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β3 Integrins Regulate Lymphocyte Migration and Cytokine Responses in Heart Transplant Rejection

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Integrin αvβ3 is important for cell survival, signaling and migration, particularly during angiogenesis and tumorigenesis, where it has been proposed as a therapeutic target. αvβ3 is up-regulated following transplantation and β3 polymorphisms are associated with increased acute kidney rejection, suggesting that αvβ3 may also play a role in transplant rejection. Here, using a model of allogeneic heart transplantation, we show that allograft survival is prolonged in β3 integrin-deficient ($\beta3^{-/-}$) mice. This is associated with Th2-type immune responses and reduced T-cell infiltration into grafts and T cells from $\beta3^{-/-}$ mice show impaired adhesion and migration, consistent with a role for αvβ3 in transmigration. These studies provide evidence that targeting β3 integrins impairs recruitment of effector cells and alters cytokine production, so prolonging graft survival. We also show that low doses of blocking antibodies against leukocyte function associated antigen-1 (LFA-1)/αLβ2 and very late antigen-4 (VLA-4)/α4β1, when combined with deletion of β3, lead to long-term survival of allografts with no evidence of chronic rejection. Hence we provide strong mechanistic evidence supporting previous genetic studies, demonstrate the involvement of β3 integrins in both acute and chronic rejection and identify β3 as a new target for immunosuppressive therapy.

Key words: Acute rejection, chronic rejection, extracellular matrix, integrin, migration

Abbreviations: LFA-1, leukocyte functional antigen-1; MLR, mixed lymphocyte reaction; VLA, very late antigen; ECM, extracellular matrix; MST, median survival time; BSA, bovine serum albumin; DCs, dendritic cells.

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Introduction

Organ transplantation holds great promise for long-term treatment of many disorders but remains plagued by the problems of acute and chronic allograft rejection. Many advances have been made in treatments to suppress rejection, although the limitations and complications of current immunosuppressive therapies reveal a need for new strategies and targets (1). Manipulating the migration and trafficking of lymphocytes provide a good potential area for therapy and, by careful target selection, may allow manipulation of specific cells or stages of the rejection response. Many developing therapies are directed toward signals that control cycling of cells from lymph to blood (such as FTY720, an antagonist of the sphingosine-1-phosphate receptor) or chemokines that stimulate the directed migration of lymphocytes. However, adhesion molecules such as integrins, which directly mediate lymphocyte activation and migration, also make good targets and integrin-directed therapies have been used with some success in a number of diseases (2).

Integrins are a family of heterodimeric cell adhesion molecules, mediating cell–cell interactions or adhesion between cells and extracellular matrix (ECM) (3). Integrins are vital to the functioning of the immune system, facilitating cell migration and cell–cell signaling. For example, T cells use specific integrins [principally leukocyte function associated antigen-1 (LFA-1)/αLβ2 and very late antigen-4 (VLA-4)/α4β1] to arrest in blood vessels and enter inflamed tissues or lymph nodes, in the generation of the immunological synapse with dendritic cells (DCs) and in co-stimulatory signaling (4). It has long been known that blockade of LFA-1 and VLA-4 dramatically reduces allograft rejection, through effects on both T-cell migration and

antigen presentation, making these molecules and their ligands good possible targets for therapeutic intervention (4).

However, lymphocytes express other integrins whose roles in transplant rejection have not been explored. One such integrin is β3 which exists as αvβ3 (the vitronectin receptor, VnR) on endothelium and leukocytes and as αIIbβ3 (platelet glycoprotein IIb/IIIa) on platelets. Genetic studies have shown that homozygosity for the common β3 polymorphism PLA1 in transplant recipients is correlated with acute renal allograft rejection (5), and these effects were attributed to putative effects on αIIbβ3 and platelet function. However, vascular endothelium and infiltrating leukocytes up-regulate expression of αvβ3 following ischemia or during transplant rejection (6,7), raising the possibility of effects on αvβ3 function also affecting rejection, particularly considering the role of αvβ3 in T-cell stimulation (8–10) and inflammatory cell recruitment (11). Hence, together these data suggest that β3 may play important roles in transplant rejection.

In this study, we have used a mouse model of cardiac allograft transplantation to determine the contribution of β3 integrins to the rejection process. We show that transplants are rejected later from recipients lacking β3 integrins than from wild-type controls, that T-cell infiltrates are greatly reduced in rejecting grafts from $\beta3^{-/-}$ recipients and that $\beta3^{-/-}$ CD8 T cells show impaired adhesion and migration on ECM. Rejection by $\beta3^{-/-}$ recipients is associated with a Th2-biased allograft response and increases in expression of Th2 cytokines in the rejecting transplant. We further show that blockade of additional integrins (VLA-4/α4β1 and LFA-1/αLβ2), when combined with β3 deficiency, leads to extended graft survival and prevention of chronic rejection. The significant prolongation of graft survival in $\beta3^{-/-}$ recipients compared with wild-type recipients identifies integrin β3 as a potential new target for suppression of both acute and chronic transplant rejection.

Materials and Methods

Mice

BALB/c $\beta3^{-/-}$ mice were generated by backcrossing $\beta3^{+/-}$ to BALB/c mice for more than seven generations, and intercrossing. Control BALB/c mice and donor C57BL/6 mice were obtained from Jackson Laboratories (Bar Harbor, ME). All mice were used at 6–12 weeks of age. All experiments were in accordance with the Institutional Animal Care and Use Guidelines.

Transplantation

Hearts from C57BL/6 (allograft) or BALB/c (isograft) donors were transplanted intra-abdominally into BALB/c wild-type or $\beta3^{-/-}$ mice as previously described (12). Data are presented as median survival time (MST) in days. Rejection was also evaluated by histological analysis on days 5 or 30 using the International Society for Heart and Lung Transplantation nomenclature system (13; see supplementary methods). For LFA-1 and VLA-4 blockade, mice received injections of 4 mg/kg anti-LFA-1 and VLA-4 antibodies (Bioexpress Cell Culture Inc., West Lebanon, NH) on day −1, 0, +1 and +2 relative to the day of transplantation. To assess donor-specific tolerance, hearts from C57BL/6 mice were first transplanted into wild-type and $\beta3^{-/-}$ recipients with LFA-1/VLA-4 treatment, followed by skin transplants from C57BL/6 (allograft) donors 5 days later, as previously described (14).

