a specific inhibitor of selectins, has been shown to reduce leukocyte adhesion and to ameliorate cell infiltration and autoimmune skin lesions in a murine model of psoriasis.[11] Treatment with the potent selectin antagonist bimosiamose was recently reported to inhibit rejection of kidney allografts in a rat model.[12] Because these strategies have indiscriminately targeted both donor and recipient selectins, specific contributions of either donor or recipient selectins in the process of allograft rejection remain to be elucidated. In this study, murine cardiac allografts were performed using mice deficient in all three selectins ($ELP^{-/-}$) as donor or recipient animals. This model enabled us to dissect the individual contributions of donor- and recipient-derived selectins in the development of acute and chronic rejection. Our data highlight a predominant role of donor selectins in the pathogenesis of acute and chronic rejection. These data could have a significant implication for clinical practice, because manipulating donor-derived selectins before transplantation could be an advantageous alternative to treating recipients with selectin antagonists, which may be associated with significant adverse effects such as an increase in risk for opportunistic infection.

RESULTS

Graft Survival and Histologic Examination

Fully MHC-mismatched hearts from wild-type (WT) or $ELP^{-/-}$ C57BL/6 donors were transplanted into BALB/c recipients (Figure 1). Survival of $ELP^{-/-}$ grafts in WT BALB/c recipients was significantly prolonged as compared with that of WT grafts (median survival time [MST] = 15 [$n = 6$] *versus* 8 d [$n = 10$]; $P < 0.01$). It is interesting that $ELP^{-/-}$ grafts were similarly rejected in both WT (MST = 15; $n = 6$) and $ELP^{-/-}$ recipients (MST = 14; $n = 7$; NS). We next transplanted hearts from WT BALB/c donors into WT or $ELP^{-/-}$ C57BL/6 recipients (Figure 2) and found that WT grafts were also rejected in both the presence (MST = 8; $n = 11$) and the absence (MST = 9; $n = 6$) of recipient selectins (NS). We also found that all grafts were eventually rejected even in the absence of both donor- and recipient-derived selectins (Figure 1). It was expected that lack of
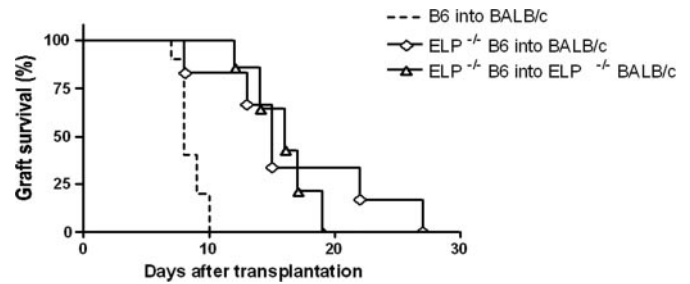
Figure 1. Cardiac allograft survival in WT or $ELP^{-/-}$ BALB/c recipients. Fully MHC-mismatched hearts from WT C57BL/6 donors were promptly rejected in WT BALB/c recipients. Survival of allografts from $ELP^{-/-}$ donors was significantly prolonged as compared with that of WT grafts; however, the allografts were similarly rejected in either the absence or the presence of recipient selectins.
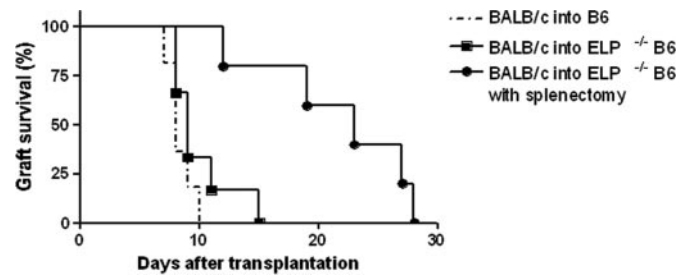
Figure 2. Cardiac allograft survival in WT or $ELP^{-/-}$ C57BL/6 recipients. Hearts from WT BALB/c donors were similarly rejected in either the presence or the absence of recipient selectins. After simultaneous splenectomy of $ELP^{-/-}$ recipients, survival of cardiac allografts was significantly prolonged.

