Original Article

Characterization of Donor Dendritic Cells and Enhancement of Dendritic Cell Efflux With cc-Chemokine Ligand 21

A Novel Strategy to Prolong Islet Allograft Survival

Paolo Fiorina,[1,2] Mollie Jurewicz,[1] Katsunori Tanaka,[1] Negin Behazin,[1] Andrea Augello,[1] Andrea Vergani,[1,2] Uli Von Adrian,[3] Neal R. Smith,[4] Mohamed H. Sayegh,[1] and Reza Abdi[1]

Dendritic cells (DCs) are the most potent antigen-presenting cells, yet little data are available on the differential characteristics of donor and recipient DCs (dDCs and rDCs, respectively) during the process of islet allograft rejection. DTR-GFP-DC mice provide a novel tool to monitor DC trafficking and characteristics during allograft rejection. We show rapid migration of dDCs to recipient lymphoid tissues as early as 3 h post–islet allotransplantation. Compared with rDCs, dDCs express different patterns of chemokine receptors, display differential proliferative capacity, and exhibit a higher level of maturity; these findings could be attributed to the effects of injury that dDCs undergo during islet cell preparation and engraftment. Intriguingly, we detected dDCs in the spleen of recipients long after rejection of islet allografts. Given that dDCs express high levels of CCR7, islets were cultured before transplant with the ligand for CCR7 (CCL21). This novel method, which enabled us to enhance the efflux of dDCs from islet preparations, resulted in a prolongation of islet allograft survival in immunocompetent recipients. This study introduces dDCs and rDCs as two distinct types of DCs and provides novel data with clinical implications to use chemokine-based DC-depleting strategies to prolong islet allograft survival. *Diabetes* 56:912–920, 2007

From the [1]Transplantation Research Center, Children's Hospital and Brigham and Women's Hospital, Harvard Medical School, Boston, Massachusetts; the [2]Department of Medicine, San Raffaele Scientific Institute, Milan, Italy; the [3]CBR Institute for Biomedical Research and Department of Pathology, Harvard Medical School, Boston, Massachusetts; and the [4]Department of Pathology, Massachusetts General Hospital, Harvard Medical School, Boston, Massachusetts.

Address correspondence and reprint requests to Reza Abdi, MD, Transplantation Research Center (TRC), Children's Hospital and Brigham and Women's Hospital, Harvard Medical School, 221 Longwood Ave., Boston, MA 02115. E-mail: rabdi@rics.bwh.harvard.edu.

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P.F. and M.J. contributed equally to this work.

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BrdU, bromodeoxyuridine; dDC, donor dendritic cell; DC, dendritic cell; DT, diphtheria toxin; FACS, fluorescence-activated cell sorting; GFP, green fluorescent protein; MCH, major histocompatibility complex; MLR, mixed lymphocyte reaction; rDC, recipient dendritic cell.

Generation of alloreactive T-cells, a pivotal event in allograft rejection, requires presentation of alloantigens to recipient T-cells. Dendritic cells (DCs) are regarded as the most potent antigen-presenting cells (1–3). During the process of direct allorecognition, recipient T-cells recognize intact allogeneic major histocompatibility complex (MHC) molecules on donor tissue resident dendritic cells (dDCs) (4–6). During indirect allorecognition, donor alloantigen shed from the allograft is released into the recipient's circulation, is processed by recipient DCs (rDCs), and finally is presented to recipient T-cells (7). DCs also express high levels of costimulatory molecules, rendering them potent initiators of alloimmune responses, and regulate immune responses by instructing T-cells and by regulating the generation of suppressive (regulatory) T-cell responses. However, many questions remain concerning DCs in transplantation, the answers to which are of fundamental importance in better comprehending the roles of DCs during the processes of engraftment or rejection. For instance, no data are yet available on distinct dDC and rDC trafficking or characteristics (maturity, longevity, proliferation, mobility, and chemokine receptor expression) during the process of islet allograft destruction. Because of the lack of animal models to easily monitor rDCs and dDCs, characterization of such cells has been a difficult task and therefore remains poorly described. B6.FVB-Tg (Itgax-DTR/GFP)57Lan (DTR-GFP-DC) mice, which have a green fluorescent protein (GFP) linked to the CD11c promoter, provide a model that can be used to circumvent this problem. Using these mice as donors of islet allografts enabled us to better study DC characteristics and trafficking (8,9). Because the capacity of DCs to proficiently present antigen to T-cells and to generate an alloimmune response relies on the ability of DC trafficking to lymphoid tissue, and given that chemokines tightly control DC migration trafficking, we also examined the expression of chemokine receptors of DCs at different time points after islet transplantation (5,10–12). In recognition of the importance of dDCs in the generation of alloimmune responses, particularly in the case of islet cell transplantation, investigators have attempted to deplete dDCs using a number of techniques with the goal of achieving prolongation of islet graft survival (13–17). Here we aim to

characterize dDCs and to establish a chemokine-based strategy to deplete islet DCs by culturing islets in the presence of the chemokine CCL21 before transplantation to enhance the efflux of DCs from islets.

