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Mechanism and Localization of CD8 Regulatory T Cells in a Heart Transplant Model of Tolerance

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Despite accumulating evidence for the importance of allospecific CD8$^+$ regulatory T cells (Tregs) in tolerant rodents and free immunosuppression transplant recipients, mechanisms underlying CD8$^+$ Treg-mediated tolerance remain unclear. By using a model of transplantation tolerance mediated by CD8$^+$ Tregs following CD40Ig treatment in rats, in this study, we show that the accumulation of tolerogenic CD8$^+$ Tregs and plasmacytoid dendritic cells (pDCs) in allograft and spleen but not lymph nodes was associated with tolerance induction in vascularized allograft recipients. pDCs preferentially induced tolerogenic CD8$^+$ Tregs to suppress CD4$^+$ effector cells responses to first-donor Ags in vitro. When tolerogenic CD8$^+$ Tregs were not in contact with CD4$^+$ effector cells, suppression was mediated by IDO. Contact with CD4$^+$ effector cells resulted in alternative suppressive mechanisms implicating IFN-γ and fibroleukin-2. In vivo, both IDO and IFN-γ were involved in tolerance induction, suggesting that contact with CD4$^+$ effector cells is crucial to modulate CD8$^+$ Tregs function in vivo. In conclusion, CD8$^+$ Tregs and pDCs interactions were necessary for suppression of CD4$^+$ T cells and involved different mechanisms modulated by the presence of cell contact between CD8$^+$ Tregs, pDCs, and CD4$^+$ effector cells. *The Journal of Immunology*, 2010, 185: 823–833.

Although CD4$^+$ regulatory T cells (Tregs) have received considerably more attention (1), CD8$^+$ Tregs have been identified both as naturally occurring cells and after different immunotherapy regimen in various pathophysiological situations (2). In organ transplantation, CD8$^+$ Tregs have been shown to play a central role in vivo in donor-specific blood transfusion-induced tolerance (3) and anti–ICOS-treated mice (4). Furthermore, we have shown in rats that blockade of CD40–CD40L interactions with CD40Ig-induced tolerogenic CD8$^+$CD45RC^{low} Tregs, which generated infectious tolerance upon adoptive transfer (5). CD8$^+$CD45RC^{low} Tregs produced IFN-γ, and tolerance was dependent on both IFN-γ and IDO (5). In human transplantation studies, CD8$^+$ Tregs have not only been described in kidney (6)-, heart (7, 8)-, and liver-intestine (9)–transplanted patients but also associated with less rejection episodes (7, 8) and even tolerance (6). Although these observations highlight an important role of CD8$^+$ Tregs in allograft tolerance, there is a little information about the cellular interactions and anatomical sites associated with their generation, migration, and tolerogenic properties.

CD4$^+$ and CD8$^+$ Treg (1, 2) function can be modulated by conventional dendritic cells (cDCs) and plasmacytoid DCs (pDCs). pDCs were shown to induce CD8$^+$ Tregs in humans (6, 10, 11), mice, and rats (12–14). In a mouse cardiac transplantation model, pDCs were reported to induce tolerance by inducing CD4$^+$CD25$^+$ Tregs in lymph nodes (LNs), and the migration of pDCs to LNs was crucial to induce tolerance (13). Obviously, different DC subsets and their migration profiles may exert different influences in the development of CD8$^+$ Tregs and their regulatory function.

The in vivo outcome of DCs and Tregs encounters depends also on the effects of CD4$^+$ effector cells which potentiate and modify the suppressive mechanisms of CD4$^+$ Tregs (15). This observation introduces an important new concept on the suppressive mechanisms of Tregs, indicating that it is not only bidirectional regulation with DC subsets, but rather a triangle modulation in which T effector cells (Teffs) play also a key role potentiating Treg function.

In the current study, using a model of transplantation tolerance mediated by CD8$^+$ Tregs following CD40Ig treatment in rats (5), we found that tolerance induction was associated with an accumulation of CD8$^+$CD45RC^{low} cells first in the graft and then in the spleen but not in LNs. The splenic compartment appeared to be sufficient at early time points and necessary at late time points for the induction and maintenance of tolerance. pDCs accumulated in the graft and spleen of tolerant animals and preferentially

supported the regulation of alloreactive CD4$^+$ T cells by CD8$^+$ CD45RC^{low} cells in vitro. Finally, we uncovered complex interactions between effector CD4$^+$ T cells, pDCs, and CD8$^+$ Tregs that resulted in distinct regulatory mechanisms depending on the extent of cell–cell contacts between the different cell populations.

Materials and Methods

Animals and cardiac transplantation models

Heart allotransplantation was performed between whole MHC-incompatible male LEW-1W (donors) and LEW-1A (recipients) rats as described previously (5). The experiments complied with the Institutional Ethical Guidelines of the Institut National de la Santé et de la Recherche Médicale (Nantes, France).

Adenovirus-mediated gene transfer and mAb administration

The recombinant noncoding adenovirus (Ad) Addl324 and the Ad encoding for the extracellular portion of mouse CD40 fused to the constant domains of human IgG1 (AdCD40Ig) with the mouse CMV promoter as well as the procedure of intragraft delivery have been described previously (5).

A neutralizing mouse anti-rat IFN-γ mAb (DB1, IgG1; provided by P. H. Van der Meisle, Amsterdam, The Netherlands) or the isotype control 3G8 mAb was injected (3 mg/kg) i.p. twice a week beginning the day of transplantation. The IDO-specific inhibitor, 1-methyltryptophan, was administered twice daily by oral gavages at 50 mg/dose, as described previously (5). A neutralizing murine anti–TGF-β mAb (2G7; provided by Dr. K. Melief, Amsterdam, The Netherlands) was injected i.p (5 mg/kg) twice a week beginning the day of transplantation as described previously (16). All treatments were continued until rejection or 40 d.

Adoptive transfer

Naive LEW-1A recipients received 4.5 Gy whole-body irradiation on the day before transplantation. Total splenocytes, bone marrow (BM) cells, LNs, or PBLs (50–100 × 10^6 cells) as well as PKH-labeled pDCs (1.5 × 10^6 cells) or PKH-labeled CD8$^+$ Tregs (2.5 × 10^6 cells) were adoptively transferred into recipients i.v. immediately after allograft implantation.

Purification of T cell subpopulations

T cells were enriched by negative selection from total splenocytes after depletion with a mixture of mAbs reactive with γδT cells (V65), B cells (His24, anti-CD45R), NK cells (3.2.3, anti-CD161), and monocytes (OX42, anti-CD11b/c) and magnetic beads (Dynal Biotech, Cergy Pontoise, France). Enriched T cells were labeled with a mixture of anti–CD45RC-biotin (OX22), anti–CD8α-PE (OX8), anti–CD6-FITC (OX56), anti-CD25-Alexa647 (OX39), and strepavidin-PE-Cy7. CD8$^+$CD45RC^{low} T cells, CD4$^+$CD25$^+$ T cells, and CD4$^+$CD25$^-$ T cells were sorted after gating of CD6$^+$ cells with a FACSAria (BD Biosciences, Mountain View, CA). Purity of sorted populations was >99%.

Purification of DC subsets

DCs subsets were prepared and isolated from spleen as described previously (17, 18). Briefly, spleens were digested by collagenase D, followed by Nycodenz gradient centrifugation and the middle layer of cells was collected as enriched cDCs. cDCs were sorted using the following Abs: anti-TCR (R73-FITC), anti-CD45RA (OX33-FITC), CD103 (OX62-APC), and anti-CD4 (OX35-PE). cDCs were defined as TCR$^-$CD45RA$^-$CD103$^+$ cells and further sorted into CD4$^+$ cDCs and CD4$^-$ cDCs subpopulations (17). The Nycodenz cell pellet was fractioned by Ficoll-Hypaque gradient centrifugation, and middle layer cells were depleted of T cells (R73 and V65 clones) and B (OX33 clone) cells, followed by sorting of pDCs using the following Abs: anti-TCR (R73-FITC), anti-CD45RA (OX33-FITC), CD45R (His24-PE), and anti-CD4 (OX35-APC). pDCs were defined as TCR$^-$CD45RA$^-$CD45R$^+$CD4$^+$ cells (18). Cell sorting was performed with a FACSAria with cell purity of sorted populations >98%.

mAbs and flow cytometry

The Abs used for sorting CD8$^+$ T cells and DC subsets were obtained from the European Collection of Cell Culture (Salisbury, U.K.). All biotin-labeled mAbs were visualized using strepavidin-PE-Cy7 (BD Biosciences). Fibroleukin-2 (Fgl-2) was detected using a murine mAbs anti–Fgl-2 (Abnova, Heidelberg, Germany), An LSR II cytometer (BD Biosciences) was used to measure fluorescence, and data were analyzed using the FlowJo software

(Tree Star, Ashland, OR). Cells were first gated by their morphology excluding dead cells, and the gates that were used to separate the populations and the percentages of gated cells are depicted in Supplemental Fig. 1.

Mixed leukocyte reaction

For MLR performed in coculture experiments, purified naive CFSE-labeled (5 μM CFSE, 3 min at room temperature) CD4$^+$CD25$^-$ T cells (2 × 10^4 cells) were cultured with DCs of different types (5 × 10^3 cells) in a round-bottom 96-well plate in a final volume of 200 μl complete RPMI 1640 medium, with or without suppressive cells for 6 d at 37C° in 5% CO$_2$.

For Transwell MLRs, purified naive CD4$^+$CD25$^-$ T cells (0.4 × 10^6 cells) were cultured with DCs (10^5 cells) in a V-bottom tube used as a lower chamber. A cell culture insert (BD Biosciences) with 0.4-μm pore size membrane was then inserted into the V tube as an upper chamber. A total of 4 × 10^5 cells CD8$^+$ Tregs with or without pDCs were seeded onto the cell culture insert as an upper chamber. The cells were cultured at 37°C in 5% CO$_2$ in a final volume of 1 ml complete RPMI 1640 medium for 6 d.

Proliferation of CFSE-labeled naive CD4$^+$CD25$^-$ T cells was analyzed by flow cytometry after gating TCR$^+$CD4$^+$ cells.

MLRs were performed in the presence of rat IFN-γ (5000 UI/ml; Roussel Uclaf, Romainville, France), mouse anti-rat IFN-γ mAb (20 or 100 μg/ml), rabbit anti-rat IL-10 (19) (20 μg/ml, provided by Dr. J. Khalife, Institut Pasteur de Lille, France), murine anti–TGF-β mAb (20 μg/ml), the IDO-specific inhibitor methyl-thiohydantoin-tryptophan (MTHT; 20 μM) (Calbiochem, Nottingham, U.K.) (20), B18R from vaccinia virus neutralizing all type I IFNs (21) (eBioscience, San Diego, CA) (250 pg/ml or 2 ng/ml), or an anti-Fgl-2 mAb (20 μg/ml). IFN-γ was measured using ELISA kits (BD Biosciences).

