Impaired selectin-dependent leukocyte recruitment induces T-cell exhaustion and prevents chronic allograft vasculopathy and rejection

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Selectin–selectin ligand interactions mediate the initial steps in leukocyte migration, an integral part of immune responses. Fucosyltransferase-VII (FucT-VII), encoded by *Fut7*, is essential for biosynthesis of selectin ligands. In an established model of cardiac allograft vasculopathy and chronic rejection, *Fut7*$^{-/-}$ recipients exhibited long-term graft survival with minimal vasculopathy compared with WT controls. Graft survival was associated with CD4 T-cell exhaustion in the periphery, characterized by impaired effector cytokine production, defective proliferation, increased expression of inhibitory receptors programmed death-1 (PD-1) and T cell Ig- and mucin-domain–containing molecule-3 (Tim-3), low levels of IL-7Rα on CD4 T cells, and reduced migration of polyfunctional CD4 memory T cells to the allograft. Blocking PD-1 triggered rejection only in *Fut7*$^{-/-}$ recipients, whereas depleting regulatory T cells had no effect in either *Fut7*$^{-/-}$ or WT recipients. Adoptive transfer experiments confirmed that this CD4 T cell-exhausted phenotype is seen primarily in *Fut7*$^{-/-}$ CD4 T cells. These data suggest that impaired leukocyte recruitment is a novel mechanism leading to CD4 T-cell exhaustion. Our experimental system serves as an excellent model to study CD4 T-cell exhaustion as a dominant mechanism of transplant tolerance. Further, targeting FucT-VII may serve as a promising strategy to prevent chronic allograft rejection and promote tolerance.

adhesion | traffic | apoptosis

With the advent of newer, more powerful immunosuppressive regimens, short-term outcomes of solid organ transplants have improved dramatically, but long-term graft survival has not changed significantly (1). Chronic allograft rejection (CR) is the leading cause of graft failure and death in patients who survive beyond the first year after heart transplantation (2). However, currently there is no effective therapy for CR (3).

Chronic allograft vasculopathy (CAV) or graft arteriosclerosis characterized by vascular inflammation, neointimal hyperplasia, and vascular occlusion is a key component of CR (4). Endothelial injury, resulting in recruitment of circulating leukocytes, release of profibrotic cytokines, proliferation of vascular smooth muscle cells, and deposition of extracellular matrix proteins, plays a central role in the pathophysiologic mechanisms underlying CAV (3). In a model of CR, the intensity of arterial intimal thickening significantly correlated with endothelial expression of P-selectin (5).

Selectins mediate attachment and rolling, the first step in the leukocyte infiltration cascade that is essential for the ensuing steps of chemokine-activated adhesion and extravasation. Targeting selectins may therefore prove more effective than targeting the subsequent stages of firm arrest or transmigration. Studies targeting selectins for prevention of experimental allograft rejection in a variety of models, however, have met with mixed results (6–12). In cardiac allograft rejection, although anti-E or P-selectin mAbs or both delayed acute rejection (AR) (8), grafts in recipients lacking all three selectins were not protected from AR or CR (12). Lácha et al. (10) took advantage of targeting fucosyltransferase-VII (FucT-VII), because FucT-VII activity is required for biosynthesis of ligands for all three selectins. However, grafts in *Fut7*$^{-/-}$ recipients were not protected from AR. In contrast, although grafts from donors lacking only P-selectin were not protected from AR or CR (9), grafts from donors lacking all three selectins underwent delayed AR and were protected from CR in WT recipients (12). These and other studies (13, 14) together suggest that selectin-dependent interaction between leukocytes and the graft endothelium is a critical component of allograft rejection; however, the optimal method of targeting this interaction for prevention of rejection, particularly CR, is unclear.

Therefore, to clarify the role of selectin-dependent leukocyte recruitment in CR and to evaluate the potential for targeting selectin-dependent leukocyte recruitment for the prevention of CR, we evaluated cardiac allograft survival in *Fut7*$^{-/-}$ recipients in the bm12 model (3, 4, 15). Mice with a targeted disruption of *Fut7* display a profound loss of selectin ligands on both leukocytes and high endothelial cells but no defect in other adhesion molecules that play critical roles in leukocyte recruitment (16). Our results show that Fut7 deficiency in recipients of cardiac allografts results in long-term graft acceptance via a novel mechanism of interruption of leukocyte recruitment leading to CD4 T-cell exhaustion.

Results and Discussion

Selectin-Dependent Leukocyte Recruitment Is Required for Mediating Cardiac Allograft Vasculopathy and Chronic Rejection. We used the well-established bm12 single MHC class II mismatch model of

Significance

T-cell exhaustion limits the immune response against chronic infections and tumors. Reinvigorating exhausted T cells promotes clearance of infections and tumors. There are several ongoing clinical trials to harness the potential of reinvigorated T cells for the treatment of chronic infections and tumors by reversing T-cell exhaustion. However, the mechanisms leading to T-cell exhaustion are unknown. Further, the role of T-cell exhaustion in transplantation or autoimmunity is not defined. Here we show that impaired leukocyte recruitment leads to CD4 T-cell exhaustion as a novel mechanism of transplant tolerance. Strategies targeting leukocyte recruitment may prevent allograft rejection and promote tolerance. On the other hand, promoting leukocyte recruitment may prevent T-cell exhaustion and enhance tumor/microbe immunity.

