Dendritic Cells Conditioned With NK026680 Prolong Cardiac Allograft Survival in Mice

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Background. Pharmacologically modulated dendritic cells (DCs) can potentially regulate alloimmune responses. We examined the characteristics of immunoregulatory DCs induced by a novel triazolopyrimidine derivative, NK026680, which has been previously shown to inhibit DC maturation.

Methods. DCs were generated from bone marrow progenitor cells from C57BL/6 (B6, H-2^b haplotype) mice with granulocyte-macrophage colony-stimulating factor and interleukin (IL)-4. DCs were cultured with allogeneic BALB/c (H-2^d) splenocyte lysates with or without NK026680. DC functions were examined in vitro after stimulation of tumor necrosis factor α and in vivo by the intravenous injection of C3He/J (C3H, H-2^k) DCs cultured with B6 cell lysates and NK026680 into C3H mice. Seven days later, DC-treated mice received B6 heart allografts, and graft survival and alloimmune responses were assessed.

Results. In NK026680-treated DCs (NK-DCs), significant inhibition of the up-regulation of surface activation markers (CD40, CD80, CD86, and major histocompatibility complex class II) and IL-12 p40 production was observed after stimulation of tumor necrosis factor α compared with that of control DCs. Furthermore, NK-DCs suppressed alloreactive T-cell proliferation. The modulation of NK-DCs was likely associated with the inhibition of phosphorylation of p38 mitogen-activated protein kinase and the up-regulation of indolamine 2,3-dioxygenase expression. Compared with both noninjected and control DC–injected mice, mice that received a single in vivo infusion of NK-DCs showed significant increases in splenocyte IL-10 production and the splenic $CD4^+$ $IL\text{-}10^{high}$ T-cell population 7 days after injection, a significantly increased splenic $CD4^+CD25^+FoxP3^+$ T-cell population 14 days after injection, and markedly prolonged cardiac allograft survival.

Conclusions. Ex vivo NK026680 conditioning allows DCs to acquire immunoregulatory properties that suppress alloimmune responses and prolong cardiac allograft survival.

Keywords: NK026680, Heart transplantation, Mouse, Immunomodulation, Regulatory dendritic cells.

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Dendritic cells (DCs) are potent antigen-presenting cells that play a major role in the regulation of immune responses to various antigens (*1–3*). DC functions bridge innate and adaptive immunity. Therefore, the modification of DCs to regulate allograft rejection in organ transplantation has great potential. Infusion therapies with pharmacologically or genetically modified DCs have become promising modalities for immunosuppression in solid organ transplantation. Both the inhibition of the mammalian target of rapamycin by rapamycin (*4*, *5*) and the regulation of nuclear factor κB (NF-κB) activity by either oligodeoxyribonucleotide decoys (*6*) or LF15-0195 (*7–9*), an analog of the immunosuppressive drug 15-deoxyspergualin, cause syngeneic DCs to acquire regulatory properties that induce regulatory T-cell (Treg) development and peripheral tolerance. Studies on injection therapies that have used such preconditioned DCs have shown that these cells facilitate long-term graft acceptance after mouse heart transplantation (HTx).

The triazolopyrimidine-derivative NK026680 developed by Nippon Kayaku Co., Ltd. (Tokyo, Japan) has been shown to inhibit interleukin (IL)-12 production and the expression of surface maturation markers stimulated by tumor necrosis factor (TNF)-α in human monocyte–derived

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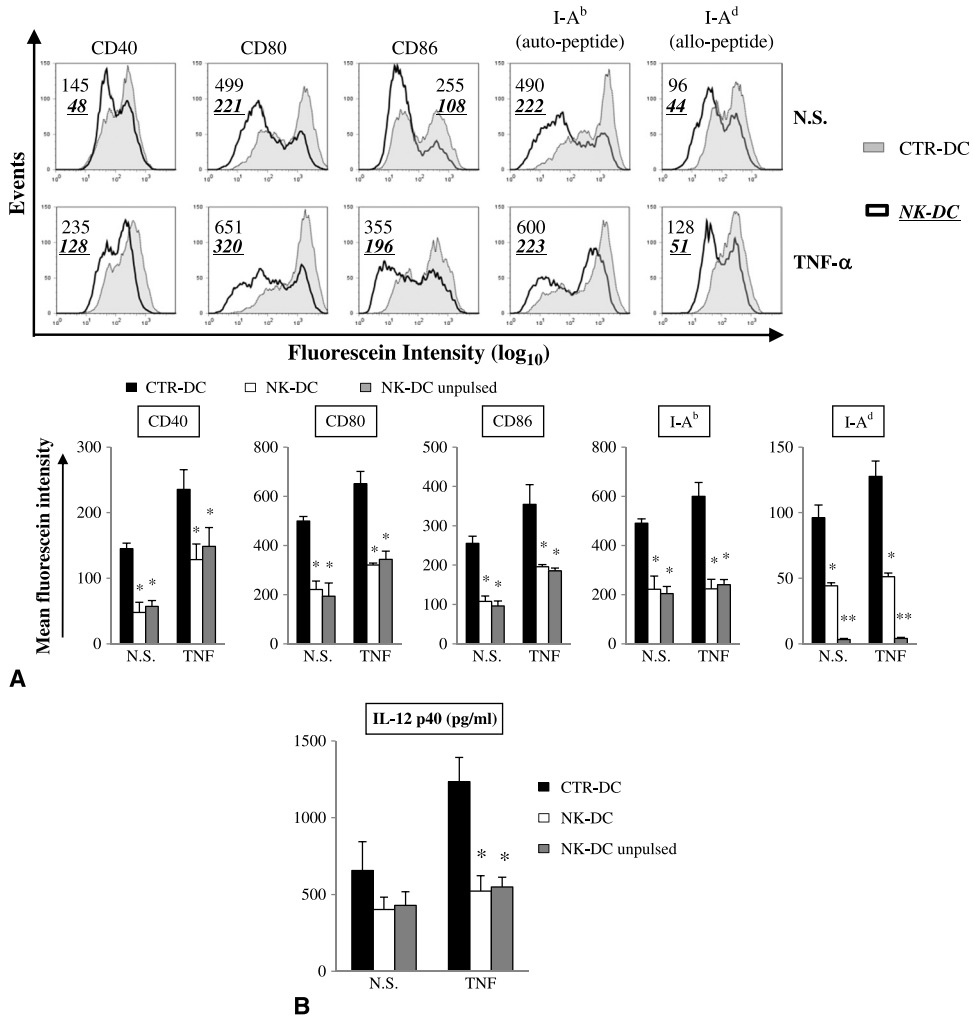