recipient selectins could potentially prevent rejection through affecting leukocytes rolling or through attenuating T cell activation, because L-selectin mediates homing of naïve T cells to the lymph node (LN). The result of acute rejection observed in the $ELP^{-/-}$ recipients, however, indicated that leukocyte activation was preserved and lymphatic organs were viable in the absence of recipient selectins. $ELP^{-/-}$ C57BL/6 recipients of WT BALB/c hearts then underwent simultaneous splenectomy on the day of surgery, which resulted in marked prolongation of graft survival (MST = 23; $n = 5$; $P < 0.01$; Figure 2) as compared with either WT recipients with splenectomy (MST = 10; $n = 5$) or without splenectomy (MST = 8; $n = 11$). These results suggest that targeting only donor selectins (but not recipient selectins) provided a mild prolongation of heart allograft survival, highlighting the existence of compensatory pathways that mount an alloimmune response even in the absence of all three selectins in the host. Neither recipient nor donor selectins seem to play a major role in acute heart allograft rejection. That splenectomy further enhanced survival in $ELP^{-/-}$ recipients to a greater degree than in splenectomized WT recipients suggests that both the spleen and

the LN are important sites for the generation of alloreactive T cells.

ELP$^{-/-}$ or WT cardiac allografts were then obtained from WT recipients at days 3, 5, and 8 after transplantation, and acute rejection was histopathologically evaluated. Whereas at day 3 heart allografts from the WT recipients began to show infiltration, less infiltration was noted in the ELP$^{-/-}$ (Figure 3, A and B, respectively). At day 5, tissue sections of WT allografts demonstrated massive mononuclear cell infiltration and vasculitis of intramyocardial coronary arteries, which were identical in their acute cellular rejection (Figure 3C). In contrast, we observed less cellular infiltration and relatively intact coronary arteries in ELP$^{-/-}$ grafts (Figure 3D). Rejection score of ELP$^{-/-}$ grafts was significantly less than that of WT grafts: 1.6 ± 0.2 ($n = 12$) versus 2.7 ± 0.4 ($n = 8$; $P < 0.01$; (Figure 3E). The contrast between WT and ELP$^{-/-}$ grafts (i.e., less vasculitis in ELP$^{-/-}$ grafts) may explain the long-term vascular protection seen in ELP$^{-/-}$ grafts in our chronic rejection model (see Figure 3). At day 8, a massive necrosis was noted in the WT heart allografts, whereas ELP$^{-/-}$ hearts demonstrated progressive infiltration (Figure 3, F and G, respectively). ELP$^{-/-}$ hearts at later time point (i.e., at the time of rejection) showed similar pattern of massive necrosis with infiltration as WT heart allografts at day 8 (data not shown).

T Cell Activation in WT Recipients Receiving ELP$^{-/-}$ Grafts

To explore roles of donor-derived selectins in mediating alloreactive T cell activity, we next performed T cell population analysis in WT recipients that received hearts from either ELP$^{-/-}$ or WT donors. Intriguing, enumeration of CD4 or CD8 effector and regulatory T cells demonstrated no statistical difference between the presence and the absence of graft selectins (Figure 4). This result indicates that activation of alloreactive T cells was intact in WT recipients that received ELP$^{-/-}$ grafts. It seems, therefore, that the survival prolongation of hearts from ELP$^{-/-}$ donors may predominantly depend on reduced attachment of

