Deletion of DOCK2, a regulator of the actin cytoskeleton in lymphocytes, suppresses cardiac allograft rejection

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Allograft rejection is induced by graft tissue infiltration of alloreactive T cells that are activated mainly in secondary lymphoid organs of the host. DOCK2 plays a critical role in lymphocyte homing and immunological synapse formation by regulating the actin cytoskeleton, yet its role in the in vivo immune response remains unknown. We show here that DOCK2 deficiency enables long-term survival of cardiac allografts across a complete mismatch of the major histocompatibility complex molecules. In DOCK2-deficient mice, alloreactivity and allocytotoxicity were suppressed significantly even after in vivo priming with alloantigens, which resulted in reduced intragraft expression of effector molecules, such as interferon-γ, granzyme B, and perforin. This is mediated, at least in part, by preventing potentially alloreactive T cells from recruiting into secondary lymphoid organs. In addition, we found that DOCK2 is critical for CD28-mediated Rac activation and is required for the full activation of alloreactive T cells. Although DOCK2-deficient, alloreactive T cells were activated in vitro in the presence of exogenous interleukin-2, these T cells, when transferred adoptively, failed to infiltrate into the allografts that were transplanted into RAG1-deficient mice. Thus, DOCK2 deficiency attenuates allograft rejection by simultaneously suppressing multiple and key processes. We propose that DOCK2 could be a novel molecular target for controlling transplant rejection.

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Abbreviations used: B6, C57BL/6J mouse; CFSE, 5,6-carboxyfluorescein diacetate succinimidyl ester; MST, median survival time; RANTES, regulated upon activation, normal T cell expressed and secreted; Tac, tacrolimus.

Graft tissue infiltration of activated T cells is a hallmark of cellular rejection of allografts (1). This process involves a complex cascade of molecular interactions and cellular responses, including the chemokine-dependent migration of T cells, the recognition by TCRs of allopeptides bound to MHC molecules, the engagement of costimulation and adhesion molecules with their ligands, and the activation of multiple intracellular signal transduction pathways which lead to the release of cytokines that are key to T cell expansion and graft tissue destruction (2–6). Thus far, targeting of TCR signaling pathways successfully controlled allograft rejection in clinical and experimental transplantation (5, 7). More recently, several attempts have been made to block chemokine–chemokine receptor interactions and costimulatory molecules for controlling transplant rejection (8–14). However, because migration and activation of T cells are critically dependent on remodeling of the actin cytoskeleton (15, 16), inhibition of the cytoskeletal reorganization in T cells is an alternative approach to attenuating allograft rejection.

Rac is one of the Rho family GTPases that are known to regulate membrane polarization and cytoskeletal dynamics in various cells (17). Rac is composed of three isoforms: Rac1, Rac2, and Rac3. Rac1 is expressed ubiquitously and Rac3 is expressed highly in the brain, whereas Rac2 is restricted largely to hematopoietic cells. The critical role of Rac activation in cell migration has been well established (15, 18). In addition, the activation of Rac has been implicated in immunological synapse formation, a large-scale molecular movement at the interface between T cells and APCs, which is considered to be critical for sustained T cell activation (16, 19–21). Therefore, Rac activation would be a

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Table I. DOCK2 deficiency prolongs survival of cardiac allografts

Group	Drug	Period	*n*	Survival days (*n*)[a]	MST	p-value
B6→B6	–	–	5	69, >100 (4)	>100	
BALB/c→B6	–	–	5	7, 8, 11 (2), 13	11	
BALB/c→*DOCK2*$^{+/-}$	–	–	7	7 (4), 8 (3)	7	NS[b]
BALB/c→*DOCK2*$^{-/-}$	–	–	7	14, 20, 34, 60, 80, 90, >100	60	<0.01[c]
BALB/c→*RAG1*$^{-/-}$	–	–	5	95, 96, >100 (3)	>100	<0.01
BALB/c→B6	Tac[d]	Day 1–10	5	9, 10, 13, 15, 26	13	NS
BALB/c→*DOCK2*$^{-/-}$	Tac	Day 1–10	6	43, 84, >100 (4)	>100	<0.01

[a]The numeral in the parentheses is the number of mice surviving to that day.
[b]No significance versus the corresponding WT B6 allograft control.
[c]$P < 0.01$ versus the corresponding WT B6 allograft control.
[d]Tacrolimus 3.2 mg/kg/d for 10 d after transplantation.

target for manipulating T cell migration and activation. However, the ubiquitous expression and redundancy of Rac isoforms preclude using Rac as a molecular target for controlling transplant rejection.

CDM family proteins, *Caenorhabditis elegans* CED-5, human DOCK180, and *Drosophila melanogaster* Myoblast City, are known to regulate the actin cytoskeleton by functioning upstream of Rac (22). DOCK2 is a new member of the CDM family and is expressed predominantly in lymphocytes (23). With the use of DOCK2-deficient (DOCK2$^{-/-}$) mice, we reported that DOCK2 is essential for chemokine- and antigen-induced Rac activation in T cells by functioning downstream of chemokine receptors and TCRs (23, 24). DOCK2$^{-/-}$ mice exhibit a migration defect of T cells in response to various chemokines in vitro (23). In addition, we found that antigen-induced translocation of TCR and lipid rafts to the APC interface is impaired severely in DOCK2$^{-/-}$ T cells, and results in a substantial reduction of alloreactivity in MLR (24). However, the effect of DOCK2 deficiency on T cell migration and activation in an in vivo situation (e.g., transplant rejection) has not been determined.

This issue was addressed in the present study by performing cardiac transplantation between BALB/c mice and C57BL/6J (B6) mice, a strain combination that is considered to have a strong histocompatibility difference, with or without DOCK2 expression. Here, we provide evidence that DOCK2 deficiency in the recipients enables long-term survival of cardiac allografts by suppressing priming and activation of naive T cells in secondary lymphoid organs, and by attenuating graft tissue infiltration of activated T cells.