FIGURE 1. Ex vivo NK026680 conditioning regulates the expression of surface molecules and cytokine production on tumor necrosis factor (TNF)-α stimulation. Bone marrow–derived dendritic cells (DCs) from B6 mice were conditioned with NK026680 (NK026680-treated DC [NK-DC]) or vehicle alone (control DC [CTR-DC]) and pulsed with BALB/c lysates. After DC propagation, cells were collected and treated with TNF-α for 24 hr. A, Expression of CD40, CD80, CD86, I-A^b, and I-A^d as measured by flow cytometric analysis. Results show representative histograms of four independent experiments that showed similar results (*upper*). N.S., no stimulation. The upper number in the panel is the mean fluorescent intensity of CTR-DCs, and the lower one that is underlined is the mean fluorescent intensity of NK-DCs. Each column represents the mean±SEM of four independent experiments (*lower*). *P<0.05 vs. CTR-DC. **P<0.05 vs. NK-DC. B, Interleukin (IL)-12 p40 production by DCs before and after stimulation with TNF-α. IL-12 p40 levels in the culture supernatants were measured by enzyme-linked immunosorbent assay. Each column represents the mean±SEM of three independent experiments. *P<0.05 vs. CTR-DC.

DCs (*10*) and to impair the DC differentiation by reducing the expression of CD86 and major histocompatibility complex (MHC) class II in rats (*11*). Treatment with NK026680 ameliorated mortality in acute lethal graft-versus-host disease after mouse bone marrow transplantation (*10*), prolonged liver allograft survival by skewing T cells toward T helper 2 polarization in rats (*11*), and prevented glomerulonephritis and perinuclear antineutrophil cytoplasmic antibodies (Abs) in SCG/Kj mice through reduction of the serum autoantibody production and proinflammatory cytokine production including TNF-α, interferon (IFN)-γ, IL-2, IL-4, and IL-5 (*12*). In addition, we have recently demonstrated that administration of NK026680 prolongs cardiac allograft survival in rats without causing a severe adverse effect (*13*). Although these immunosuppressive effects of NK026680 have been mainly attributed to impairment of DCs by the agent, the mechanism of action on how NK026680 inhibits DC activation/maturation has not been clarified.

Recently, we have reported that NK026680 inhibits T-cell function in an IL-2–dependent manner, and these effects are likely caused by the suppression of the p38 mitogen-activated protein kinase (MAPK) pathway (*13*). The activation of p38 MAPK pathway positively regulates phenotypic and functional maturation in DCs stimulated by TNF-α, and p38 inhibitors impair these processes (*14–18*). We therefore hypothesized that ex vivo conditioning with NK026680 would induce powerful regulatory DCs. To elucidate the mechanism of action of NK026680 in DCs, we studied the phenotypical and functional characteristics of NK026680-treated DCs (NK-DCs) on TNF-α stimulation. In addition, to evaluate

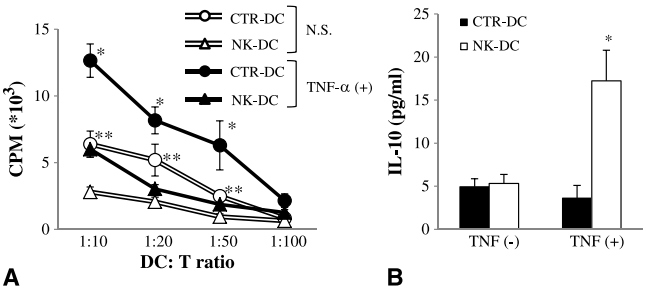

FIGURE 2. NK026680-treated dendritic cells (NK-DCs) inhibit alloantigen-driven T-cell proliferation. Bone marrow–derived DCs from B6 mice were conditioned with NK026680 (NK-DC) or vehicle alone (control DC [CTR-DC]) and pulsed with BALB/c lysates. DCs before and after stimulation with 20 ng/mL of tumor necrosis factor (TNF)-α for 24 hr were collected, extensively washed, and co-cultured with syngeneic purified T cells. A, T-cell proliferation through the indirect pathway after 72-hr stimulation with various ratios of DCs to T cells. Cells were pulsed with ^{3}H-thymidine at 8 hr before cell harvest. Thymidine incorporation was counted by a β-counter. Each symbol is representative of the mean±SEM of four independent experiments. *P<0.05, nonstimulated CTR-DC vs. nonstimulated NK-DC. **P<0.05, TNF-stimulated CTR-DC vs. TNF-stimulated NK-DC. CPM, counts per minute; N.S., no stimulation. B, Interleukin (IL)-10 production in the supernatants of mixed lymphocyte cultures. DCs were co-cultured with T cells at ratio of DC to T cell of 0.1 for 4 days. The IL-10 levels in the culture supernatants were measured by enzyme-linked immunosorbent assay. Each column is representative of the mean±SEM of four independent experiments.

the in vivo immunomodulatory effects of ex vivo conditioned DCs by NK026680, a mouse cardiac transplant model was used, and the effects of NK-DCs on alloimmune responses 7 days after NK-DC injection and allograft survival were assessed.

RESULTS

NK-DCs Were Phenotypically and Functionally Resistant to Maturation

We attempted to induce regulatory DCs by ex vivo NK026680 conditioning. To investigate the phenotypical profile of NK-DCs, the expression of cell surface maturation markers (such as CD40, CD80, CD86, and MHC class II) was analyzed (Fig. 1A). NK-DCs expressed lower levels of these markers than control DCs (CTR-DCs). Furthermore, NK-DCs retained low expression of these maturation markers after stimulation with TNF-α, whereas there was an up-regulation of these markers in CTR-DCs (Fig. 1A). Regardless of TNF stimulation, the expression of these molecules except for I-A^d showed no significant differences between the group of NK-DCs and unpulsed NK-DCs, whereas the expression of I-A^d in unpulsed NK-DCs was hardly detected (Fig. 1A). To evaluate the effect of conditioning on functional maturation, IL-12 levels in the culture supernatant were measured (Fig. 1B). In a nonstimulated state, IL-12 p40 production did not significantly differ between CTR-DCs and NK-DCs (P=0.104), although there was a trend toward less IL-12 p40 in NK-DCs. Stimulation with TNF-α induced significantly less IL-12 p40 production in NK-DCs than in CTR-DCs, whereas IL-12 p40 level of unpulsed NK-DCs did not significantly differ from that of NK-DCs (Fig. 1B). IL-12 p70 production was not detected in any of the culture supernatants (data not shown).