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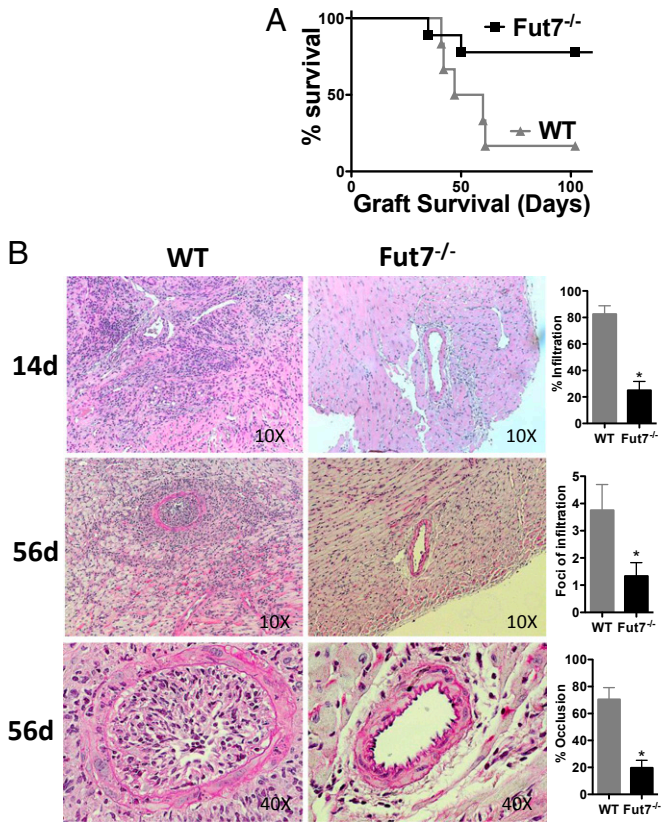

Fig. 1. Prolonged allograft survival due to protection from cardiac allograft vasculopathy and chronic rejection in Fut7$^{-/-}$ recipients. (*A*) Kaplan-Meier survival curves depicting bm12 graft survival in *Fut7*$^{-/-}$ recipients (black line; $n = 9$) compared with WT recipients (gray line; $n = 6$). (*B*) Representative light photomicrographs of bm12 grafts from WT recipients (*Left*) and *Fut7*$^{-/-}$ recipients (*Center*); and bar graphs (*Right*) depicting cellular infiltration (*Top*), foci of infiltration (*Middle*), and vascular occlusion (*Bottom*) at indicated time points.

CAV/CR (3, 4, 15). We found that bm12 cardiac allografts survived significantly longer in *Fut7*$^{-/-}$ recipients, with a median survival time (MST) of >100 d compared with 53 d in WT recipients ($P < 0.04$; Fig. 1*A*), suggesting that at least some of the *Fut7*$^{-/-}$ recipients were permanently tolerant of their grafts. Histologically, grafts from *Fut7*$^{-/-}$ recipients had significantly reduced leukocyte infiltration compared with WT recipients (25% ± 6.7% vs. 82.5% ± 6.3%, $P < 0.005$; Fig. 1*B*, *Top*) 14 d after transplantation (PT) and foci of inflammation (1.33 ± 0.95 vs. 3.75 ± 0.49, $P < 0.04$; Fig. 1*B*, *Middle*) and occlusion due to vascular endothelial proliferation (19.68% ± 5.59% vs. 70.43% ± 8.74%, $P < 0.0001$; Fig. 1*B*, *Bottom*) 56 d PT. These observations indicate that selectin-dependent leukocyte recruitment is required for mediating CAV/CR and are consistent with previous studies in diverse disease model systems demonstrating a significant role for FucT-VII–dependent leukocyte recruitment (17). Our data are consistent with results of protection from CR only when the graft rather than the recipient is deficient in expression of all three selectins (12). These studies show that nearly complete abrogation of interaction between selectins and selectin ligands as a result of *Fut7* deficiency is highly effective in preventing CAV/CR.

Functional Selectin Ligands Are Required for Recruitment of Leukocytes to the Allograft. The hallmark of allograft injury is the infiltration of leukocytes. Consistent with the decreased graft infiltration observed by histological assessment (Fig. 1*B*), the total number of graft-infiltrating leukocytes (GIL) isolated from the grafts was significantly lower in *Fut7*$^{-/-}$ recipients compared with WT recipients, both at early (day 1: $0.6 \pm 0.11 \times 10^6$ vs. $2.5 \pm 0.55 \times 10^6$, $P < 0.02$; Fig. 2*A*) and late (day 14: $0.32 \pm 0.04 \times 10^6$ vs. $0.59 \pm 0.07 \times 10^6$, $P < 0.04$; Fig. 2*B*) time points PT. The decreased early infiltration of leukocytes results in reduced ischemia reperfusion injury, similar to that seen in liver and kidney transplant recipients treated with recombinant P-selectin glycoprotein ligand-Ig (13, 14), which blocks selectin-dependent leukocyte adhesion. These lower total GIL numbers were also reflected in significantly lower numbers of CD11c$^+$ dendritic cells in the grafts in *Fut7*$^{-/-}$ recipients compared with WT recipients ($0.038 \pm 0.003 \times 10^6$ vs. $0.12 \pm 0.02 \times 10^6$, $P < 0.03$; Fig. 2*C*). Further, the grafts show significantly decreased infiltration of IFNγ/IL17A double-expressing CD4 T cells in *Fut7*$^{-/-}$ recipients compared with WT recipients ($4.05 \pm 0.8 \times 10^3$ vs. $8.5 \pm 1.4 \times 10^3$, $P < 0.05$; Fig. 2*D*), which although normally quite low in the periphery, accumulate in the graft and are critical for the pathogenesis of CAV in this model (3, 18, 19).

