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Donor-strain-derived immature dendritic cell pre-treatment induced hyporesponsiveness against allogeneic antigens

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Summary

The maturation of antigen-presenting dendritic cells (DCs) serves as an important determinant for the regulation of immunity, and overall immune response. We hypothesized that a reduced immune response to donor alloantigens and improved allograft survival could be induced by pre-treating recipients with bone-marrow-derived donor-strain fixed immature DCs (FIDCs). Donor-strain-derived mature and immature DCs were fixed before grafting to ensure that they possessed a stable immunogenic phenotype. The fixed mature DCs effectively induced allogeneic T-cell proliferation in recipients, whereas FIDCs were unable to elicit an allogeneic T-cell response. T cells that had previously been exposed to FIDCs maintained naïve phenotypes and were unable to extensively divide after injection into lethally irradiated donor-strain mice. The pre-treatment of recipients with donor-strain FIDCs markedly prolonged the survival of islet as well as skin allografts. However, T-cell hyporesponsiveness induced by FIDC injection was abrogated by the depletion of $CD4^+$ $CD25^+$ T cells. Consequently, FIDC-induced T-cell hyporesponsiveness could reflect anergy rather than specific deletion. Our findings suggest that FIDCs of donor strain could be used to induce long-term graft survival.

Keywords: clonal hyporeactivity; dendritic cells; long-term graft survival; maturation; tolerance

Introduction

Over the last four decades there has been a marked improvement in the short-term survival of allografts; however, long-term graft survival has remained relatively unchanged.[1,2] The discovery of a variety of potent immunosuppressive agents capable of preventing acute graft rejection, has been less effective against chronic graft loss. Most allograft recipients suffer from toxicities and complications associated with the use of immunosuppressive agents, ranging from susceptibility to opportunistic infection, to fatal malignancies. The induction of donor-specific allograft tolerance has increasingly demanded the need for a more definitive goal for the scientists working in this field to address.

Dendritic cells (DCs) are specialized antigen-presenting cells (APCs) that are unique in their capacity to present antigens for the activation of naïve T cells.[3] The induction of an appropriate immune response via T cells depends on DC-mediated antigen presentation. When DCs encounter antigens along with the appropriate signals, they begin to mature and express costimulatory molecules, hence triggering an immune response.[4–6] Recent studies have demonstrated that DCs play a critical role in the regulation of the immune reaction, possibly by influencing the spatial proximity between regulatory T cells

and effector T cells, and/or expressing regulatory cytokines.[7] Dendritic cells are capable of simultaneously presenting antigens to regulatory T cells and effector T cells, thereby bringing two classes of T cells into spatial proximity and facilitating 'linked suppression'.[8] Since the discovery of the immature DCs that were able to induce a state of hyporesponsiveness in T cells, the phenomenon has been used for the control or suppression of the immune response. In the fields of transplantation and autoimmune immunology, numerous attempts have been made to modify DCs to retain their immature phenotype for the induction of antigen-specific tolerance. To induce hyporesponsiveness in murine allograft models, various subtypes of DCs with an immature phenotype have been investigated.[9–11] However, previous studies have shown inconsistent results, possibly because of the spontaneous maturation of DCs *in vivo*, and the subsequent activation of an immune response.[12,13] The physical and biochemical properties of DCs perform crucial roles in dictating whether the elicited immune response is stimulatory or inhibitory. Several attempts have been made to render DCs maturation-resistant; however, only fixation of the plasma membrane has resulted in the abrogation of DC-induced T-cell proliferation. Although conflicting evidence exists, the type of APC used and the maturation status of DCs at the time of fixation are the primary factors that determine the induced alloimmune response.[14–16]

In this study, we used paraformaldehyde fixation to maintain and manage bone marrow (BM)-derived DCs in an immature state. Pre-treatment with donor strain-derived fixed immature DCs (FIDCs) was used to evaluate the efficacy of antigen presentation in allogeneic immune responses. In essence, the effects on the immune response to allograft survival and the possible immune mechanism of action were investigated.

Materials and methods

Animals

BALB/c (H-2^d), DBA2 (H-2^d), C57BL/6 (H-2^b), CB6F1 (H-$2^{b,d}$), C3H (H-2^k) and DBA1 (H-2^q) mice, aged 6–8 weeks, were purchased from Jackson Laboratories (Bar Harbor, ME) and from the Center for Animal Resource Development (Seoul National University, Seoul, Korea). The TEa $CD4^+$ T-cell receptor (TCR) transgenic mice were kindly provided by Dr Alexander D. Rudensky (University of Washington, Seattle). $CD4^+$ transgenic cells express a TCR that recognizes the peptide ASFEAQGALANIAVDKA in the context of I-A^b. This peptide corresponds to positions 52 to 68 from the α-chain of I-E class II molecules, and is expressed in all APCs from H-2^d/I-E^+ strains (CB6F1).[17] All mice were maintained in specific pathogen-free conditions at the Beth Israel Deaconess Medical Center animal facility or the Laboratory for Experimental Animal Research, the Clinical Research Institute, Seoul National University Hospital, Seoul, Korea.

Antibodies and flow cytometric analysis

The following fluorescence-conjugated antibodies were purchased from BD PharMingen (San Jose, CA); anti-CD4, anti-CD8, anti CD11c, anti-CD25, anit-CD62L, anti-CD40, anti-CD86, anti-GR1, anti TER119, RA3-3A1/6.1, anti-B220/CD45R, anti-IA^b and anti-Vβ6 TCR monoclonal antibodies (mAbs). Antibodies were used for sorting or staining surface molecules for analysis. Analyses were performed using FACSCalibur™ and FACScan flow cytometers (BD Biosciences, Franklin Lakes, NJ) with CellQuest software (BD Biosciences).