RESULTS

DOCK2 deficiency enables long-term survival of cardiac allografts

To assess the effect of DOCK2 deficiency on allograft rejection, we performed cardiac transplantation between BALB/c mice and B6 mice with or without DOCK2 expression. The cardiac grafts from B6 mice survived in B6 recipients for >100 d with no sign of rejection (Table I). When BALB/c hearts were transplanted into WT B6 or DOCK2$^{+/-}$ (heterozygote control) mice, the allografts

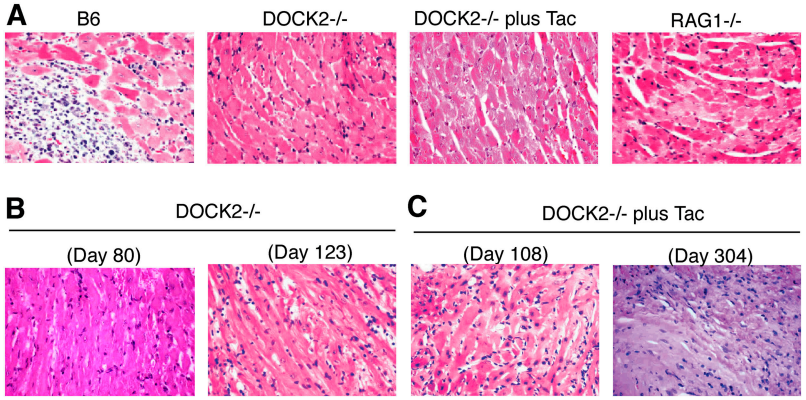

Figure 1. Histology shows no evidence of acute rejection in the allografts from DOCK2$^{-/-}$ recipients. (A) BALB/c heart tissues were harvested from WT B6, DOCK2$^{-/-}$ with or without Tac treatment, and RAG1$^{-/-}$ recipients on day 6 after transplantation and were stained with hematoxylin and eosin. (B) Tissue sections of BALB/c cardiac grafts that survived 80 or 123 d in DOCK2$^{-/-}$ recipients were stained with hematoxylin and eosin. (C) DOCK2$^{-/-}$ recipient mice that were treated with a brief course of low-dose Tac were killed on day 108 or 304 after transplantation, and BALB/c heart tissues were stained with hematoxylin and eosin.

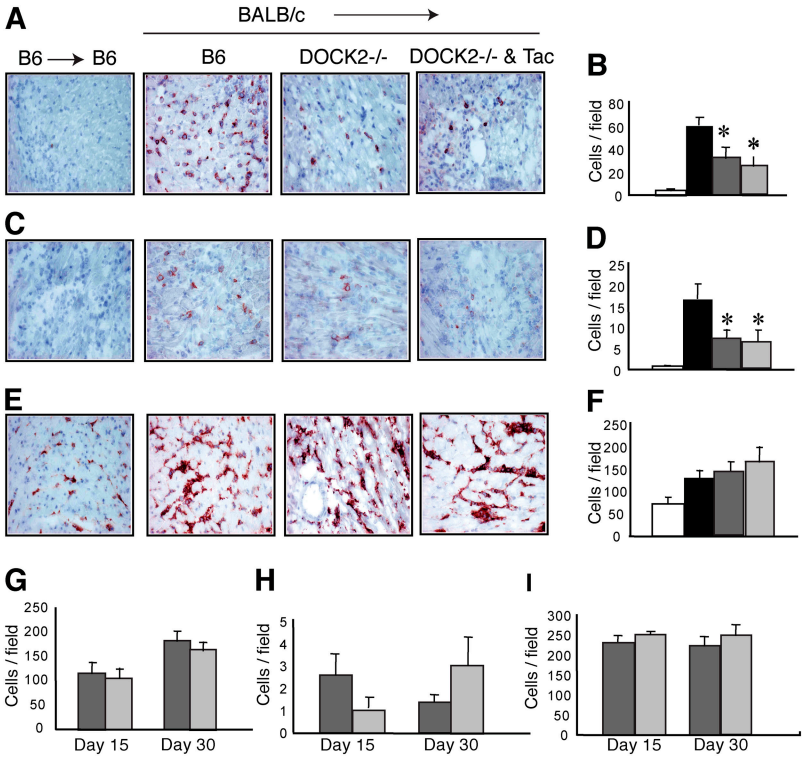

Figure 2. T cell infiltration into and T cell activation in cardiac allografts are reduced in DOCK2$^{-/-}$ recipients. BALB/c heart tissues that were harvested from the WT B6 and DOCK2$^{-/-}$ recipients with or without Tac treatment on day 6 after transplantation were stained with antibody against CD3ε (A), CD25 (C), and CD68 (E). As a control, B6 heart tissues that were harvested from B6 mice were analyzed similarly. The numbers of infiltrating CD3$^+$ (B), CD25$^+$ (D), and CD68$^+$ (F) cells were analyzed in a semi-quantitative manner. Data are mean ± SEM of at least six separate B6 grafts from B6 mice (white bar), and those of BALB/c grafts from the WT B6 (black bar) and DOCK2$^{-/-}$ recipients, with (light gray bar) and without (dark gray bar) Tac treatment. *$P < 0.05$ comparison between WT B6 and DOCK2$^{-/-}$ recipients with or without Tac treatment. (G–I) The intragraft infiltration of CD3$^+$ (G), CD25$^+$ (H), and CD68$^+$ (I) cells were compared between DOCK2$^{-/-}$ mice (dark gray bar) and those that were treated with Tac (light gray bar) on days 15 and 30 after transplantation of BALB/c hearts.

were rejected with a median survival time (MST) of 11 and 7 d, respectively. However, BALB/c cardiac allografts survived in DOCK2$^{-/-}$ mice for a median of 60 d, including one case with graft survival for more than 100 d, although this MST did not reach the level of RAG1$^{-/-}$ mice that lack all T and B cells (MST >100 d).

Treatment of DOCK2$^{-/-}$ recipient mice with a brief course of low-dose tacrolimus (Tac), a calcineurin inhibitor, resulted in a strong synergistic effect. 10 d of therapy with Tac (3.2 mg/kg/d) prolonged cardiac allograft survival by only 2 d in the WT B6 recipients, but induced long-term engraftment in four of the six DOCK2$^{-/-}$ recipient mice (Table I). These results indicate that DOCK2 deficiency effectively attenuates cardiac allograft rejection, and enables permanent graft survival when combined with certain medication. Conversely, we found no prolongation of cardiac allograft survival when DOCK2$^{-/-}$ mice were used as donors (unpublished data).