RESEARCH DESIGN AND METHODS

B6.FVB-Tg (ITgax-DTR/GF)57Lan (DTR-GFP-DC), CD45.1 (B6.SJL-Ptprca Pepcb/BoyJ), C57BL/6, and BALB/c mice were purchased from The Jackson Laboratory, Bar Harbor, Maine.

Islet transplantation. Pancreatic islets were isolated by collagenase digestion followed by density gradient separation and then handpicking, as described previously (18). For more information, please refer to the online appendix (available at http://dx.doi.org/10.2337/db06-1445).

Trafficking studies. Allogeneic islets isolated from DTR-GFP-DC mice (on a BALB/c background, H-2^d) were transplanted under the kidney capsule of streptozotocin (STZ)-induced diabetic C57BL/6 mice (H-2^b). For details of flow cytometric and immunohistological analysis, see the online appendix.

Proliferation studies. To address the proliferation of dDCs and rDCs, mice were injected intraperitoneally with 1 mg bromodeoxyuridine (BrdU) (Sigma-Aldrich) dissolved in PBS as described previously (19–22). DCs were isolated from splenocytes using anti-CD11c magnetic beads (Miltenyi Biotec, Auburn, CA) with >90% purity and were assessed for BrdU uptake (19).

Depletion of DCs using diphtheria toxin or CCL21. We established two groups of DC-depleting experiments by either administering diphtheria toxin (DT) to donor in BALB/c GFP-DTR-DT mice or by isolating islets and culturing them with CCL21. For details of both experiments, refer to the online appendix.

DC isolation, culture, and adoptive transfer. BALB/c DCs were generated as previously described from murine bone marrow (23). For details of these culture conditions as well as specifics of adoptive transfer, refer to the online appendix.

Mixed lymphocyte reaction. Splenocytes were isolated at day 7 post–islet transplantation from STZ-induced diabetic C57BL/6 mice (responders) that received BALB/c CCL21–cultured or control islets and were challenged with donor irradiated naïve BALB/c splenocyte stimulators in a fully mismatched mixed lymphocyte reaction MLR. Cells were cultured for 3 days, and proliferation was measured following pulsing for 16 h with P[3H]TdR using a liquid scintillation counter. T-cells were also stimulated with ConA as a positive control.

Statistical analyses. Survival of islet grafts has been evaluated with a survival Kaplan-Meier analysis. A Mann-Whitney test (for nonparametric data) or two-sided unpaired Student's t test (for parametric data) was used when the differences were compared among the groups cross-sectionally. A P value <0.05 (by two-tailed testing) was considered an indicator of statistical significance. Data are expressed as means ± SE. Prism Graph Pad statistical package for Windows (GraphPad Software, San Diego, CA) was used for data analysis.

RESULTS

Evaluation of dDC trafficking using DTR-GFP-DC mice. In DTR-GFP-DC mice, a GFP is linked to the CD11c promoter. As shown in Fig. 1*A*, in contrast to BALB/c WT naïve mice, BALB/c DTR-GFP-DC naïve mice show GFP^+ expression in the spleen (Fig. 1*B*). Examination of the naïve pancreas of BALB/c DTR-GFP-DC (used as donors in islet transplantation studies) showed a cluster of GFP^+ DCs surrounding the pancreatic islets (as shown by insulin staining) (Fig. 1*C*). Hence, DTR-GFP-DC mice provide a unique tool to monitor dDC trafficking by transplanting islets from DTR-GFP-DC mice (BALB/c background) into STZ-induced diabetic C57BL/6 mice. Following transplantation of islets underneath the kidney capsule, islet grafts and secondary lymphoid tissues were also examined for the presence of GFP^+ cells to evaluate the trafficking of dDCs. dDCs were shown to migrate from the islet graft (located under the kidney capsule) into the recipient renal parenchyma and spleen as early as 3 h after allogeneic islet transplantation (Fig. 1*D* and *E*, respectively). Compared with 3 h posttransplant, the number of dDCs seen in the recipient spleen increased significantly at 24 h (Fig. 1*F*). Immunostaining showed that these GFP^+ cells coexpress CD11c (Fig. 1*G*) and are found within the vicinity of $CD4^+$ cells in the spleen of islet allograft recipients (Fig. 1*H*). The earliest time point at which dDCs could be detected was at 3 h in the spleen. While at 3 h and at day 1 posttransplantation no dDCs were detected in the recipient not draining lymph nodes (data not shown), we observed very few dDCs in the draining lymph nodes at 3 h (Fig. 2*A*) with an increase at 24 h after transplantation (Fig. 2*B*). No dDCs were detected in the thymus at 3 h and at day 1. These data demonstrate a selective, robust, and rapid migration of islet dDCs into recipient spleen. Interestingly, we did detect dDCs in the bone marrow of recipients after allogeneic islet transplantation 3 h after transplant, highlighting the possible importance of bone marrow as a site of allorecognition or immunoregulation (Fig. 2*C*). The percentage of $CD11c^+GFP^+$ cells remained constant at 24 h as well (data not shown). Given that streptozotocin induces islet inflammation and could serve as a signal for dDCs to migrate, we performed islet transplantation using donor islets from DTR-GFP-DC mice into STZ-induced diabetic mice. Naïve pancreata were examined following transplantation and showed no active presence of dDCs (data not shown).