Immune cell analysis

Detailed methods are provided in figure legends and online supplementary information. For FACS analysis, cells were prepared from collagenase treated hearts or from mechanically disrupted spleens, stained with directly conjugated antibodies or biotinylated antibodies followed by fluorescent-conjugated streptavidin, and analyzed using a FACS Caliber Cytometer (Becton-Dickinson, San Jose, CA). ELISPOT assays were performed on total splenocytes as previously described (15). For proliferation assays, T cells were sorted by positive selection with anti-CD90/Thy1.2 beads and DCs sorted using anti-CD11c beads (both Miltenyi Biotech). Proliferation was assessed using Cell Titer 96 Aq reagent (Promega, Madison, WI), by loss of CFSE staining or by incorporation of $[^3H]$-thymidine. For adhesion and migration assays, effector T cells were generated from total splenocytes by culture with anti-CD3 and IL-2 (16).

Results

Integrin β 3 gene deletion prolongs graft survival in mice

Human genetic studies have linked β3 polymorphisms to transplant rejection (5). To elucidate possible roles for β3 integrins in acute transplant rejection, we used $\beta3^{-/-}$ mice as recipients for vascularized cardiac allografts. Cardiac grafts from C57BL/6 donors (H-2^b) were transplanted into fully allogeneic wild-type or $\beta3^{-/-}$ BALB/c recipients (H-2^d). The rate of allograft rejection was significantly slower in $\beta3^{-/-}$ recipients with MST increased by twofold over wild-type recipients (Figure 1A; MST 18 days vs. 9 days, $n = 5$ $\beta3^{-/-}$ and $n = 6$ wild-type, $p = 0.0014$). Histological analysis of grafts 5 days after transplantation into wild-type recipients showed cellular rejection with a strong lymphocyte infiltrate and significant myocyte death and hemorrhage (Figure 1C). Histology of allografts recovered from $\beta3^{-/-}$ recipients showed sparse lymphocyte infiltrates, few regions of focal myocyte necrosis, and a much higher proportion of viable myocytes than seen in controls (Figure 1C), reflected in a significantly reduced rejection score compared to wild-type mice (Figure 1B; $p = 0.0286$). Hence, deletion of β3 integrins significantly reduced acute rejection in allografts and prolonged graft survival.

Equivalent allograft T-cell responses in wild-type and β $3^{-/-}$ recipients

We reasoned that the delayed rejection of grafts in $\beta3^{-/-}$ recipients could be due to a failure to mount allograft responses or specific defects in subsequent lymphocyte functions. We therefore set out first to assess the allograft response in wild-type and $\beta3^{-/-}$ recipients. The immune response to the transplant can be assayed by the appearance of activated effector T-cells (defined as $CD8^+$ $CD62^{low}$ $CD44^{high}$) in the spleen (17). $\beta3^{-/-}$ mice have higher numbers of activated T-cells in the spleen before transplantation ($16.6 \pm 1.2\%$ vs. $8.4 \pm 0.9\%$; Figure 2A) and the reason for this increase in baseline remains unknown. However following transplantation the proportion of activated cells increases by equivalent amounts in both wild-type and $\beta3^{-/-}$ mice, showing that recipients of both genotypes mount

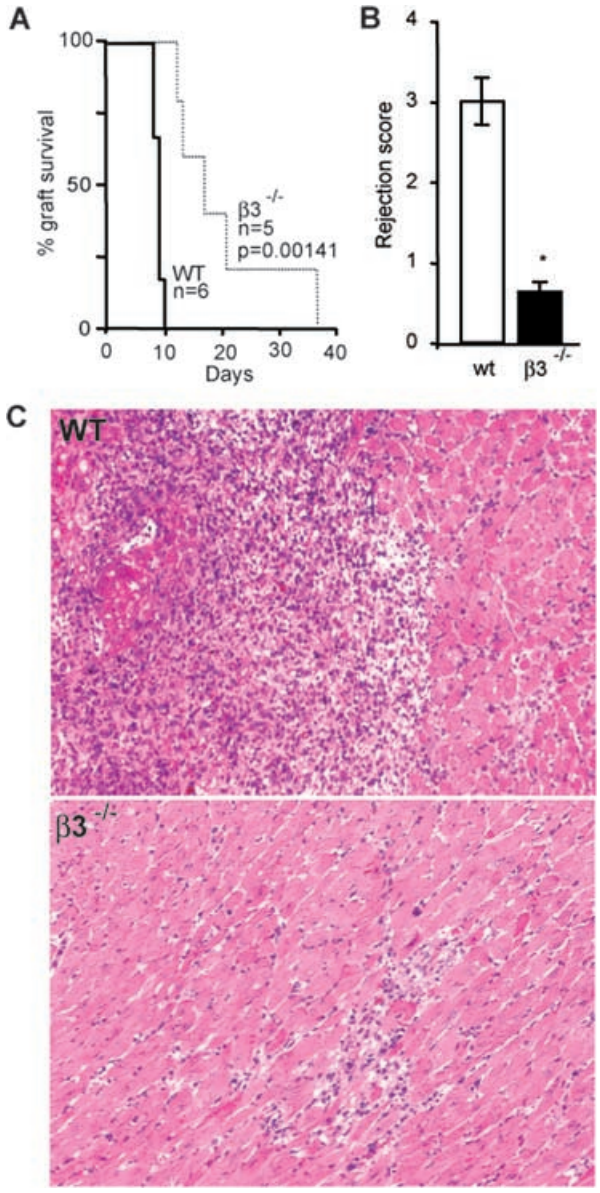

Figure 1: Cardiac allograft survival in wild-type (WT) and $\beta 3^{-/-}$ recipients. (A) C57BL/6 hearts were transplanted into WT (solid lines; n = 6) or $\beta 3^{-/-}$ (dashed line; n = 5) BALB/c recipients. Rejection was assessed by palpation daily. (B) Mean rejection score of grafts from WT and $\beta 3^{-/-}$ recipients at day 5. Bars represent s.e.m., n = 4 mice/group. *, $p < 0.05$ (Mann–Whitney test). (C) H&E staining of grafts from WT and $\beta 3^{-/-}$ recipients 5 days after transplant. WT grafts show considerable lymphocytic infiltrates which are markedly reduced in $\beta 3^{-/-}$ recipients.

robust immune responses of similar magnitude (9.8 ± 1.0% in wild-type, 8.3 ± 1.2% in $\beta 3^{-/-}$, n = 3, $p > 0.1$; Figure 2A). To assay allo-responsive T cells, we also performed MLRs following transplantation which showed similar proliferative responses in wild-type and $\beta 3^{-/-}$ recipients (Figure 2B). We also stimulated naïve T cells from wild-type and $\beta 3^{-/-}$ mice with increasing numbers of allogeneic DCs and saw no differences in cell proliferation (Figure 2C). Furthermore, wild-type or $\beta 3^{-/-}$ DCs stimulated equivalent proliferation in T cells, suggesting that there were no defects in antigen presentation by $\beta 3^{-/-}$ DCs (Figure 2D). From these results, we concluded that allograft-derived antigen was presented to T cells efficiently and that normal numbers of allograft-responsive T cells were generated in the absence of β3.