Bone-marrow DC isolation

The femurs and tibias of 6- to 8-week-old mice were harvested and the bone marrow was flushed and disrupted by pipetting with 2 ml RPMI-1640 using a syringe with a 26-gauge needle. Cells were suspended and passed through a cell strainer to remove small pieces of bone and debris, and red blood cells were lysed with ammonium chloride red blood cell lysis buffer (Sigma Aldrich, St Louis, MO). Following washing, granulocytes, B cells and red blood cell precursors were removed by magnetic adsorption cell sorting (MACS; Miltenyi Biotec, Auburn, CA) using a mAb cocktail of phycoerythrin-conjugated (PE-) anti-GR1, PE-anti-TER119, PE-anti-B220/CD45R and anti-PE microbeads according to the manufacturer's instructions. Then, 5×10^5 cells/ml were plated in 24-well plates (Nunc, Naperville, IL) in 1 ml of medium supplemented with 20 ng/ml recombinant granulocyte–macrophage colony-stimulating factor (rGM-CSF; BioSource, Camarillo, CA). The cultures were fed every 2 days by gently swirling the plates, aspirating 50% of the medium, and adding fresh medium containing rGM-CSF.

DC harvesting and manipulation

Dendritic cells were harvested 6 days after bone-marrow culture by removing $Gr1^+$ granulocytes from cultures using MACS with PE-anti-Gr1 mAb and anti-PE microbeads. To induce maturation of DCs, 1 μg/ml lipopolysaccharide (LPS; Salmonella Abortus Equi; Sigma-Aldrich) was added to the cell culture for 18 hr before harvest. To render DCs maturation-resistant, they were fixed by resuspension in 2% (weight/volume) paraformaldehyde in Dulbecco's modified Eagle's minimum essential medium (pH 8·0 buffer) for 10 min at 10°, then washed three times with Hanks' buffered salt solution (HBSS) buffer. Immature, fixed immature or fixed mature cells were used for the following experiments.

In vitro *mixed lymphocyte reaction*

T-cell-enriched single-cell suspensions were prepared from the spleens of mice using a T-cell enrichment column (R&D Systems Inc, Minneapolis, MN) according to the manufacturer's instructions. Fixed or non-fixed, immature or mature BM-derived DCs were cultured with 3×10^5 allogeneic T cells in 200 μl RPMI-1640 culture medium containing 10% fetal bovine serum, 1% penicillin/streptomycin and β_2-mercaptoethanol in U-bottom 96-well plates. In experiments where whole splenocytes were used, 5×10^5 cells were used as responders, or irradiated (25 Gy) allogeneic stimulators in the mixed lymphocyte reaction. After 3 days of culture, cells were pulsed with [^{3}H]thymidine (1 μCi per well, Amersham, UK) for 16 hr. Cells were harvested using a multi-well harvester and [^{3}H]thymidine incorporation was determined using a liquid scintillation counter. Results are expressed as the mean counts per minute ± standard deviation (SD) from triplicate cultures. In some experiments, T cells from TEa transgenic mice that were pretreated with FIDCs, were cultured for mixed lymphocyte reaction and analysed for the changes in phenotype using flow cytometry.

In vitro *culture of CD4$^+$ CD25$^+$ T cells*

Single-cell suspensions were prepared from lymph nodes of naive CB6F1 mice and cells were stained with fluorescein isothiocyanate (FITC)-conjugated anti-CD4 and PE-conjugated anti-CD25 (PharMingen). The CD4$^+$ CD25$^-$ cells and CD4$^+$ CD25$^+$ T cells were isolated by FACSAria (BD Bioscience). The purity of the selected CD4$^+$ CD25$^-$ cells and CD4$^+$ CD25$^+$ T cells was greater than 98%. Immature and mature DCs (5×10^4) were fixed with 2% paraformaldehyde and co-cultured with CD4$^+$ CD25$^+$ T cells (5×10^5) for 96 hr in a 96-well U-bottom plate. [^{3}H]Thymidine (1 μCi/well) was added for the last 16 hr. Plates were harvested and [^{3}H]thymidine incorporation was evaluated in counts per minute (c.p.m.).

In vivo *proliferation assay with CFSE staining*

Single-cell suspensions of spleen and lymph nodes were prepared from naïve mice or allogeneic DC-pretreated mice and labelled with a 5-carboxyfluorescein diacetate succinimidyl ester (CFSE; Molecular Probe, Inc. Portland, OR) tracking fluorochrome as previously described.[18] Allogeneic mice were lethally irradiated (1000 rad) and subsequently injected with 6×10^7 to 8×10^7 CFSE-labelled cells via the tail vein. The mice were killed after 3 days, and spleen and lymph node mononuclear cells were harvested and stained with directly conjugated mouse monoclonal antibodies against CD4, CD8 and H-2^k. The adoptively transferred cells were identified by gating H-2^k+. The patterns of T-cell proliferation were analysed as the intensity of CFSE. In some experiments, CD4$^+$ CD25$^+$ T cells from C3H mice that were pretreated with FIDCs, were depleted and the purified CD4$^+$ CD25$^-$ T cells were adoptively transferred into the lethally irradiated host.

Islet transplantation

The transplantation of islet cells was performed on naïve or CB6F1 DC-pretreated C3H mice as previously described.[19] Briefly, CB6F1 donor pancreata were perfused with 3·5 ml HBSS containing collagenase type IV 1·5 mg/dl (Sigma-Aldrich) through the common bile duct and incubated at 37° for 14 min. Islets were released from the pancreata and purified in discontinuous Ficoll (Sigma-Aldrich) gradients. The harvested islets were washed in HBSS, and in excess of 600 islets were transplanted under the renal capsule of each C3H recipient, which had been rendered diabetic by a single intraperitoneal injection of streptozotocin (250 mg/kg; Sigma-Aldrich). Allograft function was monitored by serial blood glucose measurements. Primary grafts were considered to be functional if the blood glucose level was < 200 mg/dl 3 days after transplantation. Grafts were considered to have been rejected if two consecutive elevations in blood glucose > 300 mg/dl occurred following a period of primary graft function. In some recipients, CD4$^+$ CD25$^+$ T cells of C3H recipients were depleted using a mAb (PC61) before and after DC pre-treatment (days –1 and 3). To evaluate the strain specificity, islets from CB6F1 (same as primary graft) or from DBA1 (third-party graft; H-2^q) mice were grafted on the right kidney of the mice that had long-term primary graft function.