Since spleen contained more dDCs than other lymphoid tissues, we compared the percentage of $CD11c^+GFP^+$ cells in the spleen of islet allograft and isograft recipients by fluorescence-activated cell sorting (FACS). While there were very few $CD11c^+GFP^+$ cells in the spleen of syngeneic recipients (islets from C57BL/6 donor DTR-GFP-DC mice into C57BL/6 recipient) (Fig. 2*D*), a large number of double-stained cells were noted in the spleen of allogeneic recipient mice (Fig. 2*E*). These data are suggestive of much more prominent migration of dDCs in the allogeneic setting and suggest that most of the GFP^+ cells also coexpress CD11c. To study the kinetics of dDC and rDC trafficking, we recovered infiltrating cells from islet grafts and subjected them to our FACS analysis. We observed only a small increase in rDCs at days 1 and 7, while a 20-fold increase in dDCs recovered from islet grafts was noted at day 1 after islet transplantation (Fig. 2*F* and *G*). This could be in part due to the proliferative capacity of dDCs (data below).

dDCs exhibit a higher proliferative ability at an earlier time point than rDCs. No data exist on the proliferative capacity of DCs in islet cell transplantation. To assess the proliferative capability of dDCs and rDCs, we injected BrdU, a proliferation marker that binds to newly synthesized DNA (19–22), into recipients of islet allografts. By analyzing recipient splenocytes by FACS and gating on GFP^+ and $BrdU^+$ cells, we show that in the allogeneic setting dDCs begin to proliferate at an early time point (3 h) and that dDCs exhibit a higher proliferative capacity than rDCs (both recovered from recipient spleens) during the time course of the study (Fig. 2*H*–*J*). To further confirm the proliferative capability of dDCs, we also used Ki67, a sensitive cycle marker for in vivo studies. Sections of the spleens recovered from islet allograft recipients of GFP^+ donors were stained with an anti-Ki67 antibody (red). dDCs (GFP^+ cells, green) undergoing proliferation were costained for Ki67 and appeared yellow (Fig. 2*K*).

Chemokine receptor and costimulatory molecule expression of dDCs and rDCs. We then examined dDC and rDC expression of chemokine receptors and of CD86/CD80 (DC maturity markers) at 3 h, day 1, and day 7 posttransplantation in recipient spleens (Fig. 3). Com-

