

Tolerogenic Donor-Derived Dendritic Cells Risk Sensitization In Vivo owing to Processing and Presentation by Recipient APCs

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Modification of allogeneic dendritic cells (DCs) through drug treatment results in DCs with in vitro hallmarks of tolerogenicity. Despite these observations, using murine MHC-mismatched skin and heart transplant models, donor-derived drug-modified DCs not only failed to induce tolerance but also accelerated graft rejection. The latter was inhibited by injecting the recipient with anti-CD8 Ab, which removed both CD8⁺ T cells and CD8⁺ DCs. The discrepancy between in vitro and in vivo data could be explained, partly, by the presentation of drug-modified donor DC MHC alloantigens by recipient APCs and activation of recipient T cells with indirect allospecificity, leading to the induction of alloantibodies. Furthermore, allogeneic MHC molecules expressed by drug-treated DCs were rapidly processed and presented in peptide form by recipient APCs in vivo within hours of DC injection. Using TCR-transgenic T cells, Ag presentation of injected OVA-pulsed DCs was detectable for ≤ 3 d, whereas indirect presentation of MHC alloantigen by recipient APCs led to activation of T cells within 14 h and was partially inhibited by reducing the numbers of CD8⁺ DCs in vivo. In support of this observation when mice lacking CD8⁺ DCs were pretreated with drug-modified DCs prior to transplantation, skin graft rejection kinetics were similar to those in non-DC-treated controls. Of interest, when the same mice were treated with anti-CD40L blockade plus drug-modified DCs, skin graft survival was prolonged, suggesting endogenous DCs were responsible for T cell priming. Altogether, these findings highlight the risks and limitations of negative vaccination using alloantigen-bearing “tolerogenic” DCs. *The Journal of Immunology*, 2013, 190: 4848–4860.

In transplantation, immunosuppressive drugs have reduced the incidence of acute rejection, but the average half life of a cadaveric kidney transplant remains about 10 years owing to chronic organ rejection (reviewed in Ref. 1). Transplantation research therefore aims to define which strategy can be employed to induce immunosuppressant-free, indefinite allograft survival, including the injection of ex vivo expanded regulatory T cells (Tregs) (reviewed in Refs. 2, 3) and the use of in vitro modified tolerogenic dendritic cells (DCs) (reviewed in Ref. 4).

Several lines of evidence suggest that immature DCs or in vitro modified DCs may be useful tools in promoting tolerance. Different agents have been used to interfere with DC differentiation, migration, Ag uptake and processing, and DC activation (reviewed in Refs. 5, 6). For example, in vitro treatment of murine-derived DCs with either dexamethasone (Dex) or 1,25-dihydroxy vitamin D3 (D3) has been shown to impair DC phenotype and function (7). D3 treatment of DCs also inhibits IL-12 production by downregulation of NF- κ B signaling (8). In vitro treatment of bone marrow (BM)-derived DCs with Dex has been shown to inhibit the proliferation of alloreactive T cells, preventing a Th1-type skewing of responses (9, 10) and promoting the generation of IL-10-producing regulatory cells (11, 12). Furthermore, Dex and D3 have been demonstrated to have synergistic effects (13).

In humans, the same drugs have shown a similar effect in vitro, with Dex-DCs secreting higher levels of IL-10 and having a reduced allostimulatory capacity for both naive and memory T cells (9, 14–18). In addition, downregulation of NF- κ B activation after treatment with D3 has been shown (19, 20). Human monocyte-derived DCs treated with a combination of Dex and D3 produce IL-10, upregulate ILT-4, and become more resistant to LPS maturation than either drug treatment alone (21). Furthermore, they elicit lower proliferative responses from CD4⁺CD25[−] T cells and can induce Tregs (21).

In vivo adoptive transfer of murine Dex-treated BM-DCs results in prolonged allograft survival in some strain combinations (10, 22). Recently, prolongation of heart allograft survival has been shown using D3-modified DCs (23). Moreover, the combination of Dex and D3 treatment of DCs reduces the development of colitis in an adoptive transfer model (24). Other studies using tolerogenic DCs have demonstrated a range of outcomes, for example, pro-

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Received for publication March 19, 2012. Accepted for publication February 25, 2013.

This work was supported by grants from the British Heart Foundation, European Union Resetting The Immune System To Establish Tolerance (RISET), and Medical Research Council. The authors acknowledge financial support from the Department of Health via the National Institute for Health Research comprehensive Biomedical Research Centre award to Guy's and St Thomas' National Health Service Foundation Trust in partnership with King's College London and King's College Hospital National Health Service Foundation Trust. The authors acknowledge the support of the Medical Research Council Centre for Transplantation.

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The online version of this article contains supplemental material.

Abbreviations used in this article: ATCC, American Type Culture Collection; BM, bone marrow; D3, 1,25-dihydroxy vitamin D3; DC, dendritic cell; Dex, dexamethasone; LN, lymph node; MST, mean survival time; Treg, regulatory T cell.

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longation of allograft survival from just a few days to > 100 d, depending on the animal model used (22, 25–30). Most importantly, additional therapy such as CTLA4-Ig or anti-CD40L Ab (MR1) combined with tolerogenic DC treatment greatly improves graft survival outcomes (31, 32).

In a rat kidney allograft model, we have been successful in achieving tolerance through the adoptive transfer of Dex-treated DCs derived from F1 rats (allowing concurrent presentation of allogeneic MHC molecules via both direct and indirect pathways) combined with low-dose CTLA4-Ig and short-term cyclosporine. This treatment induced indefinite allograft survival mediated through Treg expansion by Dex-DC-dependent IL-2 production (33). In contrast with our data, in the study of DePaz et al. (26), also in the rat system, the injection of immature DCs with antilymphocyte serum did not increase the effect observed with antilymphocyte serum alone, further demonstrating the variability of the effect of tolerogenic DC treatment.

From the aforementioned *in vivo* data, it appears that the success of negative vaccination with DCs in achieving transplantation tolerance may depend on many factors, such as the species used, the strain combination, the time of injection, the capacity of DCs to migrate to specific sites, and the additional therapies applied. Another relevant parameter for the *in vivo* effect of DCs is the way that DCs are generated *in vitro*. Yamano et al. (34) demonstrated that whereas DCs generated from BM in the presence of Flt3L induced prolonged acceptance of skin allograft in the presence of MR1, CTLA4-Ig, and anti-NK1.1 Ab, DCs generated *in vitro* with GM-CSF did not affect the time of skin rejection. In contrast, Divito et al. (23) have demonstrated that donor BM-derived D3-DCs or immature DCs internalized as apoptotic cells and presented by host APCs can delay heart transplant rejection. Evidence that MHC molecules, expressed by intact apoptotic cells, are rapidly processed and presented as peptides *in vivo* following DC transfer has been previously shown (35). However, the consequence for the immune response of the recipient was either tolerance (36) or priming (36, 37). In support of the latter statement, in a rat model of islet transplantation, recently de Kort et al. (38) have shown that injection of Dex-treated DCs induced Ab-mediated accelerated graft rejection.

We demonstrate in this study that alloantigen-expressing tolerogenic BM-derived DCs, shown to induce T cell hyporesponsiveness and the expansion of Foxp3⁺ T cells *in vitro*, once injected *in vivo* die rapidly, resulting in alloantigen presentation by recipient APCs, including recipient CD8⁺ DCs. This result leads to sensitization and activation of recipient T cells through the indirect pathway, leading to graft rejection. However, prolongation of graft survival was achieved when tolerogenic DCs were injected in the presence of anti-CD40L Ab in the absence of CD8⁺ DCs.

In summary, it appears that donor-derived tolerogenic DCs are linked to an increased risk of priming the recipient immune system to the donor graft by processing and presentation of donor Ags by recipient DCs (CD8⁺ DCs). In addition, the data presented in this article underline the caution that should be taken, when planning future clinical interventions, in extrapolating *in vitro* data that do not necessarily reflect the *in vivo* situation.

Materials and Methods

Mice

CBA/Ca (H-2^k), C57BL/6 [(B6) H-2^b], BALB/c (H-2^d), C57BL/6 × BALB/c (B6 × B/c) F1 (H-2^{b×d}), and C57BL/6 × DBA-2 (B6D2F1) F1 (H-2^{b×d}) mice were purchased from Harlan Olac (Bicester, U.K.). DO11.10 Rag^{-/-}, TCR75 Rag^{-/-}, and B6K^d mice were kind gifts from Pat Bucy (University of Alabama, Birmingham, AL); Baft3^{-/-} mice were a kind gift from Kenneth Murphy (Washington University School of Medicine, St. Louis, MO)

(39); and H-2K^{bm1} (K^{bm1}) mice were a kind gift from Dr. Sandra Diebold (King's College London, London, U.K.). All experimental procedures were performed on sex-matched mice between 6 and 12 wk of age, in accordance with the Home Office Animals Scientific Procedures Act (1986).

Cell culture medium

Cell cultures were performed in RPMI 1640 medium (Sigma-Aldrich, Poole, U.K.) supplemented with 100 IU/ml penicillin, 100 µg/ml streptomycin, 2 mM L-glutamine, 0.01M HEPES, 50 µM 2-ME (Invitrogen, Paisley, U.K.), and 10% heat-inactivated FCS (SERAQ, Sussex, U.K.). Cells were maintained at 37°C in a humidified atmosphere with 5% CO₂.

Purification of CD4⁺ T cells

A single-cell suspension was obtained by passing spleens or pooled lymph nodes (LNs) through a 70-µm cell strainer (BD Pharmingen, San Jose, CA). Erythrocytes were lysed using ACK buffer (0.15 M NH₄Cl/1 mM KHCO₃/0.1 mM Na₂-EDTA), and CD4⁺ cells were isolated by negative selection using a CD4 Dynabeads isolation kit (Dyna, Wirral, U.K.). The purity of the selected CD4⁺ population was $> 90\%$, as assessed by flow cytometry.

Generation of DCs from BM

DCs were generated as previously described (40), with some modifications. BM cells were passed through a 70-µm cell strainer to obtain a single-cell suspension. Erythrocytes were lysed as above. BM cells were incubated for 30 min, at 4°C, with supernatants from the following hybridoma cultures: YTS 191 [anti-CD4; American Type Culture Collection (ATCC), Manassas, VA], M5/114 (anti-Class II; ATCC), RA3-3A1 (anti-B220; ATCC), and YTS 169 (anti-CD8; ATCC). The cells were washed twice in RPMI, then incubated with pan anti-rat Ig Dynabeads for 30 min (4°C), followed by magnetic separation. After washing, the BM cells were seeded in a 24-well plate at 1×10^6 cells per well in 1.5 ml complete medium supplemented with 20 ng/ml murine recombinant GM-CSF (kind gift from GlaxoSmithKline R&D, Stevenage, U.K.). On days 3 and 5 of culture, plates were swirled gently, the medium containing small nonadherent cells was removed, and fresh GM-CSF-containing complete medium was added. To some wells Dex (10^{-6} M) and D3 (10^{-7} M) (Sigma-Aldrich) were added. Three different stimuli were used independently to induce DC maturation: 100 ng/ml LPS (*Escherichia coli* 026:B6; Sigma-Aldrich), 20 ng/ml TNF-α (First Link, Wolverhampton, U.K.), or 50 µg/ml α-CD40 Ab (FGK45 hybridoma, kindly provided by A. Rolink, University of Basel, Basel, Switzerland). Maturation stimuli were added to cell cultures on day 6 for the final 24 h. Purity of DCs was $> 90\%$, as measured by CD11c surface expression on flow cytometry analysis (data not shown).

Flow cytometry

Fluorochrome-conjugated (FITC, PE, Cy-Chrome, allophycocyanin) mAbs against the following mouse cell-surface Ags—CD80 and CD86, MHC class I and II, CD40, CD11c, CD8, CD4, Thy1.1, KJ126, CD90.1, CD25, and CD69—were purchased from eBioscience (San Diego, CA), along with their relevant isotype controls. For flow cytometry analysis, 1×10^5 cells were labeled with fluorochrome-conjugated mAbs for 20 min (4°C), washed twice in FACS Buffer (PBS/2% FCS/0.1% EDTA), and analyzed on a FACSCalibur, using CellQuest software (Becton Dickinson, Mountain View, CA). Foxp3 staining was performed using a murine Foxp3 kit according to the manufacturer's protocol (eBioscience). Subsequent analysis was accomplished with FlowJo software (TreeStar, Ashland, OR).

Cytokine production

Day 6 immature and DexD3-BALB/c DCs were cocultured with a CD40L-expressing murine L-cell line for 18 h, and supernatants were harvested. Control supernatants were from nonstimulated cells (20). Cytokine production was assayed on 50 µl culture supernatant, with a double Ab sandwich ELISA using purified mAbs paired with a biotinylated mAb. Murine IL-12p40/p70 and IL-10 capture and biotinylated Abs were obtained from BD Pharmingen.

Proliferation assays and Foxp3 expression

Sequential dilutions of irradiated DCs (either drug treated or not) were added to CD4⁺ T cells (2.5×10^4) in complete media. The assay was carried out in 96-well round-bottom plates, with a total volume per well of 250 µl. CD4⁺ T cells alone were used as controls. On day 2 of culture, cells were pulsed with 1 µCi per well ³H-thymidine (Amersham Pharmacia, Little Chalfont, U.K.). Proliferation was measured by ³H-thymidine incorporation after 20 h by liquid scintillation counting using a β-plate counter. DCs treated with or without DexD3 in the presence or absence of

LPS were cocultured with CFSE-labeled ($1 \mu\text{M}/10^7$ cells; Invitrogen) CD4^+ T cells or CD4^+ T cells depleted of CD25^+ cells ($\text{CD4}^+\text{CD25}^-$) derived from B6 mice, at a ratio of 1:10 for 5 d. IL-2 (200 U/ml) was added at the beginning of the coculture. On day 5, T cells were harvested and analyzed for expression of CD4, CD25, and Foxp3 by flow cytometry, and also analyzed for CFSE dilution by flow cytometry.