Figure 3. Histologic analysis of WT (A, C, and F) or ELP$^{-/-}$ (B, D, and G) grafts at days 3, 5, and 8 after surgery in a fully MHC-mismatched model. A delay in cellular infiltrates noted in the ELP$^{-/-}$ grafts as compared with the WT grafts at days 3, 5, and 8 after transplantation (A versus B and F versus G). Acute cellular rejection accompanied with endarteritis of the intramyocardial coronary artery was observed in WT grafts, whereas ELP$^{-/-}$ grafts showed relatively intact vessels and less cellular infiltration into myocardium (C and D, respectively). Quantitative analysis of acute rejection at day 5 (E) was performed as described in the Concise Methods section. Data are means $\pm$ SEM. *$P < 0.01$. Magnification, $\times 400$ (hematoxylin and eosin).
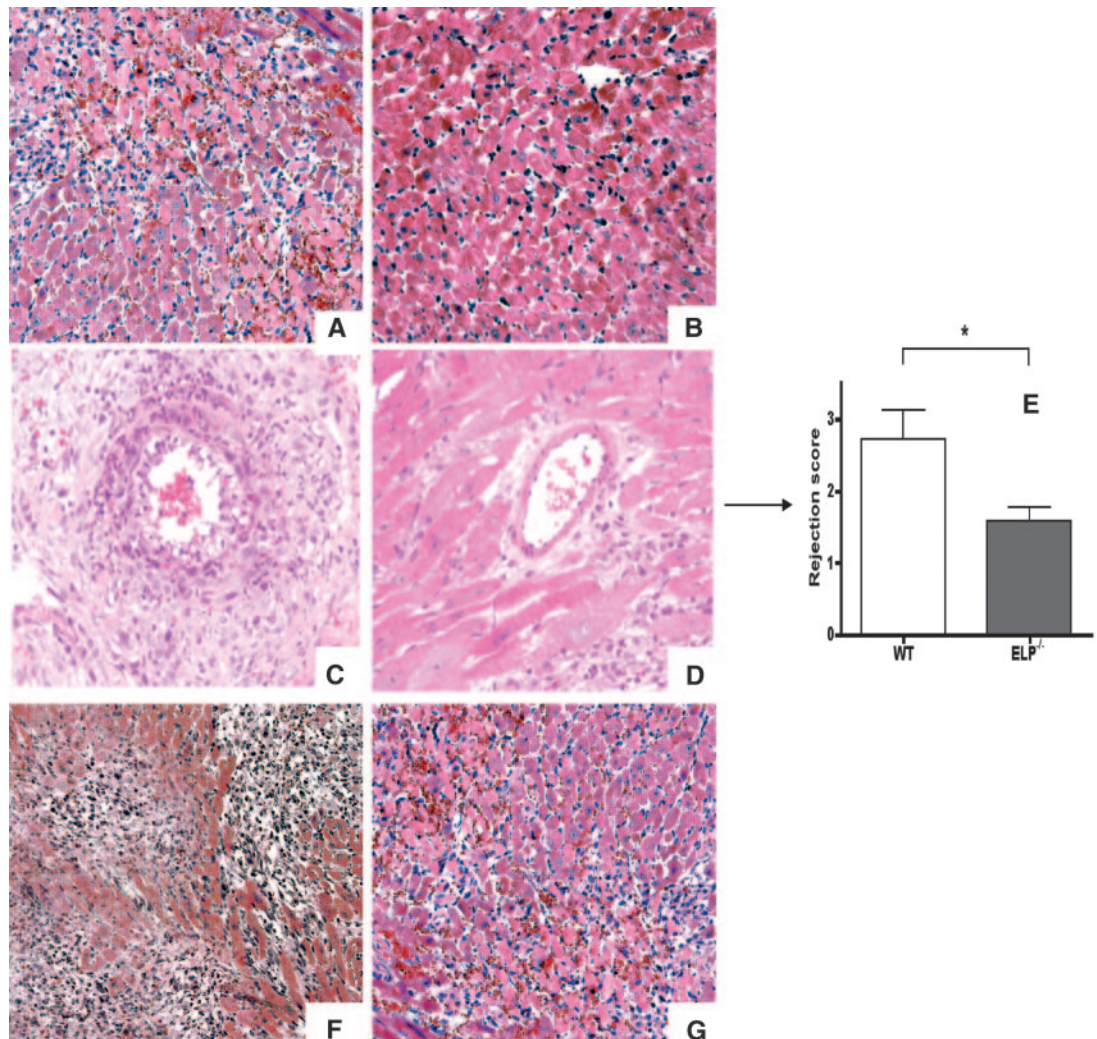
Figure 4. In WT recipients, the impact of the presence or the absence of donor-derived selectins on the generation of effector or regulatory T cells (Tregs) was also evaluated. Splenocytes were isolated 8 d after transplantation from recipients that received hearts from either ELP$^{-/-}$ or WT donors, and flow cytometry was performed as well. There was no statistical difference between the two groups.
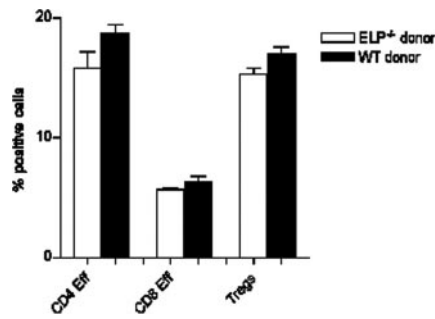
leukocytes to the graft endothelium and lesser trafficking into the graft as a result of the deficiency of donor-derived selectins.