Ag presentation by indirect pathway

Heart resident leukocytes were isolated from donor LEW.1W hearts digested with collagenase D, followed by Ficoll gradient centrifugation. Apoptosis was induced by UV irradiation (TUV 30 W/G 30 TB; wavelength 280–340 nm; Philips, Eindhoven, The Netherlands) for 3 h at room temperature. Apoptotic cells were then cultured overnight with recipient (LEW.1A) DC subsets (1:1 ratio) at 10^6 pDCs/ml. Ficoll gradient centrifugation was used to harvest DCs loaded with donor alloantigens and eliminated apoptotic cells.

Histological analysis and microscopy

Cryostat sections were harvested and fixed in acetone. Sections were mounted in Prolong Antifade Kit (Molecular Probes, Invitrogen, Cergy Pontoise, France) and analyzed by confocal microscopy (Leica TCS-SPE, Nanterre, France) with ImageJ software (National Institutes of Health, Bethesda, MD).

Fgl-2 quantitative RT-PCR

The isolation and retrotranscription of mRNA as well as the quantification of specific mRNA levels using SYBR green technology after normalization to hypoxanthine phosphoribosyltransferase values have been described previously (5). The sequence of primer pairs for rat Fgl-2 are 5′-CAAGA-ACACAACCAGCCAAATCC-3′ (forward) and 5′-CCCAGCCAAAATT-CTCGTTCAA-3′ (reverse).

Statistics

For graft survival, log-rank test was done. For numbers of DC subsets and proliferation assay, ANOVA test was done to examine individual differences versus various controls. For CD8$^+$CD45RC^{low} T cell percentages, one-way ANOVA was done.

Results

Tolerogenic CD8$^+$ Tregs sequentially accumulate in the allograft and the spleen

Treatment of graft recipients with AdCD40Ig resulted in long-term cardiac allograft survival (5). Tolerance to cardiac allografts induced by CD40Ig treatment could be adoptively transferred exclusively by CD8$^+$CD45RC^{low} Tregs, whereas CD8$^+$CD45RC^{low} T cells from naive animals failed to do so (5). Thus, CD8$^+$ CD45RC^{low} cells from naive animals versus tolerant CD40Ig-treated transplanted recipients will be hereafter referred to as natural (or naive) versus tolerogenic CD8$^+$ Tregs, respectively. In previous work and in the present paper, adoptive transfers were performed with recipient cells isolated at different time after

CD40Ig treatment and transferred to graft recipients sublethally irradiated the day before transplantation. Tolerance was induced in 50% of recipients that were transferred with splenocytes harvested after 1 wk of treatment, and this frequency was increased to 100% when splenocytes were harvested 1 or 3 mo after treatment (Fig. 1A). In contrast, graft survival was not prolonged after adoptive transfer of the same numbers (Fig. 1B) or even double numbers (data not shown) of LN cells. To examine the role of cells present within the allograft in the prevention of rejection, tolerant allografts were retransplanted into naive recipients without CD40Ig treatment. Even though they were eventually rejected in <45 d, retransplanted allografts survived significantly longer than primary allografts (Fig. 1C).

The data above suggested that CD8+ Tregs present in both spleen and allograft could contribute to graft acceptance. The presence of CD8+CD45RC^{low} T cells was thus analyzed in the spleen, LN, and allograft at 1 wk, 1 mo, and 3 mo after treatment with CD40Ig. Tolerogenic CD8+ Tregs appeared firstly in the allograft (1 wk) and later in the spleen (1 mo) but never in LNs (Fig. 1D). One week after CD40Ig treatment, the proportion of tolerogenic CD8+ Tregs increased rapidly in the allograft and then remained stable, whereas it took 3 mo to reach similar levels in the spleen (Fig. 1E). Control animals treated with Addl324 virus showed lower proportion of CD8+CD45RC^{low} cells in the spleen

at 3 mo after transplantation, comparable to that of naive animals (data not shown). To further investigate whether the organ distribution of CD8+ Tregs correlated with their tolerogenic properties, PKH-labeled natural or tolerogenic CD8+ Tregs were adoptively transferred into recipients immediately after transplantation. Tolerogenic CD8+ Tregs migrated to the cardiac graft within 5 d, whereas natural CD8+ Tregs did not (Fig. 1F). A higher proportion of tolerogenic CD8+ Tregs was observed in the spleen compared with natural CD8+ Tregs and little to none tolerogenic or natural CD8+ Tregs were observed in LNs (Fig. 1F) or the recipient native heart (data not shown). Thus, long-term allograft survival was associated with accumulation of tolerogenic CD8+ Tregs first in the allograft and later in the spleen.

*The spleen is dispensable for the generation but necessary for the long-term maintenance of tolerogenic CD8+ Tregs*

To investigate the role of the spleen in the development of tolerogenic CD8+ Tregs, we treated graft recipients with a suboptimal dose of CD40Ig to induce tolerance in ~50% of recipients. Splenectomy performed on the day of transplantation together with a suboptimal dose of CD40Ig treatment increased allograft survival rates to 100%, whereas splenectomy alone did not alter allograft survival (Fig. 2A). Splenectomy 3 mo after treatment did not abrogate long-term allograft survival (Fig. 2B). In contrast, allografts were rejected when the

FIGURE 1. Tissue distribution of tolerogenic CD8+ Tregs. To analyze the for the presence of CD8+ Tregs, allograft survival rates were compared after adoptive transfer of 50×10^6 cells isolated at different times after transplantation from LEW-1A rats treated with control Ad or AdCD40Ig and transplanted with cardiac grafts (A–C). A, Adoptive transfer of splenocytes (n = 4–6/group). *p < 0.05 compared with all other groups. B, Adoptive transfer of LN cells (n = 4–6/group). C, Tolerant allografts (survival > 3 mo) or normal donor grafts were transplanted into naive irradiated recipients (n = 3) *p < 0.05 compared with all other groups. Direct identification of CD8+CD45RC^{low} Tregs was performed by flow cytometry analysis (D, E). D, CD8+CD45RC^{low} Tregs were analyzed in spleen, LN, and allografts in recipients treated with CD40Ig at the indicated time points. Cells were gated by morphology and TCR+CD8+ cells (Supplemental Fig. 1) were analyzed for CD45RC expression. Data are representative of at least three independent experiments at each time point. E, Same experiments as in D expressed as percentage mean ± SD of the percentage of CD8+ CD45RC^{low} Tregs among TCR+CD8+ T cells from three to five animals treated with CD40Ig sacrificed at each time point. **p < 0.01 compared with LN group. The migratory capacity of CD8+ Tregs was analyzed by adoptive transfer experiments. F, Natural or tolerogenic CD8+ Tregs were sorted from naive or tolerant recipients, labeled with PKH26, and adoptively transferred into naive irradiated and transplanted recipients. The presence of PKH-labeled CD8+ Tregs was analyzed by confocal microscopy in spleen, LN, and allografts collected 5 d after adoptive transfer (original magnification ×63).
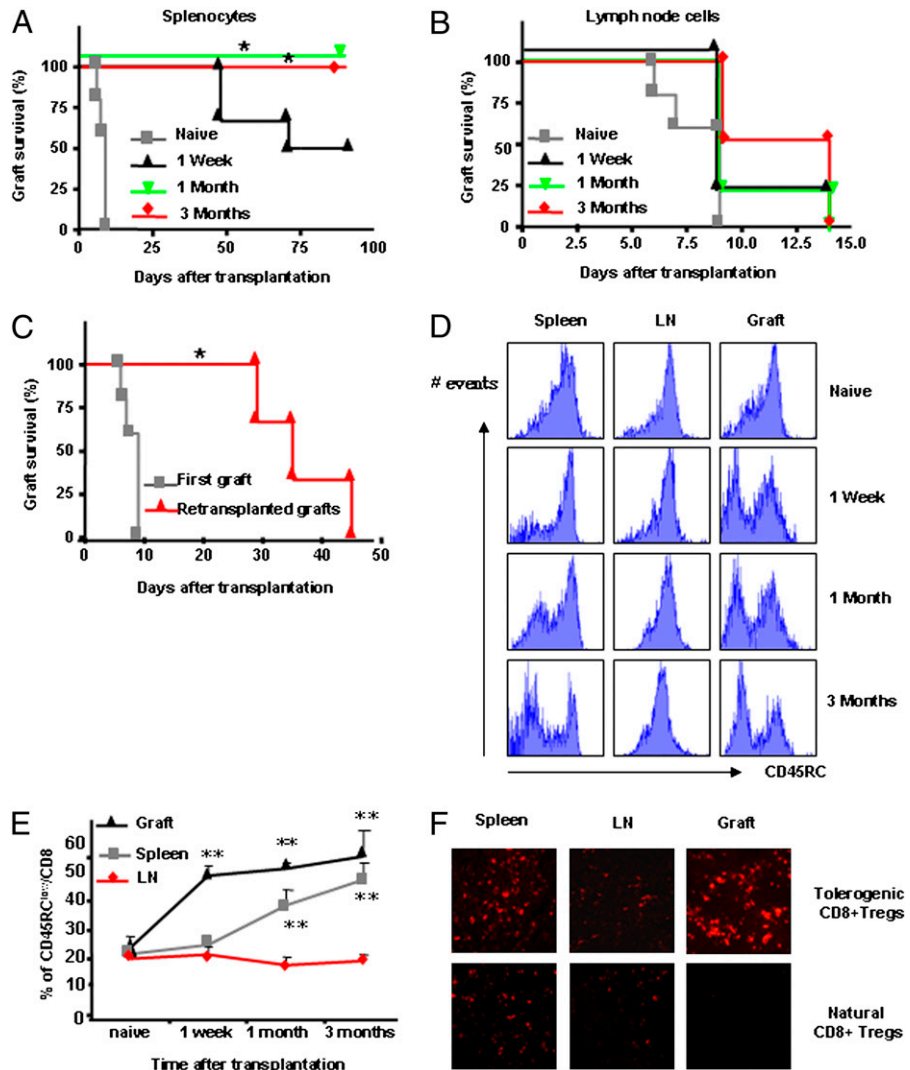
FIGURE 2. Role of lymphoid organs and BM in the induction and maintenance of tolerance by CD8^{+} Tregs. To analyze the role of spleen in graft survival was analyzed in recipients that received different doses of AdCD40Ig and were splenectomized at different time points (*A, B*). *A,* Graft survival rates in recipients treated with a suboptimal dose of AdCD40Ig associated or not with splenectomy (*n* = 4/group). *p < 0.05 compared with all other groups. *B,* Graft survival rates among recipients who received a full dose of AdCD40Ig and were splenectomized at 3 or 6 mo after transplantation. Day 0 represents the day at which splenectomy was performed (*n* = 4/group). To analyze the localization of tolerogenic CD8^{+} Tregs in splenectomized recipients with long-surviving grafts, cell adoptive transfer experiments and flow cytometry experiments were performed in other lymphoid organs or BM (*C, D*). *C,* Graft survival rates after adoptive transfer (50 × 10⁶ cells) of LN cells, PBLs, and BM cells collected from splenectomized recipients harboring long-term surviving allografts (*n* = 4 per group). BM cells from CD40Ig-treated and transplanted but not splenectomized recipients were used as control. *p < 0.05 compared with all other groups. *D,* Tolerogenic CD8^{+}CD45RC^{low} Tregs were analyzed in BM, PBL, and LN 3 mo after transplantation in recipients treated with CD40Ig and splenectomized (*upper panels*) or treated with CD40Ig only (*lower panels*). Data are representative of four independent experiments.
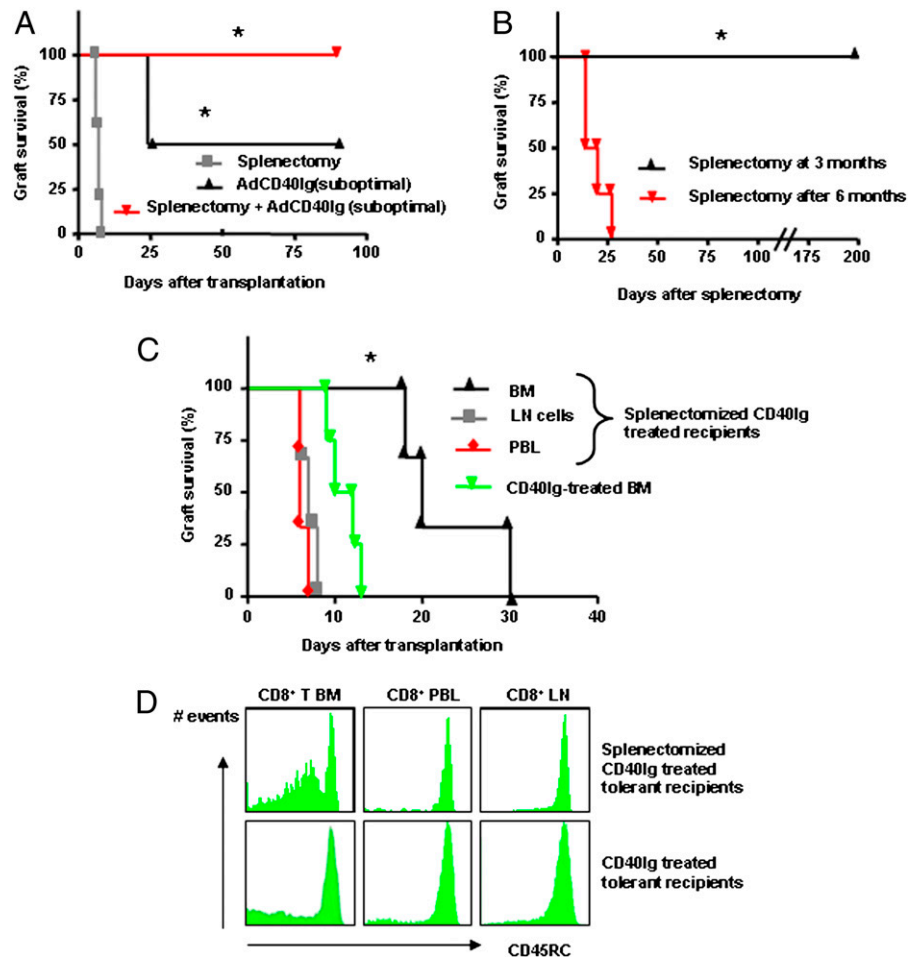
spleen was removed 6 mo after CD40Ig treatment (Fig. 2*B*). These very contrasted data following splenectomy suggest that although tolerogenic CD8^{+} Tregs were present in the spleen at earlier time points after transplantation (= 3 mo), their removal was dispensable for maintaining tolerance suggesting the presence of CD8^{+} Tregs in other organ compartments. Furthermore, because splenic tolerogenic CD8^{+} Tregs can transfer tolerance while removal of spleen on day 0 improved the survival of allografts, both rejection and tolerance mechanisms occurred simultaneously in the spleen.