In vitro and in vivo rechallenge experiments

In vitro, MLRs, comprising BALB/c DCs ($\pm$ drug treatment) and CD4^+ T cells isolated from CBA/Ca mice, were performed in 48-well plates. On day 5, the T cells were recovered and were rested for 2 d. These cells were then recultured with irradiated allogeneic BALB/c DC stimulators. Proliferation was measured after 3 d by ^3H -thymidine incorporation.

In vivo, B6 mice were challenged with 2×10^6 DexD3 + LPS-BALB/c DCs by i.v. tail vein injection. At 10 d, CD4^+ T cells were isolated from the spleen of recipient mice and rechallenged in vitro with DCs derived from BALB/c, CBA/Ca, or B6K^d mice. Proliferation was measured on days 3, 5, and 7 of culture, using ^3H -thymidine incorporation.

Skin and heart transplantation

Skin grafting was performed according to the technique described by Billingham et al. (41), with some modifications. Full-thickness donor tail skin ($0.5\text{--}1 \times 0.5\text{--}1$ cm) was grafted on the right lateral flank of recipients. The graft site was covered by an Elastoplast plaster that was removed on day 7. The grafts were observed daily and considered rejected when no viable skin remained. Intra-abdominal heterotopic heart transplantation (either B6D2F1 or BALB/c grafts) was performed in B6 mice, as previously described (42, 43). Heart allograft survival was assessed by direct abdominal palpation, where rejection was defined by complete cessation of cardiac impulses. To test the effect of DCs on graft survival, recipient B6 mice were injected i.v. with different types of donor-derived (B6K^d) DCs or K^d pulsed recipient DCs 6 d prior to the graft. Mice were either not treated or treated with 250 μg anti-CD8 Ab on day -1 and day 1 of the transplantation via i.p. injection.

In some experiments, recipient animals were treated with 20 μg CTLA4-Ig (abatacept; kind gift from Dr. Wendy Rowan, GlaxoSmithKline, Stevenage, U.K.) at day -5 pretransplantation, via i.p. injection, or received 500 μg MR1 (BioXCell, West Lebanon, NH) on days -7 , -4 , and 0.

Analysis of DC processing in vivo

B6, $\text{Batf3}^{-/-}$, or (B6 $\times$ B/c) F1 mice were injected with 2×10^6 BALB/c DCs, either drug treated or not (day 0). Some DCs were pulsed with 5 $\mu\text{g}/\text{ml}$ OVA peptide for 2 h prior to injection. At various time points, mice received 2×10^6 CFSE-labeled CD4^+ T cells purified from TCR75 $\text{Rag}^{-/-}$ or DO11.10 $\text{Rag}^{-/-}$ mice via i.v. injection. Some mice received anti-CD8 treatment or polyinosinic-polycytidylic acid (dsRNA) to decrease the number of recipient DCs or inhibit their function. Spleen and LN cells were harvested, and in some experiments CD4^+ T cells were isolated (as above). Thy1.1 and KJ126 Abs were used to distinguish CD4^+ T cells from TCR75 $\text{Rag}^{-/-}$ and DO11.10 $\text{Rag}^{-/-}$ mice, respectively. CD69 expression was measured by flow cytometry using a specific Ab.

In some experiments, animals were treated with 20 μg CTLA4-Ig via i.p. injection 1 d after DC injection.

Alloantibody detection

B6K^d DCs (2×10^6) were injected i.v. into naive mice, and sera were collected to measure alloantibody production after 7, 14, 21, and 28 d. B6 splenocytes (used as negative control) and BALB/c (K^d) splenocytes (as target cells) were incubated with PBS/2% BSA/5% goat serum containing rat Abs against CD16/32 (0.5 $\mu\text{g}/10^6$ cells; BD Biosciences, San Jose, CA) for 20 min on ice. Hamster anti-mouse CD3-PE (BD Biosciences) was then added directly to the cells to enable subsequent gating on CD3^+ T cells. After further incubation for 20 min on ice, cells were washed twice in staining buffer. Serum was added (final dilution 1/10) to appropriate wells and incubated as before. Cells were washed twice before the addition of goat anti-mouse IgG FITC (1/200; Sigma-Aldrich, Dorset, U.K.) for 20 min on ice. Cells were washed twice and analyzed on a BD Biosciences FACSCalibur running CellQuest software (BD Biosciences).

MTT cell survival assays

DCs (1×10^5) were added to each well of a 96-well plate in 250 μl complete media. Each DC type was tested in triplicate. MTT assay was performed following the manufacturer's instructions (Invitrogen) after 24 and 48 h at 37°C and 5% CO_2 . Statistical comparisons for MTT experiments were performed using paired Student *t* tests.

Statistical analysis

Statistical comparisons for experiments assessing in vitro proliferation were performed using unpaired two-tailed Student *t* tests. Mean survival time (MST) of skin and heart allografts was assessed by log rank test. Data shown are mean $\pm$ SD or SE (SEM), as indicated. Statistical significance was expressed as follows: **p* < 0.05, ***p* < 0.01, ****p* < 0.001.

Results

DexD3-DCs have an immature phenotype, are resistant to maturation, and have an impaired capacity to stimulate Ag-specific T cells in vitro

The doses of 10^{-6} M Dex and 10^{-7} M D3 were used in combination to generate tolerogenic DCs, from BM, as previously published (21). As shown in Fig. 1A, DexD3-DCs had an immature phenotype; expressed lower levels of CD40, CD80, and CD86, compared with untreated immature DCs; and were resistant to maturation, as no significant increase in maturation markers was observed after LPS, TNF- α , or anti-CD40 Ab treatments for most of the markers.

Immature DCs and DexD3-DCs were stimulated with CD40L-expressing murine L-cells for 18 h, after which supernatants were collected and tested for the presence of IL-12 (p40/p70) and IL-10. As shown in Fig. 1B, IL-12 (*left panel*) production by DexD3-DCs in response to CD40 ligation was decreased compared with that in untreated DCs (mean $\pm$ SD IL-12 production by unstimulated DCs, 199.6 ± 159.3 , versus CD40L activation, 1716 ± 1298 ; and unstimulated DexD3-DCs, 43.7 ± 75.8 , versus CD40L activation, 625.6 ± 759.6 pg/ml), whereas IL-10 levels (*middle panel*) were very similar in the two groups of DCs (mean $\pm$ SD IL-10 production by unstimulated DCs, 0.0 ± 0.0 , versus CD40L activation, 211.7 ± 102.4 ; and unstimulated DexD3-DCs, 12.1 ± 40.1 , versus CD40L activation, 214.9 ± 108.9 pg/ml). Expressing the production of both IL-12 and IL-10 as a ratio showed that drug-treated DCs, compared with untreated DCs, had an IL-10-skewed cytokine response (*right panel*).

The "tolerogenic" nature of drug-modified DCs was further analyzed in an MLR. Allogeneic CD4^+ T cells from B6 mice proliferated following coculture with both immature and LPS-matured BALB/c DCs, the latter being the more potent stimulus. In comparison, coculture with DexD3-DCs or LPS-treated DexD3-DCs (DexD3 + LPS-DCs) resulted in reduced T cell proliferation (Fig. 1C). Indeed, DexD3 + LPS-DCs induced the same level of proliferation as immature non-drug-treated DCs, confirming their attenuated stimulatory function in vitro. The reduced ability to stimulate T cells was further confirmed using T cells derived from TCR transgenic mice, TCR75 $\text{Rag}^{-/-}$, specific for K^d and restricted by H2^b (Fig. 1D).

To rule out the possibility that the lower T cell proliferation induced by drug-treated DCs compared with mature DCs was due to the reduced viability of DCs, an MTT cell death assay was performed. As shown in Supplemental Fig. 1, over 48 h of culture, immature DexD3-DCs and DexD3 + LPS-DCs had an equivalent or improved cell viability compared with mature DCs. Taken together, these data confirm that the reduced stimulatory capacity of drug-modified DCs in vitro was not due to loss of cell viability.

CD4⁺ T cells cocultured with allogeneic DexD3-DCs in vitro are hyporesponsive to alloantigen rechallenge and Foxp3⁺ T cells are expanded

Previously, it has been shown by us, and others, that drug-treated DCs induce hyporesponsiveness in vitro (14, 18, 20, 21, 44, 45). To test whether DexD3-DCs can induce T cell anergy, immature, LPS-matured DexD3-DCs, and DexD3 + LPS-BALB/c DCs were cocultured for 6 d with allogeneic CD4^+ T cells. These T cells were then purified from the primary cultures and restimulated with

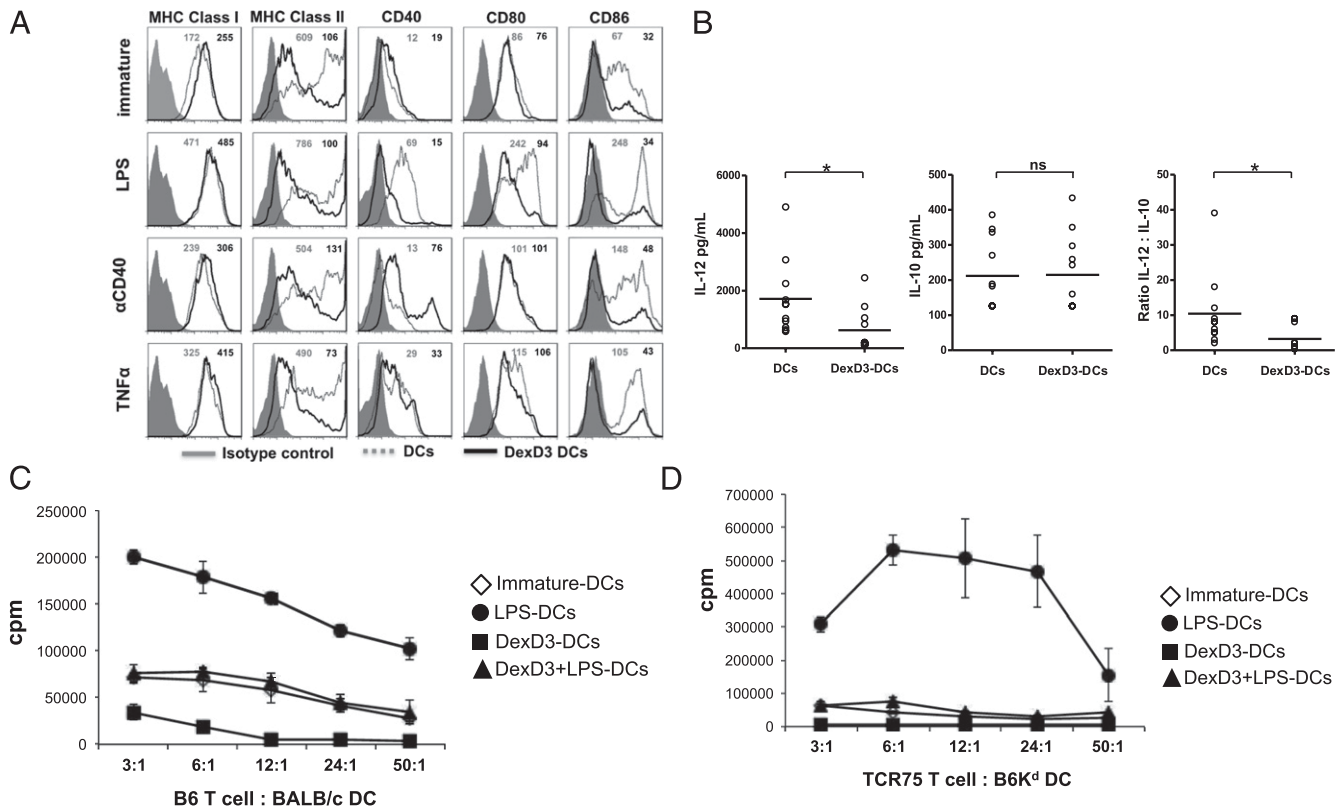


FIGURE 1. DexD3-DCs are refractory to maturation and are inefficient APCs. BALB/c-DCs were grown in the presence or absence of Dex and D3 (DexD3-DCs) for 7 d. LPS, TNF- α , or anti-CD40 Ab were added for the last 24 h of culture. Control drug-treated DCs received no maturation stimuli. (A) DCs were stained for the expression of MHC class I, class II, CD40, CD80, and CD86 molecules, using specific Abs and analyzed by flow cytometry. Isotype controls are shown as gray histograms. Untreated DexD3-treated DCs are shown as gray dashed lines, whereas DexD3-DCs are shown as solid black lines. The mean fluorescence intensity of both untreated (gray) and treated (black) is shown in the top right-hand side of each panel. One representative experiment of 13 (LPS) and 3 (TNF- α and anti-CD40 Ab) is shown. (B) Immature and DexD3-DCs were cocultured for 18 h, alone or with a CD40L-expressing cell line; supernatants from these cocultures were used to detect production of IL-10 and IL-12p40/p70 by ELISA. * $p < 0.05$. (C) DCs derived from BALB/c, treated or not with DexD3 and LPS [LPS-matured DCs (●), immature DCs (◇), DexD3 + LPS-DCs (▲), and DexD3-DCs (■)] were cocultured with 1×10^4 CD4⁺ T cells isolated from B6 mice at different ratios. T cell proliferation was measured after 16 h following addition of ³H-thymidine for the last 16 h of culture. Proliferation is expressed as cpm $\pm$ SD. One representative experiment is shown of three performed. (D) DCs derived from B6K^d mice, treated or not with DexD3 and LPS [LPS-matured DCs (●), immature DCs (◇), DexD3 + LPS-DCs (▲), and DexD3-DCs (■)] were cocultured with 1×10^4 CD4⁺ T cells isolated from TCR75 Rag^{-/-} mice at different ratios. T cell proliferation was measured after 72 h following addition of ³H-thymidine for the last 16 h of culture. Proliferation is expressed as cpm $\pm$ SD. One representative experiment is shown of three performed.

untreated BALB/c-DCs. As shown in Fig. 2A, CD4⁺ T cells isolated from a primary coculture with allogeneic immature and mature DexD3-DCs were hyporesponsive to rechallenge with BALB/c DCs. A similar response was obtained with T cells cocultured with immature DCs. In contrast, CD4⁺ T cells isolated following coculture with LPS-matured BALB/c DCs were able to respond to a second challenge with allogeneic DCs.