Peyer's Patch as a Plausible Site of T Cell Activation in the ELP$^{-/-}$ Recipients of Heart Allografts

We next compared cell numbers in the mesenteric LN and Peyer's patches between naïve mice and allograft recipients. ELP$^{-/-}$ naïve mice revealed relatively hypocellular LN and Peyer's patches as compared with those of WT mice (cell counts of a mesenteric LN were 6×10^6 *versus* 15×10^6 and cell counts of Peyer's patches were $<0.2 \times 10^6$ *versus* 1.2×10^6, ELP$^{-/-}$ *versus* WT, respectively; Figure 5, A and B). Importantly, after transplantation, although there was an increase in lymphocyte numbers in both ELP$^{-/-}$ and WT recipients, the increase in the WT group was markedly more pronounced (Figure 5A); however, examination of the cell number in the Peyer's patch after transplantation showed a four-fold increase in ELP$^{-/-}$ *versus* 1.4-fold in WT (Figure 5B). Given these data, we speculate that the Peyer's patches could be potential sites for alloreactive T cell activation in ELP$^{-/-}$ mice and therefore contribute to mounting alloimmune responses that lead to allograft rejection in ELP$^{-/-}$ recipients. We also examined the percentage of CD4 and CD8 cells in the mesenteric lymph nodes of naïve and transplanted ELP$^{-/-}$ and WT recipients. Whereas the percentage of CD4 cells remained similar in all groups, the percentage of CD8 cells was markedly increased in both groups of ELP$^{-/-}$ (by five-fold) and WT (by six-fold) in the Peyer's patches after transplantation (data not shown).

In addition, we further examined the importance of alternative adhesion molecules in the absence of selectins, focusing on the surface expression of $\alpha 4\beta 7$ integrin, because this molecule is responsible for T cell homing into gut-associated lymphoid tissues.[13] It is interesting that a dramatic upregulation of the $\alpha 4\beta 7$ complex was noted in ELP$^{-/-}$ mice before (naïve mice) *versus* after transplantation (recipients) as follows: 0.26 *versus* 28.4, 0.7 *versus* 24.2, and 0.44 *versus* 17.2% (axillary LN, mesenteric LN, and Peyer's patch, respectively), whereas those in WT mice were 2.84 *versus* 9.69, 7.19 *versus*
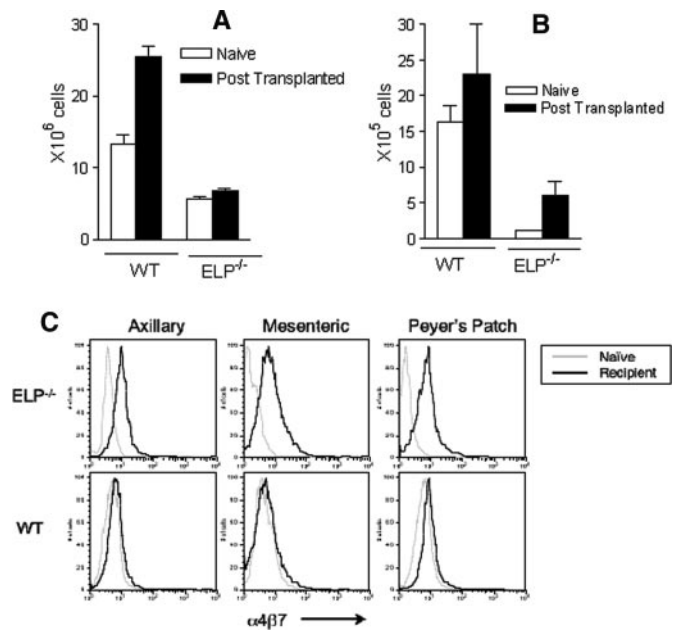

Figure 5. Mesenteric lymph nodes (A) and Peyer's patches (B) were removed from both ELP$^{-/-}$ and WT naïve mice and the ELP$^{-/-}$ and WT recipients of heart allografts at day 7 after transplantation. Compared with naïve WT mice, naïve ELP$^{-/-}$ mice lymphoid tissues were found to be significantly hypocellular. After transplantation, there was a marked increase in cell numbers in the Peyer's patches of ELP$^{-/-}$ recipients (B). Representative histograms of $\alpha 4\beta 7$ integrin expression on lymphocytes isolated from ELP$^{-/-}$ (top) or WT (bottom) mice, which were either naïve or allograft recipients. Lymphocytes from axillary and mesenteric lymph nodes and from the Peyer's patch were gated on CD4. (C) A dramatic upregulation of integrin $\alpha 4\beta 7$ expression was noted in ELP$^{-/-}$ recipients as compared with that of WT mice.