NK-DCs Inhibited Alloantigen-Driven T-Cell Proliferation Through the Indirect Pathway

To assess the ability of NK-DCs to activate naive T cells, a mixed leukocyte reaction (MLR) was performed (Fig. 2). T-cell proliferation was examined after 72 hr of culture with various ratios of DC to T cell (Fig. 2A). Without TNF-α stimulation, CTR-DCs mildly stimulated syngeneic naive T cells in a fashion dependent on the ratio of DC and T cell, and NK-DCs demonstrated a significantly reduced ability to induce T-cell proliferation compared with CTR-DCs. After TNF-α stimulation, CTR-DCs powerfully induced the proliferation of naive T cells. Compared with CTR-DCs, NK-DCs significantly inhibited the proliferation of naive T cells through the indirect pathway (Fig. 2A). We then evaluated IL-10 production in the MLR cultures (Fig. 2B). In the absence of TNF-α stimulation, IL-10 production was not significantly different between CTR-DCs and NK-DCs. After TNF-α stimulation, IL-10 levels in the NK-DC group were dramatically increased than those of the CTR-DC group (Fig. 2B).

NK-DC Modulation Was Likely Associated With the Inhibition of the MAPK Pathway and Up-regulation of Indolamine 2,3-Dioxygenase

To examine the underlying mechanisms of action of NK026680 on NK-DC biology, the MAPK signaling pathway was investigated (Fig. 3A). Intracellular levels of the active forms of total- or phospho-p38 MAPK, extracellular signal–regulated kinase (ERK), and c-Jun N-terminal kinase (JNK) were determined in CTR-DCs and NK-DCs after 30-min TNF-α stimulation. NK026680 conditioning significantly reduced the phosphorylation of p38 MAPK, ERK, and JNK compared with that in CTR-DCs (Fig. 3A). The major downstream substrates of MAPKs, activator protein 1 and NF-κB, were next examined (Fig. 3B). In NK-DCs, the nuclear levels of c-Jun and c-Fos, not the NF-κB family members (including Rel-A, p50, and p52), were significantly decreased compared with those in CTR-DCs (Fig. 3B). The nuclear protein level of Rel-B was also examined but was below the limit of detection (data not shown). We also examined the intracellular levels of indolamine 2,3-dioxygenase (IDO) in CTR-DCs and NK-DCs stimulated with TNF-α for 24 hr (Fig. 3C). NK-DCs demonstrated higher levels of intracellular IDO than CTR-DCs.

Injection Therapy of NK-DCs Induced CD4$^+$ IL-10^{high} T-cells on Day 0

To investigate the regulatory potential of NK-DCs in vivo, DCs (3×10^6) from C3H mice were pulsed with B6 cell lysates and then injected intravenously into a C3H mouse. After 7 days, the alloreactive splenocyte proliferation and the frequencies of alloreactive IFN-γ– and IL-10–producing cells were evaluated in cultures derived from the spleens of the injected mice (Fig. 4). The splenocytes of mice treated with CTR-DCs exhibited a high level of proliferation in response to an alloantigen, a high frequency of alloreactive IFN-γ–producing cells, and a low