To address whether the hyporesponsive state of T cells cultured with DexD3-DCs was accompanied by an increase in Foxp3⁺ T cells, compared with T cells cocultured with mature DCs, the percentage of Foxp3⁺ T cells was analyzed after 6 d of coculture. As shown in Fig. 2B, immature, DexD3-DCs, and DexD3 + LPS-DCs were all able to generate a significantly higher percentage of Foxp3⁺ T cells than were mature DCs following coculture in vitro. The highest percentage of Foxp3⁺ T cells was observed when T cells were cocultured with DexD3-DCs. To investigate whether the increase in Foxp3⁺ T cells was due to an expansion of CD4⁺CD25⁺ Tregs or de novo generation of Tregs, CD4⁺CD25⁻ T cells were cocultured with each preparation of DCs. As shown in Fig. 2C, the major contribution to Foxp3⁺ T cell generation was due to an expansion of naturally occurring Tregs and not due to the de novo induction of these cells. Altogether, these data demonstrate that DexD3-DCs induce T cell unrespon-

siveness in vitro that is accompanied by the expansion of Foxp3-expressing putative Tregs.

Pretreatment of recipient mice with tolerogenic allogeneic donor DCs accelerates skin allograft rejection; this effect is abolished by anti-CD8 Ab treatment

Once the "tolerogenic" potential of DexD3-DCs was demonstrated in vitro, the capacity of these cells to prolong skin allograft survival in vivo was investigated. B6 recipient mice received 2×10^6 donor DCs (from B6K^d mice) 6 d prior to transplantation. The i.v. route of injection was selected because it has been shown to be more effective at delivering DCs to the spleen than i.p. inoculation and has previously been used to induce tolerance in vivo (46). Donor-derived DexD3-DCs and DexD3 + LPS-DCs were used in these experiments; the latter condition was used because TLR activation in combination with drug treatment has been shown to induce IL-10 production and migratory capacity to draining LNs (9, 47). Control mice received PBS. In addition, control and DC-treated animals were also treated with a CD8-depleting (anti-CD8) Ab (day -1 and day +1 of transplantation), to prevent the direct response to K^d molecules by CD8⁺ T cells (42). As shown in Fig. 3A, skin allografts from mice that had received DexD3-DCs or DexD3 + LPS-DCs were rejected significantly earlier (MST $\pm$ SE,

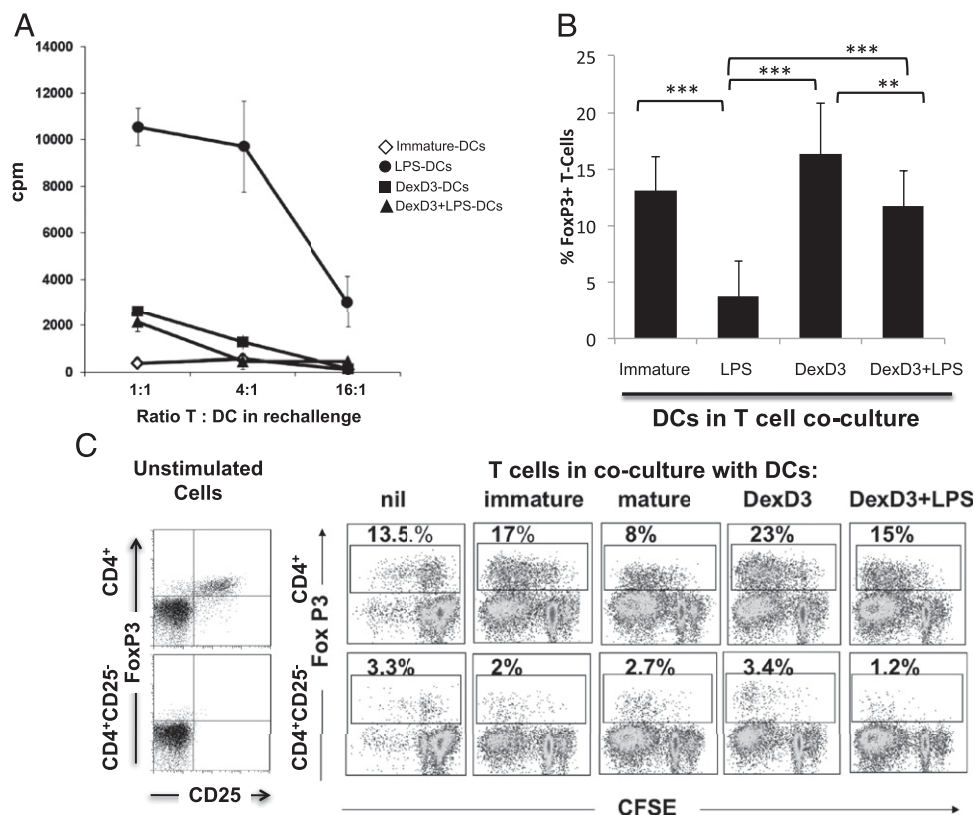


FIGURE 2. CD4⁺ T cells cocultured with allogeneic DexD3-DCs are hyporesponsive to alloantigen rechallenge in vitro and preferentially expand CD4⁺ 25⁺ T cells. **(A)** CD4⁺ T cells (2×10^5) from CBA mice were cocultured with BALB/c-derived DCs treated or not with DexD3 in the presence or absence of LPS [LPS-matured DCs (●), immature DCs (◇), DexD3 + LPS-DCs (▲), and DexD3-DCs (■)]. On day 6 of culture, the CD4⁺ T cells were purified and restimulated with immature BALB/c DCs at different ratios. T cell proliferation was measured after 72 h by addition of ³H-thymidine for the last 16 h. Proliferation is expressed as cpm $\pm$ SD. One representative experiment is shown of five performed. **(B)** BALB/c DCs treated with or without DexD3 in the presence or absence of LPS were cocultured with total CD4⁺ T cells (1×10^6), derived from B6 mice, at a ratio of 1:10 in the presence of rIL-2 (200 U/ml). On day 5, T cells were harvested and analyzed for expression of CD4, CD25, and Foxp3 by flow cytometry. Data plotted are the mean percentage of Foxp3⁺ T cells detected after coculture with DCs in four independent experiments. ** $p < 0.05$, *** $p < 0.001$. **(C)** CFSE-labeled total CD4⁺ T cells, or CD4⁺ T cells depleted of CD25⁺ cells (CD4⁺CD25⁻) derived from B6 mice, were cultured with BALB/c DCs preparations, as described in (B). On day 5, T cells were stained for expression of CD25 and Foxp3, and also analyzed for CFSE dilution by flow cytometry. Data shown are representative of three independent experiments and shows the expansion (CD4⁺ T cells, *top panels*) and induction (CD4⁺CD25⁻ T cells, *bottom panels*) of Foxp3⁺ Tregs after 5 d of coculture with DCs.

8.2 ± 0.20) than the skin grafts transplanted onto recipient mice that received PBS only (MST, 12.0 ± 1.6). Similar results were obtained following transplantation of K^{bm1} skin allografts onto B6 recipient mice that had received DexD3-DCs derived from donor K^{bm1} mice (MST $\pm$ SE, $11.2^{+/-0.66}$ versus 13.3 ± 0.48 for PBS) (Fig. 3B). These results suggest that the injection of drug-treated donor DCs expressing intact donor MHC molecules primed the recipient immune system to the alloantigens, even when only three differences in the MHC molecules were expressed between recipient and donor cells.

Next we addressed whether pulsing DexD3-DCs with an allogeneic peptide derived from the K^d molecule was enough to prime the immune system to donor alloantigens. As shown in Fig. 3A, the time of graft rejection between mice injected with PBS or DexD3-DCs pulsed with K^d peptide was not statistically different (MST, 10.5 ± 0.6), further supporting the idea that donor DCs expressing intact alloantigens need to be processed and presented by endogenous APCs to enhance graft rejection.

Of note, when the mice that received K^d skins were treated with anti-CD8 Ab, to delete CD8⁺ T cells, all the skin transplants were rejected within a similar time frame, irrespective of whether recipient mice were injected with PBS (MST, 11.6 ± 0.3), donor-derived DexD3-DCs (MST, 12.4 ± 0.8), DexD3 + LPS-DCs

(MST, 11.9 ± 0.6), or DexD3-DCs pulsed with K^d peptide (MST, 12.8 ± 0.9).

To understand the difference in skin graft outcomes between recipient animals injected with anti-CD8 Ab or left untreated, splenocytes from animals treated with anti-CD8 Ab were analyzed 2 d after Ab treatment. As shown in Fig. 3C, not only CD8⁺ T cells but also CD8⁺ DCs were effectively depleted by anti-CD8 Ab treatment. This result explains the difference in graft survival time between mice treated with anti-CD8 Ab and those left untreated because the relative contribution of the direct pathway alone in skin graft rejections may be indicated by the difference (nonsignificant $p = 0.58$) between the PBS control groups.

It has been previously shown that additional therapies, such as treatment with CTLA4-Ig or anti-CD40L Ab, in conjunction with murine tolerogenic DCs, greatly improved graft survival (27, 48). We have previously shown in a rat kidney transplant model that treatment with CTLA4-Ig was necessary to induce the indefinite survival of kidney allografts (33). We hypothesized that CTLA4-Ig inhibited presentation of alloantigens derived from "tolerogenic" DCs (24). However, when we tested the effect of CTLA4-Ig treatment of recipient mice on skin allograft rejection, at an equivalent dose applied in the rat model (33), the skin graft survival times did not significantly change compared with untreated

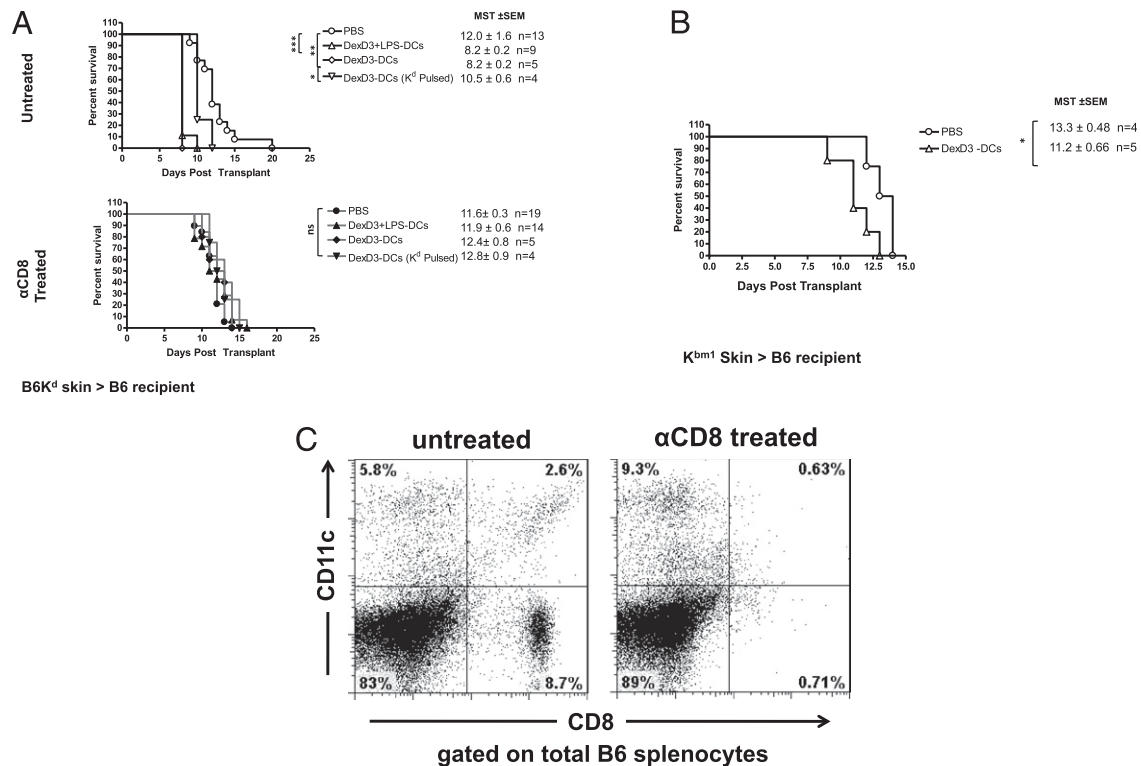


FIGURE 3. DexD3-DCs pretreatment enhances skin allograft rejection. **(A)** B6 mice were given 2×10^6 B6K^d DCs treated or not with DexD3, in the presence or absence of LPS, or K^d-pulsed B6 DCs (DexD3-DCs K^d pulsed), via i.v. injection. Control mice received PBS. At 7 d later, these mice received a B6K^d skin transplant. In addition, some mice were treated with 250 μ g YTS169, an anti-CD8-depleting Ab, 1 d before and 1 d after the transplant (filled symbols). Mice were monitored daily, and rejection was deemed to have occurred when no viable skin remained. The MST $\pm$ SEM is shown (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$) and the number of animals per condition (n). Data shown are representative of two independent experiments. **(B)** B6 mice were given 2×10^6 DCs K^{bm1} treated with DexD3 via i.v. injection. Control mice received PBS. At 7 d later, these mice received a K^{bm1} skin transplant. Mice were monitored daily, and rejection was deemed to have occurred when no viable skin remained. The MST $\pm$ SEM is shown and the number of animals per condition ($n = 5$). **(C)** Mice treated with two doses of 250 μ g of anti-CD8 Ab are depleted of both CD8⁺ T cells and also CD8⁺ DC subsets. Data show analysis of total splenocytes, using flow cytometry after CD8 and CD11c staining, 2 d after the last anti-CD8 treatment, and is representative of two independent experiments.

mice, although the effect of anti-CD8 Ab treatment described earlier was maintained (Supplemental Fig. 2). This result suggests that CTLA4-Ig in this strain combination, and at the dose used, was insufficient to prevent the activation of alloreactive T cells.