DOCK2 deficiency improves myocardial histopathology of allografts

We then compared the histopathology of the allografts harvested on day 6 after transplantation. The classic signs of acute rejection were seen in the allografts from the WT B6 and DOCK2$^{+/-}$ controls, including strong interstitial infiltration of mononuclear cells—especially concentrated in the perivascular and epicardial regions—with severe hemorrhage, edema, and necrosis (Fig. 1 A and not depicted). In contrast, the allografts from DOCK2$^{-/-}$ recipient mice and from RAG1$^{-/-}$ mice, irrespective of Tac treatment, showed only mild interstitial mononuclear cell infiltration with little evidence of hemorrhage, edema, and necrosis (Fig. 1 A). Histologic evaluation at later time points revealed certain evidences of acute rejection, including mononuclear cell infiltration and focal edema, in the allografts surviving <80 d after transplantation into DOCK2$^{-/-}$ recipients (Fig. 1 B, left). However, the allograft surviving >100 d in a DOCK2$^{-/-}$ recipient showed well-preserved myocardial structure and normal vessels with no interstitial fibrosis (Fig. 1 B, right). Similarly, no signs of rejection were found in the allograft that was harvested on day 108 after transplantation from a DOCK2$^{-/-}$ recipient that was treated with a brief course of low-dose Tac (Fig. 1 C, left). In one Tac-treated DOCK2$^{-/-}$ recipient, the allograft survived >300 d. Although the allograft that was harvested from this recipient on day 304 after

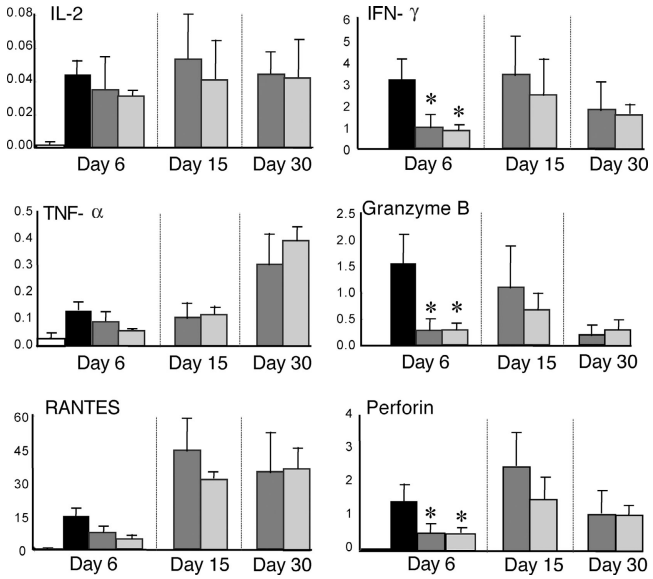

Figure 3. The gene expression profile of cytokines and cytolytic factors is altered in the allografts from DOCK2$^{-/-}$ recipients. BALB/c heart grafts harvested from WT B6 (black bar), DOCK2$^{-/-}$ (dark gray bar), and Tac-treated DOCK2$^{-/-}$ recipients (light gray bar) on day 6 after transplantation were used for measurement of IL-2, TNF-α, RANTES, IFN-γ, granzyme B, and perforin mRNA expression by real time RT-PCR. As a control, B6 grafts from B6 mice were analyzed similarly (white bar). Data are expressed relative to GAPDH level and represent mean ± SEM of three animals per group. *P < 0.05 comparison between WT B6 and DOCK2$^{-/-}$ recipients with or without Tac treatment.

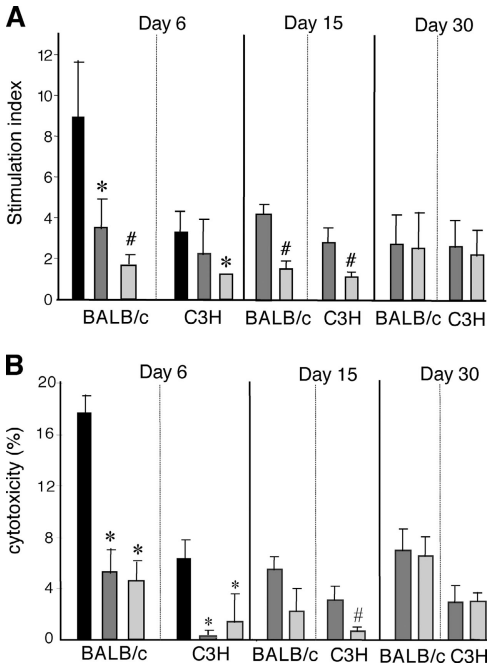

Figure 4. Alloreactivity and allocytotoxicity are suppressed in DOCK2$^{-/-}$ recipients. (A) Splenic T cells were prepared from WT B6 (black bar), DOCK2$^{-/-}$ (dark gray bar), and Tac-treated DOCK2$^{-/-}$ (light gray bar) recipients on day 6, 15, or 30 after transplantation of BALB/c hearts, and were cultured with mitomycin C–treated spleen cells of B6, BALB/c, or C3H mice. Data are shown as mean ± SEM of the stimulation index calculated by dividing the responses to BALB/c or C3H spleen cells by that to B6 spleen cells. *P < 0.05 comparison between WT B6 and DOCK2$^{-/-}$ recipients with or without Tac treatment. #P < 0.05 comparison between DOCK2$^{-/-}$ recipients and those treated with Tac. (B) Splenic T cells were prepared from WT B6 (black bar), DOCK2$^{-/-}$ (dark gray bar), and Tac-treated DOCK2$^{-/-}$ (light gray bar) recipients on day 6, 15, or 30 after transplantation of BALB/c hearts as effector cells, and were cultured with Con A–stimulated BALB/c or C3H spleen cells as target cells. Data are shown as mean ± SEM of the percentage of cytotoxicity of triplicate wells. *P < 0.05 comparison between WT B6 and DOCK2$^{-/-}$ recipients with or without Tac treatment. #P < 0.05 comparison between DOCK2$^{-/-}$ recipients and those treated with Tac.

transplantation exhibited mild to moderate interstitial infiltration of mononuclear cells and fibrosis (Fig. 1 C, right), these results provide further evidence that DOCK2 deficiency effectively attenuates cardiac allograft rejection.