Skin transplantation

Full-thickness tail skin from CB6F1 mice was grafted onto the thoracic walls of naïve or CB6F1 DC-pretreated C3H recipients. Graft survival was monitored daily and was considered to have been rejected if complete necrosis occurred.

Localization of DCs

For the localization of infused DCs in secondary lymphoid organs, DCs stained with CFSE with/without fixation were introduced into mice via the tail vein. After 3 and 24 hr, the spleen was harvested and minced into a single-cell suspension. CFSE$^+$ cells were counted using a flow cytometer.

Immunohistochemistry

Spleens were harvested from recipient mice at 3 hr after adoptive transfer, embedded in OCT medium (Miles Inc.,

Elkhart, IN), and frozen at 80°. Frozen sections 6 μm thick were fixed for 10 min in cold acetone. For immunofluorescence analysis, the sections were stained overnight at 4° with 2 μg/ml rat anti-mouse CD4 (R&D Systems Inc.) for T cells and 2 μg/ml rat anti-mouse B220 (R&D Systems Inc.) for B cells in blocking reagent. A second layer of Alexa Fluor® 488-conjugated anti-rat antibody (Molecular Probes, Eugene, OR) was applied and incubated at room temperature for 40 min. Primary antibodies were omitted from sections used as negative controls. Sections were evaluated blindly and randomly. Incorporation of DCs labelled with CM-DiI (Molecular Probes) and DAPI (Sigma Aldrich), was examined using confocal laser-scanning microscopy (Carl Zeiss, Jena, Germany) under × 200 magnification.

Statistical analysis

Statistical analyses were performed using chi-squared tests, Kaplan–Meier cumulative plots and log-rank tests where appropriate. *P*-values of $< 0{\cdot}05$ were considered to be statistically significant.

Results

The effect of fixation on BM-derived DCs.

Bone-marrow-derived DCs were harvested from CB6F1 mice and cultured in media which contained rmGM-CSF. After 6 days of culture the majority (73–84%) of cells isolated by negative selection using anti-Gr1 mAb microbeads were CD11c-positive DCs. In the absence of LPS stimulation the expression of CD40, CD86 and major histocompatibility complex (MHC) class II molecules on harvested DCs was low (Fig. 1a) indicating that these DCs were predominantly immature. Stimulation of the immature DCs with LPS led to the increased expression of CD40, CD86 and MHC class II (Fig. 1a) molecules, which were indicative of maturation. Following cell sorting and fixation of immature BM-derived DCs with 2% paraformaldehyde, stimulation with LPS did not up-regulate the expression of CD40, CD86 or MHC II, indicating that fixation rendered cells effectively resistant to changes in maturity (Fig. 1a, middle column). Again, the maturation phenotypes of DCs induced by LPS stimulation were not changed with LPS re-stimulation when these DCs were fixed with paraformaldehyde (Fig. 1a, right column).

To investigate whether fixation preserved the capacity of DCs to stimulate allogeneic T-cell proliferation, immature and mature CB6F1 (H-$2^{b.d}$) BM-derived DCs were isolated and fixed as described earlier. Following fixation, either immature or mature cells were used to stimulate C3H (H-2^{k}) T cells *in vitro*. When DCs were co-cultured with allogeneic T cells, fixed mature DCs (FMDCs) induced the proliferation of allogeneic T cells at various DC : T-cell ratios, although FMDCs were slightly less effective than irradiated BM-derived DCs at inducing allogeneic T-cell proliferation. This implies that paraformaldehyde fixation does not abrogate the capacity of DCs to stimulate allogeneic T-cell proliferation when DCs were in a matured state. Conversely, the proliferation of allogeneic T cells co-cultured with FIDCs was minimal regardless of the DC : T-cell ratio, implying that FIDCs are unable to stimulate allogeneic T-cell proliferation (Fig. 1b).

Effect of donor strain FIDC pre-treatment on allogeneic T-cell responses

The absence of an allogeneic T-cell response to FIDCs *in vitro* led us to hypothesize that FIDCs may be able to suppress the allogeneic immune response *in vivo*. To test our hypothesis, LPS-stimulated or non-stimulated BM-derived DCs isolated from CB6F1 (H-$2^{b.d}$) mice were fixed (i.e. FMDCs or FIDCs), and were injected into naïve C3H (H-2^{k}) mice (2×10^{6} DCs per mouse) via the tail vein. Immature, non-fixed DCs were also used for pre-treatment as control. After 2 weeks, splenocytes were harvested from DC-pretreated C3H mice or naïve C3H mice and labelled with CFSE before being injected into lethally irradiated CB6F1 hosts. To monitor the proliferation of T cells, splenocytes were isolated from the CB6F1 hosts after 3 days. $CD4^{+}$ T cells from naive C3H or immature, non-fixed DC-pretreated C3H mice proliferated vigorously in CB6F1 hosts in response to alloantigens, and a significant proportion of cells underwent at least eight divisions. $CD4^{+}$ T cells harvested from C3H mice that had been pre-treated with FMDCs 2 weeks earlier showed a similar proliferation pattern to $CD4^{+}$ T cells from naïve C3H mice. This implied that fixation of matured DCs did not destroy the potential of T cells to proliferate in response to alloantigen. Conversely, $CD4^{+}$ T cells harvested from C3H mice that had been pre-treated with FIDCs from CB6F1 mice 2 weeks earlier did not undergo more than two cell divisions in irradiated CB6F1 mice. This implied that the allogeneic T-cell population was hyporesponsive to CB6F1 alloantigens (Fig. 2). However, T cells from C3H mice that were pre-treated with FIDCs of DBA1 (H-2^{q}) proliferated in the irradiated CB6F1 host (as third-party control) showing similar dynamics to the naïve T cells (Fig. 2 last row). We also evaluated the homeostatic proliferation of CB6F1 T cells and confirmed the proliferation although it was not as vigorous as the allogeneic response (fifth row). $CD8^{+}$ T cells showed a similar pattern of proliferation to that observed with $CD4^{+}$ T cells.