Adoptive transfer of tolerogenic allogeneic donor DCs accelerates heart allograft rejection in both semi-mismatched and completely mismatched strain combination

The data obtained with mice that received skin transplants were further extended to heart allograft models. First, B6D2F1 hearts were transplanted into B6 mice. As shown in Fig. 4A, compared with mice treated with PBS alone (PBS, $12.6.0 \pm 1.4$), mice that received 2×10^6 donor-derived DexD3-DCs or DexD3 + LPS-DCs (day -6) rapidly rejected the allografts (MST, 6.0 ± 0.0 or 4.1 ± 0.4 , respectively), with mice that received DexD3 + LPS-DCs rejecting even earlier than mice injected with DexD3-DCs. This rapid rejection is perhaps a result of the amplification of the indirect response caused by constitutive indirect presentation by donor APCs contained within the heart. However, when donor DC treatment of mice was combined with anti-CD8 Ab treatment, as previously observed for the skin transplants, prolongation of allograft survival was observed (MST: anti-CD8 treatment with PBS, 15.3 ± 1.0 ; DexD3-DCs, 11.8 ± 1.5 ; or DexD3 + LPS-DCs, 12.9 ± 1.6). Differences in graft survival were now observed between animals treated with PBS alone or PBS in the presence of anti-CD8 Ab, suggesting that CD8⁺ T cells with direct allospecificity may contribute to heart allograft rejection.

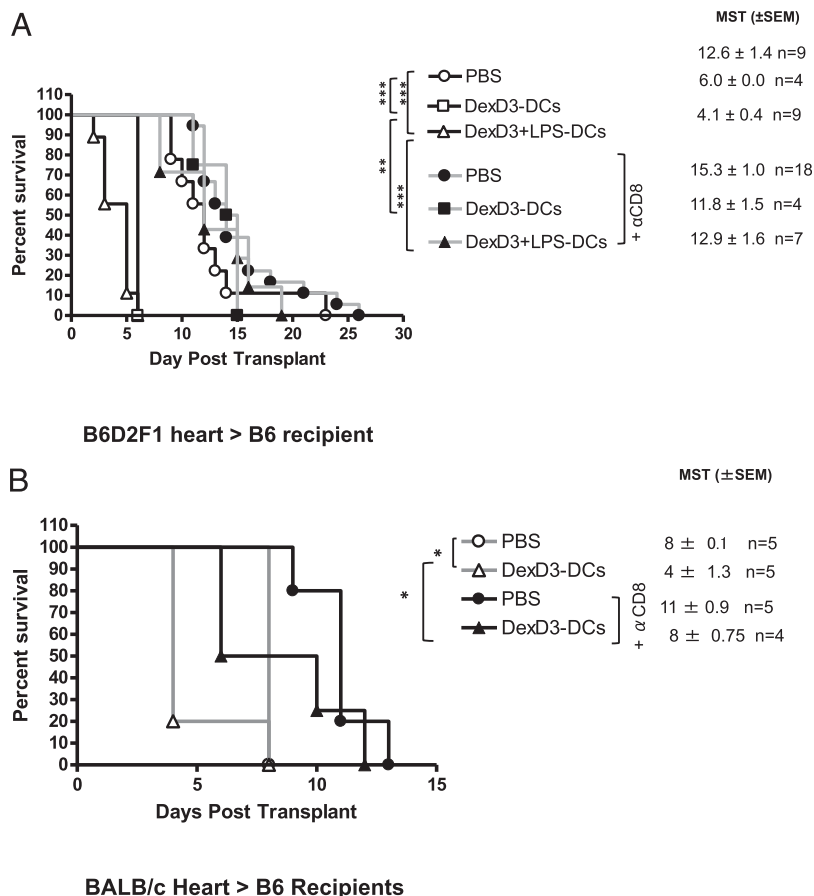
Most of the published work on tolerogenic DCs and their effect on heart transplant rejection have been performed in completely mismatched strain combination (22, 23, 49). To test whether using such a strain combination would result in a delay in graft rejection, B6 mice were transplanted with heterotopic BALB/c hearts (Fig. 4B). As previously shown in Fig. 4A, DexD3-DCs accelerated graft rejection in the absence of anti-CD8 Ab treatment (MST, 4 ± 1.3). This finding may be due to indirect presentation, which is entirely dependent on the presentation of alloantigens by recipient APCs in this combination. No differences in graft survival were observed between mice that received PBS or DexD3-DCs when CD8 cells were depleted (MST, 11 ± 0.9 and 8 ± 0.75).

With all data taken together, it appears that donor-derived drug-treated DCs, despite their tolerogenic in vitro effects, when used in vivo not only fail to promote tolerance but also can sensitize recipient mice.

T cells with indirect allospecificity are primed in vivo by injected alloantigen-bearing DCs

To directly prove that alloantigens derived from "tolerogenic" DCs can be processed and presented by endogenous APCs and can activate recipient T cells with indirect allospecificity, the following experiments were performed. B6 mice were injected with PBS or DexD3-DCs from BALB/c mice. At 10 d later, CD4⁺ T cells were isolated from these mice and challenged in vitro with DCs derived from BALB/c (direct response), B6K^d (indirect response),

FIGURE 4. DexD3-DC pretreatment enhances heart allograft rejection. **(A)** B6 mice were given 2×10^6 B6D2F1 DCs treated with DexD3, in the presence or absence of LPS or PBS, via i.v. injection, followed by a B6D2F1 heart transplantation 7 d later. Some mice received 250 μ g anti-CD8 Ab 1 d before and after the transplantation. Results plotted show the MST of allografts $\pm$ SEM and are pooled data from two independent experiments. **(B)** B6 mice were injected with DexD3-BALB/c DCs or PBS before receiving a heart BALB/c transplant 7 d later. Some mice received 250 μ g anti-CD8 Ab 1 d before and after the transplantation. Results plotted show the MST of allografts $\pm$ SEM and n = number of mice.



or CBA/Ca (third party response) mice for 3, 5, and 7 d (Fig. 5A). This time course allowed the primary (peaking on days 5 and 7) and secondary (peaking on day 3) T cell responses to be visualized. We observed that CD4⁺ T cells isolated from mice previously challenged with DexD3-DCs respond to an in vitro rechallenge to BALB/c-DCs optimally at day 7, at the same time as mice injected with PBS only, suggesting a primary direct response to alloantigens presented by BALB/c-DCs. The response to CBA/Ca-DCs (third party alloantigens) was again at day 7 and identical to the response of mice injected with PBS. However, we did observe a secondary response, started at day 3 and maintained up to day 7, to B6K^d-DCs by T cells isolated from recipient mice injected with Dex-DCs that was, although low, significantly greater than the response in the PBS-treated controls.

To further support the idea that injection of DCs leads to priming of T cells with indirect alloresponse, Ab production specific for K^d was measured in B6 mice that were injected with DexD3 B6K^d DCs (the same treatment as in Fig. 5A). As shown in Fig. 5B, Abs specific for H2^d were found at a significant level in the sera of three mice that received B6K^d DCs, with the peak of production between 14 and 21 d. Early production (between days 7 and 14) of IgM was observed in two of three mice (data not shown).

These observations further confirm the observation that treating mice with donor "tolerogenic" DCs can prime the T cell response to the alloantigen indirectly presented by self-restricted MHC.

DCs injected intravenously are rapidly processed and alloantigen is presented by recipient APCs

To visualize the extent and the kinetics of processing and presentation of DCs expressing alloantigens, CFSE-labeled CD4⁺ T cells isolated from TCR transgenic mice (TCR75 Rag^{-/-} mice, Thy1.1⁺) were adoptively transferred into B6 (H-2^b) recipient mice. At 24 h

later BALB/c (H-2K^d) immature or DexD3 + LPS-DCs were injected. Control mice received either PBS or lysed BALB/c-DCs. Proliferation of Thy1.1⁺ TCR75 cells was observed 3 d later in B6 mice given DexD3 + LPS-DCs. As shown in Fig. 6A, proliferation of Thy1.1⁺ TCR75 cells was also observed in B6 recipient mice that had received lysed BALB/c-DCs, and no proliferation was seen in mice treated with PBS. As the TCR of the adoptively transferred CD4⁺ T cells recognizes K^d antigen only when presented by H-2A^b, we can conclude that the injected BALB/c-DCs were "processed" in vivo and the alloantigen (K^d) was presented by the recipients' APCs.

To confirm directly that anti-CD8 Ab removed a fraction of the CD8⁺ DCs and by doing so decreased the presentation of alloantigens, mice were injected with anti-CD8 Ab the day before receiving allogeneic DC and at the same time as TCR75 injection. The results presented in Fig. 6B, in which treatment of the mice with anti-CD8 Ab led to a decreased proliferation of TCR75 cells, further support the contribution of CD8⁺ DCs in processing and presenting alloantigens released by injected DCs. The partial inhibition of TCR75 proliferation was further confirmed by treating recipient mice with polyinosinic-polycytidylic acid (dsRNA). The dsRNA not only inhibits the capture and processing of Ags by DCs but also reduces the CD8 α ⁺ DC population (50–52).

To further prove that CD8⁺ DCs are the major endogenous DC subset involved in the processing and presentation of the injected donor-derived DCs, Baft3^{-/-} mice were used. These mice lack CD8⁺ DCs, the subpopulation of DCs with the capacity to cross-present exogenous Ag (39). Baft3^{-/-} mice were injected with DexD3-DCs prior to transplantation; as shown in Fig. 6C, K^d skin grafts were rejected at the same time as those in mice that received PBS only and in a similar manner to B6 mice treated with anti-CD8 Ab (Fig. 3A). These data suggest that in the absence of CD8⁺

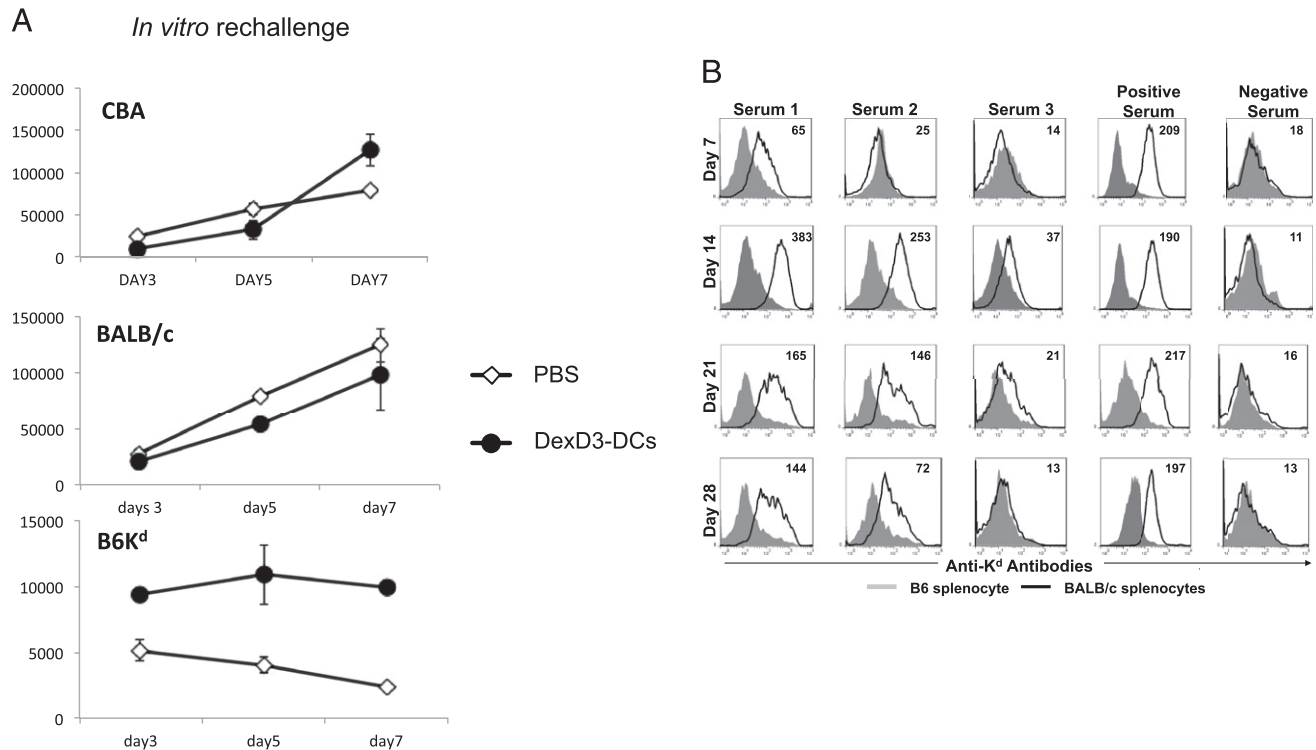


FIGURE 5. Demonstration of in vivo priming of T cell responses with indirect allospecificity induced by DexD3-DCs. **(A)** B6 mice received DexD3-BALB/c DCs or PBS (2×10^6). At 10 d later, 2×10^4 CD4⁺ T cells isolated from recipient mice were stimulated with 1×10^4 DCs from BALB/c, B6K^d, or CBA/Ca mice. Proliferation was measured on days 3, 5, and 7 of culture by addition of ³H-thymidine for the last 16 h. Proliferation is expressed as cpm $\pm$ SD. One representative experiment is shown of three performed. **(B)** B6 mice were injected with B6K^d DCs (2×10^6) treated with DexD3, and sera were collected after 7, 14, 21, and 30 d. Ab production was evaluated by flow cytometric analysis using BALB/c splenocytes as target cells (black line) and B6 splenocytes as control ones (shaded histograms). The mean fluorescence intensity of Ab response to BALB/c splenocytes (black) is shown in the top right-hand side of each panel. Experiment represents one of two experiments.