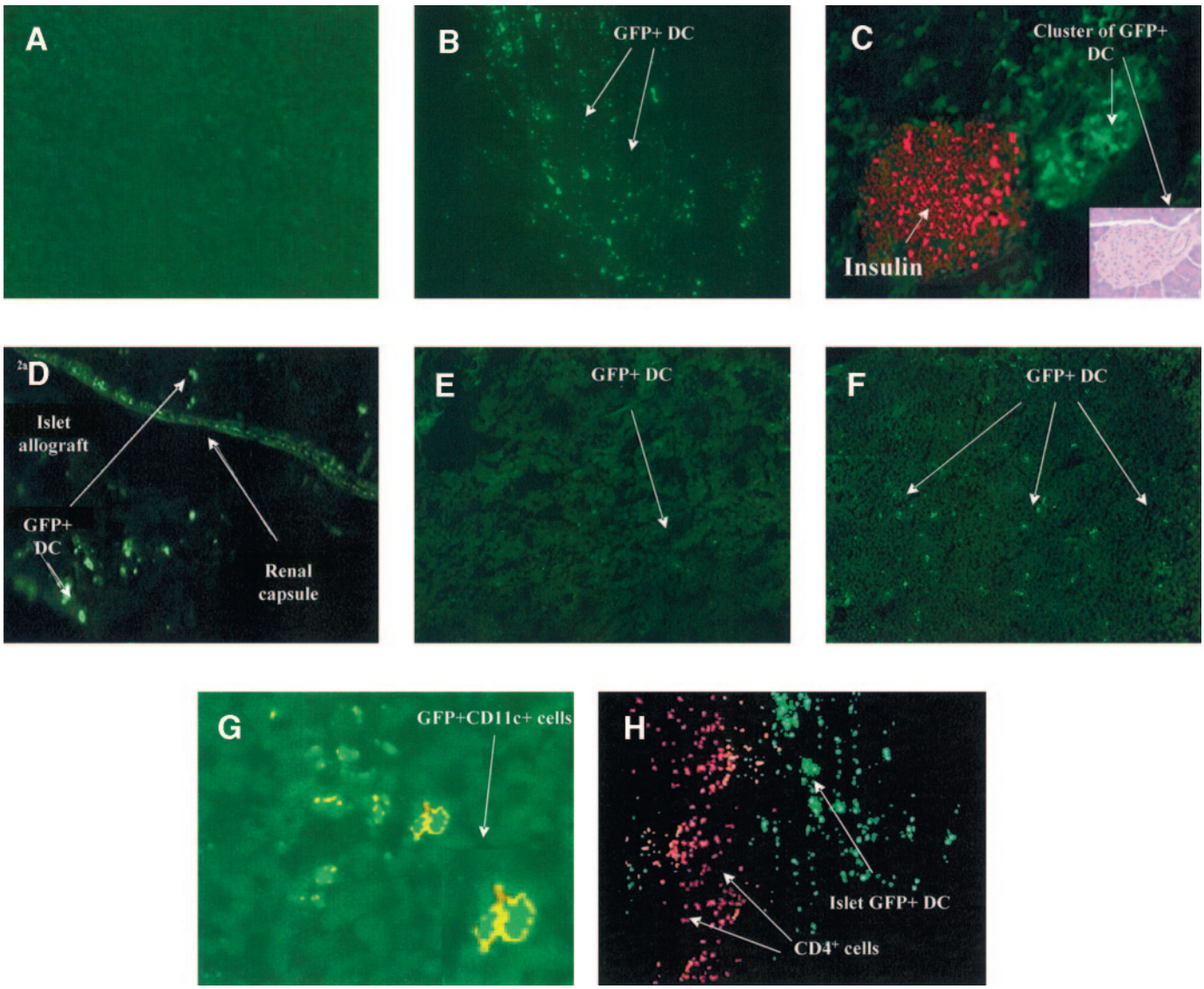

FIG. 1. Spleens were removed from naïve BALB/c (control) and BALB/c DTR-GFP-DC mice. Compared with background (autofluorescence) (*A*), DTR-GFP-DC mice showed GFP$^+$ cells in the spleen (*B*). A cluster of GFP$^+$ cells is evident in the vicinity of the islet complex, identified by insulin staining, in the pancreas of DTR-GFP-DC mice (*C*). Three hours after allogeneic islet transplantation, dDC-GFP$^+$ migrate through the recipient renal parenchyma (*D*) and into the recipient spleen (*E*) with an increase in number observed at day 1 (*F*). GFP$^+$ cells detected in the spleen of islet allograft recipients are costained with CD11c (*G*). Finally, dDC-GFP$^+$ cells (green) were noted in the CD4$^+$ cell zone (red) of recipients' spleens (*H*). All fields originally magnified at 400×. n = 4 mice per group.

pared with rDCs, dDCs displayed much higher levels of expression of the chemokine receptors CCR5, CXCR4, and CCR7 at 3 h and at day 1 (Fig. 3*A–C* and *F–H*, respectively). At day 7, although dDCs still exhibit higher levels of CXCR4 and CCR7 than rDCs, this difference subsides for CCR5 (Fig. 3*K–M*).

At 3 h and at day 1, dDCs also expressed much higher levels of CD86 and class II than rDCs (Fig. 3*D* and *E* and *I* and *J*). At day 7, dDCs and rDCs display comparable levels of CD86 and class II (Fig. 3*N* and *O*). These data are in accordance with the current thought that dDCs (direct pathway) are the first initiators of early acute rejection of islet allografts (24–27). Finally, these data could also suggest that similar to the in vitro DC maturation data previously reported (23), CXCR4 and CCR7 appear to be important chemokine receptors involved in the migration and maturation of DCs.

Oxidative/ischemic stress and DC activation. We examined the hypothesis that tissue injury during islet cell preparation and transplantation ischemia can contribute to the activation of dDC, an important aspect in the generation of alloimmune response that remains to be explored. A number of studies have highlighted the active recruitment and presence of DCs in ischemic tissue (28,29). Kostulas et al. (28) showed an increase in the number and in the activation of DCs in ischemic brain compared with sham hemispheres as early as 1 h after arterial occlusion. Ischemia associated with the surgical procedure itself was reported to recruit DCs to the site of injury in a model of renal ischemia (28,29). We evaluated the maturation of islet dDCs following 24-h culture and compared them with islet DCs analyzed immediately after isolation. The expression of CD80, MHC class II molecules, and CCR7 was upregulated on dDCs recovered from cultured islets as compared with preculture islets (Fig. 4*A–C*). Class I expression studies showed no significant upregulation (data not shown). These data support the idea that due to prolonged ischemia time and tissue