Loss of Selectin-Dependent Leukocyte Traffic Impairs Allospecific Priming in the Periphery. The lower numbers of dendritic cells in grafts in *Fut7*$^{-/-}$ recipients (Fig. 2*C*) suggest that less antigen is transported by these antigen-presenting cells (APC) to the secondary lymphoid organs, primarily spleen in this model. Consistent with this, we observed decreased priming and generation of memory (CD44^{hi}) CD4 T cells in the periphery (19.7% ± 0.3% vs. 32.2% ± 0.96% of CD4, $P < 0.005$; Fig. 3*A*). The impaired T-cell priming and memory generation in the periphery was accompanied by impaired production of inflammatory effector cytokines IFN-γ (0.51% ± 0.03% vs. 0.66% ± 0.04%, $P < 0.04$; Fig. 3*B*) and IL-17A (1.43% ± 0.03% vs. 1.86% ± 0.14%, $P < 0.05$; Fig. 3*C*) by splenic CD4 T cells in *Fut7*$^{-/-}$ recipients compared with WT recipients.

Together our data suggest that functional selectin ligands are required for efficient recruitment of host APC to the graft, subsequent allospecific priming of naïve CD4 T cells and generation of effector T cells in the spleen, and ultimately recruitment of these effector T cells to the graft. However, the fate of these effector cells in the periphery and the mechanisms of

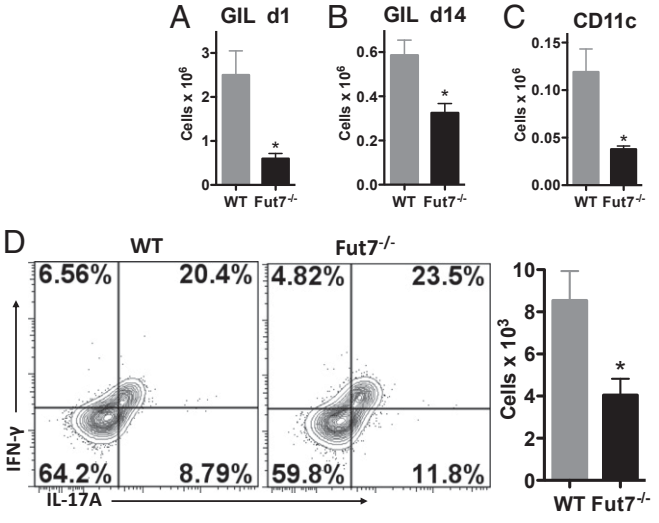

Fig. 2. Impaired leukocyte recruitment in Fut7$^{-/-}$ recipients. (*A–C*) Bar graphs depicting significant reduction in total leukocyte count at day 1 (*A*) and day 14 PT (*B*); and CD11c$^+$ cell count at day 14 PT (*C*) in the GIL from Fut7$^{-/-}$ compared with WT recipients. (*D*) Representative flow cytometry plots with adjacent bar graph depicting significant decrease in polyfunctional IFN-γ and IL-17A double producing CD4 T cells in the GIL from Fut7$^{-/-}$ compared with WT recipients 14 d PT.

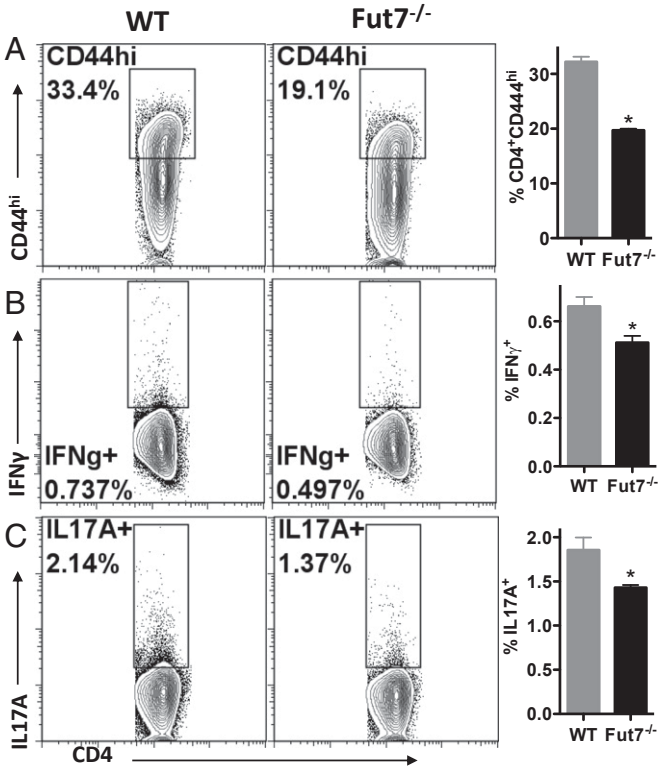

Fig. 3. Impaired allospecific priming of T cells in Fut7$^{-/-}$ recipients. (*A–C*) Representative flow cytometry plots with adjacent bar graphs depicting significant decrease in memory (*A*), IFN-γ (*B*), and IL-17A (*C*) producing CD4 T cells in the spleens from Fut7$^{-/-}$ compared with WT recipients 14 d PT. CD44, IFN-γ, and IL-17A staining of CD4 T cells from untransplanted mice were used to set the gates, and no significant differences were found between untransplanted Fut7$^{-/-}$ and WT mice (*SI Appendix*, Fig. S1).

tolerance to the allograft by impaired leukocyte recruitment remain to be elucidated.