DCs impaired processing or presentation of donor Ags expressed by injected DCs occurs. This hypothesis was confirmed by adoptively transferring CFSE-labeled TCR75 T cells into Baft3^{-/-} mice, followed by BALB/c-DCs injection. Decreased proliferation of the TCR75 cells was observed in these animals compared with wild-type controls (from 32 to 1% cell division, data not shown), further supporting the data presented in Fig. 6B.

Finally, treatment of wild-type animals with CTLA4-Ig did not alter the response of TCR75 T cells to "processed" alloantigens (Supplemental Fig. 3). This result is in line with the absence of a tolerogenic effect of CTLA4-Ig observed in the skin allograft experiments described above (Supplemental Fig. 2).

To investigate how long processed alloantigen remained available for indirect allorecognition in vivo, as well as the longevity of intact drug-treated donor DCs, the following three experiments were performed. First, DexD3 + LPS-BALB/c DCs were injected into B6 recipient mice, and CFSE-labeled CD4⁺ T cells from TCR75 Rag^{-/-} mice were adoptively transferred 1 or 4 d later. Proliferation was measured 3 d later. Second, OVA peptide-pulsed drug-treated DCs were injected into (B6 $\times$ B/c) F1 recipient mice, and CFSE-labeled CD4⁺ T cells from DO11.10 Rag^{-/-} mice were injected 1 and 4 d later. As shown in Fig. 7A (left panel) good proliferation of TCR75 cells was observed when T cells were adoptively transferred 1 d after DCs (proliferation measured at day 4), but T cell responses were lost when TCR75 cells were adoptively transferred 4 d following DC challenge (proliferation measured at day 7). Similarly, lack of proliferation was observed when CD4⁺ T cells derived from DO11.10 Rag^{-/-} mice were adoptively transferred 4 d after DC injection into (B6 $\times$ B/c) F1 recipient mice, despite a good response being observed if the T cells were given day 1 after the

DC injection (Fig. 7A, right panel). To further define how long drug-treated DCs survive in vivo, the above experiment using OVA-pulsed drug-treated DCs was repeated. However, this time CD4⁺ T cells derived from DO11.10 Rag^{-/-} mice were adoptively transferred 1, 2, or 3 d after the drug-treated DCs (Fig. 7B). Although OVA-specific CD4⁺ T cell proliferation was evident when T cells were transferred 1 or 2 d after the peptide-pulsed DCs, this response was greatly reduced after 3 d, confirming that by day 4 no donor DCs were left in vivo. No differences between drug-treated and untreated DCs were observed in these experiments, further demonstrating the similarity in viability of drug-treated and untreated DCs in vivo at least for 3 d (data not shown). Finally, to determine how rapidly "processed" alloantigen is available for presentation by endogenous APCs, CD4⁺ T cells from TCR75 Rag^{-/-} mice were adoptively transferred 1 d before DC challenge, and T cell activation was analyzed 14 h after DC challenge by measuring CD69 upregulation. We observed that CD69 upregulation occurred within 14 h on Thy1.1⁺ TCR75 cells in mice receiving DexD3 + LPS-BALB/c DCs, suggesting that the allogeneic DCs were rapidly processed after being adoptively transferred (Fig. 7C).

Donor-derived DexD3-DCs induce skin transplant survival in recipient mice lacking CD8⁺ DCs in combination with anti-CD40L Ab therapy

From all the experiments presented in this article, we concluded that drug-treated allogeneic DCs were rapidly processed and presented by recipient APCs, primarily CD8⁺ DCs. Removal of this DC subset inhibited the rapid priming caused by injection of drug-treated donor cells. Therefore, whether drug-treated tolerogenic DCs could prolong transplant survival in the presence of

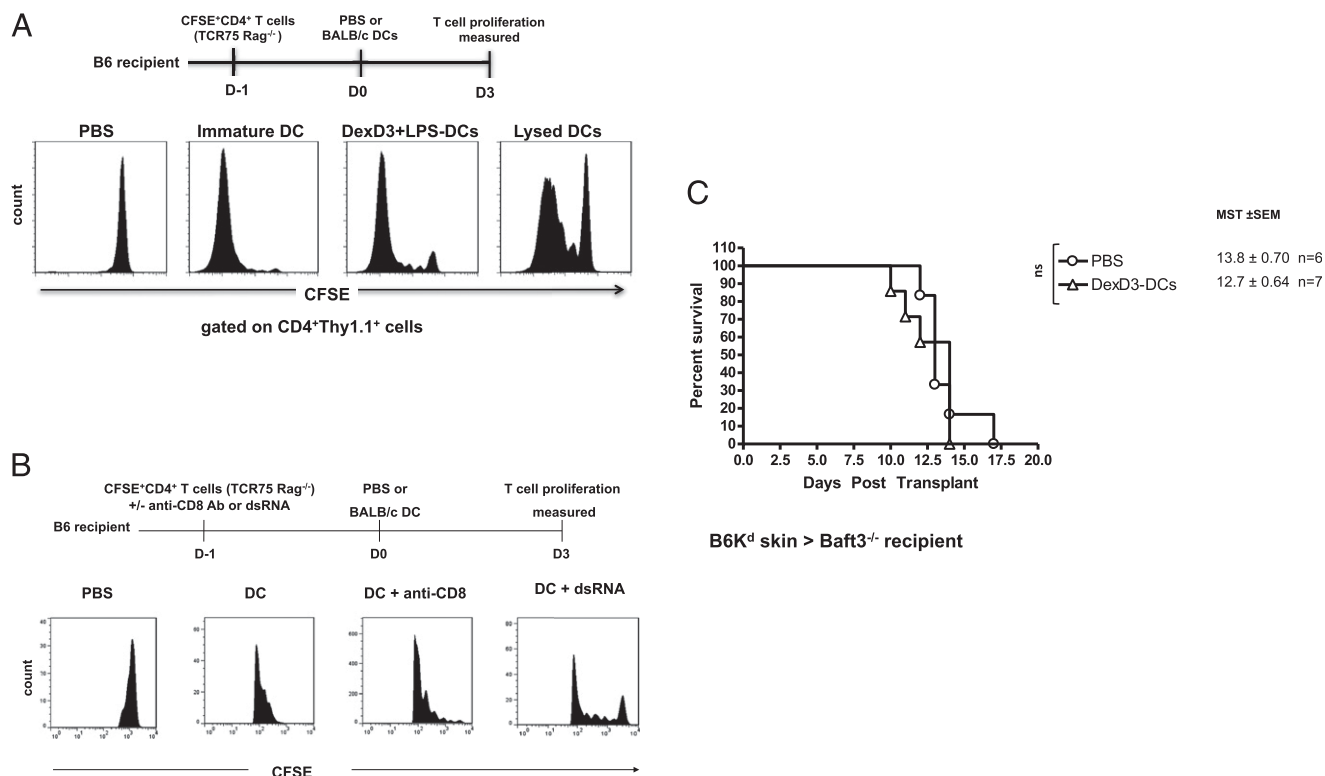


FIGURE 6. Allogeneic tolerogenic DCs injected in vivo provide alloantigens for T cells with indirect allospecificity. **(A)** B6 mice received 2×10^6 CFSE-labeled CD4⁺ T cells isolated from TCR75 Rag^{-/-} mice. At 24 h later, these mice received 2×10^6 of immature, DexD3 + LPS-BALB/c DCs, lysed BALB/c DCs, or PBS. At 3 d later, CD4⁺ T cells were isolated from spleen and LNs and stained with anti-Thy1.1 Ab to identify the adoptively transferred T cells. Cells were analyzed by flow cytometry. Data represent the expression of Thy1.1 and CFSE on CD4⁺ T cells. One representative experiment is shown of four performed. **(B)** The same protocol as above with the additional treatment of B6 mice either with 500 μ g of anti-CD8 Ab or 100 ng of dsRNA the day prior to DC injection. **(C)** Baft3^{-/-} mice were given 2×10^6 B6K^d DCs treated with DexD3 via i.v. injection. Control mice received PBS. At 7 d later, mice were transplanted with skins derived from B6K^d mice. Mice were monitored daily, and rejection was deemed to have occurred when no viable skin remained. The MST $\pm$ SEM is shown and the number of animals per condition (*n*). Data shown are representative of two independent experiments.

additional therapies in the absence of CD8⁺ DCs was addressed. Baft3^{-/-} and B6 recipient mice were injected with donor-derived DexD3-DCs in combination with three doses of anti-CD40L Ab (MR1) treatment (days -7, -4, and 0 at 500 μ g per injection) prior to K^d skin transplantation. With this protocol, prolongation of skin graft survival was observed in Baft3^{-/-} recipients using DexD3-DCs (MST, 22.1 ± 2.64) compared with PBS (MST, 15.1 ± 0.46) (Fig. 8A). However, when DCs are injected into B6 mice in the presence of MR1 Ab, a nonsignificant prolongation of skin allograft survival was observed, PBS (MST, 18.0 ± 3.8) versus DexD3-DCs (MST, 22.9 ± 3.8) (Fig. 8B).

As a whole, the results presented in this study further support the idea that transplant survival mediated by donor-derived tolerogenic DCs can be achieved only in the absence of endogenous DCs with cross-presenting function and when recipient mice are treated with additional therapies, such as MR1.

Discussion

One strategy to promote T cell regulation in transplantation is via the in vivo induction or expansion of Tregs with modified DCs. The combination of Dex and D3 has previously been shown to be synergistic, and we adopted this drug combination as the preferred approach for exploring the therapeutic potential of tolerogenic DCs (14, 53). Our in vitro findings are in keeping with other publications that demonstrate that drug modification can maintain DCs in an immature state, resistant to LPS-, CD40L-, or TNF- α -induced maturation, with reduced expression of costimulatory molecules and an impaired capacity to produce IL-12 (12, 20, 53, 54).

We also demonstrated that the phenotype of DexD3-DCs was tolerogenic owing to their capacity to induce T cell hyporesponsiveness and expansion of Foxp3⁺ T cells in vitro (12, 20, 53, 55).

However, the key and most interesting observation made in this study was that despite demonstrating "tolerogenic" potential in vitro, DexD3-treated DCs did not prolong skin or heart allograft survival; instead, they accelerated graft rejection. We have also demonstrated that although in vivo treatment with anti-CD8 Abs abolished this early rejection no "tolerance" was established. This finding was further confirmed using as recipients a strain of mice that lacks DCs with the capacity to cross-present exogenous Ag (CD8⁺ DCs). Furthermore, we observed that the injection of allogeneic DCs primed the recipient CD4⁺ T cells to alloantigens. Using T cells from TCR transgenic mice as a tool to measure the response to alloantigens presented indirectly, we demonstrated that the life span of the injected drug-treated DCs expressing alloantigens is short lived in vivo owing to efficient processing in vivo. The T cell activation by "processed" alloantigens was partially inhibited by using either anti-CD8 Ab or dsRNA pretreatment, suggesting a role for recipient DCs in amplifying the immune responses to the allograft. At present we believe that CD8 α ⁺ DCs contribute to amplification of the indirect alloresponse, as the aforementioned treatments lead to a reduction in the number of these cells (Fig. 3C) (50, 51). Taken together, the data presented in our study suggest that negative vaccination using drug-treated donor DCs confers a major risk of sensitization owing to processing and presentation of alloantigens by endogenous DCs, leading to priming of CD4⁺ T cells with indirect allospecificity.