Allo-responses in $\beta 3^{-/-}$ recipients are Th2-biased

We next examined the nature of the immune response in $\beta 3^{-/-}$ mice, looking at cytokine responses of spleen T-cells. We noted that the absolute responses (expressed as number of spots/10^6 total splenocytes) were lower in $\beta 3^{-/-}$ mice than wild-type mice which probably reflects the lower overall percentage of lymphocytes; $\beta 3^{-/-}$ mice exhibit splenomegaly with expansion of megakaryocyte and erythroblast populations (18) and consequent reduction in the proportion of lymphocytes. Despite these differences, splenocytes from $\beta 3^{-/-}$ mice showed a similar pattern of Th1/Th2 cytokine production to that seen in wild-type mice (approximately 3:1 ratio of IFN-γ : IL-4-producing spots; Figure 2E). However, after transplantation, $\beta 3^{-/-}$ recipients showed a pronounced Th2 bias in allo-responses, such that the number of IFN-γ -producing spots was reduced by 50% and IL-4-producing spots increased by twofold relative to wild-types (Figure 2F). These differences correlated with changes in cytokine production in the allograft, with less IFN-γ and more IL-5 mRNA in $\beta 3^{-/-}$ recipients (Figure 2G). Therefore, although wild-type and $\beta 3^{-/-}$ recipients generated T-cell responses of similar magnitude following transplantation, the nature of the immune responses was clearly different, with a marked Th2-bias in $\beta 3^{-/-}$ recipients.

T-cell infiltration into grafts is reduced in $\beta 3^{-/-}$ recipients

These differences in the nature of the T-cell allo-response suggested that loss of β3 may directly affect T-cell function. αvβ3 is known to be up-regulated on T cells stimulated *ex vivo* (8) but it is not known whether this occurs during allo-immune responses *in vivo*. We found that β3 is expressed on subsets of $CD4^+$ and $CD8^+$ T cells and these subsets are expanded during transplant rejection, suggesting that β3 is expressed by activated T cells *in vivo* (Figure 3A). This was confirmed by analysis of $CD8^+$ effector cells which were 89% $\beta 3^+$ compared to fewer than 10% of unstimulated splenic $CD8^+$ T cells (Figure 3A and B). We also confirmed that β3 was present as the αvβ3 heterodimer (8) as lymphocytes also stained with antibodies against the αv integrin, but not αIIb (Figure 3C). A critical property of activated effector T cells is their ability to enter tissues, which has been shown to be dependent on the expression of specific adhesion molecules such as selectins or α4 integrins (19,20). The up-regulation of αvβ3 during T-cell activation and a reported role for αvβ3 in transmigration of inflammatory cells (11,21) suggested to us that