We examined the anatomic location of tolerogenic CD8^{+} Tregs in splenectomized recipients with long-term surviving allografts. Adoptive transfer of LN cells, PBLs, and BM cells from splenectomized tolerant recipients was performed. Partial but significant prolongation of allograft survival was observed only with the transfer of BM cells (Fig. 2*C*). In contrast, adoptive transfer of BM cells from CD40Ig-treated rats with an intact spleen did not prolong allograft survival (Fig. 2*C*). Furthermore, in splenectomized recipients with long-term surviving grafts, an expansion of CD8^{+}CD45RC^{low} T cells was observed in the BM, but not in LN and peripheral blood, whereas CD8^{+}CD45RC^{low} Tregs were present at a much lower frequency in the BM of CD40Ig-treated animals (Fig. 2*D*) or naive rats (data not shown). These data suggest that, in splenectomized animals, the BM could substitute for the spleen as a reservoir of tolerogenic CD8^{+} Tregs.

Thymus was also dispensable to generate the tolerogenic mechanism because tolerance was induced in all recipients after thymectomy at the day of transplantation (Supplemental Fig. 2) and could be adoptively transferred to naive recipient by splenocytes from thymectomized animals (data not shown).

Differential accumulation of pDCs in the spleen and allograft in tolerant versus rejecting recipients

The number of cells in different DC subsets was compared in the spleen of recipients that rejected or tolerated allografts. Numbers of pDCs were mostly stable in tolerant spleen, although they slightly increased 3 mo after transplantation. In contrast, numbers of pDCs in rejected spleen were dramatically decreased in the early phase (rejection occurred between 6 and 9 d), reaching less than half at 2 wk and recovering after 3 mo (Fig. 3*A*). Numbers of CD4^{−} cDCs in tolerant spleen increased in the first week, remained high at 2 wk, and decreased to pretransplantation levels by 3 mo (Fig. 3*B*). The numbers of CD4^{−} cDCs in rejected spleen also increased in the first week and then decreased rapidly to pretransplantation levels by 2 wk. The changes in numbers of CD4^{+} cDCs were similar in rejected and tolerant spleens, increasing during the first week, and stably returning to pretransplantation levels after 2 wk (Fig. 3*C*). Thus, although changes in numbers of splenic CD4^{−} cDCs and CD4^{+} cDCs were associated temporally with the early inflammatory response in both rejecting and tolerant recipients, pDCs were the only DC subset characterized by dynamic changes that correlated with the outcome of the allograft.

The presence of pDCs was analyzed in the LN and allograft 5 d after transplantation. The percentage of pDCs in LNs was similar in rejecting and tolerant recipients (Fig. 3*D*). In contrast, in the graft, pDCs were observed in higher percentages in tolerant allografts versus rejected allografts (Fig. 3*D*) and were also present in long-surviving allografts (data not shown) suggesting that the preferential migration of pDCs to long-term surviving allografts was important to
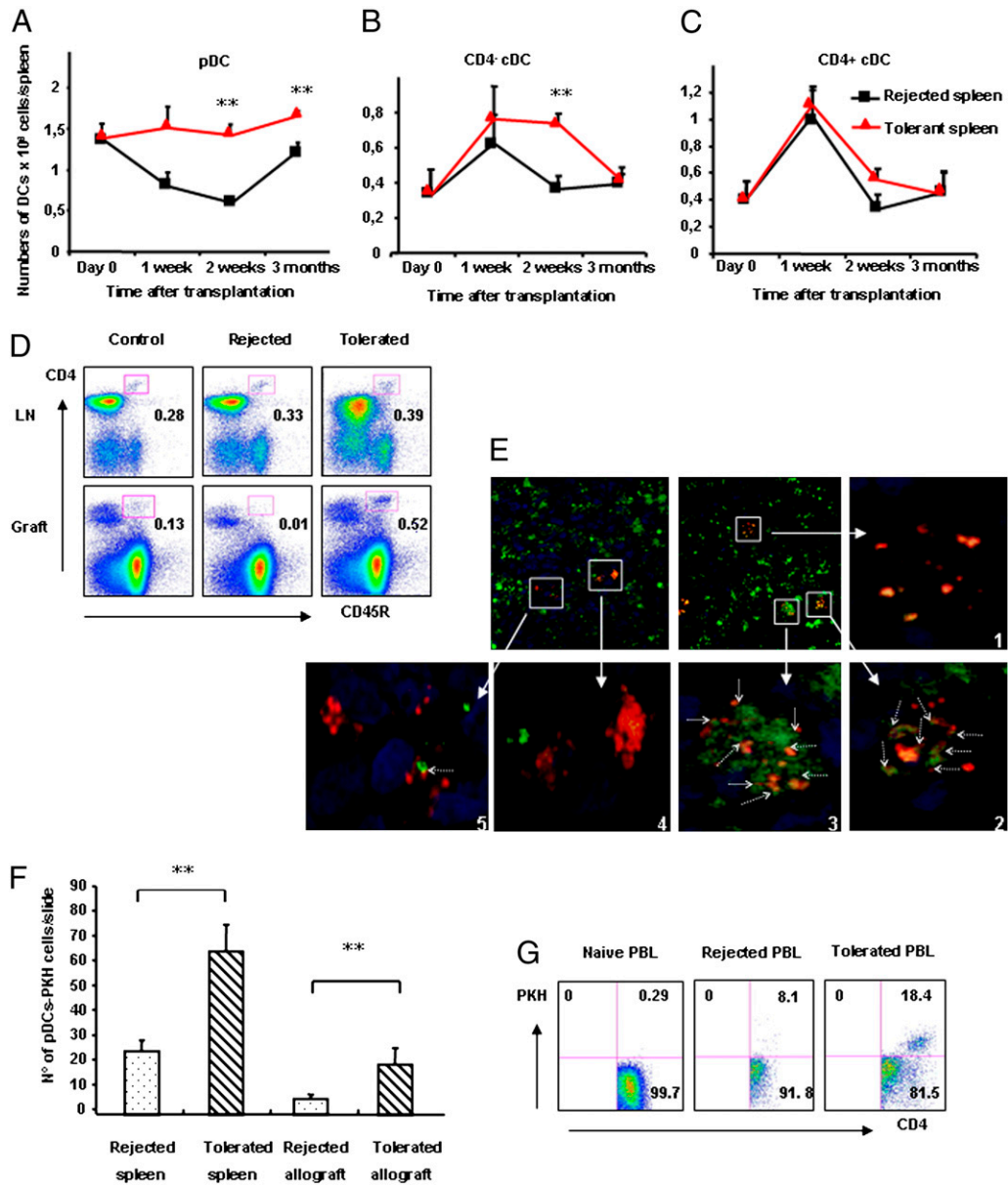
FIGURE 3. DC subsets organ distribution and pDC accumulation in long-term allograft recipients. Grafted recipients treated with CD40Ig were sacrificed at the indicated time points and analyzed for the presence of different DCs populations by flow cytometry (*A–D*). Absolute numbers of pDCs (*A*), CD4⁻ cDCs (*B*), and CD4⁺ cDCs (*C*) in spleen from rejecting (squares) or tolerant (triangles) recipients were compared at indicated times after transplantation. At least $n = 4$ in each time point and each group, total $n = 35$, generated in 15 different experiments. $**p < 0.01$. *D*, Percentage of pDCs (gated as TCR⁻CD4⁺CD45R⁺; Supplemental Fig. 1) in LNs and allografts at day 5 after transplantation in syngeneic, rejected, or tolerant recipients. Data are representative of three independent experiments. The accumulation of pDCs was evaluated by adoptive cell transfer experiments using PKH-labeled cells (*E–G*). *E*, PKH-labeled pDCs were adoptively transferred in along with CFSE-labeled natural or tolerogenic CD8⁺ Tregs to mimic rejection or long-term allograft survival conditions (samples labeled rejected and tolerated, respectively). The samples were collected on day 5 and analyzed by confocal microscopy or cytofluorimetry. The spleens were analyzed by confocal microscopy (*upper panels*, original magnification ×63; *lower panels* were digitally amplified to original magnification ×200). CD8⁺ Tregs are green, and pDCs are red. Data representative of three independent experiments and in each spleen at least five sections were analyzed. *F*, The numbers of PKH-positive pDCs in spleen and allograft were presented as cells per field. At least two sections were counted for each sample and at least six independent fields were analyzed for each section. $**p = 0.0006$ for tolerant spleen versus rejected spleen; $**p = 0.008$ for tolerant allograft versus rejected allograft. *G*, After gating TCR⁻CD4⁺ cells PKH-positive pDCs in peripheral blood were compared between rejected and tolerant groups by flow cytometry. Data are representative of three independent experiments.

prevent acute rejection and induce tolerance. cDCs populations in LNs were not different in the experimental groups at day 5 after transplantation, and it was not possible to identify them using the conditions used in lymphoid organs, probably because of inflammatory and necrotic cells during acute rejection (data not shown).