DOCK2 deficiency attenuates graft tissue infiltration of T cells

To better understand the mechanism by which DOCK2 deficiency attenuates allograft rejection, we first analyzed the number of infiltrating cells bearing CD3, CD25, or CD68 macrophage marker in the cardiac grafts on day 6 after transplantation. Whereas cells expressing CD3, CD25, or CD68 macrophage marker were scarce in the B6 grafts transplanted into B6 mice, BALB/c grafts from the WT B6 recipients exhibited infiltration of numerous mononuclear cells expressing these cell surface markers (Fig. 2, A, C, and E). However, the number of CD3$^+$ T cells in the allografts of DOCK2$^{-/-}$ recipient mice with or without Tac treatment decreased to 40% or 56%, on average, of the WT B6 level (Fig. 2 B). In addition, the allografts in DOCK2$^{-/-}$ mice, irrespective of Tac treatment, showed significantly reduced CD25$^+$ cells (Fig. 2 D). Conversely, the numbers of CD68$^+$ macrophages (Fig. 2 F) and NK cells (not depicted) infiltrating into the allografts were unchanged between WT B6 and DOCK2$^{-/-}$ mice with or without Tac treatment. When similar analysis was performed on days 15 and 30 after transplantation, no significant difference could be found in the number and the composition of the infiltrating cells between DOCK2$^{-/-}$ recipients and those that were treated with Tac (Fig. 2, G–I).

DOCK2 deficiency attenuates intragraft expression of T cell effector molecules

We then examined the intragraft expression of cytokines, chemokines, and cytolytic factors on day 6 after transplantation using real-time quantitative RT-PCR. Whereas the expression of cytokines (IL-2, IFN-γ, TNF-α), chemokines (regulated upon activation, normal T cell expressed and secreted [RANTES]), and cytolytic molecules (granzyme B, perforin) was scarcely detected in the B6 grafts that were transplanted into B6 mice, BALB/c grafts from the WT B6 recipient mice exhibited strong induction of all effector mol-

ecules tested (Fig. 3). Even when DOCK2$^{-/-}$ mice were used as recipients, with or without Tac treatment, the expression of IL-2, TNF-α, and RANTES was induced comparably in the allografts (Fig. 3). However, the expression of IFN-γ, granzyme B, and perforin was reduced significantly in the allografts from DOCK2$^{-/-}$ recipient mice, irrespective of Tac treatment (Fig. 3). The expression of all effector molecules tested was unchanged between DOCK2$^{-/-}$ recipients and those treated with Tac, not only on day 6, but also on days 15 and 30 after transplantation (Fig. 3). These results indicate that DOCK2 deficiency effectively attenuates the expression of multiple T cell effector molecules.

DOCK2 deficiency attenuates alloreactivity or allocytotoxicity

Having found that the number of activated T cells and the expression of their effector molecules were reduced in the allografts from DOCK2$^{-/-}$ recipient mice, we compared the MLR and the CTL activity among WT B6 and DOCK2$^{-/-}$ mice, with or without Tac treatment, on day 6 after transplantation of BALB/c heart grafts. Splenic T cells from WT B6 recipients showed a vigorous proliferative response to the mitomycin C–treated BALB/c spleen cells, which was considerably greater than that of the third-party control, C3H spleen cells (Fig. 4 A). In the case of splenic T cells from DOCK2$^{-/-}$ mice, however, the proliferative response was reduced to 40% of the WT level (Fig. 4 A). The more profound suppression of the MLR was found when splenic T cells from DOCK2$^{-/-}$ mice treated with a low dose of Tac for 10 d were used as responders (Fig. 4 A). Splenic T cells from Tac-treated DOCK2$^{-/-}$ mice also had significantly less proliferative response than that of the untreated DOCK2$^{-/-}$ mice on day 15, but not day 30, after transplantation (Fig. 4 A); this indicated that Tac treatment has an additional effect on suppressing the proliferative response of alloreactive T cells. Consistent with the alloreactivity results, splenic T cells from DOCK2$^{-/-}$ recipients, irrespective of Tac treatment, revealed weak allocytotoxicity against donor antigen (Fig. 4 B). Taken together, these results indicate that the T cell response to allo-MHC molecules is impaired significantly in DOCK2$^{-/-}$ mice, even after in vivo priming with alloantigens.

We then examined whether exogenous IL-2 restores the proliferative response of DOCK2$^{-/-}$ T cells in the MLR. Consistent with the results shown in Fig. 4 A, DOCK2$^{-/-}$ T cells harvested on day 15 after transplantation showed poor proliferative response to BALB/c spleen cells in the absence of exogenous IL-2 (Fig. 5 A). However, the proliferative responses of DOCK2$^{-/-}$ T cells were enhanced by adding IL-2 to the culture (Fig. 5 A), which suggested that some of the alloreactive T cells are in the anergic state in DOCK2$^{-/-}$ recipients. This led us to explore whether DOCK2 functions downstream of CD28, a costimulatory molecule that is required for the full activation of T cells. When DOCK2$^{+/-}$ T cells were stimulated with anti-CD28 mAb, GTP-bound, activated Rac was detected readily (Fig. 5 B). However, such Rac activation was totally abolished in DOCK2$^{-/-}$ T cells (Fig. 5 B), which indicated that DOCK2 also is critical for Rac activation in CD28-mediated costimulation of T cells.