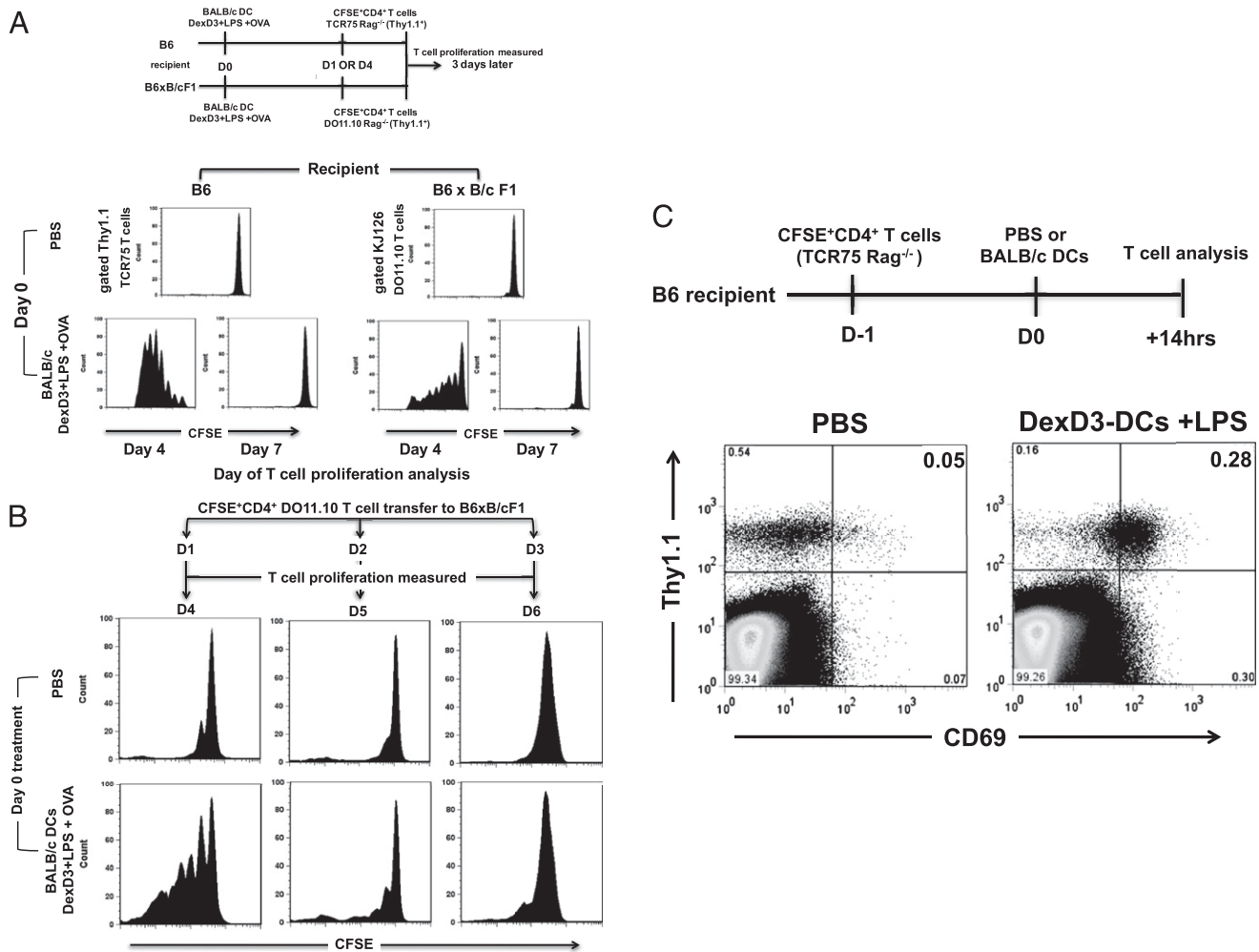


FIGURE 7. Tolerogenic donor DCs have a short life in vivo and are processed by recipient APCs. **(A)** Recipient B6 and (B6 × B/c) F1 mice were injected with DexD3 + LPS-BALB/c DCs (2×10^6) pulsed with 5 $\mu\text{g}/\text{ml}$ of OVA peptide. Control mice received PBS only. CD4⁺ T cells isolated from TCR75 Rag^{-/-} or DO11.10 Rag^{-/-} mice were CFSE labeled and adoptively transferred (1.5×10^6) on day 1 or day 4 following DC challenge into B6 or F1 mice, respectively. T cell proliferation was measured 3 d after T cell injection by flow cytometry. Histograms represent proliferation of CD4⁺ T cells identified using Thy1.1 (TCR75 Rag^{-/-}) or KJ126 (DO11.10 Rag^{-/-}) specific Abs (left and right panels, respectively). One representative experiment is shown of three performed. **(B)** Recipient F1 mice were injected with DexD3 + LPS-BALB/c DCs (2×10^6) pulsed with 2 $\mu\text{g}/\text{ml}$ of OVA peptide. Controls received just PBS. CD4⁺ T cells isolated from DO11.10 Rag^{-/-} mice were CFSE labeled and adoptively transferred (4×10^6) on days 1, 2, and 3 following DC challenge. T cell proliferation was measured 3 d later by flow cytometry. Histograms represent proliferation of CD4⁺ T cells identified using KJ126-specific Abs. One representative experiment is shown of two performed. **(C)** B6 mice received 2×10^6 CD4⁺ T cells isolated from TCR75 Rag^{-/-} mice. At 24 h later, these mice received 2×10^6 DexD3 + LPS-DCs. Controls received PBS only. At 14 h later, CD4⁺ T cells were isolated from spleen and LNs and stained with Thy1.1, to identify the adoptively transferred T cells, and CD69. Cells were analyzed by flow cytometry. Data represent the expression of Thy1.1 and CD69 on CD4⁺ T cells. One representative experiment of three performed.

Two papers were recently published in which the effect of murine DCs on allograft survival was investigated and the results obtained were conflicting. In the first paper, Divito et al. presented data showing that donor-derived immature DCs injected in vivo, in a completely mismatched strain combination, induced indefinite survival of heart allografts. These authors suggest that this survival resulted from anergy induction in alloantigen-specific recipient T cells following processing and presentation of apoptotic donor DCs by endogenous APCs (23). By contrast, Yamano et al. (34) observed that skin allografts, in mice injected with donor-derived DCs (obtained using a protocol similar to that of the Divito study), were rejected at the same time, if not slightly earlier, than those in PBS-treated mice. More recently, in a rat model of islet transplantation, the treatment of the recipient mice with donor-derived Dex-DCs led to Ab-mediated allograft rejection (38). Both the aforementioned paper, as well as the observations of Yamano et al., support our data that donor-derived tolerogenic DCs can prime rather than induce tolerance in vivo.

For a long time, apoptosis was thought to be tolerogenic, whereas necrosis was immunogenic. However, now it is clear that processing and presentation of Ag from apoptotic cells can lead to immunogenicity, particularly in the presence of proinflammatory signals. Although early evidence of immune responses to minor histocompatibility Ags demonstrated strict MHC restriction in vitro (54, 56), a responder mouse expressing H-2^b and H-2^d alleles, primed in vivo with cells homozygous for H-2^b and differing in minor histocompatibility Ags, demonstrated a strong in vitro secondary response against the minor Ags restricted not only by H-2^b but also by H-2^d (57). Bevan (58) hypothesized and subsequently demonstrated that this was the result of cross-primed cytotoxic T cells. Cross-presentation and cross-priming of anti-tumor CTL responses occur by the processing of apoptotic cells by DCs, resulting in augmented tumor immunogenicity, highlighting the significance of this immunological pathway (49). Although cross-presentation is generally regarded as a pathway by which

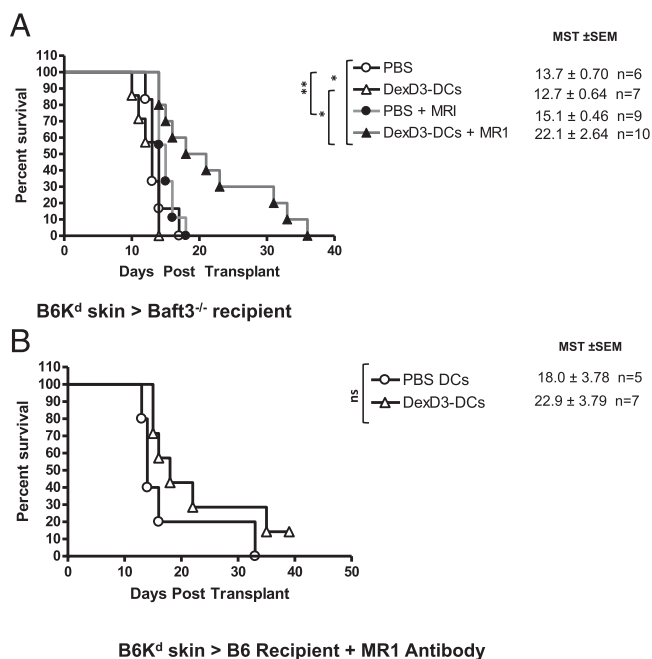


FIGURE 8. Tolerogenic donor DCs induce skin transplant survival when recipient mice that lack CD8⁺ DCs are treated with anti-CD40L Ab therapy. Baft3^{-/-} (**A**) and B6 (**B**) mice received 2×10^6 B6K^d DCs treated with DexD3. Control mice received PBS. At 7 d later, mice received a B6K^d skin transplant. In addition, some mice were treated with 500 μ g of MR1 4 and 7 d prior to, and on the day of transplantation (filled symbols). Mice were monitored daily, and rejection was deemed to have occurred when no viable skin remained. The MST $\pm$ SEM is shown (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$) and the number of animals per condition (n). Data shown represent three and two independent experiments, respectively.

exogenous Ag becomes available to CD8⁺ T cells, it has also been observed for Ag presentation on MHC class II Ags. In a corneal transplant model, it was observed that recipient APCs transported Ag from the donor cornea and cross-presented this to host Ag-specific CD4⁺ T cells, leading to activation and expansion of these cells (59).

In our study, the occurrence of cross-presentation of donor alloantigens and therefore cross-priming to CD4⁺ T cells with indirect allospecificity is suggested and, at least in part, demonstrated by the reduction of TCR75 proliferation when anti-CD8 Ab and dsRNA were used, and in CD8 α^- DC animals. Our results are different from the data recently published by Divito et al. (23) and similar to the results published by Yamano et al. (34). Others have also reported, in transplant models, that injected cells that died of apoptosis prime the recipient immune system, and factors such as the inflammatory state of the recipient may contribute to the balance between activation of the immune response and tolerance (37, 60). In our study, owing to the nature of Ag presentation and the observation that the processed alloantigen is available to responder T cells within 14 h and for up to 4 d, the likelihood is equal that an effector response will be generated, particularly in the context of an inflammatory response induced by the transplant itself (61). As demonstrated by Divito et al. (23, 62), the processing and presentation of injected DCs may be the result of NK-mediated killing (62).

The involvement of endogenous DCs in the accelerated graft rejection observed in our study was supported by the data obtained when recipient mice were treated with anti-CD8 Ab and when Baft3^{-/-} mice were used. Our data suggest that the CD8⁺ DC subset plays the major role in the priming and activation of recipient T cells with indirect allospecificity that contribute to graft

rejection. The observation that treatment with anti-CD8 Ab causes CD8⁺ DC deletion has major implications for the large number of published studies in which anti-CD8 Ab was used and thought to delete CD8⁺ T cells only.

Although it is clear that the removal of recipient CD8⁺ DCs is not enough to induce transplant tolerance when mice are treated with donor-derived tolerogenic DCs, the additional therapy with anti-CD40L Ab led to graft survival. In the study by Yamano et al. (34), the injection of MR1 (together with CTLA4-Ig and anti-NK1.1 Ab) with immature DCs into wild-type mice is not enough to induce tolerance. The difference between the two studies further supports our idea that the increased survival of an allograft is a very delicate equilibrium in which many factors play an important role. It is clear from our data that only when endogenous DCs are absent, together with interfering with CD40–CD40L interaction, this combination can induce graft survival. This result differs from the experiment in which CTLA4-Ig, rather than MR1, was used. Although this additional therapy was enough in the rat kidney allograft model (38), no improvement in transplant survival was seen using our strain combination. One possible explanation for the difference observed is the amount of CTLA-4 used (equivalent amount used in the rat model, but lower than the amount used in other murine studies). However when a combination of MR1 and CTLA4-Ig [at the dose normally used in murine models (27)] was injected during donor-derived DC treatment, lack of skin transplant survival was also observed in wild-type animals (data not shown), further confirming the need for recipient DC removal to achieve graft survival.

An alternative to the use of donor-derived DCs to achieve tolerance in the context of autoimmunity or transplantation is the adoptive transfer of recipient DCs rendered tolerogenic and expressing either autoantigens or alloantigens. This approach has already entered the clinical arena. A safety study that is currently recruiting uses autologous DC therapy for type 1 diabetes suppression; in this study autologous monocyte-derived DCs are treated ex vivo with antisense phosphorothioate-modified oligonucleotides targeting the primary transcripts of the CD40, CD80, and CD86 costimulatory molecules (immunoregulatory DCs). The hypothesis to be tested in this study is that immunoregulatory DCs are safe and without toxicity in established type 1 diabetic patients (63). The closest studies, in terms of modifying recipient DCs, to those described in this article are of locally injected DCs, with DexD3-DCs injected into the knees of patients with rheumatoid arthritis (64); this represents one of the first efficacy trials to determine whether recipient drug-modified DCs can alter the course of disease locally in a systemic disorder.

Finally, another way to achieve tolerance is by targeting recipient DCs with Ag directly in vivo—for example, in a quiescent state (65). Delivering Ag specifically to conventional DCs via DEC-205 or 33D1 leads to effective presentation by MHC class I or II molecules, respectively, which is followed by induction or expansion of Tregs and/or T cell deletion (66–69). The usefulness of this concept was shown in mouse models of type 1 diabetes in which targeting of autoantigens to CD205 could prevent the onset and progression of disease (67, 70). More recently, we have demonstrated for the first time, to our knowledge, in a skin transplant model that delivering alloantigens to cDCs via 33D1 leads to indefinite transplant survival, but only in the presence of anti-CD8-depleting Ab (71).

Altogether, the results presented in this study suggest that cellular negative vaccination with donor-derived DCs is linked to the risk of sensitization to donor Ags. These findings have major implications in clinical strategies in which donor tolerogenic DCs are to be used to induce tolerance to a graft.

Disclosures

The authors have no financial conflicts of interest.