Because numbers of pDCs in tissues correlated with induction of tolerance by CD40Ig, we examined the relationship between pDCs and tolerogenic CD8⁺ Tregs that mediate this tolerant state (5). The

presence and accumulation of pDCs were analyzed in vivo by tracing PKH-labeled naive pDCs from recipient origin adoptively transferred simultaneously with natural or tolerogenic CD8⁺ Tregs, and animals were sacrificed at day 5. The use of recipient pDCs aimed to reproduce the changes observed in the distribution of pDCs described above and also on the fact that recipient pDCs were able to present donor Ags to CD8⁺ Tregs and trigger suppression (see next section). The use of natural or tolerogenic CD8⁺ Tregs

aimed to mimic the outcome of the graft, rejection or tolerance, but the early timing of sacrifice was necessary to analyze the fate of this cells within the graft before complete rejection (~ day 7).

PKH-labeled pDCs in spleens were more frequently juxtaposed to CFSE-labeled tolerogenic CD8⁺ Tregs compared with natural CD8⁺ Tregs (Fig. 3E). The numbers of CFSE-labeled tolerogenic CD8⁺ Tregs were higher versus natural CD8⁺ Tregs in the spleen (608 ± 11 versus 365 ± 13 CFSE⁺ cells/section; $n = 12$; $p < 0.05$), and the percentage in contact with PKH-labeled pDCs was also higher with tolerogenic versus natural CD8⁺ Tregs (23.7 versus 10.4%, respectively; $p < 0.05$). Enumeration of PKH-labeled pDCs showed that numbers of pDCs were significantly higher in the spleen and graft when pDCs were cotransferred with tolerogenic compared with naive CD8⁺ Tregs (Fig. 3F).

Furthermore, PKH-labeled pDCs were not observed in LNs (data not shown). Cytofluorimetry analysis of PBMCs showed a significantly higher percentage of PKH⁺ pDCs in recipients transferred with tolerogenic CD8⁺ Tregs compared with naive CD8⁺ Tregs (Fig. 3G). PKH-labeled pDCs were also observed in the BM of recipients transferred with tolerogenic or naive CD8⁺ Tregs without significant differences (data not shown). Thus, tissue accumulation of pDCs correlated with both graft outcome and localization of tolerogenic CD8⁺ Tregs, suggesting privileged interactions between the two cell subsets.

CD8⁺ Tregs donor-specific suppression is preferentially induced by pDCs

Donor pDCs or cDCs were purified and used as stimulators in an in vitro suppression assay where CFSE-labeled CD4⁺CD25⁻ responder T cells were cocultured in the presence or absence of natural or tolerogenic CD8⁺ Tregs. Both natural and tolerogenic CD8⁺ Tregs suppressed the proliferation of CD4⁺CD25⁻ T cells in the presence of first-donor– and third-party–derived immature pDCs but tolerogenic CD8⁺ Tregs showed significantly higher suppression when using first donor-derived but not third-party pDCs (Fig. 4A). The enhanced suppressive effect of tolerogenic CD8⁺ Tregs when using first-donor pDCs was dose dependent when analyzed both at the level of percentage of nonproliferating cells (Fig. 4B) and mean fluorescence intensity of proliferating cells (Fig. 4C). When either of the two rat cDC populations (CD4⁺ or CD4⁻) (17, 22) of first donor origin were used in the suppressive MLR assay, suppression of CD4⁺ T cell proliferation was induced by natural and tolerogenic CD8⁺ Tregs in the presence of CD4⁻ cDCs, whereas no or little suppression was observed with CD4⁺ cDCs (Supplemental Fig. 3A–C). Taken together, these results show that pDCs were the most effective DC subset to mediate donor specific suppression by tolerogenic CD8⁺ Tregs and that the suppression was higher when using first

FIGURE 4. Suppressive activity of tolerogenic versus natural CD8⁺ Tregs was preferentially induced by interaction with pDCs. Regulatory function of natural and tolerogenic CD8⁺ Tregs was analyzed by measuring their capacity to suppress MLR assays. The proliferation of naive CFSE-labeled LEW.1A CD4⁺CD25⁻ T cells against alloantigens presented by LEW.1W pDCs was analyzed after 6 d of culture in the absence or presence of LEW.1A natural or tolerogenic CD8⁺ Tregs. A, Representative experiment in which natural and tolerogenic CD8⁺ Treg suppressive activity was analyzed following gating on TCR⁺CD4⁺ of CFSE-labeled naive LEW.1A CD4⁺CD25⁻ T cells after culture with medium alone or pDCs from LEW.1W or BN origin (1:1 ratio for effector/suppressor). B, Same suppressive assay performed using decreasing ratios of effector/suppressor cells. Results are depicted as the percentage of nonproliferating CFSE-labeled naive CD4⁺CD25⁻ T cells. Data are the mean ± SD of three independent experiments. Dotted line represents values obtained in the absence of CD8⁺ Tregs. ∗$p <$ 0.05 tolerogenic versus natural CD8⁺ Tregs. C, Same suppressive assay with results depicted as the MFI of CD4⁺ CD25⁻ proliferating cells. Dotted line represents values obtained in the absence of CD8⁺ Tregs. ∗$p < 0.05$ tolerogenic versus natural CD8⁺ Tregs. D, Transwell experiments were performed using CFSE-labeled naive CD4⁺CD25⁻ T cells, CD8⁺ Tregs (1:1 ratio for effector/suppressor), and donor pDCs distributed as shown in the figure and analyzed after 6 d of co-culture. Data are representative of five independent experiments. BN, Brown Norway; MFI, mean fluorescence intensity.
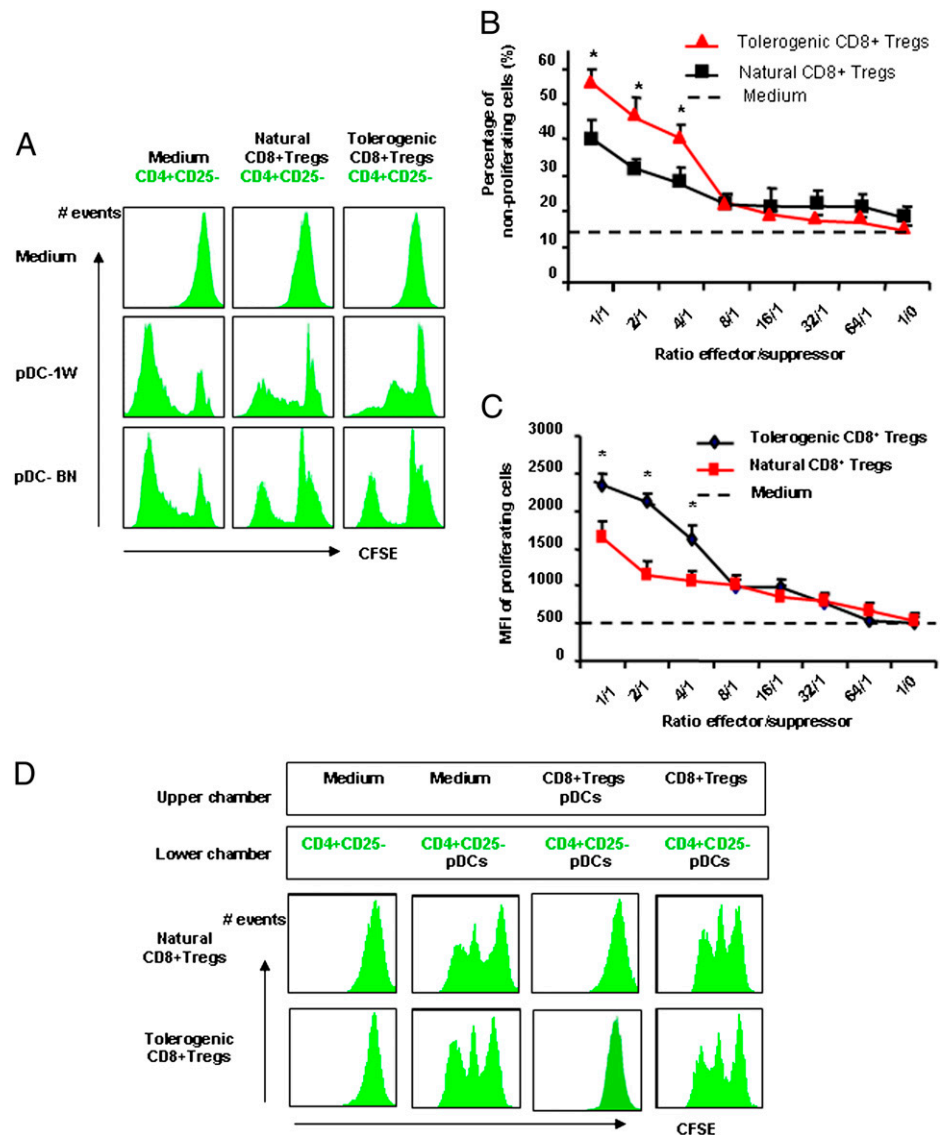
donor-derived pDCs, in keeping with the previously demonstrated alloantigen-specificity of CD8+CD45RC^{low} Tregs (5).

To analyze whether CD8+ Tregs could also suppress the indirect alloantigen presentation pathway, we used recipient pDCs loaded with apoptotic PKH-labeled donor cells (Supplemental Fig. 4A). Proliferation of CD4+CD25− T cells was observed when cultured with recipient pDCs loaded with donor alloantigens and not with apoptotic donor cells alone in the absence of recipient pDCs (Supplemental Fig. 4B). Suppression of CD4+CD25− T cell proliferation induced by indirect alloantigen presentation was most effectively achieved by tolerogenic CD8+ Tregs in the presence of recipient pDCs (Supplemental Fig. 4C, 4D). These results demonstrate that rat pDCs can cross-present Ags as was previously observed in humans and mice (23) and that both indirect and direct donor alloantigen presentation by pDCs resulted in stronger donor-specific suppressive activity by tolerogenic CD8+ Tregs compared with natural CD8+ Tregs.