13.9, and 7.49 *versus* 21.3% (Figure 5C). Baseline expression for $\alpha 4\beta 7$ was significantly less in ELP$^{-/-}$ naïve mice as compared with WT mice; therefore, the fold increases were significantly higher in the ELP$^{-/-}$ group: 109-fold increase in axillary LN, 34.6-fold in mesenteric LN, and 39.1-fold in Peyer's patch. Those in the WT group were 3.4-fold in axillary LN, 1.9-fold in mesenteric LN, and 2.8-fold in Peyer's patch. In recipients lacking selectins, therefore, $\alpha 4\beta 7$ integrin seems to be a potential therapeutic target to achieve survival prolongation.

Attenuation of Chronic Rejection in ELP$^{-/-}$ Grafts

To explore the role of selectins in the development of chronic rejection, we performed cardiac allografts using a single class II mismatch model between bm12 and ELP$^{-/-}$ or WT C57BL/6 mice. First, we transplanted hearts from bm12 into ELP$^{-/-}$ (C57BL/6 background) and WT C57BL/6 mice. Mice were killed at 4 and 8 wk after transplantation. In both combinations, histologic examination revealed evidence of chronic rejection at both time points
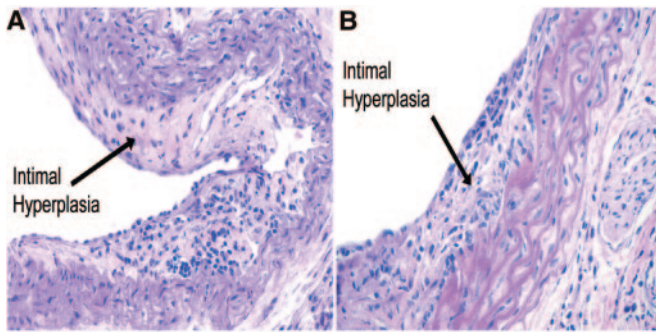
Figure 6. bm12 heart allografts were recovered from the ELP$^{-/-}$ (A) or WT C57BL/6 (B) recipients at day 60 after transplantation. Histologic analysis of allografts in both combinations demonstrate an equal degree of intimal hyperplasia consistent with chronic rejection.
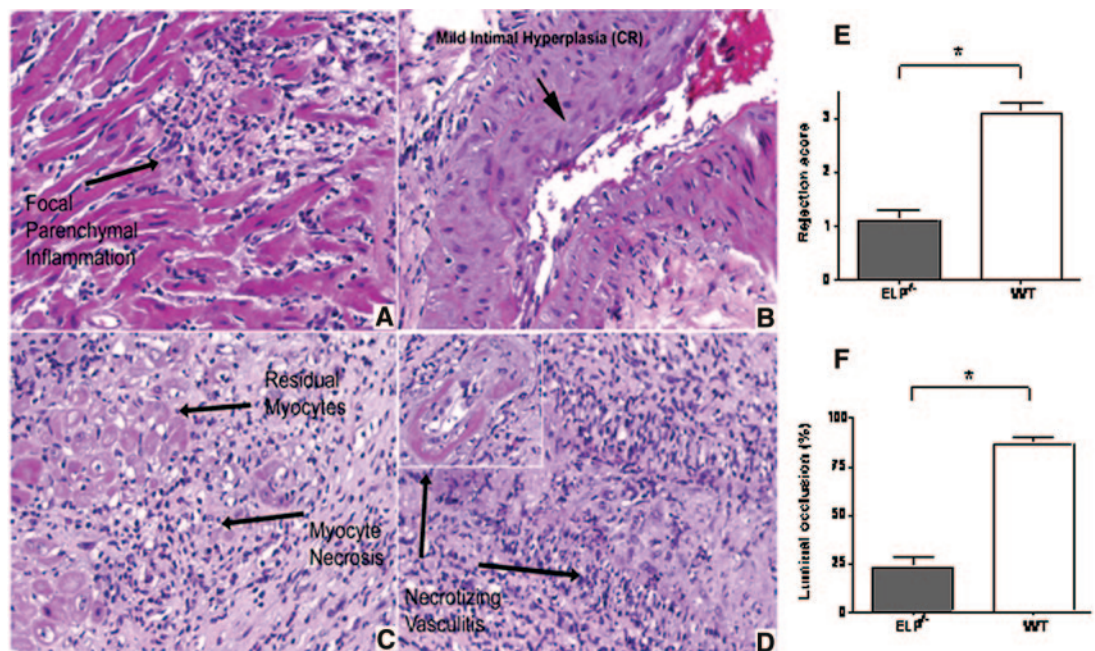
equally, suggesting that the lack of recipient selectins did not offer protection against chronic rejection. As shown in Figure 6, bm12 hearts that were recovered from ELP$^{-/-}$ (C57BL/6 background) and WT C57BL/6 mice at 8 wk showed a significant amount of parenchymal fibrosis and vascular lesions consistent with chronic rejection (Figure 6). To evaluate the role of donor-derived selectins, we transplanted hearts from ELP$^{-/-}$ or WT cardiac allografts into bm12 recipients. Heart allografts were retrieved from bm12 recipients at 8 wk after transplantation, and tissue sections of ELP$^{-/-}$ or WT cardiac allografts were examined histologically for chronic rejection. WT grafts demonstrated characteristic graft arteriosclerosis and diffuse polymor-