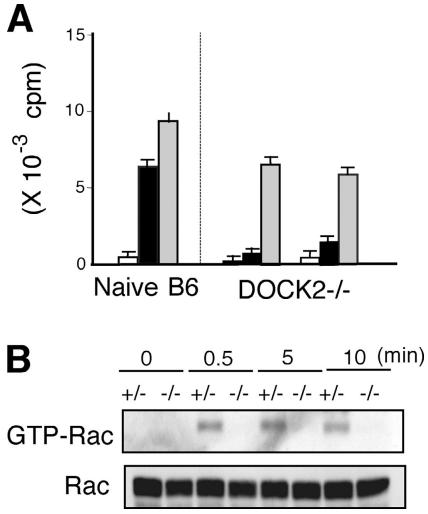

Figure 5. DOCK2 functions downstream of CD28 and is required for full T cell activation. (A) Splenic T cells were prepared from DOCK2$^{-/-}$ recipients on day 15 after transplantation of BALB/c hearts, and were stimulated with mitomycin C–treated spleen cells of B6 mice (white bar) or BALB/c mice in the absence (black bar) or presence (light gray bar) of exogenous IL-2. As a control, splenic T cells from naive B6 mice were analyzed similarly. Data are shown as mean ± SEM of ^{3}H-thymidine incorporation of triplicate wells. (B) Splenic T cells of DOCK2$^{+/-}$ and DOCK2$^{-/-}$ mice were stimulated with anti-CD28 mAb for the indicated times. Cell extracts were incubated with glutathione S-transferase–fusion (GST)-PAK1–Rac binding domain, and the activated and total Rac were analyzed by immunoblot using anti-Rac mAb.

DOCK2$^{-/-}$ naive T cells transferred into RAG1$^{-/-}$ mice exhibit poor proliferative response and fail to infiltrate into the allograft tissues

To visualize the effect of DOCK2 deficiency on activation and migration of lymphocytes in vivo, naive lymphocytes from B6 and DOCK2$^{-/-}$ mice were labeled with 5,6-carboxyfluorescein diacetate succinimidyl ester (CFSE), and adoptively transferred into RAG1$^{-/-}$ recipients with B6 or BALB/c heart grafts. On day 6 after transfer of 60 × 10^{6} lymphocytes, more than 80% of B6 T cells recovered from RAG1$^{-/-}$ recipients were CFSE-negative, irrespective of the origin of the cardiac grafts (Fig. 6 A); this indicated that B6 T cells undergo homeostasis-driven proliferation. However, under the same conditions, ~40% of the DOCK2$^{-/-}$ T cells remained CFSE-bright, undivided cells, even after transfer into RAG1$^{-/-}$ recipients having BALB/c cardiac grafts (Fig. 6 A). It is known that competition limits the extent of homeostatic proliferation. When 6 or 3 × 10^{6} CFSE-labeled DOCK2$^{-/-}$ lymphocytes were transferred into RAG1$^{-/-}$ mice, the percentage of undivided cells was reduced to

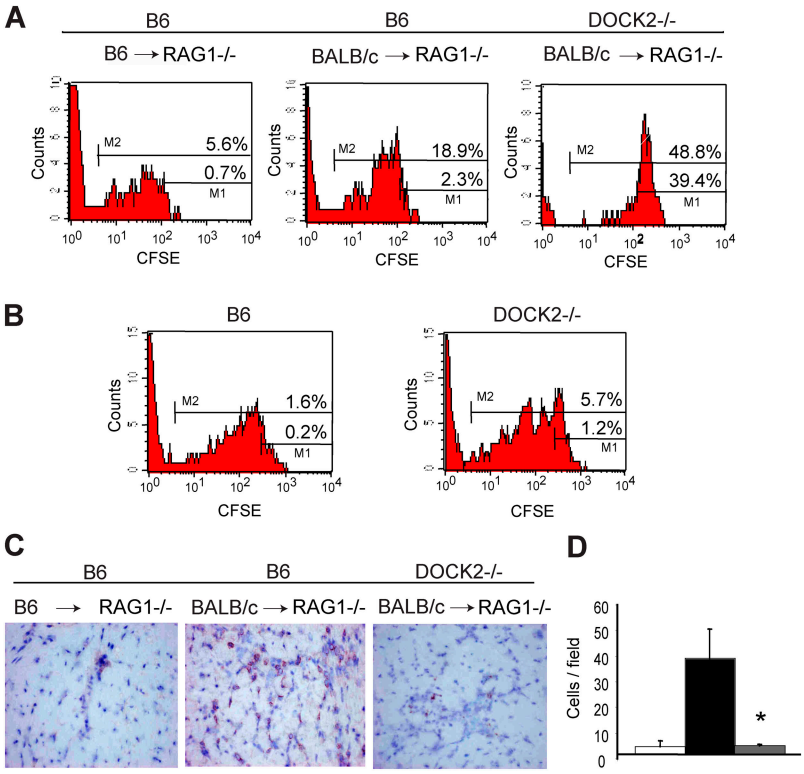

Figure 6. DOCK2$^{-/-}$ naive T cells transferred into RAG1$^{-/-}$ mice exhibit poor proliferative response and fail to infiltrate into the allograft tissues. (A) CFSE-labeled naive lymphocytes (60 ×10^6) from B6 or DOCK2$^{-/-}$ mice were adoptively transferred into RAG1$^{-/-}$ mice transplanted with B6 hearts (left panel) or BALB/c hearts (middle and right panels). On day 6 after transfer, CFSE intensity was analyzed for all CD3$^+$ T cell populations of the spleen. The percentages of CFSE$^+$ cells (M2) and CFSE$^+$ undivided cells (M1) are shown. (B) CFSE-labeled naive lymphocytes (6 × 10^6) from B6 (left panel) or DOCK2$^{-/-}$ (right panel) mice were adoptively transferred into RAG1$^{-/-}$ mice, and CFSE intensity was analyzed for all CD3$^+$ T cell populations of the spleen on day 6 after transfer. The percentages of CFSE$^+$ (M2) cells and CFSE$^+$ undivided cells (M1) are shown. (C and D) CFSE-labeled naive lymphocytes (60 × 10^6) from B6 or DOCK2$^{-/-}$ mice were adoptively transferred into RAG1$^{-/-}$ mice transplanted with B6 hearts (left panel) or BALB/c hearts (middle and right panels). On day 6 after transfer, graft tissues were stained with anti-CD3ε mAb (C), and the numbers of infiltrating T cells were analyzed in a semi-quantitative manner (D). Data are mean ± SEM of the number of CD3$^+$ cells in at least three mice per group. White bar, B6 T cell infiltration into B6 heart tissues; black bar, B6 T cell infiltration into BALB/c heart tissues; dark gray bar; DOCK2$^{-/-}$ T cell infiltration into BALB/c heart tissues. *P < 0.05 comparison between WT B6 and DOCK2$^{-/-}$ T cell infiltration into BALB/c hearts.