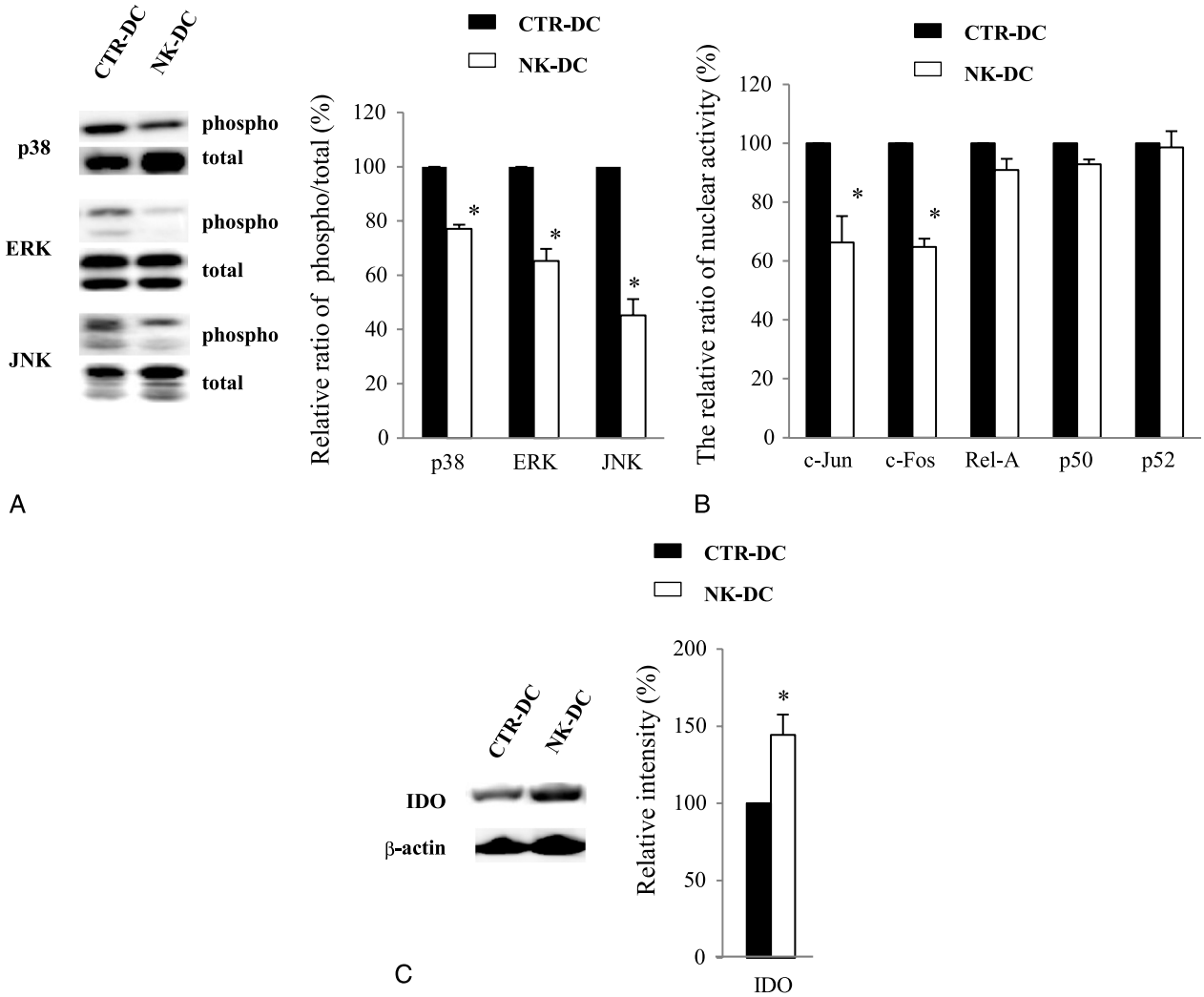

FIGURE 3. NK026680-treated dendritic cells (NK-DCs) show decreased mitogen-activated protein kinases (MAPKs) phosphorylation but increased expression of indolamine 2,3-dioxygenase (IDO) on tumor necrosis factor (TNF) stimulation. Bone marrow–derived DCs from B6 mice were cultured with NK026680 (NK-DC) or vehicle alone (control DC [CTR-DC]). A, Intracellular levels of MAPK proteins in NK-DCs after 30-min TNF stimulation. Whole cell lysates were prepared, transferred to membranes, and immunoblotted with antibodies against phospho- or total-ERK1/2 (extracellular signal–regulated kinase [ERK]), stress-activated protein kinase/JNK (c-Jun N-terminal kinase [JNK]), and p38 MAPK (p38). The bands were visualized by chemiluminescence. Representative immunoblots of three independent experiments (*left*) and the densitometry results and the ratios of phospho/total MAPKs (*right*). Each column is representative of the mean±SEM of three independent experiments with similar results. **P*<0.05 vs. CTR-DC. B, Nuclear activities of activator protein 1 and nuclear factor κB in DCs stimulated by TNF-α for 1 hr. Nuclear proteins were extracted and quantified using the TransAM kit. The relative activity ratios of nuclear Rel-A, p50, p52, c-Jun, and c-Fos are shown. Each column represents the mean±SEM of three independent experiments. **P*<0.05 vs. CTR-DC. C, Intracellular IDO expression after 24-hr TNF stimulation. After the cell lysates were prepared and immunoblotted with antibodies against purified IDO and β-actin, the bands were visualized by chemiluminescence. The representative immunoblot of three independent experiments (*left*) and the relative intensity of the specific IDO band (*right*) are shown. Each column is representative of the mean±SEM of three independent experiments with similar results. **P*<0.05 vs. CTR-DC.

percentage of IL-10–producing cells. In contrast, the infusion of NK-DCs inhibited alloreactive splenocyte proliferation but did not suppress alloreactive IFN-γ production. Moreover, NK-DC infusion increased the number of IL-10–producing cells on stimulation with donor antigen (Fig. 4A–C). The number of alloreactive IL-10–producing cells was also increased by injection of unpulsed NK-DCs as compared with that of CTR-DC; however, it was lower than that of alloantigen-pulsed NK-DCs. In contrast, the alloreactive splenocyte proliferation and frequency of IFN-γ–producing cells in the unpulsed NK-DC group did not differ from those of CTR-DC or alloantigen-pulsed NK-DC group (Fig. 4A–C). Analysis of the T-cell phenotypes in the cultures indicated that the $CD4^+$ $IL\text{-}10^{high}$ T-cell population (Tr1 cells) was significantly increased in splenocyte cultures derived from NK-DC–injected mice (7.5%±0.7%) compared with cultures derived from untreated (4.1%±0.7%, $P<0.05$) and CTR-DC–injected (4.2%±0.4%, $P<0.05$) mice