Transwell experiments were performed to test whether the suppressive function of CD8+ Tregs was dependent on cell contact. Proliferation of CFSE-labeled CD4+CD25− T cells stimulated with allogeneic pDCs in the lower chamber was strongly suppressed by the addition of tolerogenic CD8+ Tregs together with pDCs in the upper chamber (Fig. 4D). In the Transwell assay, we did not observe higher suppression by tolerogenic versus naive CD8+ Tregs, as we did in the coculture system, suggesting different suppressive mechanisms in each situation. However, tolerogenic CD8+ Tregs without direct contact with allogeneic pDCs in the upper chamber did not exert suppression of CD4+ effector T cells in the lower chamber (Fig. 4D). The same results were obtained with recipient pDCs loaded with donor Ags (data not shown). These data indicate that the suppressive function of CD8+ Tregs required contact-dependent stimulation at least by pDCs, whereas suppression itself occurred independently of regulatory/effector T cells contacts.

Contact with CD4+ T cells modifies the suppressive mechanisms of tolerogenic CD8+ Tregs

Because suppression by CD4+CD25+ Tregs is modified and potentiated by cell contact with CD4+ effector cells (15), we tested whether contact with CD4+ cells would modify the suppressive mechanisms of tolerogenic CD8+ Tregs.

In vitro, Transwell experiments showed that the suppressive function of tolerogenic CD8+ Tregs was abrogated by an IDO inhibitor when Tregs were not in direct contact with CD4+ Teffs (Fig. 5A). In contrast, although IFN-γ can induce IDO (24), IFN-γ
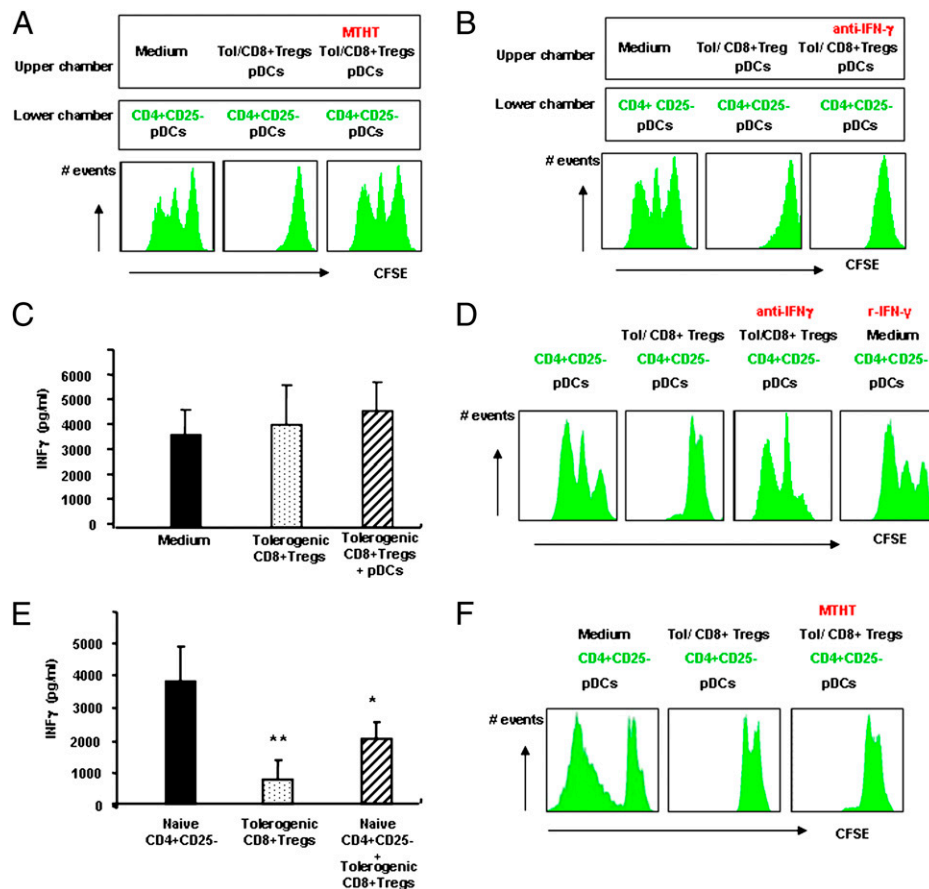
FIGURE 5. Suppressive mechanisms by tolerogenic CD8+ Tregs are modified by cell contact with CD4+ T cells. Recipient tolerogenic CD8+ Tregs were cultured with donor pDCs, and recipient CFSE-labeled naive CD4+CD25− T cells either in coculture or Transwell MLRs in the presence of different blocking molecules and proliferation were analyzed by CFSE dilution. *A*, The effect of the IDO inhibitor MTHT (20 μM) on the suppressive function of CD8+ Tregs was determined by analysis of the dilution of CFSE-labeled naive CD4+CD25− T cells in a Transwell MLR. Data representative of five or three independent experiments, respectively. *B*, The effect of the neutralizing IFN-γ Ab (100 μg/ml) on the suppression of proliferation of naive CD4+CD25− T cells by tolerogenic CD8+ Tregs analyzed in a Transwell MLR. Data are representative of three independent experiments. *C*, Concentration of IFN-γ in the supernatant of Transwell MLRs. *D*, The effect of neutralizing anti–IFN-γ mAb (100 μg/ml) and rIFN-γ (5000 UI/ml) was analyzed in a coculture MLR. Data are representative of five independent experiments. *E*, Concentration of IFN-γ in the supernatant of coculture MLR (*n* = 3). **p = 0.0007; *p = 0.03 versus naive CD4+CD25−. *F*, The effect of inhibition of IDO by MTHT (20 μM) was analyzed in a coculture MLR. Data are representative of five independent experiments.

was not responsible for the contact-independent suppression of CD4+ T cells, because a neutralizing anti–IFN-γ Ab did not reverse suppression (Fig. 5B), and concentrations of IFN-γ in the supernatant did not correlate with suppression (Fig. 5C). These results also indicate that IDO-mediated suppression by CD8+ Tregs inhibited the proliferation but not the effector function of CD4+ T cells assessed by IFN-γ production. Because IDO is also induced by type I IFNs (24), we tested the effect of the viral molecule B18R (which neutralizes all type I IFNs) (21). Suppression was still observed in the presence of B18R (data not shown), indicating that production of IDO was controlled by IFN-independent mechanisms.

We examined whether the suppressive mechanisms of tolerogenic CD8+ Tregs could be modified after contact with CD4+ cells by analyzing the suppressive function of CD8+ Tregs in a coculture MLR containing CD8+ Tregs, pDCs, and CD4+ cells. Although tolerogenic CD8+ Tregs suppressed CD4+ T cell proliferation as efficiently as in the Transwell system, the addition of neutralizing anti–IFN-γ Ab abrogated the suppression by CD8+ Tregs in the coculture MLR (Fig. 5D). In contrast, addition of soluble IFN-γ protein alone did not suppress the proliferation of CD4+CD25− T cells (Fig. 5D). Furthermore, the addition of tolerogenic CD8+ Tregs to the coculture system significantly suppressed the production of IFN-γ (Fig. 5E), indicating that tolerogenic CD8+ Tregs inhibited both the proliferation and effector function of CD4+ T cells and suggesting that the mechanisms of suppression were distinct from the Transwell experiments. In support of this, suppression by CD8+ Tregs in the coculture MLR was not due to the induction of IDO, because IDO inhibitors did not restore CD4+ proliferation (Fig. 5F). Thus, contacts with CD4+ T cells modified the suppressive mechanisms of CD8+ Tregs to an IFN-γ–dependent mechanism, different from the IDO-dependent mechanism observed when cells were not in contact in the Transwell system.

Our previous in vivo data supports a role for both IDO- and IFN-γ–dependent mechanisms in allograft tolerance. We previously reported that both IDO and IFN-γ were crucial in the maintenance phase of long-term allograft survival after adoptive transfer of CD8+ Tregs (5). We now further confirm that IDO and IFN-γ are necessary in the induction phase of long-term allograft survival by CD40Ig treatment, because graft rejection was triggered after administration at the day of transplantation of an IDO inhibitor in 60% of recipients (Supplemental Fig. 5A) and in 75% of recipients after administration of an anti–IFN-γ Ab (Supplemental Fig. 5B).

Fgl-2 is involved in suppression when CD8+ Tregs are in contact with CD4+ T cells

To identify other mediators potentially involved in suppression by CD8+ Tregs, we performed microarray analysis to compare tolerogenic versus naive CD8+ Tregs from spleen from recipients of long-surviving allografts (>100 d) and naive animals and showed increased expression of several potentially regulatory molecules in tolerogenic CD8+ Tregs. Among them was Fgl-2, which has been shown to have suppressive functions on CD4+ effector T cells through action on DCs (25). We confirmed that Fgl-2 expression was significantly increased in tolerogenic compared with naive CD8+ Tregs by quantitative RT-PCR (Fig. 6A) and at protein level at the membrane of cells by FACS analysis (Fig. 6B) and by confocal microscopy within the cells (Fig. 6C). Importantly, inclusion of a neutralizing anti–Fgl-2 Ab restored CD4+ T cell proliferation in the coculture MLR suppression assay (Fig. 6D). These results suggest that Fgl-2, a molecule induced by IFN-γ (25), was one of the mediators of CD8+ Treg suppression when CD4+ and CD8+ Tregs were in contact. Altogether, although both

IFN-γ– and IDO-dependent mechanisms coexisted in vivo for tolerance induction by tolerogenic CD8+ Tregs, these pathways occurred independently and differentially depending on cell contacts with CD4+ T cells, which switched suppression from an IDO-dependent mechanism in the absence of contacts to an IFN-γ– and Fgl-2–dependent mechanism in the presence of contacts.

Discussion

In this model of vascularized heart transplantation in rats, we have defined the kinetics, tissue distribution, and cellular interactions underlying tolerance induction by CD8+CD45RClow Tregs following CD40Ig treatment. We demonstrate that the accumulation of tolerogenic CD8+ Tregs first occurs within the allograft, whereas maintenance of long-term tolerance requires the splenic compartment. pDCs appear to play a predominant role in the tolerogenic process as their dynamics correlate with both allograft outcome and tissue distribution of tolerogenic CD8+ Tregs in vivo, and they preferentially induce Tregs suppression in vitro. Distinct mechanisms of suppression occurred depending on cellular interactions taking place in the MLR in vitro. In particular, the presence or absence of contacts between CD8+ Tregs and CD4+ Teffs resulted in a switch between an IFN-γ– and Fgl-2–dependent mechanism and an IDO-dependent mechanism of suppression, respectively. Because both IFN-γ and IDO are important for tolerance mediated by CD8+CD45RClow Tregs in vivo (5), these results suggest that CD8+ Tregs suppress alloreactive CD4+ responses by both contact-dependent and -independent mechanisms after CD40Ig treatment.