1.2% or 0.3%, respectively (Fig. 6 B and not depicted). Taken together, these results suggest that DOCK2 deficiency does not abolish, but reduces the susceptibility to, homeostatic proliferation.

We then compared T cell infiltration into B6 or BALB/c cardiac grafts on day 6 after adoptive transfer of 60 × 10^6 lymphocytes, by staining the graft tissue sections with anti-CD3 mAb. Whereas B6 T cells were scarcely detected in B6 cardiac grafts, considerable numbers of B6 T cells infiltrated into BALB/c heart grafts (Fig. 6, C and D); this indicated that alloreactivity is required for graft tissue infiltration of T cells. In contrast to the results on B6 T cells, DOCK2$^{-/-}$ T cells failed to infiltrate into the BALB/c heart grafts (Fig. 6, C and D).

DOCK2 deficiency affects graft tissue infiltration of activated, alloreactive T cells

To examine whether DOCK2 deficiency affects graft tissue infiltration of activated, alloreactive T cells, T cells from B6 and DOCK2$^{-/-}$ mice were stimulated with the mitomycin C–treated BALB/c spleen cells in vitro in the presence of exogenous IL-2, and were adoptively transferred into RAG1$^{-/-}$ recipients with BALB/c heart grafts. Functional and flow cytometric analysis confirmed that T cells from both strains were comparably activated under this condition (unpublished data). On day 6 after adoptive transfer of B6 T cells, numerous CD3$^+$ T cells were found in BALB/c cardiac grafts (Fig. 7, A and B). However, CD3$^+$ T cells infiltrating into the allografts were scarcely detected in the case of DOCK2$^{-/-}$ T cells (Fig. 7, A and B). These results indicate that DOCK2 deficiency attenuates graft tissue infiltration of T cells, even when the priming defect is bypassed.

DISCUSSION

T lymphocyte migration and activation are important for initiating allograft rejection and are critically dependent on remodeling of the actin cytoskeleton (15, 16). Therefore,

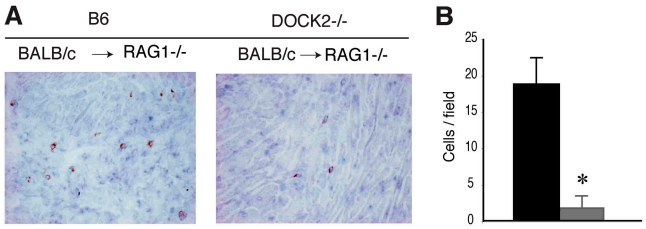

Figure 7. DOCK2 deficiency affects graft tissue infiltration of activated, alloreactive T cells. T cells from B6 or DOCK2$^{-/-}$ mice were cultured with mitomycin C–treated BALB/c spleen cells in the presence of exogenous IL-2, and adoptively transferred into RAG1$^{-/-}$ mice transplanted with BALB/c hearts. On day 6 after transfer, graft tissues were stained with anti-CD3ε mAb (A), and the number of infiltrating T cells was analyzed in a semi-quantitative manner (B). Data are mean ± SEM of the number of CD3^{+} cells in at least six sections obtained from two mice per group. Black bar, B6 T cell infiltration into BALB/c heart tissues; dark gray bar, DOCK2$^{-/-}$ T cell infiltration into BALB/c heart tissues. *$P < 0.05$ comparison between WT B6 and DOCK2$^{-/-}$ T cell infiltrations.

provided that remodeling of the actin cytoskeleton could be manipulated in lymphocytes, such manipulation would be a useful approach to attenuate allograft rejection. In the present study, we have provided evidence that DOCK2, a regulator of the actin cytoskeleton in lymphocytes, could be a novel molecular target for controlling transplant rejection.

DOCK2 deficiency enabled significant prolongation of cardiac allograft survival across a complete mismatch of MHC with an MST of 60 d when compared with the MSTs of 11 d in the WT B6 and 7 d in the DOCK2$^{+/-}$ controls. Myocardial histopathology revealed that the classic signs of acute rejection were seen in the allografts from the WT B6 controls, but not in the allografts from the DOCK2$^{-/-}$ recipients on day 6 after transplantation. This histologic feature of DOCK2$^{-/-}$ recipients was similar to that of allografts from RAG1$^{-/-}$ recipient mice, lacking all T and B cells, under the same conditions. Although there is a need to pinpoint the sources of the variation in the allograft survival times and the exact causes of eventual loss of allografts in some DOCK2$^{-/-}$ recipients, these results indicate that DOCK2 deficiency effectively attenuates allograft rejection.

The expression of IFN-γ, granzyme B, and perforin, all of which are known to be involved in the induction of cytotoxicity and direct damage of allograft tissues (25–28), was reduced significantly in the allografts from DOCK2$^{-/-}$ recipients. Considering that allografts normally are rejected, even in IL-2$^{-/-}$, IFN-γ$^{-/-}$, or perforin$^{-/-}$ mice (29–31), it is unlikely that the decreased expression of IFN-γ, granzyme B, or perforin alone contributes to survival of the cardiac allografts in DOCK2$^{-/-}$ recipients. Therefore, the combined reduction of these effector molecules or the changes in gene expression profiling might be responsible for prevention of allograft rejection in this transplant setting. Alternatively, DOCK2 deficiency might attenuate the expression of some other undetermined molecules that are critical for graft rejection. Nonetheless, together with the evidence of the decreased number of CD25^{+} cells in the allografts, these results suggest that DOCK2 deficiency affects the activation of alloreactive T cells, and leads to allograft survival.