Donor strain FIDC pre-treatment and allograft survival

The hyporesponsiveness of allogeneic T cells in response to pre-treatment with FIDCs implied that donor strain

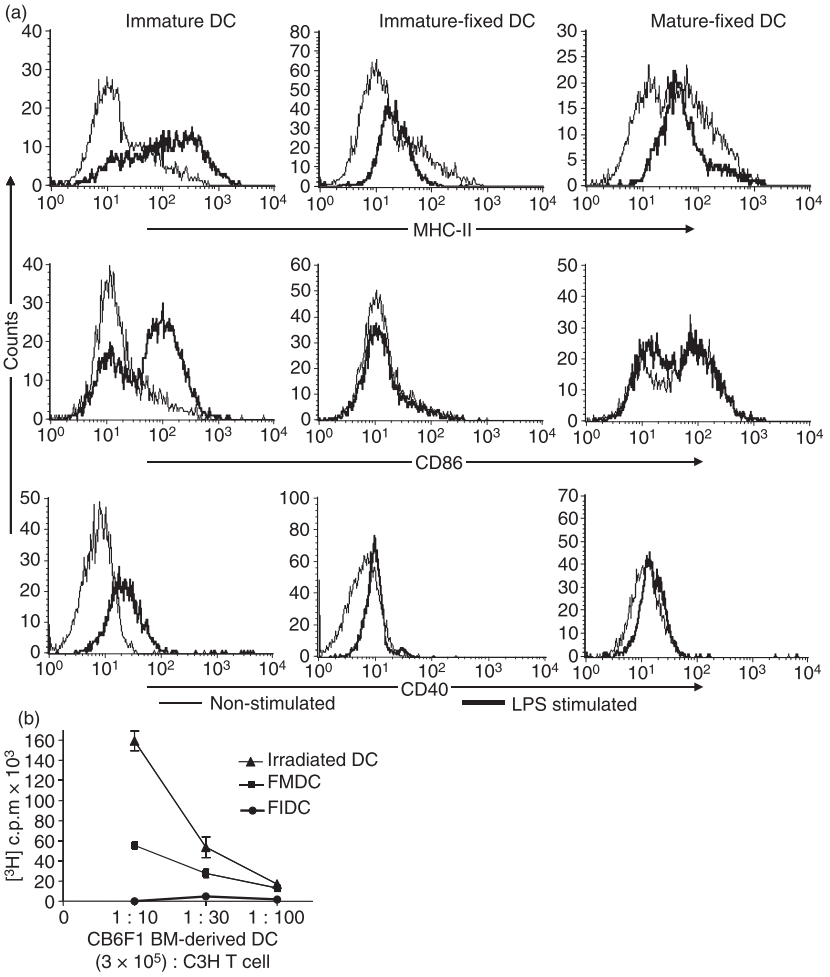

Figure 1. Fixed dendritic cells (DCs) remain biologically effective. Bone-marrow (BM)-derived DCs harvested from CB6F1 mice were used to analyse cell surface molecules (a) or to stimulate allogeneic T cells for proliferation (b). (a) Expressions for I-A^b (major histocompatibility complex class II; MHC-II), CD86 and CD40 were assayed using BM-derived DCs. When BM-derived immature DCs were stimulated with lipopolysaccharide (LPS; left column, thick line), the expression of all three markers increased significantly, compared with non-stimulated BM-derived DCs (left column, thin line). However, after fixation, LPS stimulation did not enhance the expression of these costimulatory molecules or MHC II molecules on BM-derived DCs (middle column). When BM-derived DCs were matured and fixed, re-stimulation with LPS did not change the expressions of these molecules (right column). (b) Different numbers of irradiated DCs (triangular dot), fixed mature DCs (FMDCs; square dot) and fixed immature DCs (FIDCs; round dot) from CB6F1 mice were co-cultured with allogeneic C3H T cells. FIDCs did not induce the proliferation of C3H T cells, while FMDCs retained the stimulatory effect on allogeneic C3H T cells comparable to irradiated DCs. T cells were cultured for 96 hr and [^{3}H]thymidine (1 μCi/well) was added for the last 16 hr.

FIDC pre-treatment may improve the long-term survival of allogeneic grafts. To test this hypothesis, an MHC-mismatched islet and skin transplant model was utilized. Immature or mature BM-derived DCs were harvested from CB6F1 mice, fixed and injected into C3H recipients. Some recipients were injected with unfixed immature CB6F1 BM-derived DCs. After 14 days, the C3H mice were grafted with CB6F1 islets after being rendered diabetic with streptozotocin. Pre-treatment with donor strain FIDCs markedly prolonged islet allograft survival, and some grafts survived for more than 100 days (Fig. 3), whereas naïve C3H mice rejected the islet allograft (mean survival time; MST, 13 days, $n = 4$). Conversely, pretreatment with donor strain FMDCs or unfixed immature donor strain BM-derived DCs did not prolong islet allograft survival (MST < 20 days) in both groups. Surgical removal of the left kidney bearing the islet allograft was performed after 120 days of engraftment. In the absence of further immunosuppressive therapy, a second CB6F1 islet allograft was successfully engrafted without evidence of rejection. In contrast, islet transplants from the third party (DBA1; H-2^q) were acutely rejected.

To confirm the increased allograft tolerance following FIDC treatment in a more stringent model, skin grafts from CB6F1 donors were transplanted onto C3H mice that had been pre-treated with FIDCs from CB6F1 mice. Long-term skin allograft survival of more than 120 days MST was accomplished in five of nine recipients. The untreated control C3H mice rejected the skin allograft within 14 days (Fig. 4). Third-party skin grafts from DBA1 (H-2^q) mice onto C3H mice that had been pre-treated with CB6F1 FIDCs were rejected within a time-frame similar to that for grafts transplanted onto naïve mice. This implied that donor strain FIDC-induced hyporesponsiveness to allografts was alloantigen-specific.