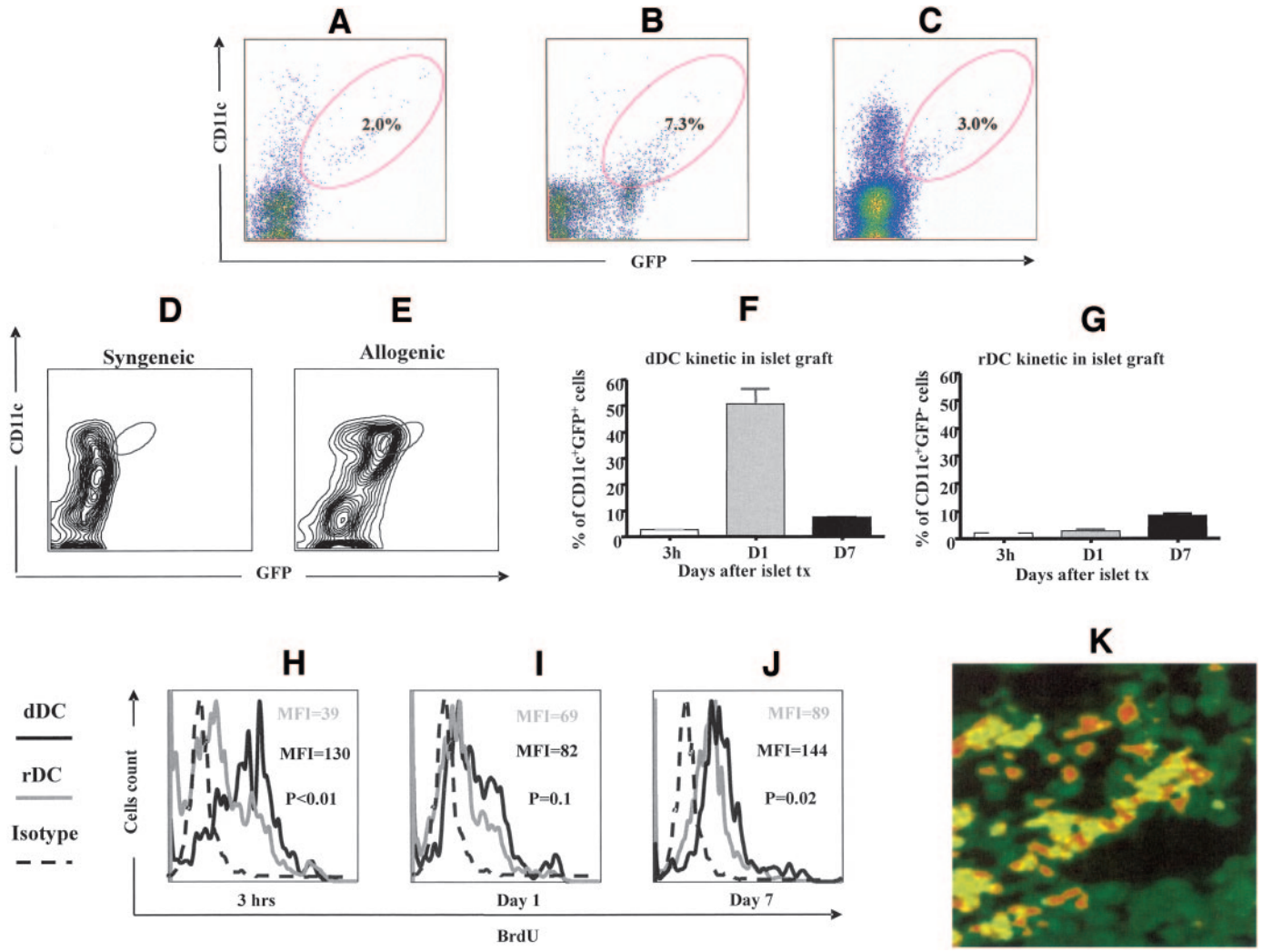

FIG. 2. Islet grafts, DLN, pancreata, and bone marrow were recovered from recipients of islet allografts from DTR-GFP-DC donors. Compared with 3 h (*A*), a significant increase in the number of dDC-GFP$^+$ cells can be detected in the recipient DLN 24 h after transplantation (*B*); dDC-GFP$^+$ cells are detected in the recipient bone marrow as well at 3 h (*C*). More CD11c$^+$GFP$^+$ cells were found in the allogeneic model compared with syngeneic (*D* and *E*) ($P < 0.01$). The percentage of dDC-GFP$^+$ cells in islet grafts increases significantly at 24 h compared with 3 h (*F*), while rDCs show a slight increase at day 7 only (*G*) ($P < 0.01$). BrdU was administered to the islet allograft recipients, and then proliferative capacity of dDCs and rDCs was studied. dDCs in the spleen of allogeneic islet recipients showed a higher proliferative capacity than rDCs posttransplantation (*H–J*) ($P < 0.01$ at 3 h and $P = 0.02$ at day 7). $n = 4$ mice per group. Sections of the spleens recovered from islet allograft recipients of GFP$^+$ donors were stained with an anti-Ki67 antibody (red). dDCs (GFP$^+$ cells, green) undergoing proliferation were costained for Ki67 and appear yellow (*K*).