Because cardiac allograft survival is prolonged remarkably in mice lacking LNs, Peyer's patches, and spleen (32–34), activation of alloreactive T cells is considered to occur mainly in secondary lymphoid organs. We reported that DOCK2 deficiency severely impairs the chemotactic responses of naive lymphocytes to homeostatic chemokines, such as CCL19 (EBI1-ligand chemokine), CCL21 (secondary lymphoid tissue chemokine), and CXCL13 (B-lymphocyte chemoattractant) (23). Consistent with this report, homing of adoptively transferred DOCK2$^{-/-}$ lymphocytes to peripheral LNs, mesenteric LNs, Peyer's patches, and white pulp of the spleen is reduced remarkably, compared with that of WT lymphocytes (35). Therefore, it is suggested that DOCK2 deficiency attenuates alloreactivity, at least in part, by reducing the number of potentially alloreactive T cells in secondary lymphoid organs. Recently, Beckmann et al. (36) reported that the deficiency of CCR7, a receptor for CCL19 and CCL21, has only a modest effect on cardiac allograft survival. Different from the case of CCR7 (37), DOCK2 functions downstream of various, if not all, chemokine receptors and critically regulates T cell homing, T cell egress from the thymus, and B cell homing (23, 35). This might account, in part, for the differential effect on cardiac allograft survival between DOCK2$^{-/-}$ and CCR7$^{-/-}$ mice. However, because of its nature to regulate the actin cytoskeleton, DOCK2 deficiency is expected to have additional effects on suppression of T cell activation.

We found that splenic T cells from DOCK2$^{-/-}$ recipients showed significantly lower MLR response and CTL activity toward donor antigens than those of the WT B6 controls. Because the proliferative response of DOCK2$^{-/-}$ T cells in the MLR was much enhanced in the presence of exogenous IL-2, it seems likely that in DOCK2$^{-/-}$ recipients, some of the alloreactive T cells are in the anergic state. Thus far, costimulatory molecules, such as CD80/CD86 and CD28, have been the major targets for blocking T cell full activation and inducing allograft tolerance (11–14). Although the nature of costimulation is not understood fully, it was reported that delivering the signal that induces lipid raft clustering in T cells is related to costimulatory signaling–induced T cell full activation (38). We reported earlier that DOCK2 plays a crucial role in antigen-induced TCR clustering and lipid raft clustering (24). In addition, the present study reveals that DOCK2 also is critical for CD28-mediated Rac activation. Therefore, it is suggested that DOCK2 deficiency impairs T cell activation by inhibiting TCR-mediated and CD28-dependent cytoskeletal reorganization.

Because tissue infiltration of activated T cells is a crucial step for induction of allograft rejection, significant efforts were made recently to block this process, especially focusing on chemokine–chemokine receptor interactions (2, 39, 40). For example, targeting of the chemokine re-

ceptors CXCR3, CX_3CR1, or CCR1 suppressed cardiac allograft rejection with or without cyclosporin A treatment (8–10). We found that graft tissue infiltration of $DOCK2^{-/-}$ T cells was impaired severely, even when alloreactive T cells activated in vitro were used for adoptive transfer. These results indicate that DOCK2 deficiency attenuates priming and activation of potentially alloreactive T cells in secondary lymphoid organs, and graft tissue infiltration of activated, alloreactive T cells.

The present study also suggests that DOCK2 deficiency causes resistance to homeostatic proliferation if the space is relatively limited. Although costimulatory molecules do not play a major role in this type of T cell activation (41, 42), several lines of evidence indicate that homeostatic proliferation requires the engagement of TCRs by MHC–self-peptide complexes and cytokine/chemokine stimulation, particularly IL-15 and -7 in the case of $CD8^+$ T cells, and CCL21 for $CD4^+$ T cells (43). At this stage, we are unable to determine the precise mechanism by which DOCK2 deficiency affects homeostatic proliferation. However, homeostatic proliferation is considered to be a barrier to transplantation tolerance (42, 43). In this respect, DOCK2 deficiency might be of benefit for controlling transplant rejection.

We also found that the treatment of $DOCK2^{-/-}$ recipient mice with a suboptimal dose (3.2 mg/kg/d) of Tac for 10 d resulted in a remarkable synergism, with almost permanent engraftment of cardiac allografts. Although mononuclear cell infiltration and gene expression profiles were unchanged in $DOCK2^{-/-}$ recipient mice, with or without Tac treatment, the proliferative T cell response of the Tac-treated $DOCK2^{-/-}$ mice was significantly lower than that of the untreated $DOCK2^{-/-}$ mice on days 6 and 15 after transplantation. Therefore, this effect is suggested to be caused by the combined actions of DOCK2 deficiency, which reduces the alloreactivity and allocytotoxicity to a "manageable" level, and Tac treatment, which further silences the remaining alloreactivity of T cells.

In conclusion, we have shown that DOCK2 deficiency enables long-term survival of cardiac allografts across a complete MHC mismatch by simultaneously suppressing multiple and key processes that lead to allograft rejection. Therefore, DOCK2 may be a novel therapeutic target for controlling transplant rejection.

MATERIALS AND METHODS

Mice. B6 (H-2^b), BALB/c (H-2^d), and $RAG1^{-/-}$ B6 mice were purchased from The Jackson Laboratory. $DOCK2^{-/-}$ mice have been described elsewhere (23, 24). $DOCK2^{-/-}$ mice were backcrossed with B6 mice for more than seven generations, and $DOCK2^{-/-}$ and $DOCK2^{+/-}$ littermates that were obtained by intercrossing $DOCK2^{-/-}$ males and $DOCK2^{+/-}$ females were used in this study. All animals were kept under specific pathogen-free conditions in the animal facility at Kyushu University or Evanston Northwestern Healthcare. Animals were used at 6–10 wk of age. All experiments were done in accordance with the guidelines of the committee of Ethics on Animal Experiment, Faculty of Medical Sciences, Kyushu University and Evanston Northwestern Healthcare Institutional Animal Care and Use Committee.

Mouse cardiac transplantation. Vascularized cardiac allografts were transplanted using microsurgical techniques as described by Ono and Lindsey (44). In brief, donor and recipient mice were anesthetized with ketamine and xylazine. Donor hearts were harvested and placed in chilled physiologic saline, during which time the recipient mice were prepared. The donor hearts were heterotopically transplanted into recipient mice by end-to-side anastomosis of the donor aorta and pulmonary artery to the recipient's abdominal aorta and inferior vena cava, respectively. The technical success rate was ~84%. Heart allograft survival was determined by daily palpation. Rejection was considered complete at the time of cessation of a palpable heart beat, and was confirmed visually after laparotomy.

Immunosuppression. Tacrolimus (Tac; an oral formulation) was suspended in water and administered in a volume of 10 ml/kg/d. The $DOCK2^{-/-}$ recipients were treated with Tac at a predetermined suboptimal dose of 3.2 mg/kg/d for 10 d beginning at transplantation.