Impaired Selectin-Dependent Leukocyte Recruitment Leads to CD4 T-Cell Exhaustion. The impaired inflammatory effector cytokine production (Figs. 2*D* and 3 *B* and *C*) and the absence of any detectable increase in regulatory T cells (Tregs) (4.84% ± 1.3% in *Fut7*$^{-/-}$ vs. 7.46% ± 1.1% in WT, $P > 0.2$) together suggested that graft tolerance observed in *Fut7*$^{-/-}$ recipients in this CR model might be due to T-cell exhaustion. Indeed, a significantly higher proportion of splenic CD4 T cells expressed PD1, a molecular marker of exhausted T cells, (15.57% ± 1.13% vs. 10.43% ± 0.52%, $P < 0.02$; Fig. 4*A*), with a significantly higher density of this inhibitory receptor expressed on a per-cell basis [median fluorescence intensity (MFI) of PD1$^+$ cells: 199.7 ± 5.6 vs. 172.7 ± 7.9, $P < 0.05$; Fig. 4*A*], whereas there was no significant difference in PD1 expression by CD4 T cells between *Fut7*$^{-/-}$ and WT recipients of syngeneic heart transplants (9.72% ± 0.50% vs. 10.05% ± 1.09%, $P = 0.799$; MFI of PD1$^+$ cells: 4,008 ± 232.7 vs. 4,217 ± 414.3, $P = 0.682$; *SI Appendix*, Fig. S2) and between untransplanted *Fut7*$^{-/-}$ and WT mice (7.12% ± 1.95% vs. 4.93% ± 0.49%, $P = 0.391$; MFI of PD1$^+$ cells: 146.5 ± 15.5 vs. 145.5 ± 5.5, $P = 0.957$; *SI Appendix*, Fig. S3). We also found that expression of other inhibitory receptors associated with T-cell exhaustion (20), including Tim-3 and KLRG1, was essentially limited to CD4 T cells that were PD1$^+$ (Tim-3: 8.32% ± 1.3% vs. 1.36% ± 0.1%, $P < 0.004$, Fig. 4*B*; and KLRG1: 9.81% ± 0.5% vs. 0.31 ± 0.03%, $P < 0.0001$, Fig. 4*C*). Furthermore, CD127, the α-chain of IL-7 receptor, which is down-regulated on exhausted T cells, where its low expression correlates with enhanced apoptosis (21), was also lower on PD1$^+$ compared with PD1$^-$ CD4 T cells (7.447% ± 0.4% vs. 13.47% ± 0.4%, $P < 0.001$ and MFI 5.847 ± 0.2 vs. 9.213 ± 0.05, $P < 0.001$; Fig. 4*D*), and the PD1$^+$ cells were indeed undergoing more apoptosis compared with PD1$^-$ CD4 T cells (19% ± 3.7% vs. 1.9% ± 0.4%, $P < 0.02$; Fig. 4*E*). Similar results with respect to Tim-3, KLRG1, and CD127 expression were found in PD1$^+$ vs. PD1$^-$ CD4 T cells from WT recipients (*SI Appendix*, Fig. S4), and there was no significant difference between WT and *Fut7*$^{-/-}$ recipients of syngeneic heart transplants (*SI Appendix*, Figs. S5 and S6) and between untransplanted WT and *Fut7*$^{-/-}$ mice (*SI Appendix*, Figs. S7 and S8).

Coexpression of inhibitory receptors with low expression of CD127 correlates with impaired proliferative capacity (22), and impaired proliferative potential is a key feature of T-cell exhaustion. Using Ki67 as a marker of proliferation, we noted that a significantly lower proportion of CD4 T cells from *Fut7*$^{-/-}$ recipients were proliferating in vivo in response to alloantigen encounter after transplantation compared with those from WT recipients (8.37% ± 0.8% vs. 11.1% ± 0.4%, $P < 0.04$; Fig. 4*F*), whereas there was no significant difference in Ki67 expression by CD4 T cells between *Fut7*$^{-/-}$ and WT recipients of syngeneic heart transplants (6.46% ± 0.56% vs. 4.66% ± 0.64%, $P = 0.10$; *SI Appendix*, Fig. S9) or between untransplanted *Fut7*$^{-/-}$ and WT mice (1.67% ± 0.31% vs. 5.61% ± 1.37%, $P = 0.107$; *SI Appendix*, Fig. S10). We confirmed the proliferative defect in an ex vivo carboxyfluorescein succinimidyl ester (CFSE) assay, where we found that CD4 T cells from *Fut7*$^{-/-}$ recipients proliferated significantly less compared with those from WT in response to allogeneic restimulation (1.1% ± 0.1% vs. 2.7% ± 0.6%, $P < 0.04$, Fig. 4*G*; and 1,104 ± 466.7 vs. 5,135 ± 1,541 per 1×10^5 cells, $P < 0.04$, Fig. 4*H* and *SI Appendix*, Fig. S11 *C* and *G*) and somewhat less with syngeneic restimulation (0.94% ± 0.2% vs. 1.96% ± 0.76%, P = ns, Fig. 4*I*; and 983.7 ± 634.3 vs. 2,746 ± 1,095 per 1×10^5 cells, P = ns, Fig. 4*J* and *SI Appendix*, Fig. S11 *B* and *F*). However, there was no difference in proliferation with syngeneic or allogeneic restimulation in either WT (*SI Appendix*, Fig. S11 *B* and *C*) or *Fut7*$^{-/-}$ CD4 cells (*SI Appendix*, Fig. S11 *F* and *G*). This might be due to the fact that the CD4 T cells were activated in vivo after transplantation and therefore continued to proliferate in the absence of antigen but in the presence of APC, as has been described before (23). Further, this exhausted phenotype was not associated with a defect in IFN-γ (6.98% ± 1.55% vs. 6.217% ± 0.794%, $P = 0.684$; *SI Appendix*, Fig. S12*A*) or IL17A (6.63% ± 2.12% vs. 6.46% ± 0.52%, $P = 0.942$; *SI Appendix*, Fig. S12*B*) production by CD4 T cells from *Fut7*$^{-/-}$ recipients compared with WT in response to allo-antigen rechallenge ex vivo. This is consistent with the notion that T cells undergo exhaustion in a hierarchical manner with loss of proliferative potential preceding loss of cytokine production (24). Taken together, these data show that significantly increased proportions of alloreactive CD4 T cells in *Fut7*$^{-/-}$ recipients exhibit multiple phenotypes associated with exhausted T cells, including proliferative defects.