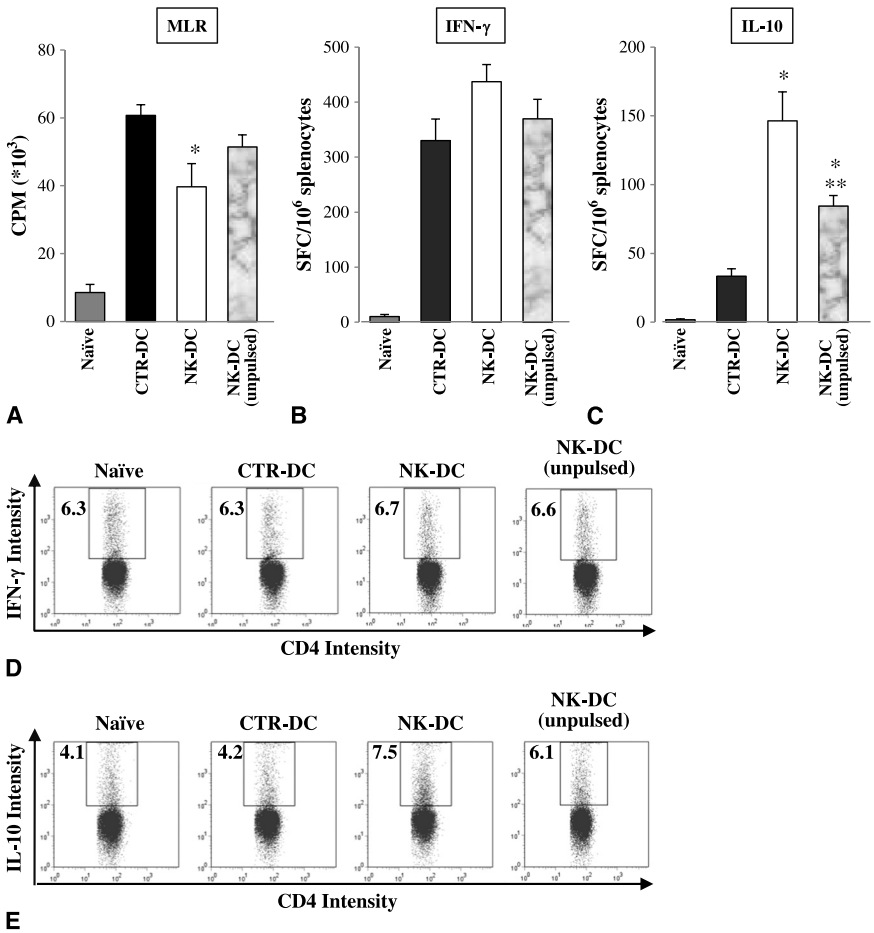

FIGURE 4. NK026680-treated dendritic cells (NK-DC) injection therapy enhances immunoregulatory activities 7 days after injection. Control DCs (CTR-DCs) or NK-DCs (3×10^6 cells) from C3H mice that were pulsed with B6 lysates were injected intravenously into C3H mice. Seven days later, alloimmune responses were evaluated in the splenocytes of the injected mice. Splenocytes from the injected C3H mice were co-cultured with irradiated B6 splenocytes. The alloreactive lymphocyte proliferation (A) after 48 hr of co-culture was measured by ^{3}H-thymidine uptake. The frequencies of alloreactive interferon (IFN)-γ–producing cells (B) and interleukin (IL)-10–producing cells (C) after 24-hr stimulation were measured by enzyme-linked immunospot assay. Each column represents the mean±SEM of four independent experiments. *P<0.05 vs. CTR-DC. **P<0.05 vs. unpulsed NK-DC. The expression of intracellular IFN-γ–producing T cells (D) (*upper*) and IL-10–producing T cells (*lower*). After 6 hr of co-culture, cells were stained intracellularly and then analyzed by flow cytometry after the gating on 3×10^4 CD4$^+$ lymphocytes. Representative histograms of four independent experiments are shown. CPM, counts per minute; SFC, spot-forming cells.

(Fig. 4D). The CD4$^+$ IL-10^{high} T-cell population in the unpulsed NK-DC–injected mice was 6.1%±0.5%, which was higher than that in the untreated mice or in mice treated with CTR-DCs but lower than that in alloantigen-pulsed NK-DCs (Fig. 4D). No differences were observed in CD4$^+$ IFN-γ–producing T cells among these four groups (Fig. 4D).

NK-DC Injection Therapy Permitted Long-term Cardiac Allograft Survival With the Induction of Tregs

To evaluate the immunosuppressive effects of NK-DCs, a fully MHC incompatible (B6 to C3H) heterotopic vascularized heart transplant model was used (Fig. 5). In this model, the naive C3H recipient acutely rejects the B6 heart graft, with the graft having a median survival time (MST) of 13 days. Three million CTR-DCs or NK-DCs from C3H mice were pulsed with B6 donor lysates and then intravenously injected into recipient C3H mice 7 days before HTx. A single injection of CTR-DCs failed to prevent cardiac allograft rejection (MST, 11 days). In contrast, the NK-DC–treated mice showed markedly prolonged graft survival with an MST of 65.5 days. In fact, one (12.5%) of the eight allografts achieved long-term graft survival that lasted for more than 100 days (Fig. 5A). Although the recipient mice injected with unpulsed-NK-DCs prolonged allograft survival to an MST of 29 days, the immunomodulatory effect as assessed by graft survival prolongation was not potent as compared with that of NK-DCs with donor-derived alloantigen (Fig. 5A). Likewise, graft MST was 29 days when B6 heart was transplanted to C3H mice that were treated with NK-DCs pulsed with third-party BALB/c splenocyte lysates (Fig. 5A). To understand how the injection of NK-DCs facilitated long-term allograft survival,