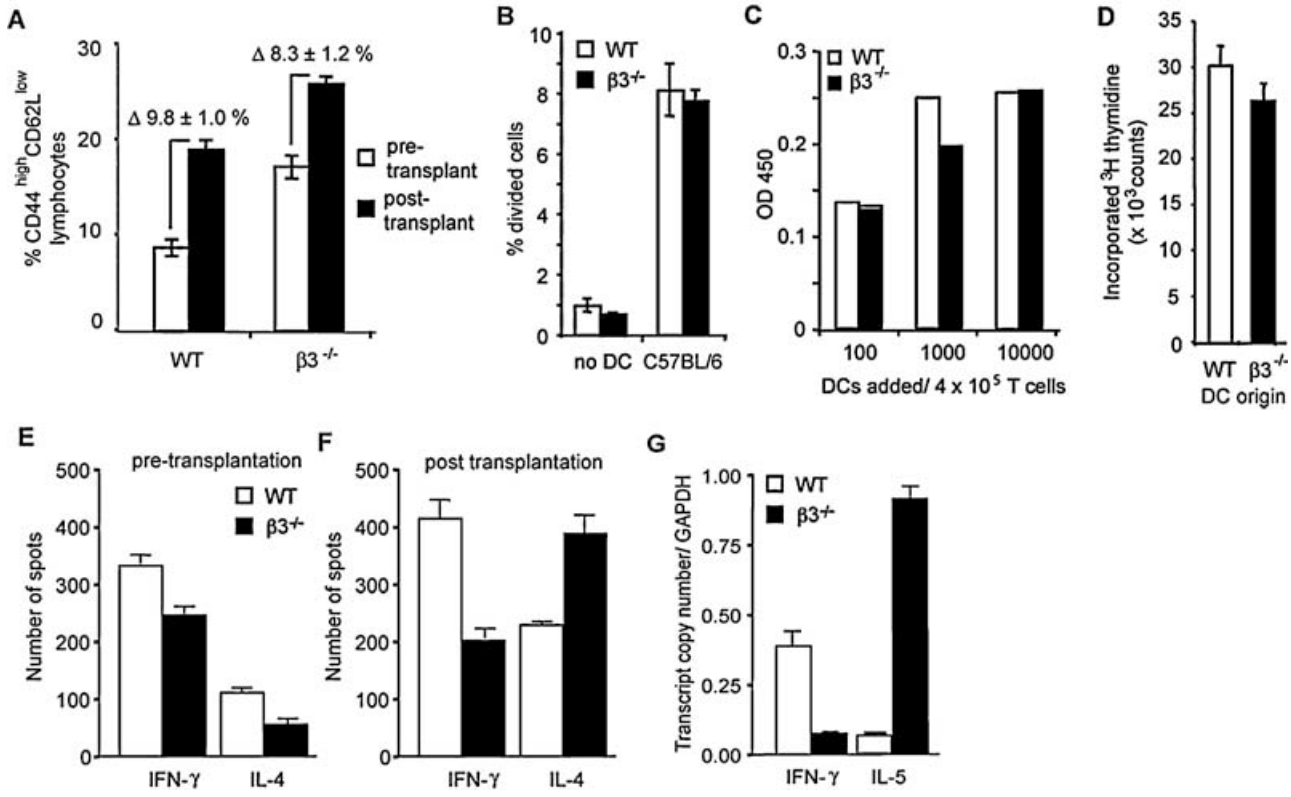

Figure 2: Allograft immune responses in wild-type (WT) and β3$^{-/-}$ recipients. (A) Analysis of splenic T cells from WT and β3$^{-/-}$ mice before (white bars) and 5 days following (filled bars) transplantation. Data are expressed as the percentage of CD8^{+} cells that are CD44^{high} CD62L^{low}, and are means ± s.e.m., n = 4/group. Δ, difference between pre- and posttransplantation percentage. (B) MLRs of purified splenic T cells from WT and β3$^{-/-}$ transplant recipients incubated with syngeneic (BALB/c) or allogeneic (C57BL/6) DCs for 4 days. Data show % divided cells, from FACS analysis of CFSE labeling, and are from triplicate measurements of four (WT) or two (β3$^{-/-}$) transplant recipients. (C) Purified splenic T cells from naïve WT or β3$^{-/-}$ mice were incubated with DCs from C57BL/6 mice. Cell number was measured at day 5 of culture by Cell Titer 96 Aq reagent. Data are means of duplicate measurements from two mice/genotype. (D) [^{3}H]-thymidine incorporation by WT T cells incubated with allogeneic WT or β3$^{-/-}$ DCs. Data are mean ± s.e.m. (E, F). ELISPOT analysis of allo-responsive spleen T cells prior to transplantation (E) and at day 5 following transplantation (F). Data are means ± s.e.m., n = 4/group. (G) qPCR analysis of cytokine expression in allogeneic grafts in WT (open bars) or β3$^{-/-}$ (filled bars) recipients 5 days following transplantation, expressed as copy number relative to GAPDH. Data are means ± s.e.m., n = 4.

αvβ3 may be important for recruitment of activated T cells. Histological analysis showed infiltration of the graft by both CD4^{+} and CD8^{+} T cells in wild-type recipients at day 5 following transplantation (Figure 4A). The mean CD4^{+} T-cell count per mm^{2} in allografts transplanted into wild-type recipients was 134 ± 6 cells compared to 54 ± 5 in grafts transplanted into β3$^{-/-}$ mice. CD8^{+} T cells were similarly reduced (34 ± 9 in wild-type recipients vs. 21 ± 7 in β3$^{-/-}$ mice). The differences were statistically significant for both CD4^{+} and CD8^{+} T cells ($p < 0.01$) and were confirmed by quantifying the infiltrating cells by FACS (Figure 4B). These results suggested that effector T-cell recruitment to tissues is compromised in β3$^{-/-}$ mice.

β 3 integrins regulate T-cell migration

To test the role of β3 in T-cell migration, we studied adhesion and migration *in vitro* by T cells from wild-type or β3$^{-/-}$ mice. Effector cells were generated by culture of activated splenic T cells with IL-2 (16) and wild-type or β3$^{-/-}$ cells showed similar surface phenotypes (CD44^{high} CD25^{+} and CD62L$^{high/low}$, data not shown) except for the expression of αvβ3 integrins (Figure 5A). αvβ3 integrin is a major receptor for the matrix protein vitronectin and, in common with many β1 integrins, also binds fibronectin. Wild-type effector T cells showed increased adhesion to fibronectin and vitronectin compared to control protein (BSA) or laminin. β3$^{-/-}$ T cells also adhered to fibronectin, but adhesion to vitronectin was reduced to the level of adhesion to control proteins (Figure 5B).

Wild-type and β3$^{-/-}$ CD8 T cells responded and migrated normally to the inflammatory chemokine CCL2, suggesting that lack of β3 did not directly affect T-cell chemokine signaling (Figure 5C). Fibronectin and vitronectin stimulated cell migration in the absence of chemokines and supported increased migration in the presence of CCL2. However, β3$^{-/-}$ T cells migrated less well than wild-type cells on

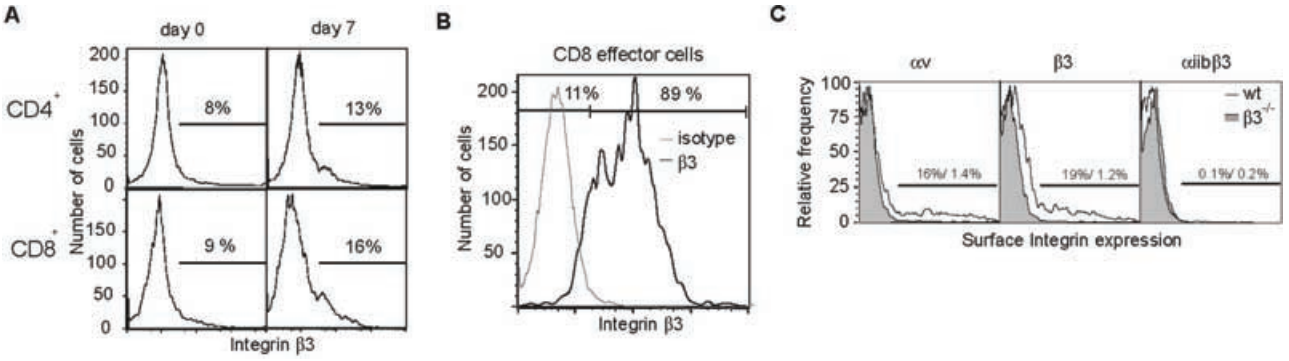

Figure 3: T-cell expression of β3 integrins. (A) β3 integrin expression on gated $CD4^+$ or $CD8^+$ cells from spleens of unmanipulated mice and at day 7 following allograft transplantation. Gates were set using cells stained with isotype-matched control antibodies and numbers indicate $\beta3^+$ cells. Note the increase in $\beta3^+$ T cells with time post-transplantation. (B) β3 expression on effector cells ($CD8^+$ $CD44^{high}$ $CD62L^{low}$). Numbers represent $\beta3^-$ and $\beta3^+$ cells, with regions set using an isotype-matched control antibody (gray line). (C) αv,β3 and αIIbβ3 expression on total spleen cells from WT (open histograms) or $\beta3^{-/-}$ (filled histograms) mice. Numbers represent percentage of positive cells from WT or $\beta3^{-/-}$ mice, respectively. Regions were set by isotype-control antibody staining.

fibronectin in the absence of chemokines and showed no increased migration on vitronectin compared to BSA or laminin (Figure 5D). These results therefore show that both adhesion and subsequent migration of T cells is impaired in the absence of β3 integrins. Hence, the lower numbers of T cells in rejecting grafts in $\beta3^{-/-}$ mice are likely to be due in part to deficient T-cell infiltration.