Immunohistology and histology. Heart graft tissues were harvested from recipients for detection of intragraft cell infiltration by immunohistochemistry. 4-μm frozen sections of heart tissues were cut, fixed with acetone, and stained with primary anti–mouse antibodies against CD3ε (145-2C11), CD25 (7D4) (both obtained from BD Biosciences), and CD68 macrophage marker (FA-11) (Serotec Ltd.) at optimal dilutions. After incubation, the sections were stained with peroxidase-conjugated secondary antibodies, and visualized using 3-amino-9-ethylcarbazole as a chromogen. Immunoperoxidase-stained tissue sections were evaluated semi-quantitatively in blinded fashion by a minimum of two of the authors. In each section, more than six different representative fields were counted by visual microscopy (400×) to determine the average number of stained cells per field of view. Average ± SEM was determined from at least three separate grafts. For morphologic analysis of heart grafts, all specimens were fixed in 10% buffered formalin and embedded in paraffin. Sections were cut and stained with hematoxylin and eosin. Light microscopic analysis was performed to assess overall cellularity and myocardial damage (45).

Quantitative real-time RT-PCR. Total RNA of heart grafts from recipients was used to detect quantitatively the mRNA levels of GAPDH, IL-2, IFN-γ, TNF-α, RANTES, granzyme B, and perforin with the ABI PRISM 7700 sequence detection system (Applied Biosystems). The specific primers and probes to detect IL-2, granzyme B, and IFN-γ have been described elsewhere (46, 47). GAPDH was detected by using Taqman rodent GAPDH control reagents (Applied Biosystems). To detect TNF-α, RANTES, and perforin, RT-PCR was performed using the following primers and probes: TNF-α: 5′-AAGGCTGCCCCGACTATGT-3′ (sense), 5′-GCGGAGAGGAGGCTGACTT-3′ (antisense), and 5′-6FAM-CTCACCCACACCGTCAGCCGATT-TAMRA-3′ (probe); RANTES: 5′-GTCGTCTTTGTCACTCGAAGGA-3′ (sense), 5′-GATGTATTCT-TGAACCCACTTCTTCTC-3′ (antisense), and 5′-6FAM-CCGCCAA-GTGTGTGCCAACCC-TAMRA-3′ (probe); perforin: 5′-GCAGGT-CAGGCCAGCATAAG-3′ (sense), 5′-GCAGTCCTGGTTGGTGAC-CTT-3′ (antisense), 5′-6FAM-ATTCATGCCAGTGTGAGTGCCAG-GATT-MGBNFQ-3′ (probe). Gene expression values were normalized to GAPDH.

T cell alloreactivity and allocytotoxicity in cardiac allografted recipients. Spleens were collected from recipients for MLRs on day 6, 15, or 30 after grafting. After T cell subpopulations were enriched with the Mouse T Cell Recovery Column Kit (Cedarlane), T cell–enriched splenocytes (2×10^5/well) were cultured with mitomycin C–treated splenocytes (5×10^5 /well) from B6, BALB/c, or the third-party control C3H mice in 96-well round-bottom plates for 72 h. 1 μCi of ^{3}H-thymidine was added during the final 6 h of culture, and incorporated radioactivity was measured with a liquid scintillation counter (Packard TRI CARB 2500). In some experiments, IL-2 was added to the culture at 10 ng/ml.

For the CTL assay, spleens were collected from recipients on day 6, 15, or 30 after grafting, and T cell–enriched splenocytes were prepared as effector cells using the Mouse T Cell Recovery Column Kit. Target cells were prepared by culturing BALB/c or C3H splenocytes (2.5×10^6/ml) with Con A (2.5 μg/ml) for 2 d. The cultures of target cells were pulsed with 2.5 μCi/ml of ^{3}H-thymidine during the final 16 h. The 10^6/well effector cells were cultured with 10^4/well of ^{3}H-thymidine–labeled target cells in 96-well round-bottom plates for 4 h at 37°C in a humidified atmosphere of 5% CO_2. The percentage of cytotoxicity was calculated as described in a previous protocol (48).

In vivo assessment for proliferation and infiltration of lymphocytes. Lymphocytes were prepared from B6 and DOCK2$^{-/-}$ mice, and incubated with 5 μM 5,6-carboxyfluorescein diacetate succinimidyl ester (Invitrogen). Labeled cells (60×10^6) were injected i.v. through the tail vein into RAG1$^{-/-}$ recipients immediately after transplantation of B6 or BALB/c heart grafts. On day 6 after transfer, spleen and heart grafts of RAG1$^{-/-}$ recipient mice were harvested and subjected to flow cytometric or histologic analysis. In some experiments, various amounts of the labeled lymphocytes were injected directly into RAG1$^{-/-}$ mice and were analyzed similarly.

For transfer of activated T cells, T cell–enriched splenocytes (2×10^6/ml) from B6 and DOCK2$^{-/-}$ mice were cultured with mitomycin C–treated BALB/c splenocytes (8×10^6/ml) in the presence of IL-2 (10 ng/ml). The cells were harvested on day 4, and equal amounts of T cells (6×10^6) from each group were adoptively transferred into RAG1$^{-/-}$ recipients with BALB/c heart grafts for histologic analysis. The activation status was confirmed by functional or flow cytometric analysis.

Pull-down assay. T cells from DOCK2$^{-/-}$ and DOCK2$^{+/-}$ mice were incubated with anti-CD28 mAb (37.51, 5 μg/ml; BD Biosciences) at 4°C for 20 min, and stimulated with goat anti–hamster IgG Ab (G94-56, 20 μg/ml, BD Biosciences) at 37°C for the specified times. Cell extracts were incubated with glutathione S-transferase (GST)–fusion, Rac binding domain (RBD) of PAK1 at 4°C for 60 min. The GST-PAK1-RBD–bound proteins were analyzed by SDS-PAGE, and blots were probed with the Rac-specific mAb 23A8 (Upstate Biotechnology).

Statistical analysis. To calculate graft survival, Kaplan-Meier survival graphs were constructed. Comparisons between groups were evaluated by Student's *t* test. Data were expressed as mean ± SEM, and were considered to be statistically significant if p-values were ≤ 0.05.

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