Distribution of transferred BM-derived DCs

Dendritic cells are known to interact and present antigens to T cells in lymphoid organs. To test whether fixed BM-derived DCs could localize to lymphoid organs and interact with alloreactive T cells, CFSE labelled FIDCs, non-fixed CFSE-labelled DCs, or phosphate-buffered saline controls were injected into C3H mice. After 3 hr,

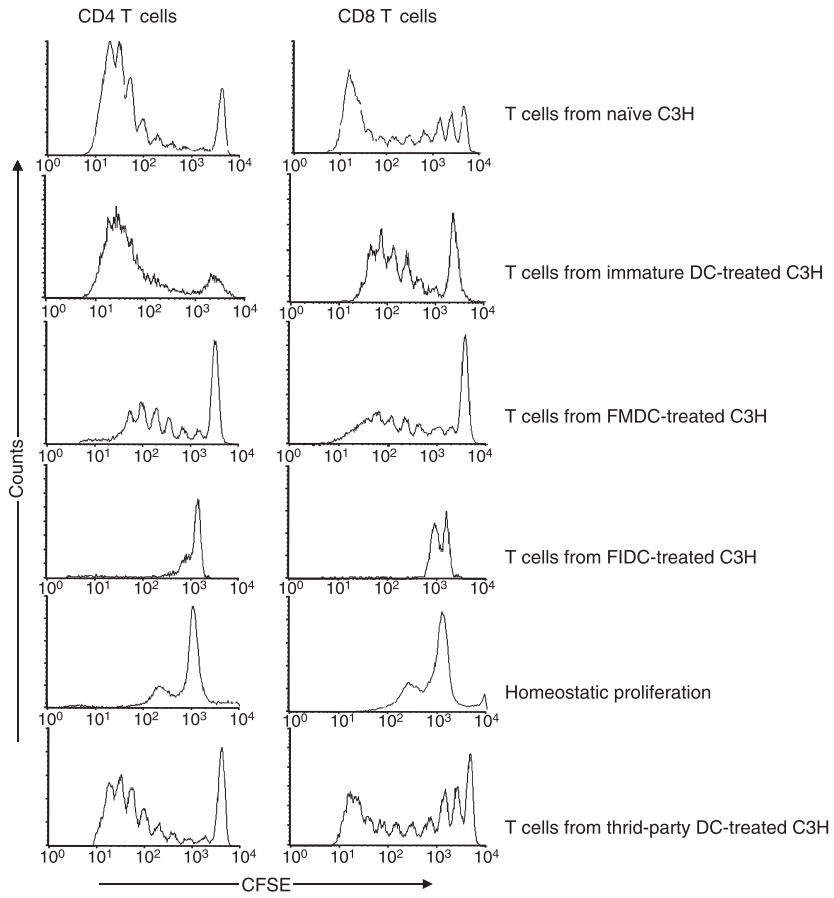

Figure 2. Fixed immature dendritic cell (FIDC) pre-treatment suppressed alloreactive T-cell proliferation *in vivo*. Splenocytes were recovered from naïve C3H, CB6F1 fixed mature dendritic cell (FMDC)-prereated C3H mice, CB6F1 FIDC-pretreated C3H mice and DBA1 FIDC-pretreated C3H mice and labelled with 5-carboxyfluorescein diacetate succinimidyl ester (CFSE). These cells were adoptively transferred into lethally irradiated CB6F1 hosts and then allowed to proliferate. T cells from CB6F1 FIDC pre-treated C3H did not proliferate in CB6F1 hosts, while T cells from naïve and CB6F1 FMDC-pretreated mice showed significant proliferation. FIDC from third-party donor (DBA1; H-2^q) was not effective to show hyporesponsiveness of T cells against allogeneic antigens (fifth row). Homeostatic proliferation of T cells from CB6F1 was evaluated as control (fourth row).

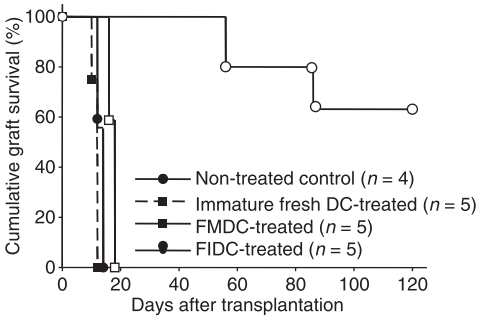

Figure 3. Donor strain fixed immature dendritic cell (FIDC) pre-treatment prolonged islet allograft survival. Bone marrow (BM)-derived DCs were prepared from CB6F1 mice and transplanted into C3H recipients with or without stimulation and/or fixation. After 2 weeks, CB6F1 islets were transplanted into diabetic C3H recipients. While naïve C3H recipients (filled dot), C3H hosts pre-treated with immature unfixed CB6F1 BM-derived DCs (filled square) and C3H hosts pre-treated with fixed mature dendritic cells (FMDCs; empty square) rejected the islet allografts within 20 days, the C3H recipients pre-treated with CB6F1 FIDC (three out of five recipients) showed a significantly prolonged allograft survival. This is one of two independent transplantation experiments and the other experiments showed the same result.

spleens were harvested, and analysed by flow cytometry. As expected, $CFSE^+$ cells were detectable outside the lymphocyte fraction by flow cytometric analysis. Splenocytes harvested from mice that were injected with non-fixed immature CB6F1 BM-derived DCs comprised 1·2% $CFSE^+$ cells, and splenocytes harvested from mice injected with FIDCs comprised 0·6% $CFSE^+$ cells (Fig. 5a). At 24 hr after DC injection, CFSE fluorescence was detected in 0·44% of non-fixed DC-treated splenocytes and 0·43% of fixed DC-treated splenocytes. Immunohistochemical localization of infused DCs was performed using confocal microscopy. CM-DiI labelled DCs were detectable along with T and B cells in the spleen irrespective of prior fixation (Fig. 5b). These DiI^+ DCs were nucleated intact cells as confirmed by DAPI positivity (Fig. 5b third row).