Long-term survival of allografts in β3$^{-/-}$ mice

Despite these initial differences in lymphocyte infiltration and changes in cytokine production, grafts transplanted into $\beta3^{-/-}$ recipients were eventually rejected (Figure 1). Multiple adhesion molecules are known to be important in allograft rejection and the effects of β3 deletion may be reduced by compensatory use of other integrins. We therefore explored the expression of other integrins in grafts recovered from wild-type syngeneic and wild-type and $\beta3^{-/-}$ allogeneic recipients using rtPCR. We noted increased expression of integrins VLA-4 and LFA-1 in allografts in wild-type recipients and even higher expression in $\beta3^{-/-}$ recipients (Figure 6A). To attempt to overcome this we administered low doses of monoclonal antibodies to LFA-1 and VLA-4 (0.1 mg/mouse IP, day 0, 1, 2 posttransplant) to wild-type and $\beta3^{-/-}$ mice receiving cardiac allografts. At the doses used, LFA-1/VLA-4 blockade slowed the rate of acute rejection in all recipients, such that all grafts survived for greater than 10 days. Hearts transplanted into wild-type recipients were eventually rejected with a MST of 33 days (n = 7) (Figure 6B). Surviving grafts removed from all wild-type recipients at day 30 or later showed graft vasculopathy

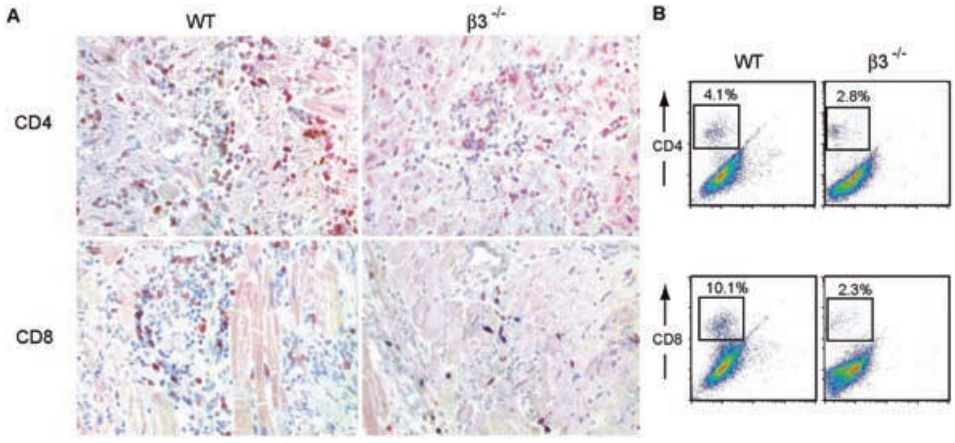

Figure 4: T-cell recruitment to grafts in wild-type (WT) and β3$^{-/-}$ recipients. (A) Sections of grafts from WT or $\beta3^{-/-}$ recipients at day 5 following transplantation stained with antibodies to CD4 or CD8. Positive cells are red/brown in sections. Microscope magnification is 400×. (B) FACS analysis of lymphocytic infiltrates in WT and $\beta3^{-/-}$ recipients at 5 days. Numbers represent the frequency of $CD4^+$ or $CD8^+$ cells, expressed as a percentage of total graft-derived cells and are representative of three experiments.

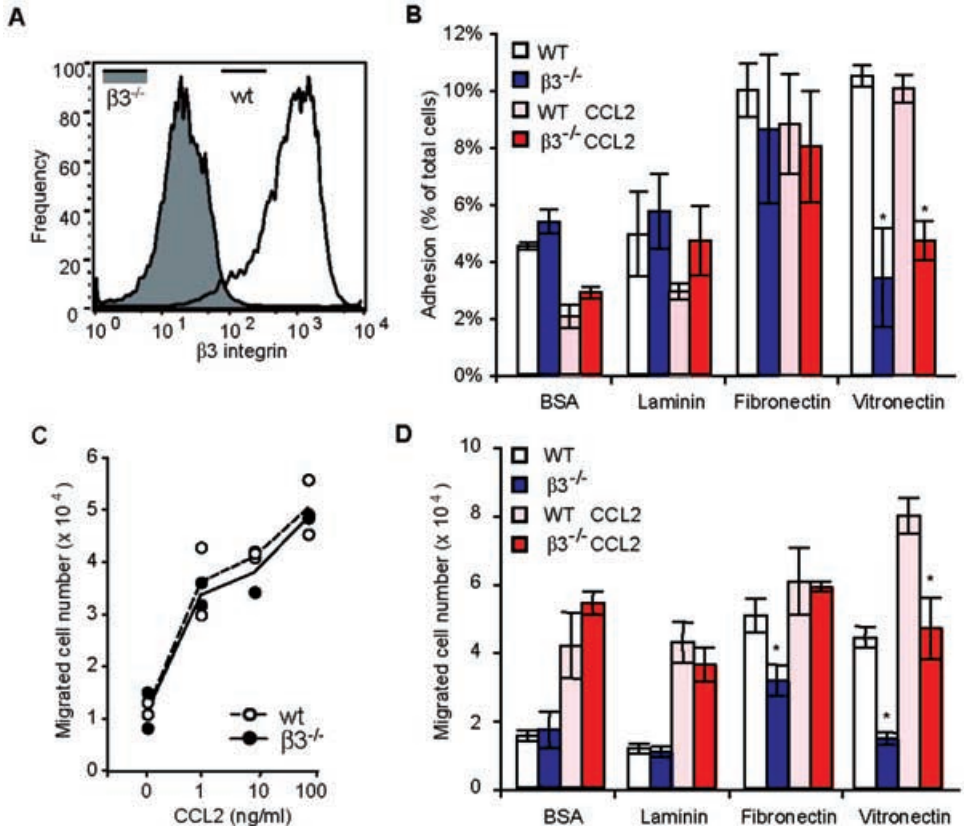

Figure 5: Adhesion and migration of effector cells from wild-type (WT) and β3$^{-/-}$ mice. (A) β3 expression by CD8^{+} effector T cells generated *in vitro* from WT and β3$^{-/-}$ mice. β3$^{-/-}$ cells stained at the same intensity as cells stained with isotype-control antibody. (B) Adhesion of WT and β3$^{-/-}$ CD8^{+} effector cells to ECM proteins in the absence or presence of 10 ng/mL CCL2. Data are expressed relative to the total number of cells added to each well and are means ± s.e.m. of three wells. *, β3$^{-/-}$ significantly different from WT, $p < 0.01$ Student's t-test. (C) Migration of activated CD8^{+} effector cells cultured from WT and β3$^{-/-}$ mice toward increasing concentrations of CCL2. Data are expressed as number of migrating cells (from addition of 1×10^5 cells) and each point represents data from one transwell insert. (D) Migration of CD8^{+} cells in the absence of chemokines or with 10 ng/mL CCL2, in the presence of ECM components. Data are expressed as number of migrating cells (from addition of 1×10^5 cells) and are the mean and s.e.m. of three samples. *, β3$^{-/-}$ significantly different from WT, $p < 0.01$ Student's t-test. In all experiments, similar results were seen with three independent T-cell cultures.

with stenotic intimal hyperplasia, medial inflammation and smooth muscle dropout (Figure 6C–F), findings characteristic of active chronic rejection.