T-Cell Exhaustion Is the Dominant Regulatory Mechanism in Allograft Tolerance Induced by Impaired Leukocyte Recruitment. The PD-1:PD-L1 pathway is recognized to be important in autoimmunity, allergy, allograft rejection, chronic viral infections, and antitumor immunity (25, 26). This pathway plays a major role in regulating CD8 T-cell exhaustion during chronic viral infections, because blocking this pathway reinvigorates the CD8 T cells and facilitates viral control (27). Although there are reports demonstrating a role for CD4 T-cell exhaustion in chronic viral infections and cancers (24), the majority of the studies are on the involvement of this pathway in controlling CD8 T-cell responses. Moreover, although the importance of the PD-1:PD-L1 pathway in CAV and rejection has been studied (28), the relationship of PD-1 expression on CD4 T cells to T-cell exhaustion in transplantation, and its induction by alteration of leukocyte traffic, has not been described previously. Previous studies used anti-PD-L1

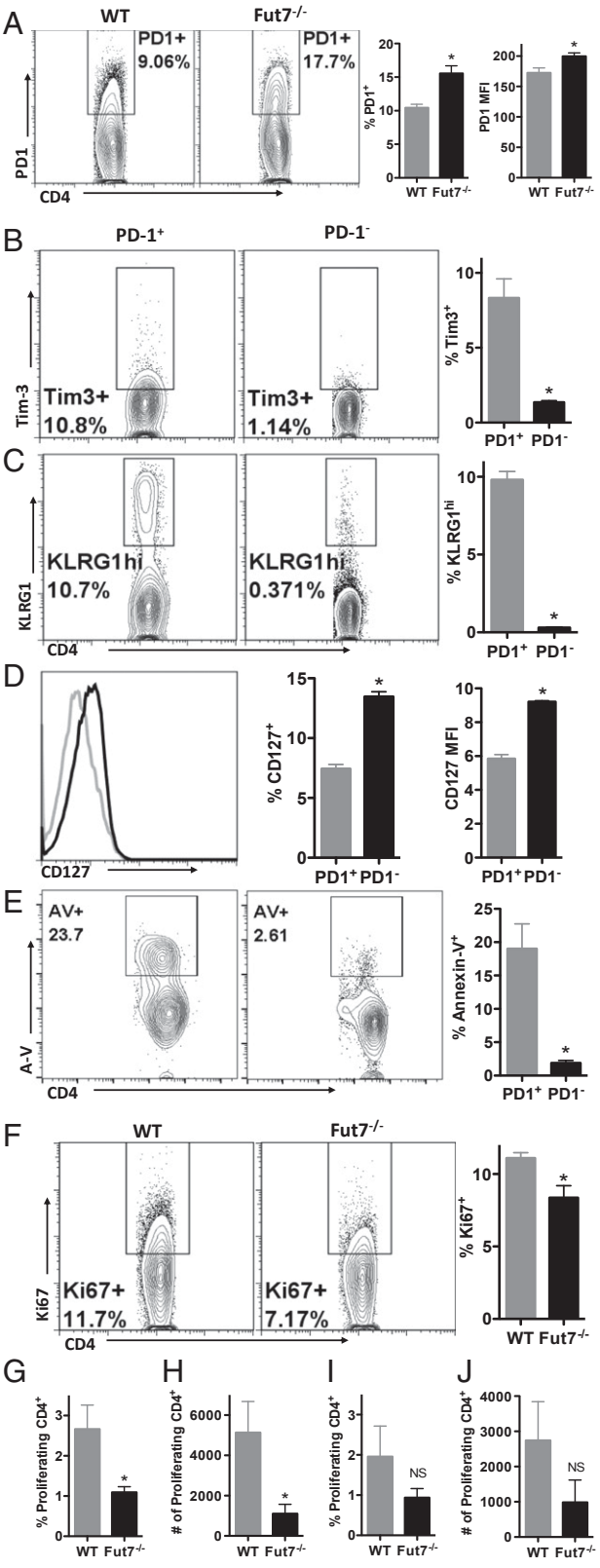

Fig. 4. CD4 T cells from Fut7$^{-/-}$ recipients exhibit an exhausted phenotype. (*A–J*) Representative flow cytometry plots with adjacent bar graphs depicting significantly increased PD-1 expression (% and MFI) by CD4 T cells in spleens from Fut7$^{-/-}$ compared with WT recipients 14 d PT (*A*); increased Tim-3 (*B*) and KLRG1^{hi} (*C*) and decreased CD127 (% and MFI) (*D*) and increased annexin-V (AV) (*E*) expression by PD-1^{+} vs. PD-1^{-} CD4 T cells from Fut7$^{-/-}$ recipients 14 d PT; decreased proliferation of CD4 T cells from Fut7$^{-/-}$ compared with WT recipients in vivo by Ki67 expression (*F*) and in vitro by CFSE dilution, upon allospecific restimulation (percentages, *G* and numbers, *H*) and syngeneic restimulation (percentages, *I* and numbers, *J*).