preparation, islet DCs become more activated and migrate faster because of chemokine receptor upregulation. Furthermore, to address the direct effect of oxidative stress on DC activation, we cultured bone marrow–derived DCs from BALB/c mice (our islet donors) with 200 μmol H_2O_2 for 30 min as described previously (in which B-cells were treated with a similar strategy) (30). FACS analysis using 7-AAD and AnnexinV showed no significant decrease in cell viability (<2%) following H_2O_2 treatment (data not shown). As shown in Fig. 4*D–E*, treating dDCs with H_2O_2 significantly upregulated the expression of CD86/CD80 (Fig. 4*D–E*). These data suggest the potential role of ischemia/injury in the induction of chemokine receptors and in the maturation of dDCs and could in part explain the higher activity of islet DCs and their immunogenicity.

dDCs were found in recipient spleen long after islet allograft rejection. One important unexpected observation was the detection of dDCs in the spleen of the recipients long after rejection of islet allografts. This was observed when islets were transplanted from BALB/c GFP-DTR-DC donors into C57BL/6 recipients. Our FACS data showed the presence of GFP$^+$ cells in the spleen of islet allograft recipients 30 days after islet transplantation (2 weeks after rejection of islet allografts) (Fig. 4*F*). Similarly, when we transplanted islets from the CD45.1$^+$ congenic strain into a CD45.2$^+$ recipient and spleens of recipients were recovered 30 days after transplantation, persistent dDCs (CD11c$^+$CD45.1$^+$ cells) were demonstrated by FACS in the islet allograft recipient spleens (Fig. 4*G*). This transplant combination has been one of the classical models to investigate allografted donor cells in recipients (31,32).

Depletion of islet DCs using DTR-GFP-DC mice. DTR-GFP-DC mice have a fusion protein that expresses both the simian DT receptor and GFP and is linked to the CD11c promoter. Murine cells, unlike primate cells, are insensitive to death by DT; transfer of primate DTR into mice via a transgene therefore confers DT sensitivity to murine cells (8,33). DT was administered to BALB/c DTR-GFP-DT mice, and islets isolated from DT-treated