In contrast, combined LFA-1/VLA-4 therapy allowed long-term survival of allografts in β3$^{-/-}$ recipients for the duration of the experiment (140 days; $n = 7$; $p < 0.01$ compared to wild-type mice). Grafts from β3$^{-/-}$ recipients showed considerably less infiltration of mononuclear cells (Figure 6G–I) and a significantly lower mean rejection score (Figure 6J) than wild-type recipients. Furthermore, quantitative analysis of graft arteriosclerosis demonstrated that luminal stenosis of graft coronary arteries in β3$^{-/-}$ recipients was markedly reduced compared to grafts in wild-type recipients (Figure 6J). Histologically, the vasculopathy was much reduced in four of six and absent in two of six of the hearts examined by histology. Hence, the inhibition of LFA-1/VLA-4 in β3$^{-/-}$ mice inhibited chronic rejection and allowed long-term survival of heart allografts. However, donor-specific tolerance was not induced as skin allografts from C57BL/6 mice transplanted 5 weeks after C57BL/6 heart allografts were rejected in both wild-type and β3$^{-/-}$ recipients, although at different rates (13 and 19 days MST, respectively, $n = 5$, $p < 0.004$). These results therefore implicate β3 integrins in longer-term chronic rejection processes also, and suggest that β3 blockade may provide a useful therapy in combination with established anti-rejection treatments.

Discussion

The identification of new molecules involved in transplant rejection is important for insight into the process of

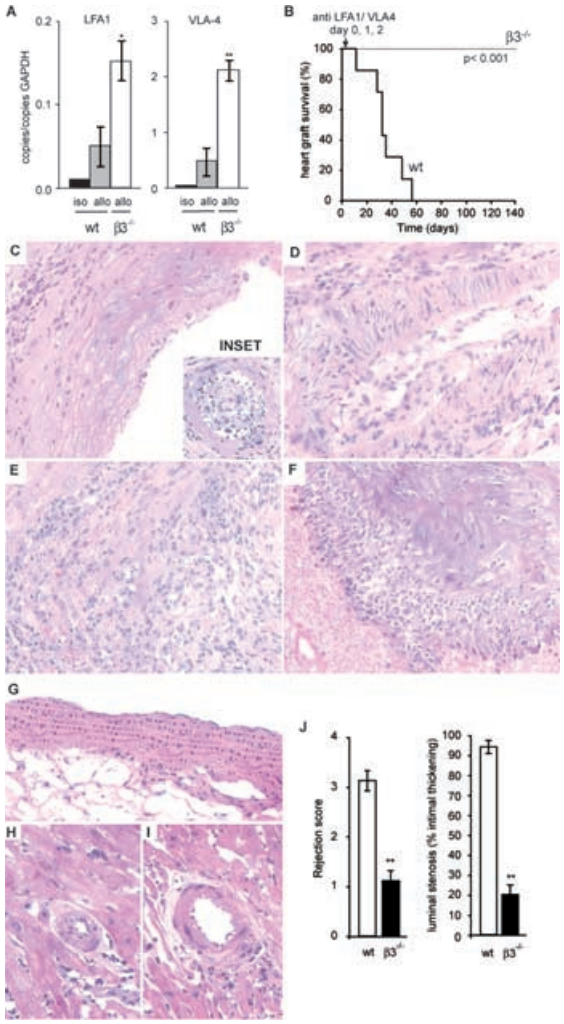

Figure 6: Relative contributions of integrins to rejection responses. (A) qPCR analysis of LFA-1 and VLA-4 expression in cardiac grafts in wild-type (WT) isogeneic and allogeneic recipients (solid and gray bars, respectively) or $\beta3^{-/-}$ allogeneic recipients (white bars) at 5 days post-transplantation. Data are expressed as copy number relative to the housekeeping gene GAPDH. (B) Cardiac allograft survival in WT (solid lines) and $\beta3^{-/-}$ (dashed line) recipients following anti-LFA-1/VLA-4 treatment. (C–F) H&E staining of grafts from WT recipients 30 days after transplant, 400× magnification. (C) Smooth muscle loss from aorta near anastomotic site with endarteritis of a small coronary artery (inset). (D) Endothelialitis and intimal hyperplasia of a coronary artery. (E) Destructive parenchymal inflammation. (F) Endothelialitis and intimal proliferation of aorta near anastomotic site. These findings are those of acute (D) and active chronic rejection (vasculopathy). (G–I) H&E staining of grafts from $\beta3^{-/-}$ recipients 30 days after transplant, 400× original magnification. (G) Aorta near anastomotic site without endothelialitis or intimal hyperplasia. (H, I) Small artery without endothelialitis or intimal hyperplasia and parenchyma without inflammation. (J) Mean rejection score and luminal stenosis of anti-LFA-1/VLA-4-treated WT or $\beta3^{-/-}$ recipients at day 30 after transplant. **, $\beta3^{-/-}$ significantly different from WT, $p < 0.01$ Student's t-test.

rejection and in the development of new therapeutic agents. In these studies, we set out to determine the contribution of β3-integrins to immune responses during allograft rejection and we have shown that allografts are rejected more slowly in $\beta3^{-/-}$ recipients than in wild-type mice. The allograft response in $\beta3^{-/-}$ mice generates similar numbers of allo-responsive T cells to those observed in wild-type recipients but is associated with greater production of Th2 cytokines and less IFN-γ. Furthermore, $\beta3^{-/-}$ T cells have impaired adhesion and migration and grafts in $\beta3^{-/-}$ mice have fewer infiltrating T cells. Hence, we have shown that β3 integrins contribute to both the nature of the allograft response (Th1 vs. Th2) and the subsequent migration of effector T cells. These results uncover new roles for β3-integrins in immune responses and identify a potential new therapeutic target in preventing transplant rejection.

The data presented here show that activated T cells express αvβ3 integrins, that the adhesion and migration of these cells are impaired in the absence of β3 and that lymphocyte numbers in rejecting allografts are reduced in $\beta3^{-/-}$ recipients. Taken together, these data are consistent with a role for αvβ3 in lymphocyte recruitment to inflamed tissue *in vivo*. Lymphocyte recruitment is a multi-step process involving sequential involvement of adhesion molecules (2): selectins and α4 integrins bind ligands on activated endothelium to slow circulating cells; the engagement of chemokine receptors stimulates firm adhesion though integrin αLβ2 (LFA-1) binding to ICAM-1; and finally integrins and other adhesion molecules mediate migration on the surface of the endothelial cell and transmigration into the underlying tissue. Our experiments show that chemokine-directed migration occurs normally in cells without β3 and suggest that αvβ3 is most likely to contribute to the migration of lymphocytes on and through endothelial layers and/or interstitial tissue, either directly or through modulation of other integrins or adhesion molecules (21,22), consistent with studies using blocking antibodies to αvβ3 (11). Neither fibronectin nor vitronectin is expressed on the surface of endothelial cells, but are found in the basement membrane and interstitial matrix, supporting a role for β3 in migration into and through tissue. Furthermore, activated immune cells express fibronectin and other RGD-containing matrix proteins that may promote migration at inflamed sites. Both fibronectin and vitronectin are also abundant as soluble proteins in plasma, however, and could therefore bind circulating immune cells or endothelium to contribute to initial stages of transmigration. Additional β3-independent mechanisms for inflammatory cell recruitment potentially explain the absence of more severe immune phenotypes in the $\beta3^{-/-}$ mice (23).