The spleen was dominant among lymphoid organs to generate and maintain tolerogenic CD8+ Tregs because tolerance could be transferred with splenocytes early after transplantation and rejection occurred upon splenectomy long-term after transplantation. Alloantigen priming from vascularized organs has been shown to occur both in the spleen and the LN (26). In a rat heart allotransplantation model similar to ours (27), blood was the preferential route for migration of recipient DCs into the graft and later for both donor and recipient DC migration into the spleen with no migration into regional LNs. This is opposed to islets grafted under the kidney capsule where lymphatic drainage is largely predominant. In our model, at early time points, spleen could be partially replaced by BM, which contained CD8+ Tregs and prolonged allograft survival upon transfer. BM is part of the lymphocyte recirculation pool, and CD4+CD25+ Tregs can accumulate in the BM (28). Our results showing accumulation of tolerogenic CD8+ Tregs first in the graft and later in the spleen are concordant with a recent model of islet transplantation where CD4+ Tregs first transit to the graft before migrating to lymphoid organs (29). CD8+ Tregs have also been shown to accumulate in tumor sites (11), allografts (4), or inflamed lymphoid tissue (30). The observation that transferred tolerogenic CD8+ Tregs were found in higher numbers in the graft and spleen compared with natural CD8+ Tregs may represent increased migration, as previously observed for CD4+ Tregs (29). In this regard, a pangenomic microarray analysis showed an increase (fold increase versus naive CD8+ Tregs) of several molecules, which could explain different migratory profiles, such as CXCR3 (2.55), CCR5 (9.63), CCR2 (6.6), and CCL5 (4.75), that were confirmed by quantitative RT-PCR (data not shown).

In tolerant animals, transferred pDCs accumulated in the graft, spleen, blood, and BM but not in LNs, and this distribution is concordant with the preferential hematogenous route for pDC migration (31). In contrast, during rejection, numbers of pDCs in
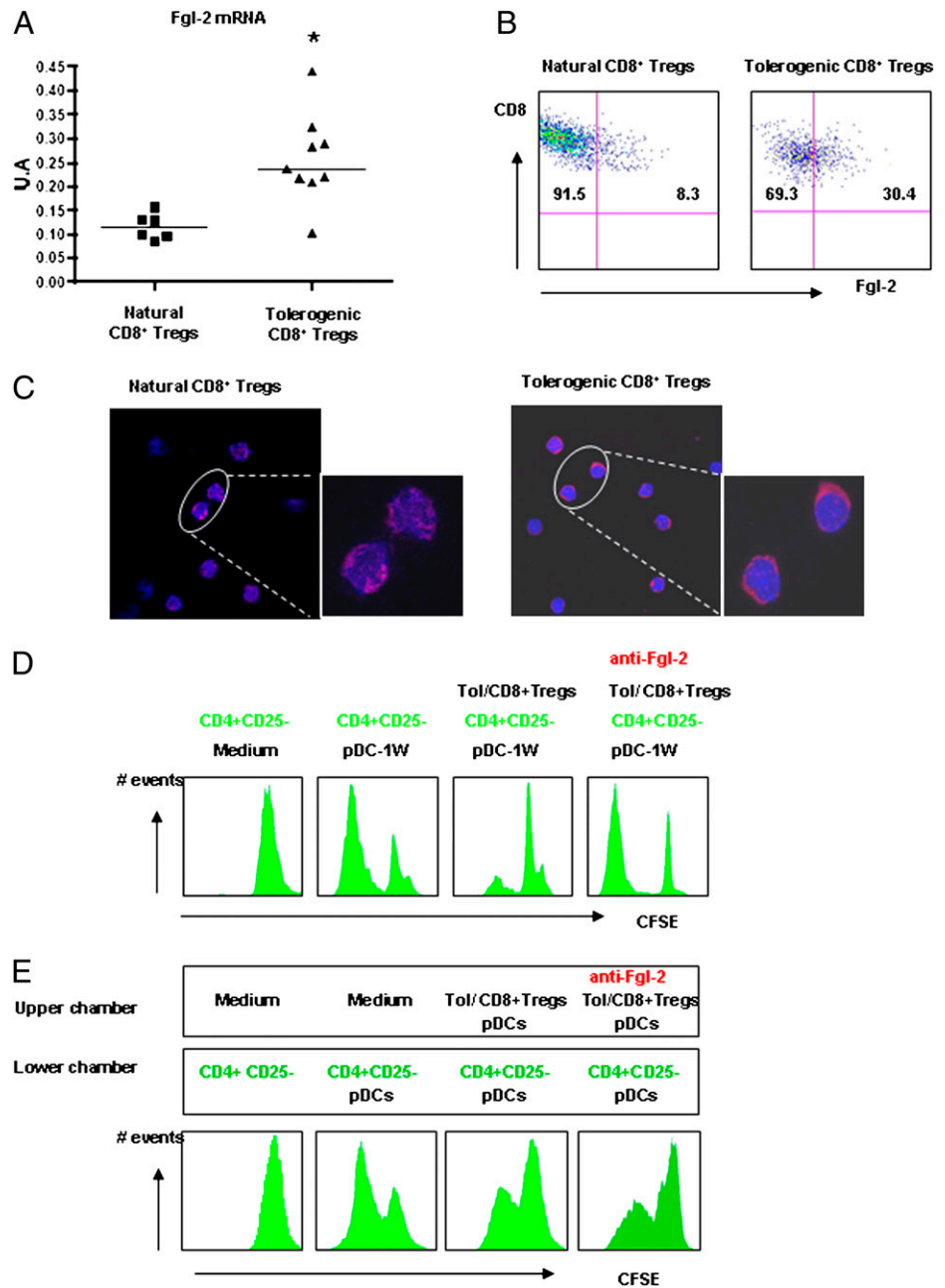
FIGURE 6. Fgl-2 is involved in the suppressive effect of tolerogenic CD8^{+} Tregs. Tolerogenic and natural CD8^{+} Tregs were isolated from spleen from recipients of CD40Ig-treated long-surviving allografts (>100 d) and naive animals and analyzed for Fgl-2 expression and its role in suppressive function. *A*, Fgl-2 mRNA levels by quantitative RT-PCR. Symbols represent cells from individual animals. *$p < 0.05$. *B* and *C*, Fgl-2 protein levels in naive and tolerogenic CD8^{+} Tregs as analyzed by FACS (*B*) and confocal (*C*) analysis (original magnification ×63). Data are representative of three independent experiments. The effect of neutralizing anti–Fgl-2 Abs (20 μg/ml) on the suppression of naive CD4^{+}CD25^{−} T cells by tolerogenic CD8^{+} Tregs analyzed in coculture MLR (*D*) and Transwell MLR (*E*). Data are representative of three independent experiments for each type of MLR.

the spleen decreased dramatically. The intragraft accumulation of pDCs in CD40Ig-treated recipients is reminiscent of tolerance (anti-CD40L plus donor-specific blood transfusion) in a cardiac graft model in mice (13), but pDC migration differed between the two models because tolerance was associated with accumulation of pDCs to LNs in the mouse model and to the spleen in our model. Furthermore, in this mouse model, tolerance was associated with CD4^{+}CD25^{+} Tregs (13). These differences may be explained by the use of distinct species and tolerance protocols. The suppressive function of CD8^{+} Tregs was observed in the presence of pDCs and CD4^{−} cDCs but not CD4^{+} cDCs, so we cannot exclude a role for CD4^{−} cDCs in tolerance induction in our model. Nevertheless, donor-specific suppression was only mediated by pDCs. CD8^{+} Tregs displayed donor alloantigen recognition in both direct and indirect Ag presentation pathways. CD4^{+}CD25^{+} Tregs with both specificities are more efficient to inhibit allograft rejection (32), suggesting that both pathways could participate in suppression by CD8^{+} Tregs.

The tolerance induced by CD8^{+} Tregs in our model could be explained by the action of Tregs on DCs (1), as previously reported for CD4^{+} Tregs (33, 34) and CD8^{+} Tregs (7, 35). Conversely, pDCs can induce allospecific CD8^{+} Tregs (6, 11) and CD4^{+} Tregs through mechanisms, such as IDO (12). IDO exists as two isoforms, IDO1 and IDO2 (36, 37), and although IDO1 was expressed by pDCs (data not shown), the expression of IDO2 and, more importantly the function of IDO1 and/or IDO2 in pDCs in this model, requires further research. Contact with pDCs was necessary to trigger suppression by tolerogenic CD8^{+} Tregs in our model. Although CTLA4 (33, 34) or the programmed death-1 (PD-1) ligand (4) have been involved in suppression by subsets of CD4^{+} or CD8^{+} Tregs, they did not play a role in suppression by our tolerogenic CD8^{+} Tregs because anti-CTLA4 or the PD-1 Fc did not reverse suppression in vitro (data not shown). Fgl-2 participated in the suppressive mechanism of CD8^{+}CD45RC^{low} Tregs because Fgl-2 was selectively increased in tolerogenic CD8^{+} Tregs, and suppression was abrogated by Fgl-2 blockade

in coculture MLR. Fgl-2 is a membrane and/or secreted molecule induced by IFN-γ and produced by CD4⁺CD25⁺ Tregs (38) and TCR⁺CD8⁺αα intraepithelial lymphocytes (39). Fgl-2 has been reported to inhibit T cell proliferation and DC maturation (25) through binding to T cells (25) or FcγRIIB on DCs (40). We have observed that, similar to human, rat pDCs express FcγRIIB (data not shown). Thus, IFN-γ–induced Fgl-2 expressed by tolerogenic CD8⁺ Tregs could inhibit CD4⁺ proliferation through interaction with FcγRIIB on pDCs. Enough neutralizing anti–Fgl-2 Ab to administrate to CD40Ig-treated rats was not available, but gene transfer in vivo using adeno-associated vectors encoding for rat Fgl-2 significantly promoted allograft survival (unpublished observation), giving indirect support to a role for Fgl-2 inhibiting allogeneic immune responses in vivo.

Interestingly, cell contact with CD4⁺ effector cells modified the suppressive mechanisms used by CD8⁺ Tregs because IFN-γ and Fgl-2 became predominant, in contrast to Transwell MLR where suppression was IDO-dependent but IFN-γ and Fgl-2 independent. A complete anti–Fgl-2 effect in coculture but not Transwell and Fgl-2 in the supernatant was detected in MLR the presence of CD8⁺ Tregs coculture but not Transwell (data not shown). As previously described (33, 34), it is possible that CD4⁺ cells inhibit IDO enzymatic activity through CD40–CD40L interactions with pDCs. This modification in suppression mechanisms by contact with CD4⁺ Teffs is reminiscent of recently published data showing that contact of CD4⁺CD25⁺ Tregs with conventional CD4⁺ T cells not only increased suppression by Tregs but also altered the suppression mechanism (15). CD8⁺ Tregs have been shown to exert suppression through contact-dependent (7, 35, 41, 42) or -independent mechanisms (4, 43). Contact-dependent mechanisms include cytotoxicity (41) but not TGF-β (7, 35, 42) or IL-10 (7). Contact-independent mechanisms can involve IFN-γ (43), TGF-β (43), and PD-1 (4) but not IL-10 (44). Donor-specific cytotoxicity was reduced in CD40Ig-treated recipients (5). The inclusion of IL-10– or TGF-β–neutralizing Abs in coculture or Transwell MLR did not modify suppression and long-term allograft survival was unchanged by anti–TGF-β Ab administration (data not shown).