References

- Lechler, R. I., and A. J. T. George. 2006. Transplantation and rejection. In *Immunology*, 7th Ed., D. Male, J. Brostoff, D. B. Roth, and I. Roitt, eds. Mosby Elsevier, Philadelphia, p. 383–399.
- Long, E., and K. J. Wood. 2009. Regulatory T cells in transplantation: transferring mouse studies to the clinic. *Transplantation* 88: 1050–1056.
- Feng, G., T. Chan, K. J. Wood, and A. Bushell. 2009. Donor reactive regulatory T cells. *Curr. Opin. Organ Transplant* 14: 432–438.
- Silk, K. M., and P. J. Fairchild. 2009. Harnessing dendritic cells for the induction of transplantation tolerance. *Curr. Opin. Organ Transplant* 14: 344–350.
- Hackstein, H., and A. W. Thomson. 2004. Dendritic cells: emerging pharmacological targets of immunosuppressive drugs. *Nat. Rev. Immunol.* 4: 24–34.
- Morelli, A. E., and A. W. Thomson. 2007. Tolerogenic dendritic cells and the quest for transplant tolerance. *Nat. Rev. Immunol.* 7: 610–621.
- Griffin, M. D., W. H. Lutz, V. A. Phan, L. A. Bachman, D. J. McKean, and R. Kumar. 2000. Potent inhibition of dendritic cell differentiation and maturation by vitamin D analogs. *Biochem. Biophys. Res. Commun.* 270: 701–708.
- Dong, X., T. Craig, N. Xing, L. A. Bachman, C. V. Paya, F. Weih, D. J. McKean, R. Kumar, and M. D. Griffin. 2003. Direct transcriptional regulation of RelB by 1 α ,25-dihydroxyvitamin D₃ and its analogs: physiologic and therapeutic implications for dendritic cell function. *J. Biol. Chem.* 278: 49378–49385.
- Rea, D., C. van Kooten, K. E. van Meijgaarden, T. H. Ottenhoff, C. J. Melief, and R. Offringa. 2000. Glucocorticoids transform CD40-triggering of dendritic cells into an alternative activation pathway resulting in antigen-presenting cells that secrete IL-10. *Blood* 95: 3162–3167.
- Roelen, D. L., D. H. Schuurhuis, D. E. M. van den Boogaardt, K. Koekkoek, P. P. M. C. van Miert, J. J. van Schip, S. Laban, D. Rea, C. J. M. Melief, R. Offringa, et al. 2003. Prolongation of skin graft survival by modulation of the alloimmune response with alternatively activated dendritic cells. *Transplantation* 76: 1608–1615.
- Adorini, L., G. Penna, N. Giarratana, and M. Uskokovic. 2003. Tolerogenic dendritic cells induced by vitamin D receptor ligands enhance regulatory T cells inhibiting allograft rejection and autoimmune diseases. *J. Cell. Biochem.* 88: 227–233.
- Matyszak, M. K., S. Citterio, M. Rescigno, and P. Ricciardi-Castagnoli. 2000. Differential effects of corticosteroids during different stages of dendritic cell maturation. *Eur. J. Immunol.* 30: 1233–1242.
- Xing, N., M. L. L. Maldonado, L. A. Bachman, D. J. McKean, R. Kumar, and M. D. Griffin. 2002. Distinctive dendritic cell modulation by vitamin D₃ and glucocorticoid pathways. *Biochem. Biophys. Res. Commun.* 297: 645–652.
- Woltman, A. M., S. W. van der Kooij, J. W. de Fijter, and C. van Kooten. 2006. Maturation-resistant dendritic cells induce hyporesponsiveness in alloreactive CD45RA+ and CD45RO+ T-cell populations. *Am. J. Transplant.* 6: 2580–2591.
- Canning, M. O., K. Grotenhuis, H. de Wit, C. Ruwhof, and H. A. Drexhage. 2001. 1- α ,25-Dihydroxyvitamin D₃ (1,25(OH)₂D₃) hampers the maturation of fully active immature dendritic cells from monocytes. *Eur. J. Endocrinol.* 145: 351–357.
- Rozkova, D., R. Horvath, J. Bartunkova, and R. Spisek. 2006. Glucocorticoids severely impair differentiation and antigen presenting function of dendritic cells despite upregulation of Toll-like receptors. *Clin. Immunol.* 120: 260–271.
- Piemonti, L., P. Monti, M. Sironi, P. Fraticelli, B. E. Leone, E. Dal Cin, P. Allavena, and V. Di Carlo. 2000. Vitamin D₃ affects differentiation, maturation, and function of human monocyte-derived dendritic cells. *J. Immunol.* 164: 4443–4451.
- Penna, G., and L. Adorini. 2000. 1 α ,25-dihydroxyvitamin D₃ inhibits differentiation, maturation, activation, and survival of dendritic cells leading to impaired alloreactive T cell activation. *J. Immunol.* 164: 2405–2411.
- Penna, G., S. Amuchastegui, N. Giarratana, K. C. Daniel, M. Vulcano, S. Sozzani, and L. Adorini. 2007. 1,25-Dihydroxyvitamin D₃ selectively modulates tolerogenic properties in myeloid but not plasmacytoid dendritic cells. *J. Immunol.* 178: 145–153.
- Buckland, M., C. B. Jago, H. Fazekasova, K. Scott, P. H. Tan, A. J. George, R. Lechler, and G. Lombardi. 2006. Aspirin-treated human DCs up-regulate ILT-3 and induce hyporesponsiveness and regulatory activity in responder T cells. *Am. J. Transplant.* 6: 2046–2059.
- Pedersen, A. E., M. Gad, M. R. Walter, and M. H. Claesson. 2004. Induction of regulatory dendritic cells by dexamethasone and 1 α ,25-dihydroxyvitamin D₃. *Immunol. Lett.* 91: 63–69.
- Emmer, P. M., J. van der Vlag, G. J. Adema, and L. B. Hilbrands. 2006. Dendritic cells activated by lipopolysaccharide after dexamethasone treatment induce donor-specific allograft hyporesponsiveness. *Transplantation* 81: 1451–1459.
- Divito, S. J., Z. Wang, W. J. Shufesky, Q. Liu, O. A. Tkacheva, A. Montecalvo, G. Erdos, A. T. Larregina, and A. E. Morelli. 2010. Endogenous dendritic cells mediate the effects of intravenously injected therapeutic immunosuppressive dendritic cells in transplantation. *Blood* 116: 2694–2705.
- Pedersen, A. E., E. G. Schmidt, M. Gad, S. S. Poulsen, and M. H. Claesson. 2009. Dexamethasone/1 α ,25-dihydroxyvitamin D₃-treated dendritic cells suppress coxitis in the SCID T-cell transfer model. *Immunology* 127: 354–364.
- Bonham, C. A., L. Peng, X. Liang, Z. Chen, L. Wang, L. Ma, H. Hackstein, P. D. Robbins, A. W. Thomson, J. J. Fung, et al. 2002. Marked prolongation of cardiac allograft survival by dendritic cells genetically engineered with NF-kappa B oligodeoxynucleotide decoys and adenoviral vectors encoding CTLA4-Ig. *J. Immunol.* 169: 3382–3391.
- DePaz, H. A., O. O. Oluwole, A. O. Aderoyi, P. Witkowski, M. X. Jin, M. A. Hardy, and S. F. Oluwole. 2003. Immature rat myeloid dendritic cells generated in low-dose granulocyte macrophage-colony stimulating factor prolong donor-specific rat cardiac allograft survival. *Transplantation* 75: 521–528.
- Lan, Y. Y., Z. Wang, G. Raimondi, W. Wu, B. L. Colvin, A. de Creus, and A. W. Thomson. 2006. "Alternatively activated" dendritic cells preferentially secrete IL-10, expand Foxp3+CD4+ T cells, and induce long-term organ allograft survival in combination with CTLA4-Ig. *J. Immunol.* 177: 5868–5877.
- Zhang, X., M. Li, D. Lian, X. Zheng, Z. X. Zhang, T. E. Ichim, X. Xia, X. Huang, C. Vladau, M. Suzuki, et al. 2008. Generation of therapeutic dendritic cells and regulatory T cells for preventing allogeneic cardiac graft rejection. *Clin. Immunol.* 127: 313–321.
- Xu, D. L., Y. Liu, J. M. Tan, B. Li, C. P. Zhong, X. H. Zhang, C. Q. Wu, and X. D. Tang. 2004. Marked prolongation of murine cardiac allograft survival using recipient immature dendritic cells loaded with donor-derived apoptotic cells. *Scand. J. Immunol.* 59: 536–544.
- Turnquist, H. R., G. Raimondi, A. F. Zahorchak, R. T. Fischer, Z. Wang, and A. W. Thomson. 2007. Rapamycin-conditioned dendritic cells are poor stimulators of allogeneic CD4+ T cells, but enrich for antigen-specific Foxp3+ T regulatory cells and promote organ transplant tolerance. *J. Immunol.* 178: 7018–7031.
- Pearson, T. C., D. Z. Alexander, R. Hendrix, E. T. Elwood, P. S. Linsley, K. J. Winn, and C. P. Larsen. 1996. CTLA4-Ig plus bone marrow induces long-term allograft survival and donor specific unresponsiveness in the murine model. Evidence for hematopoietic chimerism. *Transplantation* 61: 997–1004.
- Wang, Z., A. E. Morelli, H. Hackstein, K. Kaneko, and A. W. Thomson. 2003. Marked inhibition of transplant vascular sclerosis by in vivo-mobilized donor dendritic cells and anti-CD154 mAb. *Transplantation* 76: 562–571.
- Mirenda, V., I. Berton, J. Read, T. Cook, J. Smith, A. Dorling, and R. I. Lechler. 2004. Modified dendritic cells coexpressing self and allogeneic major histocompatibility complex molecules: an efficient way to induce indirect pathway regulation. *J. Am. Soc. Nephrol.* 15: 987–997.
- Yamano, T., S. Watanabe, H. Hasegawa, T. Suzuki, R. Abe, H. Tahara, T. Nitta, N. Ishimaru, J. Sprent, and H. Kishimoto. 2011. Ex vivo-expanded DCs induce donor-specific central and peripheral tolerance and prolong the acceptance of donor skin grafts. *Blood* 117: 2640–2648.
- Fonteneau, J. F., D. G. Kavanagh, M. Lirvall, C. Sanders, T. L. Cover, N. Bhardwaj, and M. Larsson. 2003. Characterization of the MHC class I cross-presentation pathway for cell-associated antigens by human dendritic cells. *Blood* 102: 4448–4455.
- Inaba, K., S. Turley, F. Yamaide, T. Iyoda, K. Mahnke, M. Inaba, M. Pack, M. Subklewe, B. Sauter, D. Sheff, et al. 1998. Efficient presentation of phagocytosed cellular fragments on the major histocompatibility complex class II products of dendritic cells. *J. Exp. Med.* 188: 2163–2173.
- Knudsen, S., A. Schardt, T. Buhl, L. Boeckmann, M. P. Schön, C. Neumann, and H. A. Haenssle. 2010. Enhanced T-cell activation by immature dendritic cells loaded with HSP70-expressing heat-killed melanoma cells. *Exp. Dermatol.* 19: 108–116.
- de Kort, H., C. Crul, A. M. van der Wal, N. Schlagwein, A. M. Stax, J. A. Bruijn, C. van Kooten, and E. de Heer. 2012. Accelerated antibody-mediated graft loss of rodent pancreatic islets after pretreatment with dexamethasone-treated immature donor dendritic cells. *Transplantation* 94: 903–910.
- Hildner, K., B. T. Edelson, W. E. Purtha, M. Diamond, H. Matsushita, M. Kohyama, B. Calderon, B. U. Schraml, E. R. Unanue, M. S. Diamond, et al. 2008. Batf3 deficiency reveals a critical role for CD8 α + dendritic cells in cytotoxic T cell immunity. *Science* 322: 1097–1100.
- Inaba, K., M. Inaba, N. Romani, H. Aya, M. Deguchi, S. Ikehara, S. Muramatsu, and R. M. Steinman. 1992. Generation of large numbers of dendritic cells from mouse bone marrow cultures supplemented with granulocyte/macrophage colony-stimulating factor. *J. Exp. Med.* 176: 1693–1702.
- Billingham, R. E., L. Brent, and P. B. Medawar. 1953. Actively acquired tolerance of foreign cells. *Nature* 172: 603–606.
- Tsang, J. Y., Y. Tanriver, S. Jiang, S. A. Xue, K. Ratnasothy, D. Chen, H. J. Stauss, R. P. Bucy, G. Lombardi, and R. Lechler. 2008. Conferring indirect allospecificity on CD4+CD25+ Tregs by TCR gene transfer favors transplantation tolerance in mice. *J. Clin. Invest.* 118: 3619–3628.
- Tsang, J. Y., Y. Tanriver, S. Jiang, E. Leung, K. Ratnasothy, G. Lombardi, and R. Lechler. 2009. Indefinite mouse heart allograft survival in recipient treated with CD4(+)CD25(+) regulatory T cells with indirect allospecificity and short term immunosuppression. *Transpl. Immunol.* 21: 203–209.
- Hackstein, H., T. Taner, A. J. Logar, and A. W. Thomson. 2002. Rapamycin inhibits macropinocytosis and mannose receptor-mediated endocytosis by bone marrow-derived dendritic cells. *Blood* 100: 1084–1087.
- Hackstein, H., A. E. Morelli, A. T. Larregina, R. W. Ganster, G. D. Papworth, A. J. Logar, S. C. Watkins, L. D. Falo, and A. W. Thomson. 2001. Aspirin inhibits in vitro maturation and in vivo immunostimulatory function of murine myeloid dendritic cells. *J. Immunol.* 166: 7053–7062.
- Creusot, R. J., S. S. Yaghoubi, P. Chang, J. Chia, C. H. Contag, S. S. Gambhir, and C. G. Fathman. 2009. Lymphoid-tissue-specific homing of bone-marrow-derived dendritic cells. *Blood* 113: 6638–6647.
- Anderson, A. E., D. J. Swan, B. L. Sayers, R. A. Harry, A. M. Patterson, A. von Delwig, J. H. Robinson, J. D. Isaacs, and C. M. U. Hilkens. 2009. LPS activation is required for migratory activity and antigen presentation by tolerogenic dendritic cells. *J. Leukoc. Biol.* 85: 243–250.
- Lu, L., W. Li, F. Fu, F. G. Chambers, S. Qian, J. J. Fung, and A. W. Thomson. 1997. Blockade of the CD40-CD40 ligand pathway potentiates the capacity of