The association of $CD4^+$ $CD25^+$ T cells for hyporesponsiveness after FIDC pretreatment

Investigators have recently begun to elucidate the role of DCs in the active regulation of the immune response, particularly with regard to $CD4^+$ $CD25^+$ regulatory T cells. We hypothesized that donor strain FIDC pre-treatment abrogated the allogeneic T-cell response via $CD4^+$ $CD25^+$ regulatory T cells. To test our hypothesis, we harvested splenocytes from C3H mice that had been pre-treated with FIDCs from CB6F1 mice and depleted the population of $CD25^+$ cells using MACS with a biotin-conjugated anti-CD25 mAb and streptavidin beads. The

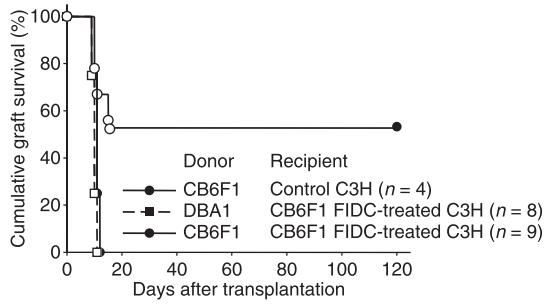

Figure 4. Fixed immature dendritic cell (FIDC) pre-treatment prolonged skin allograft survival in a donor-specific manner. C3H recipients pretreated with CB6F1 FIDCs were grafted with skin from CB6F1 or DBA1 mice. Skin grafts from CB6F1 mice showed rejection-resistance (open circle, five out of nine recipients), while the recipients rejected the third-party skin grafts promptly (open square, i.e. DBA1; H-2^q) irrespective of pre-treatment as non-treated controls (filled circle). This is one of two independent transplantation experiments and the other experiment showed similar result.

CD25-depleted cells were labelled with CFSE and injected into irradiated CB6F1 mice. The proliferation pattern of the FIDC-pre-treated, CD25-depleted C3H T cells was similar to that of naïve C3H T cells, implying that FIDC-induced hyporesponsiveness was mediated, at least in part, by $CD25^+$ regulatory T cells (Fig. 6a). Similar proliferation patterns were observed in other experiments with C3H mice that had been depleted of $CD25^+$ T cells using an *in vivo* anti-CD25 antibody before DC introduction. These mice had been injected with FIDCs from CB6F1 mice, and splenocytes harvested after 2 weeks to analyse CFSE-stained C3H T-cell proliferation in lethally irradiated CB6F1 mice (data not shown). The association of $CD4^+$ $CD25^+$ regulatory T cells in FIDC-induced hyporesponsiveness was further probed using *in vitro* proliferation capacity. When $CD4^+$ $CD25^+$ regulatory T cells (C3H) were isolated and co-cultured with immature DCs (CB6F1), there was obvious clonal expansion of regulatory T cells. However, fixed DCs did not induce the proliferation of $CD4^+$ $CD25^+$ regulatory T cells during 4 days of co-culture (Fig. 6b). To evaluate the effect of $CD4^+$ $CD25^+$ regulatory T cells on graft survival, we depleted these cells of C3H recipients using mAbs before the FIDC pre-treatment. As shown in Fig. 6(c), the diabetic recipients that were depleted of $CD4^+$ $CD25^+$ T cells at days – 1 and 3 of FIDC pre-treatment, rejected the islet graft promptly, but the presence of $CD4^+$ $CD25^+$ T cells at the time of DC pre-treatment was effective in maintaining the allogeneic graft.

To evaluate the effects of incomplete antigen presentation on the responding T-cell clones, we analysed the activation status of T cells using *in vitro* mixed lymphocyte reaction. The FIDCs from CB6F1 mice were injected into TEa transgenic mice. $CD4^+$ T cells from Tea transgenic mice express a TCR that recognizes the peptide ASFEAQGALANIAVDKA in the context of $I\text{-}A^b$ (peptide corresponds to positions 52 to 68 from the α-chain of I-E class II molecules).[17] After 2 weeks, purified T cells were cultured with irradiated splenocytes from CB6F1 mice. $CD4^+$ T cells from TEa transgenic mice that had been pre-treated with FIDCs from CB6F1 mice showed a significant decrease in proliferation (65% reduced at 5 days of culture, $P < 0{\cdot}05$; Fig. 6d), and retained the expression of CD62L (Fig. 6e). $CD4^+$ T cells taken from naive TEa transgenic mice readily responded to the cognate antigen and showed down-regulation of CD62L expression. To assess the relationship between hyporesponsiveness induced by FIDC pre-treatment and cellular apoptosis, T-cell apoptosis was evaluated by Annexin-V staining. Although the rate of apoptosis varied between experiments, the number of Annexin-V-positive T cells was consistently less in co-cultured systems when T cells from pre-treated transgenic mice were primed with FIDCs (Fig. 6f) than when T cells from naïve transgenic mice were used.

Discussion

In this study we examined the allogeneic immune response of recipients following pre-treatment with donor-strain-derived FIDCs. Since the immaturity of DCs is important for the maintenance of self tolerance,[5] many investigators have tried to enhance allograft tolerance using immature DCs.[4,6,20–22] However, previous attempts have not shown consistent results, possibly because the immature DCs mature spontaneously following the *in vivo* state and so can activate the immune response.[12] To overcome the plasticity associated with the DC phenotype, we treated cells with paraformaldehyde to render them unable to mature. We used 2% paraformaldehyde as the fixative agent in our experiments because lower concentrations can be ineffective.[15] Previous studies have used rGM-CSF at low concentrations in culture,[23] or blocking nuclear factor-κB oligodeoxyribonucleotide[24] to prevent DC maturation. Both of these studies produced excellent results, but demonstrated limitations because of the low number of DCs used, and activation of the nuclear factor-κB-independent pathway of DC stimulation. In our study, we were able to obtain sufficient numbers of DCs from donor bone marrow, as previously described.[20] The fixation of cell membranes rendered the cells incapable of up-regulating surface proteins, including costimulatory molecules, irrespective of the activation signals or pathways involved (Fig. 1).