mAb to inhibit the PD-1:PD-L1 pathway, but PD-L1, in addition to binding to PD-1, also binds to B7-1 (25), suggesting that previously observed effects of anti-PD-L1 mAb treatment could have been due in part to inhibiting the negative signal delivered by B7-1. Therefore, we chose to block the PD-1:PD-L pathway by targeting PD-1. We found that anti-PD-1 mAb overcame tolerance and precipitated AR in all *Fut7*$^{-/-}$ recipients by day 34 (MST: 22 d in anti-PD1 *Fut7*$^{-/-}$ vs. >100 d in control *Fut7*$^{-/-}$, $P < 0.0001$; Fig. 5*A*), whereas none of the WT recipients treated with anti-PD1 rejected their grafts by day 40 (MST: *Fut7*$^{-/-}$ 22 d vs. WT >40 d, $P < 0.03$; *SI Appendix*, Fig. S13*A*). In contrast, depleting regulatory T cells had no significant effect in either *Fut7*$^{-/-}$ (MST: >100 d for both anti-CD25 *Fut7*$^{-/-}$ and control *Fut7*$^{-/-}$, $P = 0.77$; *SI Appendix*, Fig. S13*B*; compare with Fig. 1*A*) or WT recipients (MST: 56 d in anti-CD25 WT vs. 60 d in control WT, $P = 0.85$; *SI Appendix*, Fig. S13*C*; compare with Fig. 1*A*). These data indicate that maintenance of allograft tolerance induced by inhibition of leukocyte traffic is independent of FoxP3^{+} regulatory T cells.

Precipitation of AR with PD-1 blockade was accompanied by increased IFN-γ (2.4% ± 0.3% vs. 1.41% ± 0.04%, $P < 0.04$; Fig. 5*B*) and IL-17A (1.56% ± 0.23% vs. 0.74% ± 0.03%, $P < 0.03$; Fig. 5*C*) production by CD4 T cells in the spleen. In the absence of selectin-dependent leukocyte recruitment other adhesion molecules, particularly VLA-4, are still functional and can contribute to leukocyte recruitment (29, 30). Thus, reversing T-cell exhaustion and thereby clonal expansion with PD1 blockade results in a net increase in recruitment of alloreactive T cells to the graft, as evidenced by increased infiltration of IFN-γ– and IL-17A–expressing CD4 T cells in the grafts of *Fut7*$^{-/-}$ recipients compared with WT recipients ($29.5 \pm 3.4 \times 10^3$ vs. $6.0 \pm 1.7 \times 10^3$, $P < 0.004$). These data are consistent with previous observation that *Fut7*$^{-/-}$ recipients, with a large alloreactive clone size against full-MHC mismatched heart allografts, reject their grafts promptly (10). Together, these data indicate that PD-1 blockade reinvigorates exhausted CD4 T cells in *Fut7*$^{-/-}$ recipients and that T-cell exhaustion is the dominant regulatory mechanism in allograft tolerance induced by targeting leukocyte recruitment.

T-Cell Exhaustion Is Seen Primarily in CD4 T Cells with Impaired Migration. In mice and humans harboring tumors or chronic viral infections, antigen persistence is associated with the development of CD8 T-cell exhaustion (24), but it remains unclear whether antigen persistence is a consequence or a cause of exhaustion. We designed an experiment to examine the development of exhaustion in T cells with normal or impaired migration capability, in response to alloantigen encounter in vivo after transplantation, in an otherwise normal environment. To this end, we adoptively transferred CD45.2^{+}GFP^{-} *Fut7*$^{-/-}$ and CD45.2^{+}GFP^{+} WT leukocytes into congenic CD45.1^{+} WT recipients that were subsequently transplanted with a bm12 heart (Fig. 6*A*). On day 14 PT, we observed that a significantly higher proportion of *Fut7*$^{-/-}$ CD4 T cells compared with WT CD4 T cells expressed PD1 (39.6% ± 3.6% vs. 4.0% ± 0.89%, $P < 0.0001$; Fig. 6*B*), with significantly higher density (MFI of PD1^{+} cells: 346.5 ± 22.75 vs. 132.5 ± 10.37, $P < 0.001$; Fig. 6*B*). Interestingly, the percentages of *Fut7*$^{-/-}$ CD4^{+}PD1^{+} cells in the adoptive transfer experiments (Fig. 6*B*) are much higher than the *Fut7*$^{-/-}$ CD4^{+}PD1^{+} cells in the *Fut7*$^{-/-}$ recipients of bm12 hearts (Fig. 4*A*) (39.6% ± 3.6% vs. 15.57% ± 1.13%, $P < 0.003$). One possible explanation is that the *Fut7*$^{-/-}$ CD4 T cells in the adoptive transfer experiments are in a normal (increased compared with that in Fut7$^{-/-}$ environment) antigen presentation environment [i.e., *Fut7*$^{-/-}$ CD4 T cells interact with more (WT)