$\beta3^{-/-}$ mice have increased numbers of activated T cells in the spleen prior to transplantation, which is likely to be due to defects in cell trafficking. Our data show that β3 integrins are not required for T-cell activation, as the numbers

of activated T cells increased following transplantation into $\beta3^{-/-}$ recipients, and $\beta3^{-/-}$ splenocytes mounted normal MLRs. However, we do see an increase in IL-4 and IL-5 production in $\beta3^{-/-}$ mice and a reduction in IFN-γ production relative to wild-type recipients. This difference may reflect a role for β3 in T-cell-receptor signaling, as reported for γδ and CD8 T cells (9,10). In these cases, β3 recruits key components of the T-cell-receptor signaling cascades, and hence $\beta3^{-/-}$ T cells would be predicted to generate weaker T-cell-receptor responses. Such suboptimal stimulation is often associated with Th2-responses. In addition, loss of fibronectin from areas of lymphocyte infiltration correlates with a reduction in Th1 cytokine production and maintenance of tolerance (24). Therefore, disruption of lymphocyte-ECM binding following deletion of β3 may also affect Th1/Th2 cytokine production in the rejecting graft, or preferentially affect longer-term survival of lymphocytes in grafts (25).

Hence, our results demonstrate that T cells from $\beta3^{-/-}$ mice show Th2-biased allograft responses and have impaired migration and recruitment. Either of these changes would be likely to lead to delayed allograft rejection and it is impossible in this model to separate the relative contributions of these two potential components of graft rejection. Indeed the effects may be linked, as Th1 and Th2 cells are thought to show different thresholds for recruitment to tissue through the use of distinct adhesion molecules (26). However, the likely effects of a Th2-dominated immune response on transplant rejection remain unpredictable despite many studies (27) whereas the failure of $\beta3^{-/-}$ T cells to infiltrate allografts efficiently is very likely to contribute significantly to graft survival. Thus, although we cannot definitively assign the increased survival to one or other effect, it is likely that this is due to migration defects.

Although graft survival was prolonged compared to wild-type recipients, the majority of $\beta3^{-/-}$ recipients eventually rejected transplants. The up-regulation of intragraft expression of VLA-4 and LFA-1 expression in $\beta3^{-/-}$ recipients suggested compensatory use of other integrins during rejection, either at the level of integrin expression by individual cells to overcome loss of β3, or the engagement of an alternative pathway of rejection that is independent of β3 integrins. VLA-4 and LFA-1 are known to participate in entry to the lymph organs and in recruitment of activated cells to inflamed tissue and LFA-1/ICAM-1 interactions are also pivotal in T cell: DC communication. Targeting LFA-1 can promote tolerance (28) although treatments directed at LFA-1 are often combined with additional therapies to achieve more robust prolongation of allograft survival (29). In our study, we used low doses of antibodies to LFA-1 and VLA-4 which delayed rejection until 30 days in wild-type mice but led to much more prominent results in $\beta3^{-/-}$ recipients, with grafts surviving until the end of the experiment (>140 days). Notably, grafts in $\beta3^{-/-}$ recipients also showed little evidence of chronic rejection. Chronic rejection is a common finding of many models of mouse transplantation tolerance induced by immunosuppressive protocols and in humans chronic rejection remains the main barrier to long-term graft survival (30,31). The greatly prolonged graft survival and lack of discernible chronic rejection following combination LFA-1/VLA-4/β3 therapy, therefore indicates an accumulative effect in antagonizing multiple integrin pathways and highlights the potential for blockade of β3 integrins in securing long-term transplant survival.

It should be noted that in this study we have concentrated on the characterization of $\beta3^{-/-}$ T cells, rather than other β3-expressing cells. β3 integrins are also expressed by platelets as both αIIbβ3 and αvβ3, are important in platelet activation and thrombosis (18). We did not see evidence of long-term hemorrhage in hearts transplanted into $\beta3^{-/-}$ mice, but it remains possible that the protection from rejection provided by β3 deletion is due, in part, to loss of pathogenic effects of platelets. Determining the contribution to transplant rejection of β3 expression by platelets is beyond the scope of this manuscript, but may be addressed in the future by use of αv conditional knockout mice (32) or selective pharmacological blockade of αvβ3 and αIIbβ3.

β3 integrins are also expressed by macrophages and DCs and have long been implicated in the acquisition and cross-presentation of antigens from apoptotic cells (33,34), processes likely to be important in transplant rejection. The generation of normal numbers of activated T cells and robust MLRs using $\beta3^{-/-}$ DCs show that APC function is not profoundly affected by β3 deletion, in agreement with cross-presentation experiments in mice lacking either β3 or β5 integrins (35). However, it remains possible that subtle differences in antigen recognition and processing by DCs, or DC migration, contribute to the delayed graft rejection or Th2-bias in allograft responses.

In summary, together these data provide strong supportive evidence for the involvement of β3 integrins in transplant rejection, as suggested by genetic association studies. Lack of β3 integrins was associated with increased survival of allografts, defective recruitment of activated T cells and altered immune responses, and in conjunction with blockade of additional integrins promoted long-term survival of transplants without chronic rejection. Our data demonstrate that $\beta3^{-/-}$ mice can provide a useful system to study the role of β3 integrins in transplantation, which may have direct implications for human treatment, and identify β3 as a novel target for therapeutic intervention in transplant rejection.

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Online Supplementary Data

Expanded materials and methods

Histological analyses: Heart allografts were removed from recipients and bisected to create two four-chamber views of the heart, exposing the aortic anastomotic site for histological review. Samples were fixed in 10% buffered formalin and stained with hematoxylin and eosin (H&E). Rejection of cardiac allografts was evaluated and graded using the International Society for Heart and Lung Transplantation nomenclature system (1,2). According to the scale, 0 indicates no rejection; 1, mild lymphocytic infiltration without myocyte necrosis; 2, diffuse infiltration and focal necrosis; 3, diffuse inflammation with necrosis; 4, diffuse polymorphous infiltrates with edema, hemorrhage and necrosis. Coronary arteries were digitally photographed, and blindly scored in an image analysis system (NIH image, version 1.62). Luminal occlusion was calculated as percent intimal thickening as follows: The area encompassed by internal elastic lamina (IEL) and luminal area (square pixels) were carefully analyzed, and the cross-sectional area of luminal stenosis was calculated as previously described: luminal occlusion = (IEL area-luminal area)/IEL area × 100 (%) (3).