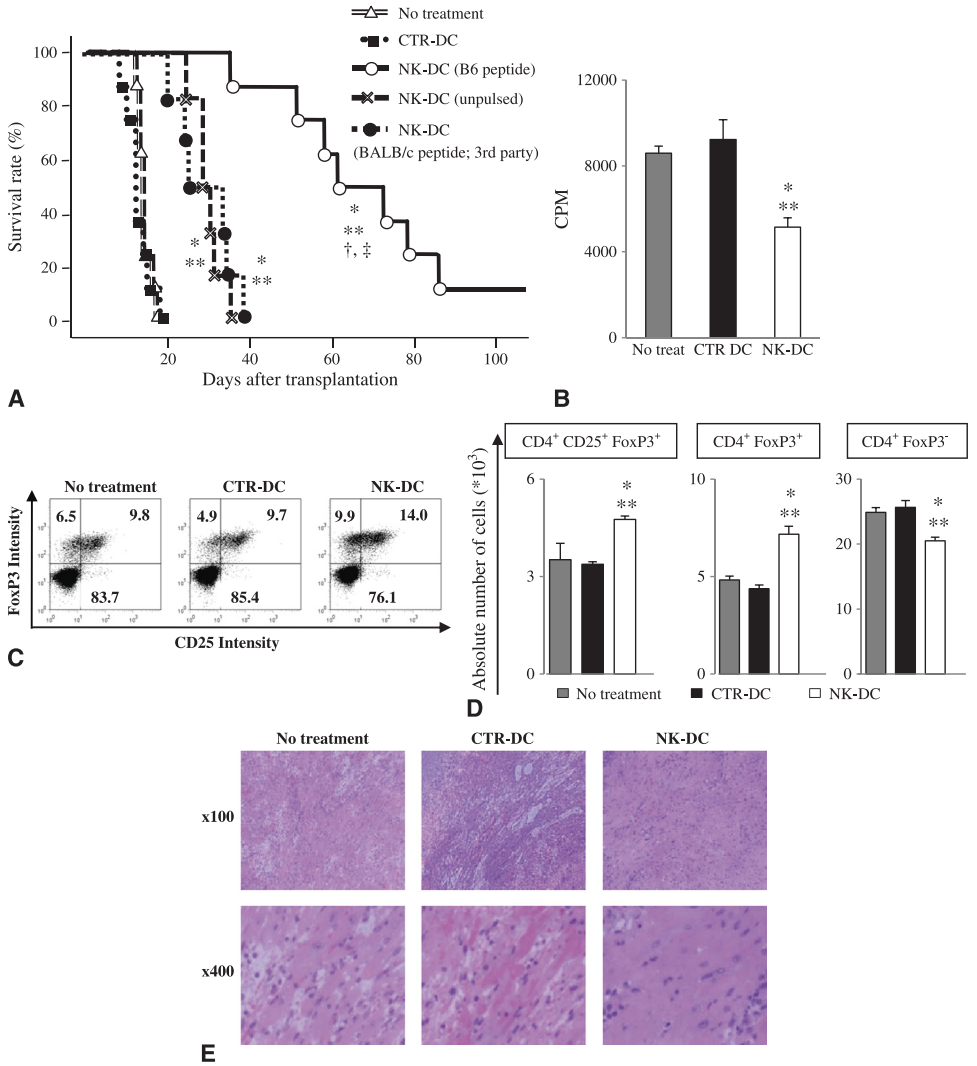

FIGURE 5. NK026680-treated dendritic cells (NK-DC) treatment markedly prolongs cardiac allograft survival and increases spleen regulatory T cells. A, The graft survival rate. Control DCs (CTR-DCs) or NK-DCs (3×10^6 cells) from C3H mice were injected intravenously into C3H mice. NK-DCs were pulsed with B6 (donor) or BALB/c (third party) lysates or without alloantigen. Seven days later, the injected C3H mice received heart allografts from B6 mice (n=6–8 in each group). *P<0.01 vs. untreated group. **P<0.01 vs. CTR-DC group. † P<0.01 vs. unpulsed NK-DC group. ‡ P<0.01 vs. BALB/c-pulsed NK-DC group. B–E, Recipient mice were sacrificed 14 days after heart transplantation, and the spleens and cardiac grafts were evaluated. B, ^{3}H-thymidine uptake of alloreactive lymphocyte proliferation. Splenocytes obtained from cardiac recipients were co-cultured with irradiated B6 splenocytes for 48 hr. Cells were pulsed with ^{3}H-thymidine 8 hr before cell harvest, and thymidine incorporation was counted with a β-counter. Each column represents the mean±SEM of four independent experiments. *P<0.05 vs. untreated group. **P<0.05 vs. CTR-DC group. C and D, $CD4^+CD25^+FoxP3^+$ T cells in the spleens of transplant recipients are measured by flow cytometric analysis. C, A representative histogram of four independent experiments is shown. D, The absolute number of $CD4^+CD25^+FoxP3^+$ T cells (*left*), $CD4^+FoxP3^+$ T cells (*middle*), and $CD4^+FoxP3^-$ T cells (*right*) are shown. Each column represents the mean±SEM of four independent experiments. *P<0.05 vs. untreated group. **P<0.05 vs. CTR-DC group. E, Histology of the cardiac grafts on day 14 after heart transplantation. Cardiac allografts were stained with hematoxylin-eosin (original magnification, ×100 or ×400). The representative photographs of four independent grafts are shown in the panel. CPM, counts per minute.

additional recipient mice were sacrificed, and the alloreactive lymphocyte response (Fig. 5B) and the $CD4^+CD25^+FoxP3^+$ T-cell population (Fig. 5C, D) were evaluated 14 days after HTx. Compared with untreated mice and those injected with CTR-DCs, splenocytes from mice treated with NK-DCs showed significantly reduced alloreactive lymphocyte proliferation (Fig. 5B). Meanwhile, the splenic $CD4^+CD25^+FoxP3^+$ T-cell population in the NK-DC group was significantly increased compared with that of the other groups (Fig. 5C). Such an increase was noted out to day 28 after transplantation (14.5%±1.3%). In addition, the absolute numbers of splenic $CD4^+FoxP3^+$ and $CD4^+CD25^+FoxP3^+$ T cells in NK-DC–injected mice significantly increased when compared with those in nontreated or CTR-DC–injected mice (Fig. 5D). Finally, the antirejection effect of NK-DCs was confirmed by histologic examination

(Fig. 5E). Cardiac grafts stained with hematoxylin-eosin illustrated heavy acute cellular rejection on day 14 in untreated or CTR-DC–treated mice, whereas the grafts in NK-DC–injected mice did not (Fig. 5E).

DISCUSSION

Our findings in vitro demonstrate that NK026680 conditioning has inhibitory effects on the phenotypical and functional maturation of DCs in response to TNF-α stimulation (Fig. 1). These results corroborate with the previous findings that NK026680 has inhibited the expression of CD80, CD86, and MHC class II and suppressed IL-12 production in human monocyte–derived DCs (*10*) and that it has impaired the differentiation of bone marrow–derived DCs by reducing the expression of CD86 and MHC class II in rat (*11*). In addition, NK-DCs inhibited alloantigen-driven T-cell proliferation (Fig. 2). These results agree with the premise that DCs that weakly express costimulatory molecules induce T-cell anergy when they present antigen to T cells (*19–21*). Although the mechanism of action on how NK026680 inhibits DC maturation and differentiation has not been elucidate in previous studies (*10*, *11*), our current findings indicate that these modulatory effects by ex vivo NK026680 conditioning are likely associated with the inhibition of the MAPK pathways including ERK, JNK, and p38 MAPK (Fig. 3). Each MAPK group is typically linked to a distinct role in the DC maturation process. The activation of the p38 MAPK pathway positively regulates phenotypic maturation, cytokine production, and allostimulatory capacity (the ability to induce alloreactive T cells) in response to TNF-α (*14–18*). JNK also upregulates the expression of MHC class II and costimulatory molecules, but it has little effect on IL-12 p40 and allostimulatory capacity (*16*). In contrast, ERK negatively regulates IL-12 production (*16–18*, *22–24*) and phenotypic maturation (*17*, *18*) and acts in an allostimulatory capacity (*15*, *18*, *23*). In this study, NK-DCs inhibited the phosphorylation of p38 MAPK, JNK, ERK, and their downstream substrates, c-Jun and c-Fos (not NF-κB). These findings partly agree with our previous study on T cells, which stated that NK026680 inhibits phosphorylation of p38 MAPK and subsequently suppresses nuclear activation of c-Jun and c-Fos (*13*), and may indicate a mechanism for the induction of immunomodulatory effects by NK026680 in NK-DCs. However, our study has not demonstrated whether NK026680 inhibits directly and specifically MAPK pathway. To clarify these issues, further studies are warranted.