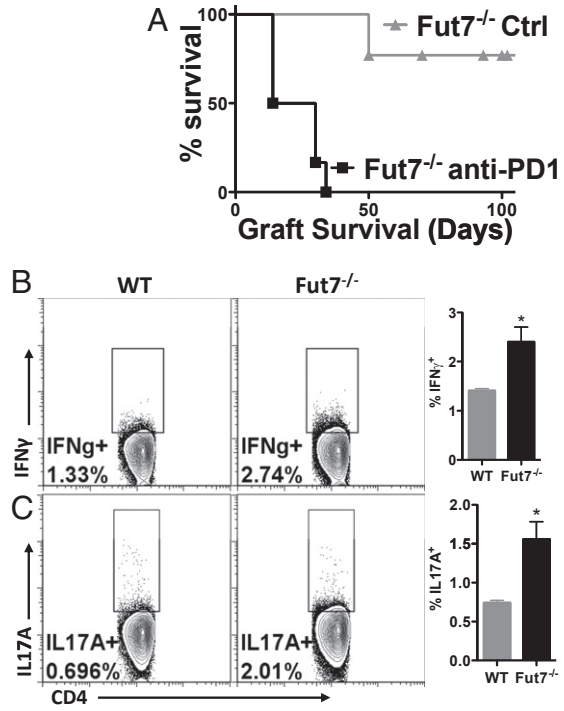

Fig. 5. Prolonged allograft survival in Fut7$^{-/-}$ recipients is dependent on T-cell exhaustion. (*A*) Kaplan-Meier survival curves depicting bm12 graft survival in anti-PD1 treated Fut7$^{-/-}$ recipients (black line; $n = 6$) compared with control Fut7$^{-/-}$ recipients (gray line; $n = 13$). (*B* and *C*) Representative flow cytometry plots with adjacent bar graphs depicting significant increase in IFN-γ (*B*) and IL-17A (*C*) producing CD4 T cells in the spleens from Fut7$^{-/-}$ compared with WT recipients treated with anti-PD-1 mAb; compare with cytokine production in untransplanted mice (*SI Appendix*, Fig. S14).

APC]. Coupled to the impaired migration capability of the *Fut7*$^{-/-}$ CD4 T cells, this leads to greater T-cell exhaustion. These findings indicate that the *Fut7*$^{-/-}$ T cells that are unable to migrate to the target organ consequently undergo continued activation and eventual exhaustion, whereas WT T cells experiencing the same amount of antigen are recruited to the target organ, mediate effector function, and do not show features of exhaustion. These data demonstrate that in a single mouse with a single transplant (which by definition presents the same antigen load and persistence), WT and *Fut7*$^{-/-}$ T cells behave very differently. Taken together, our data showing increased PD-1 expression selectively in *Fut7*$^{-/-}$ T cells in an otherwise normal environment suggest that T-cell exhaustion is a T cell-intrinsic phenomenon that is largely independent of antigen load or persistence. Recent studies show that a pathogen-specific inflammatory milieu enhances proliferative and effector capacity of memory T cells (31–33). Our data further suggest that T-cell exhaustion results from the inability of the T cells to migrate to the target organ, leading us to speculate that T cells activated in secondary lymphoid organs receive some further "instruction" in the target organ to become fully activated, mediate effector function, generate effective memory, and escape exhaustion. In our system this "additional programming" could be occurring at three mutually nonexclusive levels: (*i*) selectin-dependent interaction between T cell and endothelium of the graft, (*ii*) upon exposure to inflammatory cytokines in the graft, and (*iii*) upon execution of productive effector function.

We propose a model in which inhibition of leukocyte recruitment blunts ischemia/reperfusion injury early after transplant, as well as recruitment of host APC to the graft. This results in lower antigen levels in the draining lymphoid organ, leading to impaired T-cell priming and diminished generation of alloreactive memory T cells. Further, the few primed alloreactive T cells that do result are unable to access the graft owing to their lack of functional selectin ligands, thus limiting damage to the tissue and promoting long-term graft survival. The primed CD4 T cells, because of their inability to be recruited to the target organ and execute effector function, undergo proliferative arrest, functional exhaustion, and ultimately apoptotic clonal deletion.

Intriguingly, immunosuppressive medications used in transplantation have differential effects on PD-1 expression by T cells. For example, cyclosporine inhibits induction of PD-1 (34), whereas mycophenolate induces PD-1 expression (35), leading us to speculate that the decreased incidence of CAV with mycophenolate compared with calcineurin inhibitor-based immunosuppression in human heart transplant recipients (36) may be due to promotion of PD-1 expression and T-cell exhaustion. Thus, our present results suggest a novel mechanism for T-cell exhaustion in the context of transplantation and a rationale for designing better immunosuppressive protocols.

To our knowledge, this is the first report that impaired leukocyte recruitment leads to CD4 T-cell exhaustion as a novel mechanism of transplant tolerance and suggests the possibility that this mechanism may be operative in other settings of organ- or tissue-specific immunity. Our experimental system serves as an excellent model to study CD4 T-cell exhaustion as a dominant mechanism of transplant tolerance. Further, targeting FucT-VII with novel metabolic inhibitors (37) may serve as a promising strategy to prevent CR and promote tolerance.

Materials and Methods

Animals. C57BL/6, B6.C-H2^{bm12} (bm12), and *Fut7*$^{-/-}$ mice (16) were bred in our facility. Experiments were approved by the Institutional Animal Care and Use Committee at the Northwestern University Feinberg School of Medicine.

Heart Transplantation and Antibody Treatment. Vascularized heart grafts from bm12 donor mice were transplanted heterotopically, using a standard microsurgical technique, in C57BL/6 WT or *Fut7*$^{-/-}$ on a C57BL/6 background (38). To block PD-1:PD-L signaling, blocking anti-PD-1 mAb (clone J43; Bio-X-cell)