FACS analysis: To prepare lymphocytes from grafts, hearts were minced and incubated with stirring at 37°C for 30 min in Hank's balanced salt solution with 1.2 mM EDTA, followed by treatment with collagenase (150 U/mL: Gibco-BRL/Invitrogen, Carlsbad, CA) as previously described (4). Splenocytes were prepared by mechanical disaggregation followed by lysis of red cells and resuspended in FACS Buffer (PBS containing 1% FCS/0.1% sodium azide) at a concentration of 2×10^6/mL. Cells were incubated at 4°C for 10 min with 2.4G2 anti-Fcγ III/II receptor (BD PharMingen, San Diego, CA) to block nonspecific antibody binding. Cells were stained with anti-CD3-FITC, anti-CD8-FITC, anti-CD4-FITC, anti-CD11c-FITC, anti-CD11b-APC, anti-CD62L-APC, anti-CD44-PE, anti-β3 integrin-biotin/strepatavidin-PE and isotype controls (all from BD PharMingen). Cytofluorimetry was performed using a FACS Caliber Cytometer (Becton-Dickinson) and analyzed using CellQuest software. $CD8^+$ effector cells expressing $CD44^{high}$ and $CD62L^{low}$ were enumerated as previously described (5,6).

ELISPOT analysis: The ELISPOT assay was performed as previously described (7). Briefly, ImmunoSpot Plates (Cellular Technology, Cleveland, OH) were coated with capture antibodies against IL-4 or IFN-γ (BD PharMingen). Splenocytes in complete HL-1 medium (Biowhittaker, Walkersville, MD) were then added to each well with an equal number of irradiated syngeneic or allogeneic splenocytes. The resulting spots, or cytokine-producing cells per million splenocytes, were counted on a computer-assisted ELISPOT Image Analyzer (Cellular Technology). The number of spots in the wells with medium alone or syngeneic cells was subtracted from allo-responses to take into account the background when analyzing the data.

Gene expression: Total RNA was extracted from the cardiac grafts using Trizol reagent (Invitrogen) according to the manufacturer's protocol. After DNase I (Invitrogen) treatment, the extracted RNA was reverse transcribed to synthesize 100 μL of cDNA. The following 25 μL qPCR reaction contained 2 μL of cDNA, 12.5 μL of 2× SYBR Green master mix (Stratagene, La Jolla, CA), and 250 nmol of sense and anti-sense primer. Primers were designed using Primer Express Software (Applied Biosystems, Foster City, CA). The reaction conditions were as follows: 50°C for 2 min, 95°C for 10 min, then 40 cycles of 95°C for 15 s and 60°C for 1 min. Emitted fluorescence for each reaction was measured during the annealing/extension phase. The calculated number of copies was divided by the number of copies of the housekeeping gene GAPDH.

Proliferation assays: T cells were sorted from total splenocytes from 8–12-week wild-type (BALB/c) and $\beta3^{-/-}$ mice by positive selection with anti-CD90/Thy 1.2 beads (Miltenyi Biotech). Cells were routinely >95% $CD3^+$ T cells as determined by FACS analysis. For some experiments, T cells were labeled with CFSE (5 μM, 5 min; Molecular Probes, Carlsbad, CA). T cells were resuspended in T-cell medium (RPMI supplemented with 10% fetal calf serum, 100 U/mL penicillin/streptomycin, 10 mM glutamate, 10 mM nonessential amino acids, 10 mM pyruvate and 10 mM HEPES; all from Gibco/BRL) and added to wells previously coated with anti-CD3 antibody (10 μg/mL; Clone 145-2C11, BD PharMingen). For some wells anti-CD28 antibody was also added (5 μg/mL; Clone 37.51, BD PharMingen). Proliferation was assayed on day 4 of culture by measurement of cell number using Cell Titer 96 Aq reagent and reading absorbance at 450 nm, by FACS analysis of CFSE staining or by overnight incorporation of [^{3}H]-thymidine. For MLR assays, DCs were positively sorted from splenocytes using anti-CD11c beads (Miltenyi Biotech) and treated with 0.5 mg/mL mitomycin C for 20 min to block DC proliferation. DCs were then added to purified T cells and proliferation measured as for CD3 stimulation.

Adhesion and migration assays: Splenocytes from 8–12-week wild-type (BALB/c) and $\beta3^{-/-}$ mice were incubated with anti-CD3 (BD PharMingen) in RPMI supplemented with 10% fetal calf serum, penicillin/streptomycin, 10 mM glutamate and 10 mM HEPES for 2 days, and then with IL-2 (Sigma-Aldrich) for an additional 6 days, to generate effector cells (8). FACS analysis of CD8, CD62L and CD44 was performed to confirm the production of effector cells. For adhesion assays, 96-well tissue culture plates (Corning, NY) were coated with mouse fibronectin (10 μg/mL; Gibco BRL), rat vitronectin (1 μg/mL; Sigma-Aldrich), mouse laminin (10 μg/mL; Gibco BRL) or BSA (5%; Fraction V, heat shock, Roche) at 37°C for 2 h, blocked with 5% BSA

for 30 min and washed three times. Lymphocytes were resuspended in RPMI with 0.3% BSA and 10 mM HEPES and 2×10^5 cells added to wells containing either binding medium alone or binding medium with CCL2/MCP-1 (1 nM final concentration; R & D Systems). After 1 h unbound cells were removed by inverting plates and allowing medium to drain. Plates were gently washed twice with binding buffer. Bound cells were counted by incubating plates with RPMI medium and Cell Titer 96 Aq reagent overnight followed by measurement of absorbance at 450 nm, and expressed as adhesion relative to total number of cells added to the plates. Bound cells were independently counted by crystal violet staining and gave equivalent results (not shown). For migration assays, transwell inserts (5 μm pore size; Corning) were coated with matrix proteins as for adhesion assays, or left untreated. The 2×10^5 differentiated cells in RPMI with 0.3% BSA and 10 mM HEPES were added to the transwell insert and incubated over wells containing CCL2/MCP-1 (R & D Systems) at 0.1–100 ng/mL. Transmigrating cells were recovered after 2 h, and counted by flow cytometry by comparison to a fixed number of fluorescent beads (flow-check microspheres; Beckman Coulter).

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