One particular concern regarding this approach was that fixation might have destroyed the ability of DCs to function as APCs, thereby abrogating their ability to stimulate allogeneic T cells to proliferate. However, fixation did not eliminate the potential for allogeneic T-cell proliferation (Fig. 1b). When mature DCs were fixed and used

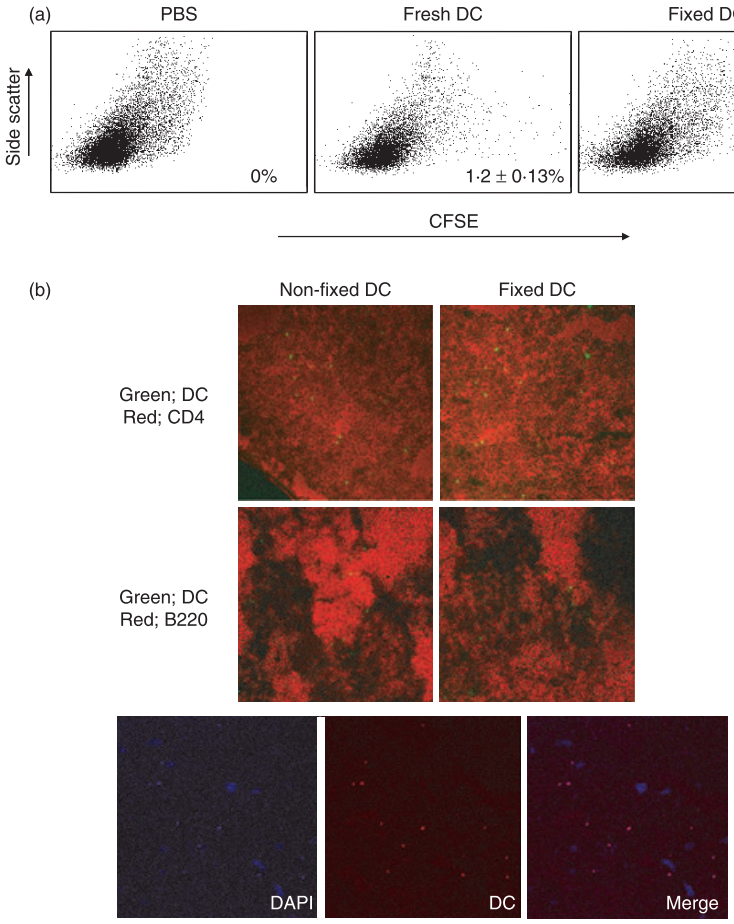

Figure 5. Transplanted, fixed dendritic cells (DCs) were incorporated into host spleen. (a) Three hours after injection with 5-carboxyfluorescein diacetate succinimidyl ester (CFSE)-labelled fresh or fixed DCs, the presence of transplanted DCs in spleens was analysed. Control mice were injected with phosphate-buffered saline. CFSE intensity was analysed by fluorescence-activated cell sorter; 0·6–1·2% of splenocytes were CFSE positive. (b) Cryosectioned spleen tissue was examined by confocal microscopy (original magnification × 200). CM-DiI labelled DCs and Alexa Fluor® 488-conjugated antibody labelled T or B cells were traced. Labelled DCs were nucleated cells as confirmed by DAPI staining.

for allogeneic stimulation, T cells actively proliferated, although the magnitude of proliferation was less than the magnitude observed using irradiated DCs. This finding suggests that fixed cell surface proteins are able to present antigens to T cells, and that the maturation status of DCs was the crucial factor for the induction of an immune response. Yamazaki *et al.* demonstrated that fixed DCs could stimulate the proliferation of T cells using *in vitro* culture systems, although unfixed cells were more effective in activating T-cell expansion.[25] Although the results of chemical fixation of APCs on the allogeneic immune response are conflicting, the determining factors that lead fixed APCs to be stimulatory or inhibitory were shown to be the cell types used and the membrane properties at the time of fixation.[14–16]

The observation of *in vitro* hyporesponsiveness of allogeneic T cells to FIDCs encourages us to investigate this phenomenon using an *in vivo* allogeneic immune response model. By monitoring T cells in irradiated donor hosts using CFSE fluorescence, it was revealed that FIDC pre-treatment abrogated donor-antigen-induced T-cell proliferation (Fig. 2). Consistent with these results, the pre-treatment of recipients with donor-strain FIDCs enabled the prolonged survival of engrafted skin and islet cells (Figs 3, 4). The immunomodulatory effect of FIDCs was also donor specific; third-party grafts were rejected within a similar time-frame to grafts rejected by naïve recipients. These data suggest that pretreatment of recipients with fixed DCs did not render hosts immunocompromised, instead it induced antigen-specific hyporesponsiveness presented by the immature DCs. The finding that claimed that fixed DCs were able to reach the spleen after *in vivo* transfer (Fig. 5) also supported our hypothesis. Dendritic cells are expected to interact with T cells in secondary lymphoid organs to direct the immune response against antigens. We confirmed that the introduced DCs reached lymphoid organs in intact-cell state because CM-DiI$^+$ cells were also DAPI$^+$ (Fig. 5b). To induce the full activation of the immune response, a period of around 24–48 hr of DC–T-cell interaction is required, however, for aborted activation of cognate T cells the duration of the DC–T-cell interaction appears to be shorter.[26,27] We demonstrated that FIDCs were present in the spleen following intravenous injection for at least 24 hr as reported previously.[23,24] We therefore speculate that the introduced FIDCs remain in secondary lymphoid organs long enough to elicit a DC–T-cell interaction, or suppression of the immune response.