phous cell infiltration with parenchymal necrosis, which were comparable to the levels seen in acute cellular rejection (Figure 7, C and D). In contrast, ELP$^{-/-}$ grafts showed considerably less infiltration of mononuclear cells, which were predominantly observed in perivascular areas (Figure 7, A and B). The rejection score of ELP$^{-/-}$ grafts was significantly lower than that of WT grafts: 1.4 ± 0.1 ($n = 10$) *versus* 2.9 ± 0.2 ($n = 9$; $P < 0.01$; Figure 7E). Furthermore, quantitative analysis of graft arteriosclerosis demonstrated that luminal stenosis of coronary arteries in ELP$^{-/-}$ grafts was markedly reduced as compared with that of WT grafts. The percentage of luminal stenosis for ELP$^{-/-}$ and WT grafts was 22.9 ± 4.9 and $87.0 \pm 3.3\%$, respectively ($n = 3$ to 4; $P < 0.01$; Figure 7F). Donor-derived selectins, therefore, played key roles in the development of chronic rejection of cardiac allografts. This result was consistent with the findings observed in acute rejection, which potentially contributed to the pathophysiologic process of graft arteriosclerosis.

DISCUSSION

The endothelial cell layer of coronary arteries is the initial contact site between the immune system and the cardiac allograft. Recent studies have demonstrated that endothelial cells express MHC and co-stimulatory molecules on their surfaces, enabling them to activate T cells.[14–16] Attachment of recipient leukocytes to the surface of the graft endothelium, therefore, is a pivotal step in cardiac allograft rejec-

Figure 7. Histologic analysis of ELP$^{-/-}$ or WT grafts into bm12 recipients at 60 d after surgery in a model of a single class II mismatched combination. Diffuse inflammation accompanied with myocardial necrosis (C), typical graft arteriosclerosis, and necrotizing vasculitis were observed in WT grafts (D), whereas a small amount of perivascular cell infiltration (A) and relatively intact vessels were observed in ELP$^{-/-}$ grafts with only intimal hyperplasia (B). Quantitative analyses of rejection scores (E) and percentage of luminal occlusion (F) were performed as described in the Concise Methods section. Data are means $\pm$ SEM. *$P < 0.01$. Magnification, $\times400$ (hematoxylin and eosin).

tion. Various selectin ligands have been reported, including sialylated Lewis X, glycoproteins, glycolipids, and proteoglycans. In particular, E- and P-selectins on the endothelium bind to P-selectin glycoprotein ligand-1 on leukocytes, whereas leukocyte L-selectin binds either to P-selectin glycoprotein ligand-1, which is presented by leukocytes attached on the endothelium, or to peripheral node addressins and heparan sulfate on the endothelium itself.[3,17–19] Thus, our target molecules, which could mediate allograft rejection in this model, were donor-derived E- and P-selectins on the graft endothelium and recipient-derived L-selectin on leukocytes.