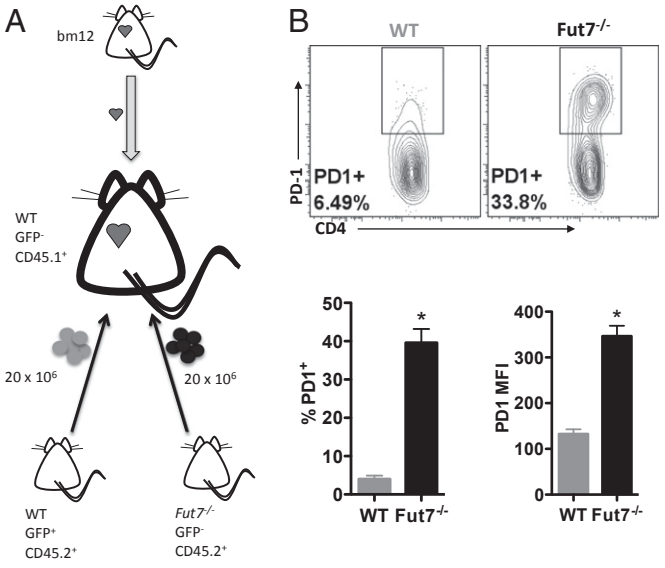

Fig. 6. Exhausted phenotype is seen primarily in Fut7$^{-/-}$ CD4 T cells. (*A*) Diagram depicting experimental scheme of adoptive transfer of splenocytes from naïve Fut7$^{-/-}$ and WT-GFP mice into a WT congenic host subsequently transplanted with a bm12 heart 1 d later. (*B*) Representative flow cytometry plots with adjacent bar graphs depicting significantly increased PD-1 expression (% and MFI) by Fut7$^{-/-}$ CD4 T cells compared with WT CD4 T cells recovered 14 d PT from spleens of adoptively transferred congenic hosts transplanted with a bm12 heart.

was administered i.p., 0.5 mg on day 10 and 0.25 mg on day 12 and day 14 PT or 0.5 mg on day 14 and 0.25 mg on days 16, 18, 20, 22, and 24 PT. To deplete Tregs, anti-CD25 mAb (clone PC61; Bio-X-cell) was administered i.p., 0.5 mg on day 28 and day 30 PT. Graft function was assessed by biweekly palpation of the abdomen. Rejection was defined as complete cessation of cardiac contractility as determined by direct visualization.

Adoptive Transfer Experiments. Twenty million splenocytes each from naïve CD45.2$^+$GFP$^-$*Fut7*$^{-/-}$ and CD45.2$^+$GFP$^+$ WT were together transferred into naïve congenic CD45.1$^+$ WT recipients that were subsequently transplanted with a bm12 heart (Fig. 6*A*). On day 14 PT, spleen cells from these recipients were analyzed by flow cytometry by gating on CD45.2$^+$GFP$^+$ (WT) or CD45.2$^+$GFP$^-$ (*Fut7*$^{-/-}$) cells.

Histology. Grafts harvested at day 14 or day 56 PT were sectioned transversely, fixed in 4% buffered formalin, and stained by hematoxylin and eosin and periodic acid schiff stains. Leukocytes and foci of infiltrate were counted in five high-power fields and four transverse sections respectively per slide, one slide each from five to six heart grafts per group. Vascular occlusion was quantified by calculating the percentage of vascular lumen occluded by intimal proliferation in each of the arteries in four transverse sections per slide, one slide each from five to six heart grafts per group. Results are presented as mean number of cells, foci of infiltration, or as percentage vascular occlusion ± SEM.

Isolation of GIL. Cardiac grafts were harvested at the designated time points, incubated at 37 °C with collagenase-II/DNase I mixture for 20 min, then passed gently through a 40-μm filter (BD Biosciences). Cells were washed, counted, and directly stained with fluorochrome-conjugated antibodies, and analyzed by flow cytometry.

Flow Cytometry. Fluorochrome-conjugated antibodies were purchased from Biolegend (FITC anti-IFNγ and -Foxp3; PE anti-TIM-3; PerCP-Cy5.5 anti-CD4; PECy7 anti-PD-1; APC-Cy7 anti-CD11c and -CD44; AF700 anti-IL17A), BD Biosciences (V500 anti-Annexin-V; V450 anti-CD127), eBioscience (PE anti-KLRG1; AF660 anti-Ki67), and Invitrogen (Live/Dead stain).

One million splenocytes in suspension in PBS with Ca^{2+}/Mg^{2+} were stained with fluorochrome-conjugated antibodies to surface markers for 30 min then washed twice. After 20 min of incubation at room temperature with 4% (wt/vol) paraformaldehyde, cells were washed with 1 mL of permeabilization buffer (Biolegend), stained intracellularly with fluorochrome-conjugated antibodies, washed once with the permeabilization buffer then once with PBS, and suspended in 200 μL PBS. Data were acquired using a BD LSRFortessa analyzer (BD Biosciences) at the Northwestern University Flow Core Facility and analyzed by Flowjo software (Tree Star).

Allospecific Stimulation Assay. Half a million WT or *Fut7*$^{-/-}$ responder spleen cells were cultured in complete DMEM with sonicate from 0.5×10^6 bm12 spleen cells per well in triplicate for 16 h. Detection of cytokine production was performed by flow cytometry.

Allospecific Proliferation Assay. CD4 T cells from bm12-heart recipients 7–14 d PT were stained with CFSE at 2.5 μM CFSE/1×10^6 cells according to the manufacturer's instructions (Invitrogen). CFSE-labeled WT or *Fut7*$^{-/-}$ responder CD4 T cells (0.1×10^6) were cultured in complete DMEM alone, with 0.1×10^6 bm12, WT, or *Fut7*$^{-/-}$ irradiated (3000 Rad) T-cell depleted splenocytes (APC), or 2.5 μg/mL of anti-CD3/CD28 per well for 3 d. Detection of proliferation was performed by flow cytometry.

Statistics. For graft survival analysis, Kaplan-Meier graphs were constructed, and log–rank comparison of the groups was used to calculate *P* values. For cell counts and percentages, data are presented as mean ± SEM, and comparisons between the values were performed using the two-tailed Student *t* test. For all statistical analyses, the level of significance was set at a probability of $P < 0.05$. All experiments were repeated at least two to five times with at least two to three animals per group. Asterisks on top of error bars indicate statistically significant differences between the two groups being compared.

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