- donor-derived dendritic cell progenitors to induce long-term cardiac allograft survival. *Transplantation* 64: 1808–1815.
49. Pêche, H., B. Trinité, B. Martinet, and M. C. Cuturi. 2005. Prolongation of heart allograft survival by immature dendritic cells generated from recipient type bone marrow progenitors. *Am. J. Transplant.* 5: 255–267.
 50. Smyth, L. A., C. Hervouet, T. Hayday, P. D. Becker, R. Ellis, R. I. Lechler, G. Lombardi, and L. S. Klavinskis. 2012. Acquisition of MHC:peptide complexes by dendritic cells contributes to the generation of antiviral CD8+ T cell immunity in vivo. *J. Immunol.* 189: 2274–2282.
 51. Fuertes Marraco, S. A., C. L. Scott, P. Bouillet, A. Ives, S. Masina, D. Vremec, E. S. Jansen, L. A. O'Reilly, P. Schneider, N. Fasel, et al. 2011. Type I interferon drives dendritic cell apoptosis via multiple BH3-only proteins following activation by PolyIC in vivo. *PLoS ONE* 6: e20189.
 52. Wilson, N. S., G. M. Behrens, R. J. Lundie, C. M. Smith, J. Waithman, L. Young, S. P. Forehan, A. Mount, R. J. Steptoe, K. D. Shortman, et al. 2006. Systemic activation of dendritic cells by Toll-like receptor ligands or malaria infection impairs cross-presentation and antiviral immunity. *Nat. Immunol.* 7: 165–172.
 53. Chamorro, S., J. J. García-Vallejo, W. W. J. Unger, R. J. Fernandes, S. C. M. Bruijns, S. Laban, B. O. Roep, B. A. 't Hart, and Y. van Kooyk. 2009. TLR triggering on tolerogenic dendritic cells results in TLR2 up-regulation and a reduced proinflammatory immune program. *J. Immunol.* 183: 2984–2994.
 54. Gordon, R. D., E. Simpson, and L. E. Samelson. 1975. In vitro cell-mediated immune responses to the male specific(H-Y) antigen in mice. *J. Exp. Med.* 142: 1108–1120.
 55. Edinger, M., P. Hoffmann, J. Ermann, K. Drago, C. G. Fathman, S. Strober, and R. S. Negrin. 2003. CD4+CD25+ regulatory T cells preserve graft-versus-tumor activity while inhibiting graft-versus-host disease after bone marrow transplantation. *Nat. Med.* 9: 1144–1150.
 56. Bevan, M. J. 1975. Interaction antigens detected by cytotoxic T cells with the major histocompatibility complex as modifier. *Nature* 256: 419–421.
 57. Bevan, M. J. 1976. Cross-priming for a secondary cytotoxic response to minor H antigens with H-2 congenic cells which do not cross-react in the cytotoxic assay. *J. Exp. Med.* 143: 1283–1288.
 58. Bevan, M. J. 1976. Minor H antigens introduced on H-2 different stimulating cells cross-react at the cytotoxic T cell level during in vivo priming. *J. Immunol.* 117: 2233–2238.
 59. Kuffová, L., M. Netuková, L. Duncan, A. Porter, B. Stockinger, and J. V. Forrester. 2008. Cross presentation of antigen on MHC class II via the draining lymph node after corneal transplantation in mice. *J. Immunol.* 180: 1353–1361.
 60. Liu, K., T. Iyoda, M. Saternus, Y. Kimura, K. Inaba, and R. M. Steinman. 2002. Immune tolerance after delivery of dying cells to dendritic cells in situ. *J. Exp. Med.* 196: 1091–1097.
 61. Williams, M. A., T. M. Onami, A. B. Adams, M. M. Durham, T. C. Pearson, R. Ahmed, and C. P. Larsen. 2002. Cutting edge: persistent viral infection prevents tolerance induction and escapes immune control following CD28/CD40 blockade-based regimen. *J. Immunol.* 169: 5387–5391.
 62. Yu, G., X. Xu, M. D. Vu, E. D. Kilpatrick, and X. C. Li. 2006. NK cells promote transplant tolerance by killing donor antigen-presenting cells. *J. Exp. Med.* 203: 1851–1858.
 63. Giannoukakis, N., B. Phillips, D. Finegold, J. Harnaha, and M. Trucco. 2011. Phase I (safety) study of autologous tolerogenic dendritic cells in type 1 diabetic patients. *Diabetes Care* 34: 2026–2032.
 64. Isaacs, J. D., and G. M. Bell. 2011. Autologous Tolerogenic Dendritic Cells for Rheumatoid Arthritis (AutoDECRA). ClinicalTrials.gov.
 65. Finkelman, F. D., A. Lees, R. Birnbaum, W. C. Gause, and S. C. Morris. 1996. Dendritic cells can present antigen in vivo in a tolerogenic or immunogenic fashion. *J. Immunol.* 157: 1406–1414.
 66. Bonifaz, L., D. Bonnyay, K. Mahnke, M. Rivera, M. C. Nussenzweig, and R. M. Steinman. 2002. Efficient targeting of protein antigen to the dendritic cell receptor DEC-205 in the steady state leads to antigen presentation on major histocompatibility complex class I products and peripheral CD8+ T cell tolerance. *J. Exp. Med.* 196: 1627–1638.
 67. Bruder, D., A. M. Westendorf, W. Hansen, S. Prettin, A. D. Gruber, Y. Qian, H. von Boehmer, K. Mahnke, and J. Buer. 2005. On the edge of autoimmunity: T-cell stimulation by steady-state dendritic cells prevents autoimmune diabetes. *Diabetes* 54: 3395–3401.
 68. Yamazaki, S., D. Dudziak, G. F. Heidkamp, C. Fiorese, A. J. Bonito, K. Inaba, M. C. Nussenzweig, and R. M. Steinman. 2008. CD8+ CD205+ splenic dendritic cells are specialized to induce Foxp3+ regulatory T cells. *J. Immunol.* 181: 6923–6933.
 69. Dudziak, D., A. O. Kamphorst, G. F. Heidkamp, V. R. Buchholz, C. Trumpfheller, S. Yamazaki, C. Cheong, K. Liu, H. W. Lee, C. G. Park, et al. 2007. Differential antigen processing by dendritic cell subsets in vivo. *Science* 315: 107–111.
 70. Mukhopadhyaya, A., T. Hanafusa, I. Jarchum, Y. G. Chen, Y. Iwai, D. V. Serreze, R. M. Steinman, K. V. Tarbell, and T. P. DiLorenzo. 2008. Selective delivery of beta cell antigen to dendritic cells in vivo leads to deletion and tolerance of autoreactive CD8+ T cells in NOD mice. *Proc. Natl. Acad. Sci. USA* 105: 6374–6379.
 71. Tanriver, Y., K. Ratnasothy, R. P. Bucy, G. Lombardi, and R. Lechler. 2010. Targeting MHC class I monomers to dendritic cells inhibits the indirect pathway of allorecognition and the production of IgG alloantibodies leading to long-term allograft survival. *J. Immunol.* 184: 1757–1764.

Supplementary Figures

Supplementary Figure 1. Cell survival analysis by MTT assay

BALB/c derived DCs were treated with DexD3 +/- LPS (DexD3-DCs, DexD3+LPS-DCs), or left untreated +/-LPS, (mature DCs, immature DCs). On day 7 cells were harvested and plated at a cell density of 1×10^5 cells/well of a 96 well plate. Cell viability was assessed over 48 hours using an MTT assay. The assay was done in triplicates for each DC type. Data plotted is the mean $\pm$ SD of 2 independent experiments. The mean optical density (OD) readings for each type of BM-DC were statistically compared by paired Students T tests and only statistically significant comparisons are shown.

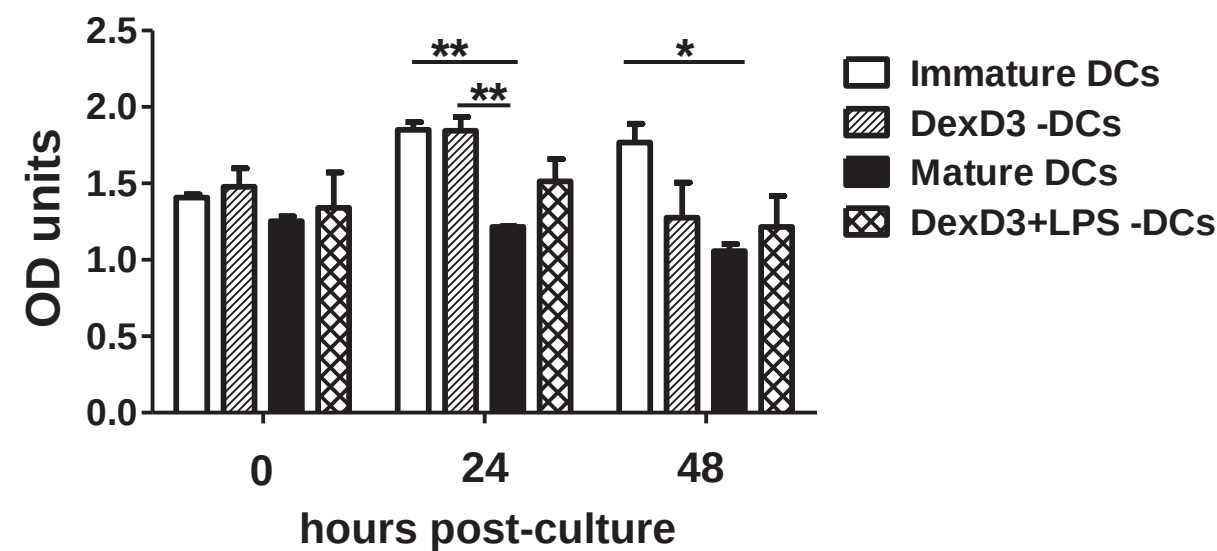
Supplementary Figure 2: CTLA4-Ig dose not affect skin graft survival time.

The skin transplant experiment described in **Figure 3(A)** was repeated with the additional treatment of mice with 20 μ g of CTLA4-Ig (squares), which was injected i.p one day after injection of the DCs (day 6 pre-transplant). Data shown is representative of 2 independent experiments.

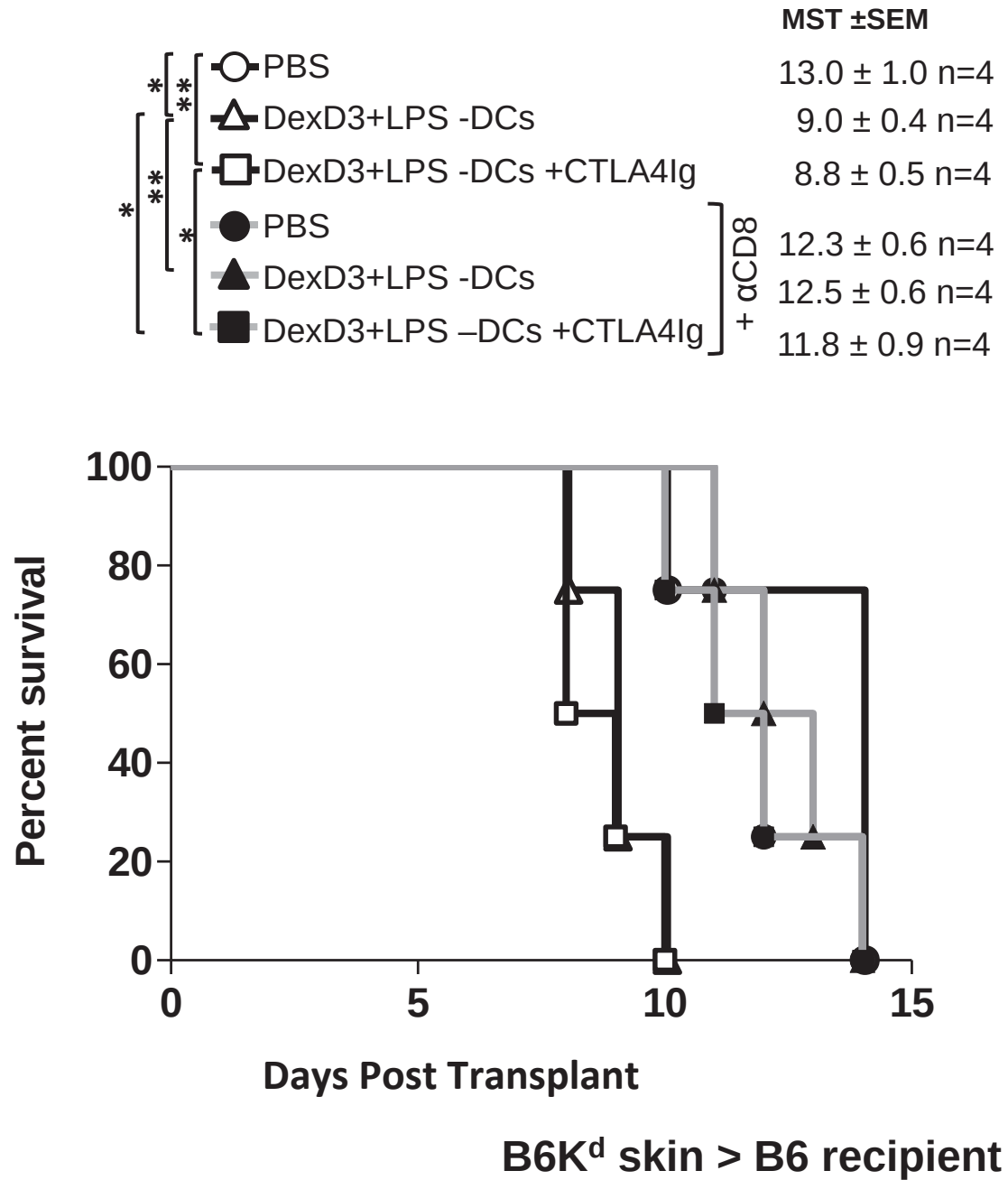
Supplementary Figure 3: CTLA4-Ig dose not affect TCR75 proliferation to injected alloantigen-bearing DCs.

The experiment described in **Figure 6** was repeated with the additional treatment of recipient mice with CTLA4-Ig (20 μ g), which was administered i.p 24 hours before and after DC injection. Splenocytes were harvested for the analysis of TCR75 proliferation at day 5 after treatment with DCs. Data shown is representative of 2 independent experiments.

Supplementary Figure 1



Supplementary Figure 2



Supplementary Figure 3