We also have demonstrated that one of the immunoregulatory functions of NK-DCs is the induction of an increase in the Treg population. In our experiments, NK-DCs inhibited antigen-driven T-cell proliferation and induced IL-10 production through the indirect pathway when reacted with T cells in vitro (Fig. 2). Even in vivo, a single infusion of NK-DCs prevented alloreactive T-cell proliferation and promoted IL-10 production (Fig. 4). These findings seem to be causally involved in the elevation of Tr1 cell numbers. We also have shown that the injection of NK-DCs increased the $CD4^+CD25^+FoxP3^+$ T-cell population and inhibited alloreactive lymphocyte proliferation 14 days after HTx (Fig. 5). However, $CD4^+CD25^+FoxP3^+$ T cells in mice treated with NK-DCs were not increased 7 days after injection (data not shown). This may be explained by the hypothesis that it takes weeks for alloantigen-specific Tregs to develop and exert their suppressive function (*25–27*). The immunoregulatory effects induced by NK-DCs are considered to occur in two phases: (1) increased Tr1 cells contribute in the prevention of acute allograft rejection and (2) the consequent increase in $CD4^+CD25^+FoxP3^+$ T cells maintains the regulatory effects and prolongs allograft survival. Furthermore, our study indicated that NK026680-conditioned DCs upregulate IDO production when stimulated with TNF-α (Fig. 3), a finding also observed when DCs are modulated with LF15-0195 (*8*). IDO is an intracellular rate-limiting enzyme that catalyzes tryptophan degradation (*28*) and is considered an important enzyme for regulatory DCs (*29*). Recent data provide evidence that IDO-expressing DCs have the potential to convert naive $CD4^+$ T cells into Tregs (*30*) and activate mature Tregs (*31*). IDO up-regulation likely plays an important role in the regulatory functions of NK-DCs.

Several experiments have demonstrated the efficacy of an infusion of preconditioning host DCs in murine allogeneic heart transplant models. In a C57BL/10 ($H\text{-}2^b$) to C3H mouse heart transplant model, a single injection of host DCs conditioned with rapamycin (*4*) or modulated by an NF-κB oligodeoxyribonucleotide (*6*) led to a prolongation of allograft MST. As in these reports, a single treatment with NK-DCs facilitated long-term allograft survival but did not lead to the induction of tolerance in recipient mice. However, the approach that we presented followed by a short postoperative course of immunosuppressive therapies may potently induce extended, long-term allograft survival, like as the previous study on rapamycin-conditioned DCs (*5*). In addition, we believe that NK-DC therapy may be a useful adjunctive therapy for chronic rejection when used in conjunction with postoperative immunosuppressants because NK-DCs are recipient derived and inhibit alloantigen-driven T-cell proliferation through the indirect pathway, a pathway that plays a pivotal role in chronic rejection (*32*).

The characteristics of NK-DCs did not differ between the allopeptide-pulsed and allopeptide-unpulsed ones (Fig. 1). However, injection therapy of allopeptide-pulsed NK-DCs induced a potent immunomodulatory effect when compared with that of unpulsed NK-DCs (Figs. 4 and 5). These findings indicate that addition of alloantigen on DC conditioning has a little impact on ex vivo DC modulation itself but plays an important role in allogeneic T-cell responses to acquire immunomodulatory properties against alloantigen in vivo. Furthermore, our findings with NK-DCs pulsed with third-party antigens (Fig. 5A) suggested the importance of antigen specificity on the immunomodulatory effect of the NK-DC therapy.

In conclusion, infusion therapy with DCs modulated by ex vivo NK026680 conditioning has great potential as a treatment modality for the prevention of allograft rejection by enhancing immunoregulatory function.

MATERIALS AND METHODS

Animals

Male C57BL/6 (B6, $H\text{-}2^b$ haplotype), BALB/c ($H\text{-}2^d$), and C3H/HeJ (C3H, $H\text{-}2^k$) mice were purchased from SLC, Inc. (Shizuoka, Japan). Animals were maintained in a pathogen-free facility and used at 9 to 12 weeks

of age. All experiments were approved by the Institutional Animal Care Committee and conducted under the Guidelines for the Care and Use of Laboratory Animals of Hokkaido University.

Reagents and Abs

NK026680 was provided by Nippon Kayaku Co., Ltd. The powder form of NK026680 was dissolved in 0.05% dimethyl sulfoxide (Sigma-Aldrich, St. Louis, MO). Murine recombinant granulocyte-macrophage colony-stimulating factor, IL-4, and TNF-α were purchased from PeproTech (Rocky Hill, NJ). Antimouse fluorescein isothiocyanate–, phycoerythrin-, and peridinin-chlorophyll-protein–conjugated and biotinylated monoclonal Abs (mAbs)—against CD4 (RM4-5), CD25 (7D4), CD80 (16-10A1), CD86 (GL1), IL-10 (JES5-2A5), IFN-γ (XMG1.2), I-A^b (AF6-120.1), and I-A^d (39-10-8)—and streptavidin–peridinin chlorophyll protein were obtained from BD Pharmingen (San Diego, CA). An antimouse FoxP3 mAb and the FoxP3 staining set were purchased from eBioscience (San Diego, CA). Antimouse phycoerythrin-conjugated CD40 mAb (3/23), antimouse purified IDO, and anti–β-actin Abs were obtained from BioLegend (San Diego, CA). Abs against phospho-p38 MAPK (Thr180/Tyr182), p38 MAPK, phospho-p44/p42 MAPK (ERK, Thr202/Tyr204), p44/p42 MAPK, phospho-stress–activated protein kinase/JNK, stress–activated protein kinase/JNK, and horseradish peroxidase–conjugated antirabbit IgG were purchased from Cell Signaling Technology (Beverly, MA).