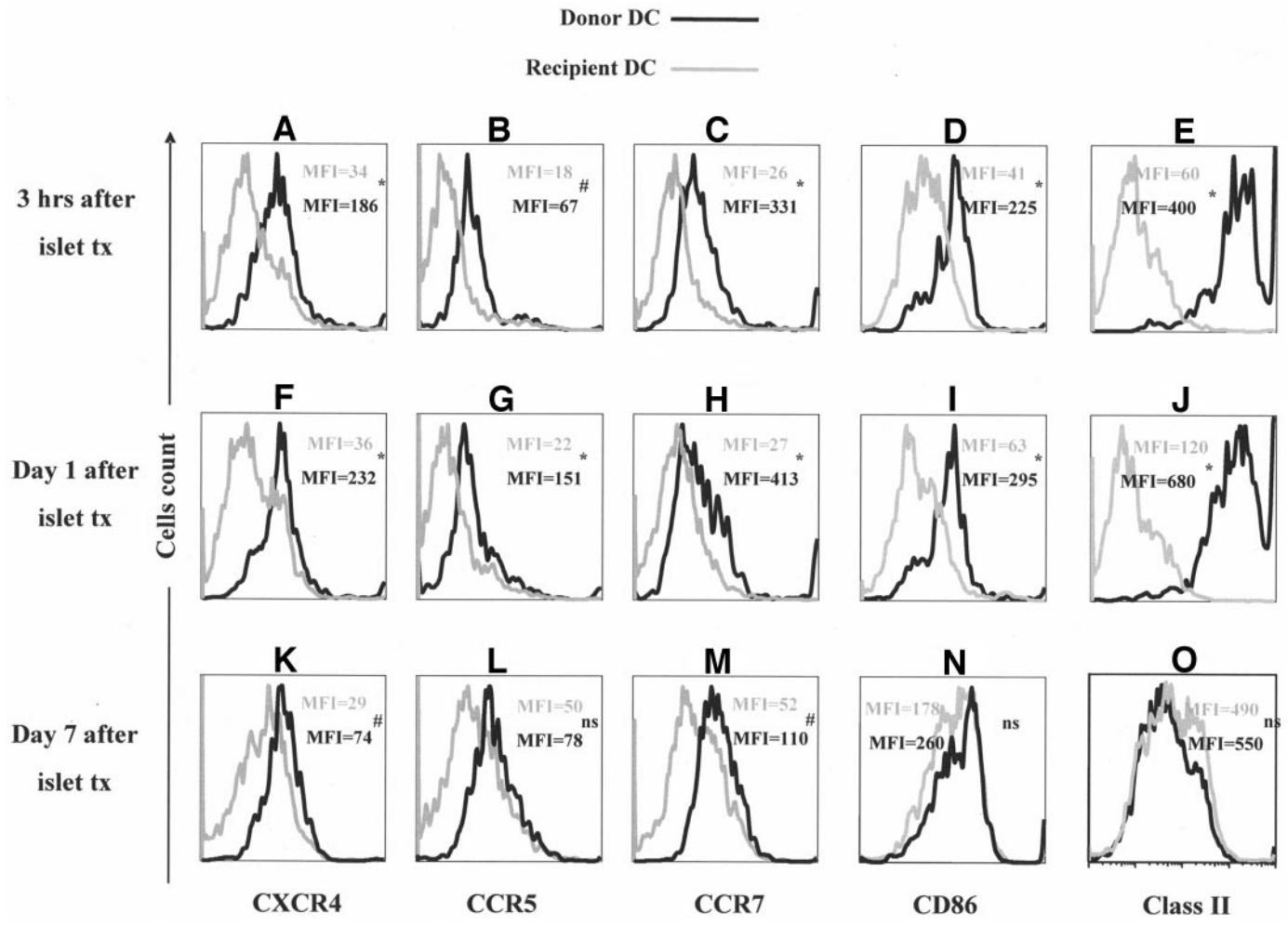

FIG. 3. Spleens of islet allograft recipients were removed and splenocytes were subjected to FACS analysis. dDCs ($CD11c^+GFP^+$) and rDCs ($CD11c^+GFP^-$) were studied for chemokine receptor and CD86/class II expression. A higher level of CXCR4, CCR5, CCR7, CD86, and class II is noted in dDCs extracted from recipient spleens at 3 h after allogeneic islet transplantation (Tx) (*A–E*). This higher expression of chemokine receptor and maturity markers (CD86/class II) in dDCs vs. rDCs is maintained at day 1 (*F–J*), while at day 7, dDCs and rDCs showed comparable levels of expression of CCR5 and maturity markers (*K–O*). All experiments $n = 4$ mice per group. *$P < 0.001$, #$P < 0.05$. ns, not statistically significant.

mice were transplanted into STZ-induced diabetic C57BL/6 recipients. At baseline, $CD11c^+GFP^+$ cells are evident in the spleen and in the pancreas of DTR-GFP-DC mice (Fig. 4*H*), but $CD11c^+GFP^+$ cells can be depleted with 250 ng DT. Depletion is evident in the spleen and pancreas at 6 h and more efficiently occurs at 48 h postinjection (Fig. 4*H*). Surprisingly, transplanting islets from DT-depleted mice into an allogeneic host resulted in a slight delay of islet allograft rejection (mean survival time [MST] 11 vs. 14 for control and DT-depleted, respectively, $P = 0.01$) (Fig. 4*I*). Examination of recipient spleens transplanted with DT-treated donors revealed that GFP^+ cells could still be detected at day 7 after islet transplantation (data not shown). The presence of dDCs in recipient spleens following depletion could explain modest prolongation of islet allograft survival. We speculate that a more robust depletion could be achieved by administering a higher dose of DT; however, a high mortality rate associated with increasing doses of DT has been a limiting factor in achieving this end.

Establishing a chemokine-based DC depletion strategy for islet transplantation. Previous studies to deplete dDCs of islets have led to prolongation of islet allograft survival. Data from these studies indicate that endocrine tissues such as islets are highly dependent on the function of dDCs to develop an acute rejection response (13–17). Here, we have established a novel strategy to enhance migration of dDCs out of islets during preparation for transplantation. Given the overexpression of CCR7 in dDCs during the alloresponse, we hypothesized that culturing islets with CCL21 (CCR7 ligand) would cause efflux of dDCs from the islets. We cultured islets for 24 h with CCL21 and performed FACS analysis of cells recovered from the aspirated medium above the islets. Medium obtained from islets cultured for 24 h with 200 ng/ml CCL21 showed that the percentage of $CD11c^+$ cells (after gating on $CD45^+$ cells) was almost threefold higher than in control cultures, and when 800 ng/ml CCL21 was used, the percentage of $CD45^+CD11c^+$ cells was sevenfold higher than in controls (Fig. 5*A*). In parallel, a depletion of $CD11c^+$ cells was evident in the islets cultured with CCL21 compared with islets cultured with medium alone; islets were treated with collagenase, homogenized, and subjected to FACS analysis (Fig. 5*B*). Transplanting BALB/c islets cultured with medium containing 800 ng/ml CCL21 into STZ-induced diabetic C57BL/6 recipients resulted in significant prolongation of islet allograft survival (MST 11