Important roles of L-selectin have been suggested in the pathophysiology of graft rejection, and a prolongation of murine skin allografts has been reported in L-selectin–deficient recipients.[20–24] As previously reported in animals that were treated with either selectin inhibitors or blocking antibodies for selectins, it was expected that less infiltration of leukocytes and survival prolongation of cardiac allografts would be observed in mice lacking selectins.[9–12] Nevertheless, our important finding was that recipient-derived L-selectin was not essential for the mechanism of acute rejection. Consistent with our results, it has also been reported that L-selectin–deficient T cells differentiate into effectors in lymphoid organs and subsequently traffic to inflammatory sites, which is due in part to their increased expression of other proinflammatory adhesion molecules.[25] In addition, given a sufficient priming stimulus by alloantigen, L-selectin–deficient mice are fully capable of rejecting allogeneic skin grafts.[25] Furthermore, induction of tolerance in recipients lacking selectins has been challenging.[26] These findings support results of this study, which demonstrate insignificant roles for recipient-derived selectins in the development of cardiac allograft rejection.

It is interesting that allograft survival was significantly prolonged after simultaneous splenectomy of ELP^{−/−} recipients, indicating that alloimmune function of the spleen was preserved and recipient leukocytes became activated even in the absence of all selectins. The grafts were nonetheless eventually rejected. We can speculate, therefore, that leukocytes may use alternative pathways or other adhesion molecules to attach to the graft endothelium or to traffic into the spleen. In fact, marked upregulation of integrin $\alpha 4\beta 7$-expressing CD4 T cells was observed in ELP^{−/−} recipients. This may highlight the importance of targeting multiple pathways including $\alpha 4\beta 7$ as a plausible dual strategy alongside selectin blockade to achieve more robust prolongation. Interactions between integrins on T cells and their respective counter receptors are also functional in both acute and chronic cardiac allograft rejection, such as lymphocyte function antigen-1 ($\alpha L\beta 2$ integrin) and very late activation antigen-4 ($\alpha 4\beta 1$ integrin), which bind to intracellular adhesion molecule-1 and vascular cellular adhesion molecule-1, respectively.[27,28] In addition, long-term allograft survival was not achieved even in the complete absence

of both donor- and recipient-derived selectins, indicating again the capability of leukocytes to migrate into cardiac allografts *via* alternative pathways (*e.g.*, integrins).

We next explored the role of selectins in chronic cardiac allograft rejection. Previous reports[29,30] demonstrated that the intensity of arterial intimal thickening is significantly correlated with the intensity of endothelial expression of P-selectin and vascular cellular adhesion molecule-1 in a chronic rejection model of rat cardiac allografts. In human recipients of lung allografts, P-selectin expression in transbronchial biopsy specimens is strongly associated with acute rejection and the development of obliterative bronchiolitis.[31] These findings are consistent with our results in a single class II mismatch model, which clearly demonstrate the importance of donor-derived selectins in the development of graft arteriosclerosis. In addition, early-phase vasculitis (Figure 3) and late-phase graft arteriosclerosis (Figure 7) were similarly attenuated in ELP^{−/−} grafts, suggesting that the acute inflammatory response in cardiac allografts may contribute to the pathogenesis of graft arteriosclerosis. Because chronic rejection has become a major obstacle to achieving long-term allograft acceptance, the marked protection observed against chronic rejection in the immunocompetent recipients that received ELP^{−/−} hearts renders the idea of targeting donor selectins even more desirable.

We have demonstrated that donor-derived selectins play crucial roles in the development of both acute and chronic cardiac allograft rejection. These results advocate strategies to manipulate donor-derived selectins to achieve better allograft outcome.

CONCISE METHODS

Animals
Mice were purchased from Taconic (Germantown, NY) and Jackson Research Laboratory (Bar Harbor, ME). For more information, please refer to the online supplement.

Murine Cardiac Transplantation
Vascularized intra-abdominal heterotopic cardiac transplantation was performed as described previously.[32] Please refer to the online supplement for details.

Histologic Analyses
ELP^{−/−} or WT heart grafts were removed, and rejection was histologically examined. Please see online supplement for details.

Mixed Lymphocyte Reaction
Please refer to the online supplement for details of our mixed lymphocyte reaction assay.

Fluorescence Labeling and Flow Cytometric Analysis

Splenocytes were recovered from ELP$^{-/-}$ or WT C57BL/6 recipients of hearts from BALB/c donors, and cells were stained for various surface markers. For more detail, please see online supplement.

DISCLOSURES

None.

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