Despite its recognized effects promoting immune responses, IFN-γ also has tolerogenic effects (45). IFN-γ upregulates Foxp3 expression in CD4⁺CD25⁺ Tregs (46), and it has been implicated in suppression mediated by CD8⁺ Tregs (43, 47–49). In a model of CD137 costimulation blockade, inhibition of immune responses was dependent on IFN-γ, yet IFN-γ alone did not induce suppression and acted through induction of TGF-β production by CD8⁺ Tregs (43). Similarly, in another model of CD8⁺ Tregs, IFN-γ did not directly inhibit CD4⁺ responses but rather licensed CD8⁺ Tregs to inhibit these responses (49). IFN-γ has been shown to induce a weak and transient increase of IDO functional activity in DCs, but neutralization of IFN-γ or IFN type I with B18R did not completely reverse IDO-dependent suppression, suggesting additional mechanisms inducing IDO, such as ligation of CD200, TLRs, or TNF-α and PGE₂ (24, 34). In our model, IFN-γ could be tolerogenic by inducing Fgl-2 on CD8⁺ Tregs. The involvement of IFN-γ only when CD4⁺ cells were in contact with DCs and Tregs could be explained by the directional secretion of IFN-γ to cells in close contact rather than diffuse production (50). Although IFN-γ promotes IDO expression on allografts (5) and allografts from IFN-γ–deficient donors are rejected more rapidly than wild-type grafts (51), intracoronary infusion of IFN-γ caused accelerated acute rejection in large animals (52), indicating that the effects of IFN-γ must take place both in the graft and in the lymphoid organs where close contact between CD4⁺, CD8⁺ Tregs, and DCs occur.

DC–T cell interaction in the absence of CD40L–CD40 results in cross-tolerization of CD8⁺ T cells, and IDO was shown to be essential (53). We found that IFN-γ was not responsible for IDO activity in vitro and other potential molecules that could induce IDO, such as IFN-I, TGF-β, and CTLA4 (33, 34), were also excluded. Further work is thus needed to identify the mechanism by which tolerogenic CD8⁺ Tregs induce IDO activity.

In conclusion, our results suggest that migration of pDCs and CD8⁺ Tregs to the graft and spleen allowed in vivo interactions necessary for suppression of CD4⁺ T cells. This suppression involved different mechanisms modulated by the presence of cell contact with CD4⁺ T cells, and IDO, IFN-γ, and Fgl-2 were the main mediators in vitro. In vivo, IDO and IFN-γ were essential for long-term allograft survival, suggesting that alloreactive CD4⁺ Teffs are suppressed by tolerogenic CD8⁺ Tregs through both contact-dependent and -independent mechanisms.

Acknowledgments

Disclosures

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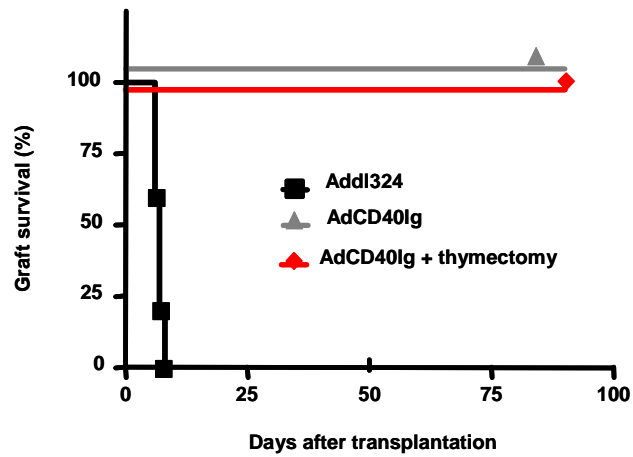
SUPPLEMENTARY FIGURES

Supplementary Figure 1.

a

b

c

d
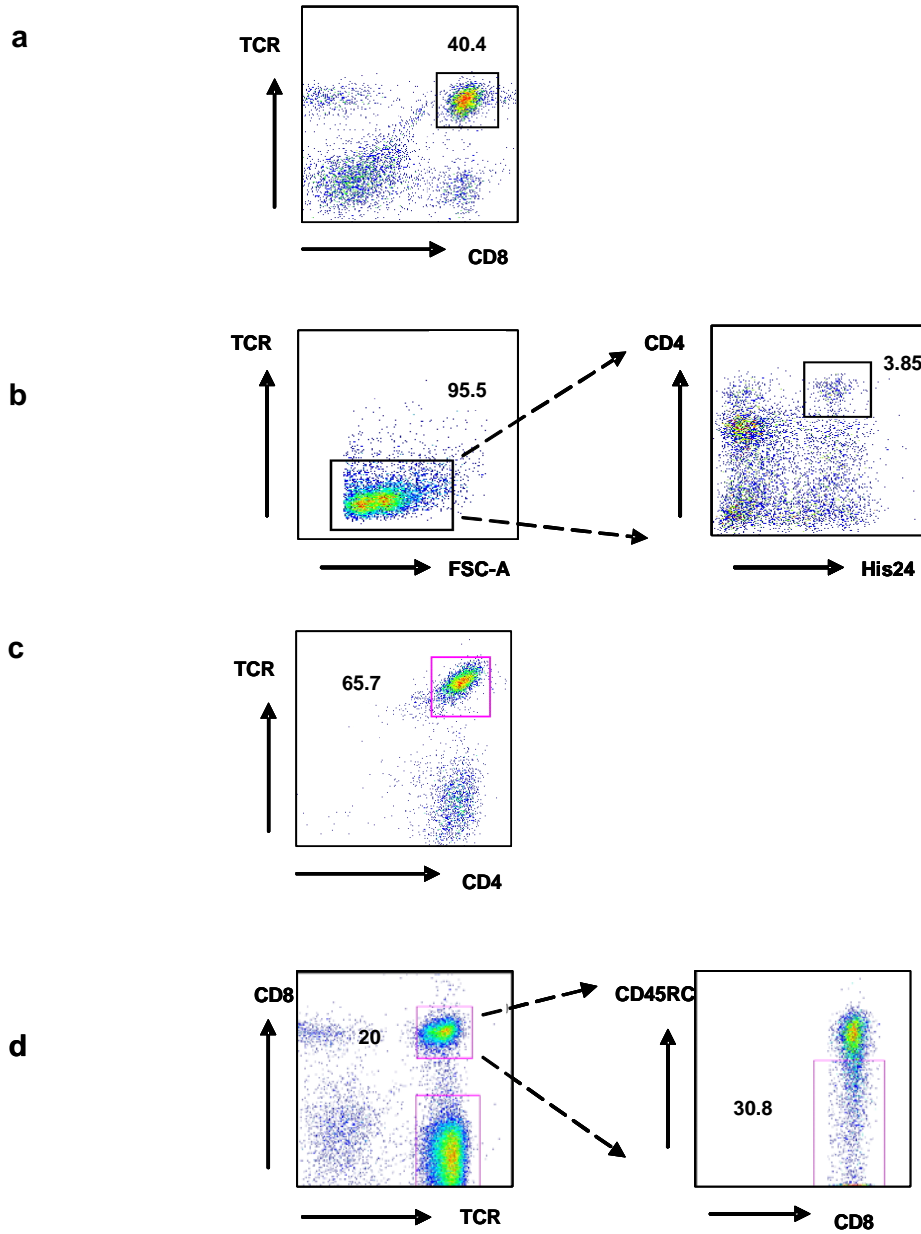
Supplementary figure 1. Gates used for cytofluorimetry analysis.

After gating cells by their morphology, cells were double labelled with the indicated antibodies, gated as indicated and used for analysis of additional markers. **a)** Gate used in figures 1d and 2d. **b)** Gates used in Figure 3d. **c)** Gate used in figures 4a, 5a-b, 5d, 5f, 6d-e as well as in supplementary figures 2a, 3b and 3c. **d)** Gate used in figure 6 b.
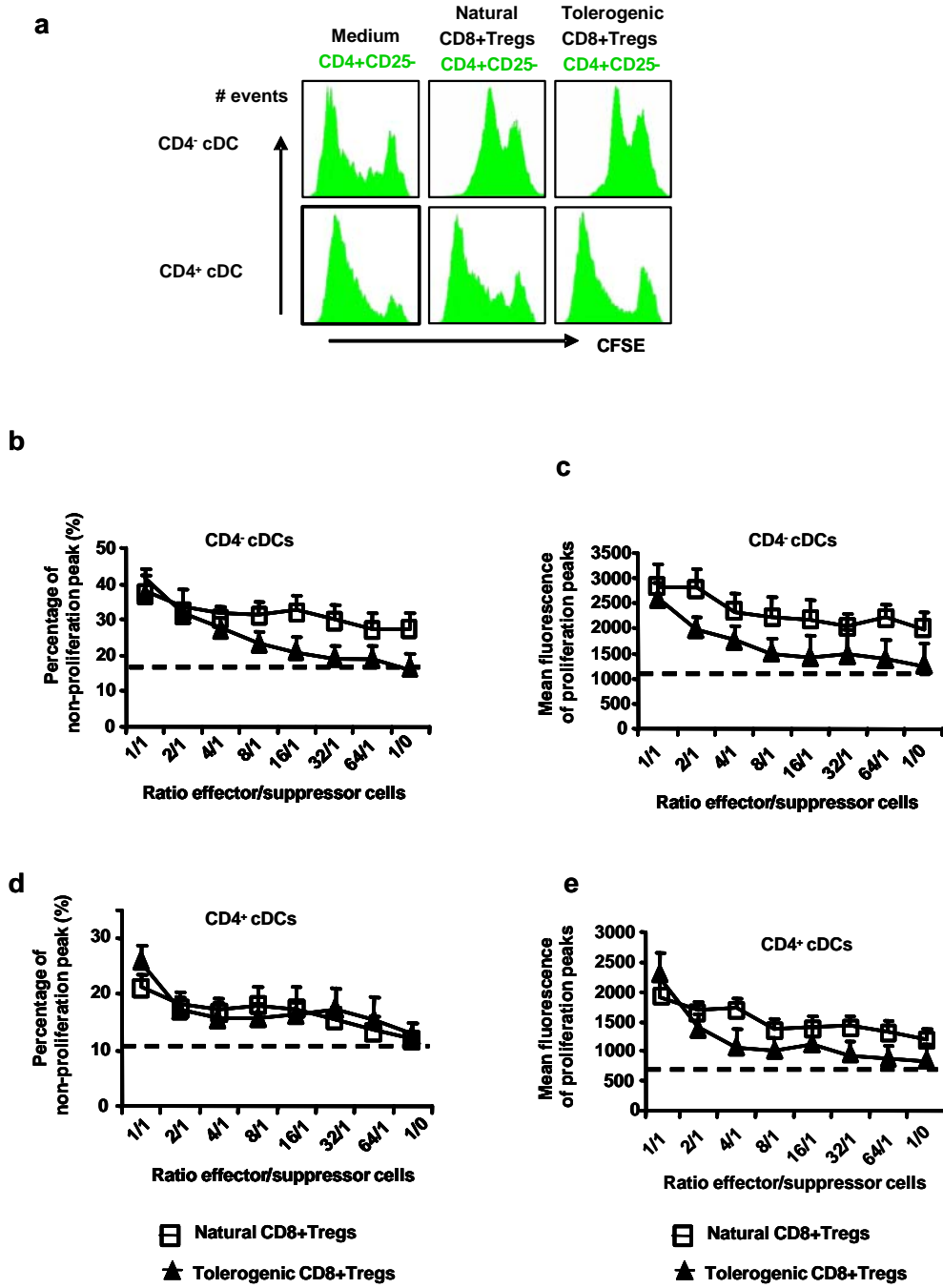
Supplementary Figure 2. Development of CD8$^+$ Tregs in CD40Ig-treated animals is independent of the thymus.