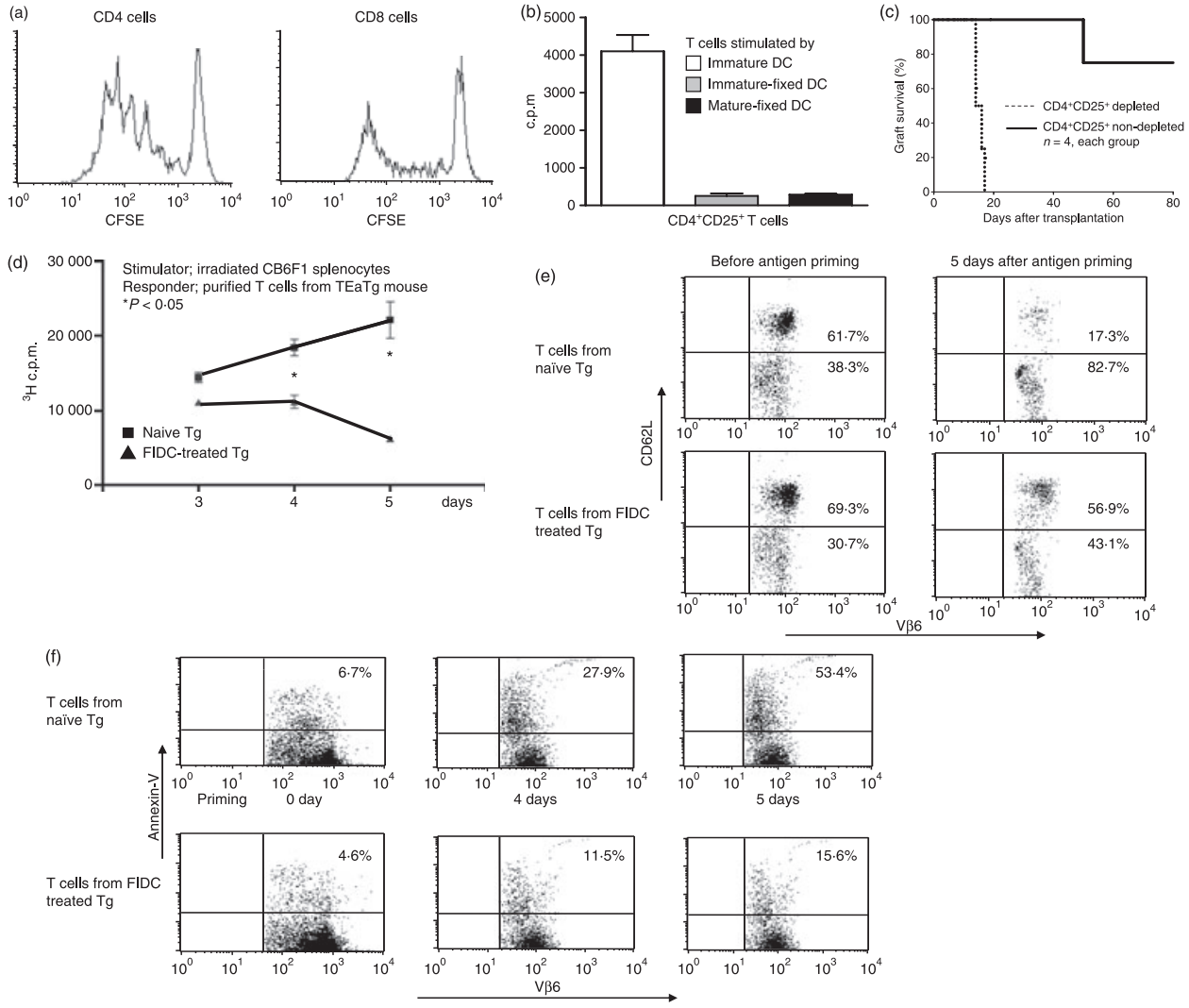

Figure 6. T-cell hyporesponsiveness induced by fixed immature dendritic cells (FIDCs) is dependent upon $CD4^+$ $CD25^+$ regulatory T cells and shows clonal hyporeactivity. (a) Splenocytes were harvested from C3H mice pretreated with CB6F1 FIDC and $CD4^+$ $CD25^+$ cells were shown to be depleted. The proliferation pattern of CB6F1 FIDC pre-treated, $CD25^+$ cell depleted C3H T cells was similar to that of naïve C3H T cells. (b) $CD4^+$ $CD25^+$ T cells from C3H mice were stimulated by either un-fixed or fixed dendritic cells from CB6F1 mice. Allogeneic DCs induced proliferation of $CD4^+$ $CD25^+$ T cells but fixed DCs did not induce cell proliferation in 4 days of culture system. T cells were cultured for 96 hr and [^{3}H]thymidine (1 μCi/well) was added for the last 16 hr. (c) The recipients whose $CD4^+$ $CD25^+$ T cells were depleted at the time of FIDC pre-treatment rejected the graft promptly, whereas the recipients with intact $CD4^+$ $CD25^+$ T cells during FIDC pre-treatment retained the graft successfully (n = 4, each group). (d) $CD4^+$ T cells from TEa transgenic mice that had been pre-treated with FIDCs from CB6F1 mice showed a significant decrease in proliferation upon cognate antigen priming, while $CD4^+$ T cells from naïve TEa transgenic mice proliferated actively. (e) $CD4^+$ T cells from TEa transgenic mice that had been pre-treated with FIDCs from CB6F1 mice retained the expression of CD62L in significant portions of Vβ6 T cells, but T cells from naïve Tea transgenic mice were activated after cognate antigen priming. (f) When T cells from pre-treated transgenic mice were primed with FIDCs, the number of annexin-V^+ cells was less than in the co-cultured system with T cells from naïve transgenic mice.

The precise mechanisms of the induction of donor-specific tolerance using DC transplantation remain to be elucidated.[28,29] Dendritic cells have been shown to play a role in the deletion of alloreactive T cells.[30,31] Considering the remarkably high frequency of alloreactive T cells, it seems possible that deletion mechanisms play a key role in the induction of tolerance to MHC-mismatched allografts.[32–35] In addition, recent investigations have revealed that DCs are involved in the expansion of immunoregulatory T cells such as $CD4^+$ $CD25^+$ T cells, which are currently being intensely investigated for their role in the active regulation of both self and allograft tolerance.[9,36,37] These findings suggest that DCs have the potential to regulate the immune response via effector T

cells and/or regulatory T cells, and reinforce trials expanding regulatory T cells.[38] Our study focused on the role of $CD4^+$ $CD25^+$ regulatory T cells in the immunomodulatory effects of fixed immature DCs. In the absence of $CD25^+$ T cells the immunomodulatory effect of FIDC pre-treatment was abolished, exemplifying the importance of $CD25^+$ regulatory T cells in mediating the immunomodulatory effect of FIDCs (Fig. 6). The hyporesponsiveness of T cells can be explained by the nature of anergic T cells, which is characterized by inefficient T-cell activation.[39] Our qualitative study clearly demonstrates that the exposure of functional cognate antigens to T cells is critical for the induction of T-cell activation (Fig. 6).

This study has demonstrated that fixed immature donor-derived DCs can be used to induce donor-specific immune modulation in transplant recipients, thereby revealing their potential use in transplantation medicine. As the mechanisms underlying the effects of donor-derived DC pre-treatment are elucidated, their clinical application may provide a dependable therapeutic modality.

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