DC Cultures

Murine bone marrow–derived DCs were generated as previously described (*33*), with minor modifications. Bone marrow cells were prepared from the femoral and tibial bone marrow of 10-week-old B6 or C3H mice. After lysis of the erythrocytes, MHC class II^+, $CD45R^+$ (B220), $CD4^+$, and $CD8^+$ cells were removed by the addition of mAbs against these surface markers (1E4, RA3-6B2, GK1.5, and 53.4.9, respectively) and rabbit complement (Cedarlane, Burlington, Canada). The cells were cultured in complete medium (CM), RPMI-1640 medium that contained 5% fetal calf serum, 20-ng/mL granulocyte-macrophage colony-stimulating factor, 20-ng/mL IL-4, 50-μM 2-mercaptoethanol, 100-IU/mL penicillin, and 100-μg/mL streptomycin, at a density of 1×10^6 cells per mL per well (24-well plate). On day 2, the medium was gently exchanged with fresh CM. On day 4, nonadherent granulocytes were removed without the dislodging of clusters of developing DCs, and fresh CM was added. On day 6, free-floating and loosely adherent cells were collected (>90% CD11c) and cultured in CM at a density of 1×10^6 cells per mL per well in 24-well untreated plates. For NK-DC preparation, 50-ng/mL NK026680 was added on days 2 and 4. On day 6, 250-ng/mL NK026680 and allogenic mouse splenocyte donor lysates that were derived by the sonicating, freezing, and thawing of cells were added for the last 24 hr of DC culture. The cells were detached with 3-mM ethylenediaminetetraacetic acid for 5 min at 37°C and used for analysis or treatment.

DC Stimulation

After 7 days of culture, untreated DCs and NK-DCs were collected, washed in phosphate-buffered saline, and recultured with TNF-α (20 ng/mL) for 24 hr. Then, cells were collected and analyzed.

Surface Marker Staining

Cells were incubated for 15 min with antimouse CD16/CD32 mAb (2.4G2; BD Pharmingen) to prevent binding to FcRII/III and then stained with fluorescein isothiocyanate–, phycoerythrin-, or biotin-conjugated mAbs and streptavidin–peridinin chlorophyll protein.

Intracellular Staining

For the staining of IFN-γ and IL-10, splenocytes obtained from DC recipients were stimulated in medium with donor splenocytes for 6 hr in the presence of 20-ng/mL phorbol 12-myristate 13-acetate and 1-μg/mL ionomycin. Brefeldin A (5 μg/mL; Sigma-Aldrich) was added for the last 4 hr of stimulation. After surface marker staining, cells were fixed in 2% paraformaldehyde, permeabilized by incubation with 0.5% saponin, and then stained with IFN-γ and IL-10 mAb for 30 min at room temperature. Intracellular staining for FoxP3 was performed according to the manufacturer's instructions (eBioscience).

Flow Cytometric Analysis

Flow cytometric analyses were performed on a FACSCalibur flow cytometer with CellQuest software (BD Biosciences, San Jose, CA). For each analysis, 10,000 DCs or 30,000 T cells were acquired.

Cytokine Measurement

IL-12 in the supernatants of DC cultures and IL-10 in MLR supernatants were subjected to quantification at the protein level by enzyme-linked immunosorbent assay using OptEIA Sets (BD Pharmingen). Measurements were performed in duplicate. The IL-10 and IFN-γ production of splenocytes obtained from transplant recipients were examined by enzyme-linked immunospot assay as previously described (*34*).

Mixed Leukocyte Reaction

Splenic B6 T cells were used as responder cells after they were enriched to greater than 90% purity by filtration of the cell suspension through a nylon-wool mesh column (R&D Systems, Minneapolis, MN). After TNF-α stimulation, untreated and pretreated B6 DCs (2×10^3–2×10^4 cells per well) were irradiated (30 Gy, cesium-137) and then co-cultured with the responder cells (2×10^5 cells per well) in 96-well round bottom plates at 37°C and 5% carbon dioxide plus air for 72 hr in RPMI-1640 media that contained 10% fetal calf serum, 50-μM 2-mercaptoethanol, 100-IU/mL penicillin, and 100-μg/mL streptomycin. Cells were pulsed with ^{3}H-thymidine (1 μCi per well) 8 hr before the analysis of thymidine incorporation with a β-counter (Perkin Elmer, Boston, MA).

Immunoblotting

Cells were lysed with radioimmunoprecipitation assay buffer (50-mM Tris HCl, 150-mM NaCl, 1% NP40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate, 1-mM ethylenediaminetetraacetic acid, and a protease inhibitor cocktail). Protein (30 μg) was resolved by sodium dodecyl sulfate–polyacrylamide gel electrophoresis on a 10% gel and then transferred to nitrocellulose (Invitrogen, Carlsbad, CA). The membrane was blocked with 5% dry milk and 0.1% Tween (Sigma-Aldrich) in phosphate-buffered saline, incubated with primary Ab, and then incubated with horseradish peroxidase–conjugated secondary Ab. Bands were detected by enhanced chemiluminescence.

Nuclear Protein Extraction and Quantification of Transcription Factor Activity

The nuclear proteins of 1×10^7 DCs were extracted with a nuclear extraction kit according to the manufacturer's instructions (Active Motif, Carlsbad, CA). The levels of NF-κB (Rel-A, p50, p52, and Rel-B) and activator protein 1 (c-Jun and c-Fos) DNA binding activity in the nuclear extracts were examined with the TransAM Kit (Active Motif).

Cardiac Transplantation

Heterotopic cardiac transplantations with B6 mice as the donors and C3H mice as the recipients were performed as previously described (*35*). The beating of the cardiac graft was monitored by daily palpation through the abdominal wall of the recipient mouse. Rejection was defined as the time of graft beating cessation as confirmed by direct inspection and histologic examination.

Graft Histologic Analysis

Cardiac graft tissue was excised at the time of animal death or sacrifice. Tissues were fixed in 10% formalin, embedded in paraffin, and stained with hematoxylin-eosin.

Statistical Analysis

Graft survival time was plotted with the use of a method based on the Kaplan-Meier estimator, and a log-rank test was applied for comparison between the groups. Other results were expressed as mean±SEM. The Student *t* test was used for the statistical analysis of paired comparisons, whereas analysis of variance with the Tukey-Kramer post hoc test was used for multiple comparisons. $P<0.05$ was considered statistically significant.

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