Cardiac allograft survival in the control group (non-coding Addl324, n=4) or after AdCD40Ig administration in recipients with (n=4) or without (n=4) the thymus
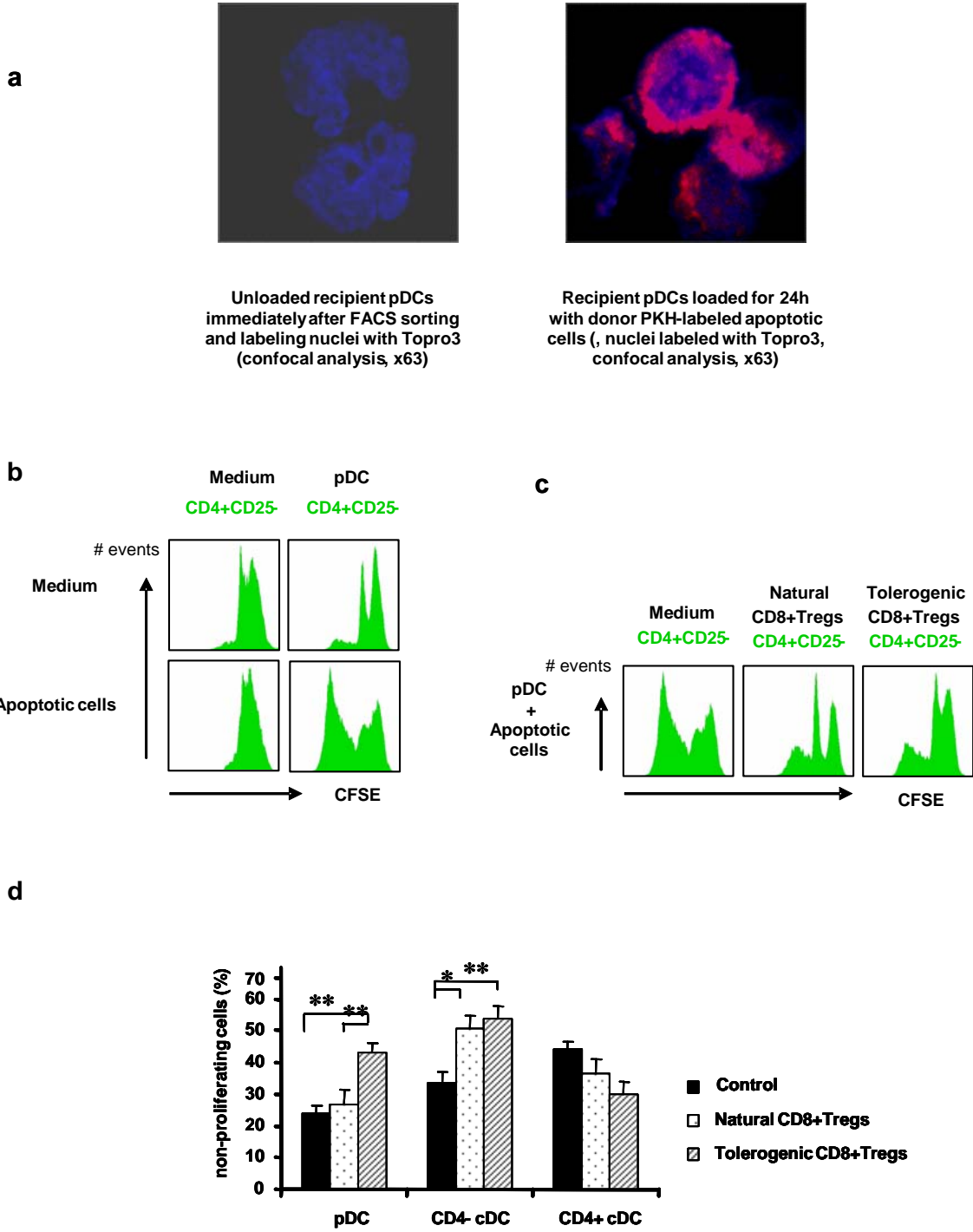
Supplementary Figure 3. CD8$^+$ Tregs suppress CD4$^+$ T cell response to direct alloantigen presentation by CD4$^-$ cDCs but not CD4$^+$ cDCs.

(a) CD4$^+$CD25$^-$ CFSE-labelled T cells were cocultured with or without natural or tolerogenic CD8$^+$ Tregs as well as CD4$^-$ or CD4$^+$ cDCs for 6 days. One representative experiment out of 3 is shown. (b) The same suppressive assay performed using decreasing ratios of effector/suppressor cells with results depicted as the percentage of non-proliferating CFSE-labelled naïve CD4$^+$CD25$^-$ T cells. Data is the mean ± SD of 3 independent experiments. Dotted lines represent values obtained in the absence of CD8$^+$ Tregs. * p< 0.05 tolerogenic vs. natural CD8$^+$ Tregs. (c) The same suppressive assays with results depicted as the MFI of CFSE-labelled naïve CD4$^+$CD25$^-$ proliferating cells. Dotted lines represent values obtained in the absence of CD8$^+$ Tregs. * p< 0.05 tolerogenic vs. natural CD8$^+$ Tregs.

Supplementary Figure 4.

a

Unloaded recipient pDCs immediately after FACS sorting and labeling nuclei with Topro3 (confocal analysis, x63)

Recipient pDCs loaded for 24h with donor PKH-labeled apoptotic cells (, nuclei labeled with Topro3, confocal analysis, x63)

b

Medium CD4+CD25-   pDC CD4+CD25-

events

Medium

Apoptotic cells

CFSE

c

Medium CD4+CD25-   Natural CD8+Tregs CD4+CD25-   Tolerogenic CD8+Tregs CD4+CD25-

events

pDC + Apoptotic cells

CFSE

d

non-proliferating cells (%)

pDC   CD4- cDC   CD4+ cDC

■ Control
□ Natural CD8+Tregs
▨ Tolerogenic CD8+Tregs

6

Supplementary Figure 4. CD8$^+$ Tregs suppress CD4$^+$ T cell response to indirect alloantigen presentation by pDCs and CD4$^-$ cDCs but not CD4$^+$ cDCs.

The ability of recipient DCs to cross-present alloantigen was tested. **(a)** pDCs from LEW-1A before (left) and after (right) loading with PKH-labelled donor LEW.1W apoptotic heart resident leukocytes were analyzed by fluorescence microscopy. **(b)** The capacity of pDCs to present donor alloantigens through the indirect pathway was analyzed by cytofluorimetry. CFSE-labelled LEW.1A naïve CD4$^+$CD25$^-$ T cells were stimulated by recipient LEW.1A pDCs after loading with medium or apoptotic donor heart resident leukocytes. Data are representative of 3 independent experiments. **(c)** After gating on TCR$^+$CD4$^+$ cells, CFSE-labelled CD4$^+$ T cells were analyzed after 6 days of coculture with natural or tolerogenic CD8$^+$ Tregs and recipient LEW.1A pDCs loaded with alloantigen (1:1 ratio for effector/suppressor). Data are representative of 3 independent experiments. **(d)** CD4$^+$CD25$^-$ CFSE-labelled T cells and indicated subpopulations of recipient DCs loaded with donor antigens were cocultured with or without natural or tolerogenic CD8$^+$ Tregs for 6 days. After gating on TCR$^+$CD4$^+$ cells, the percentage of non-proliferating CFSE-labelled cells was determined (mean ± SD of triplicates from one experiment representative out of 3) (** p = 0.001 for tolerogenic CD8$^+$ Tregs group vs. control MLR in the presence of pDCs, p = 0.0026 for tolerogenic CD8$^+$ Tregs group vs. natural CD8$^+$ Tregs group in the presence of pDCs and p = 0.006 for tolerogenic CD8$^+$ Tregs group vs. natural CD8+ Tregs group in the presence of CD4$^-$ cDCs; * p = 0.01 for natural CD8$^+$ Tregs group vs. control group in the presence of CD4$^-$ cDCs).

Supplementary Figure 5.

a

b
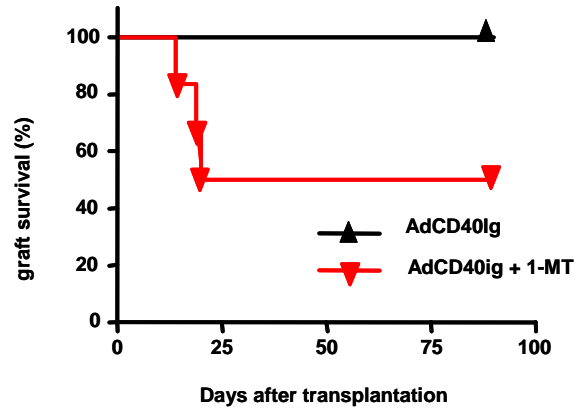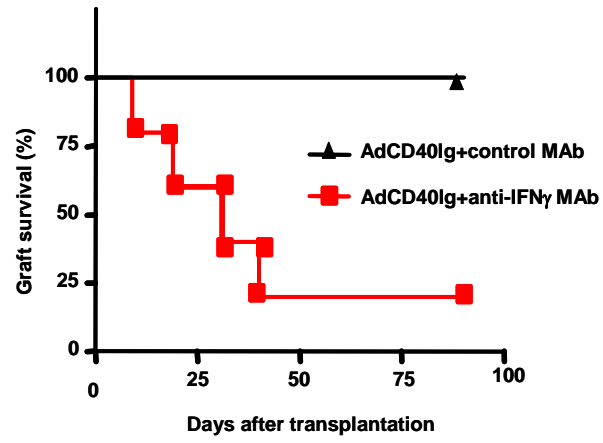
Supplementary Figure 5. Tolerance induction by CD40Ig treatment at least in part mediated by IDO and IFN-γ.

(a) Graft survival in animals that received an adenovirus encoding for CD40Ig alone or together with the IDO inhibitor 1-MT (twice daily by oral gavages at 50 mg/dose) on the day of transplantation (n = 4, each group). (b) Graft survival rates were compared after administration of CD40Ig and neutralizing anti-IFN-γ or isotype control mAb (at 3mg/kg i.p./2 week) (n=5 and n=4, respectively, p = 0.0081 between the two groups).