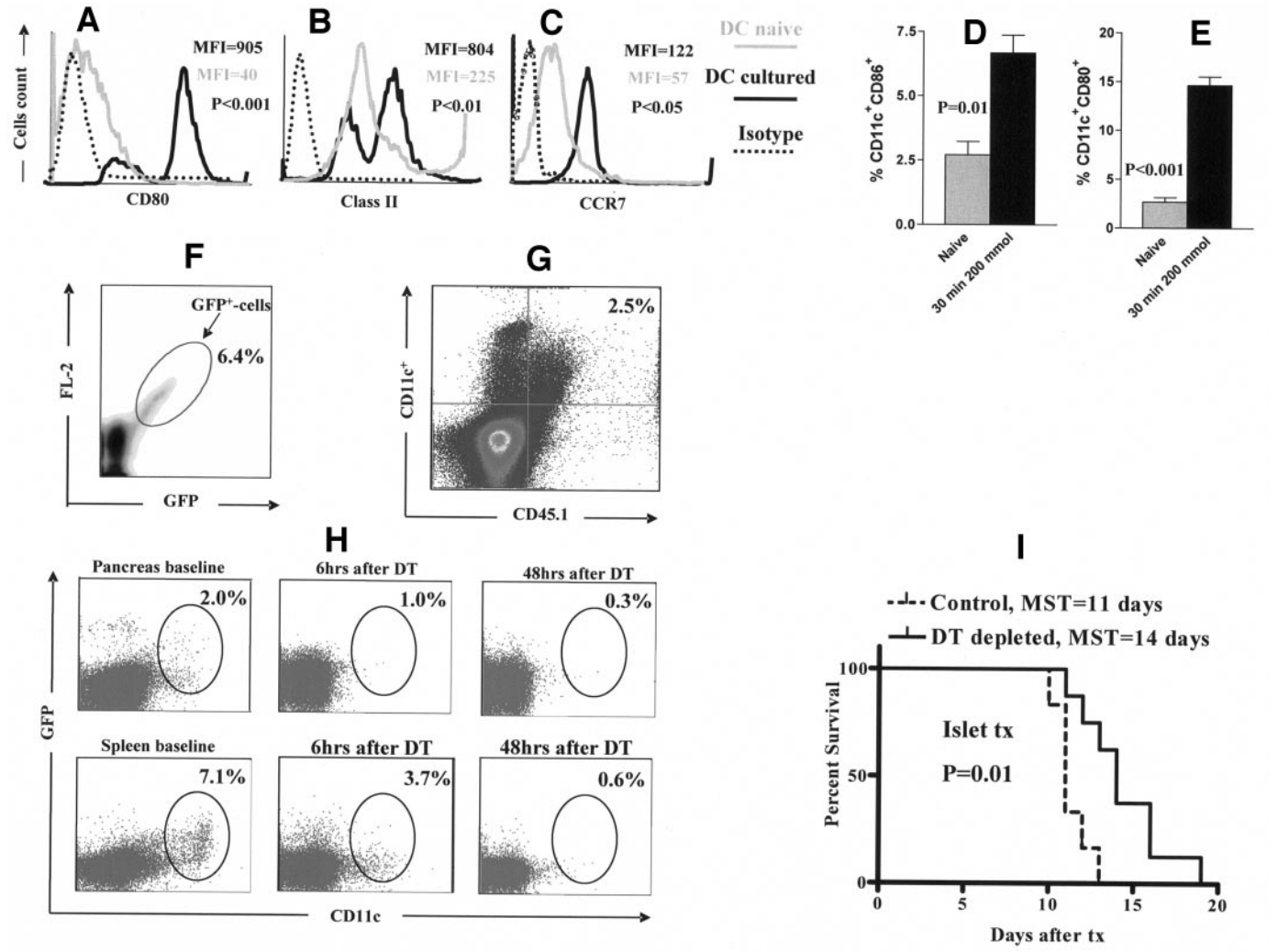

FIG. 4. Compared with freshly isolated islet DCs, the expression of maturity markers such as CD80, class II molecules, and CCR7 increases following 24 h culture of islets (*A–C*) (n = 4 mice per groups). Bone marrow–derived DCs cultured with H_2O_2 showed an upregulation of CD86/CD80 compared with untreated DCs (*D* and *E*) (n = 4 experiments per group). Spleens were recovered from islet allograft recipients of DTR-GFP-DC or CD45.1 donors. GFP$^+$ cells (*F*) or CD11c$^+$CD45.1$^+$ cells (*G*) can be detected in recipient spleen 30 days after islet allograft transplantation. DTR-GFP-DC mice were given 250 ng DT i.p., and spleen and pancreata were recovered for FACS analysis at 6 h and day 48 post-injection. Depletion of pancreatic and splenic DCs was demonstrated (*H*). Modest prolongation of islet allograft survival was observed using islets from DT-treated BALB/c mice as donors (*I*).

and 19 days for control and CCL21-cultured islets, respectively, $P < 0.001$) (Fig. 5*C*) with 20% of mice free from rejection for >30 days.

To elucidate the mechanism behind the observed prolongation of islet allograft survival, we studied and compared the status of host immune responses in two groups of recipients that received either CCL21-cultured or control islets (control islets are cultured for 24 h without CCL21). At day 7, spleens and DLN were harvested from recipients, and percentages of CD4$^+$CD25$^+$ cells (activated T-cells), CD4$^+$CD44^{hi} cells (T effectors), and CD4$^+$CD25$^+$Foxp3$^+$ cells (T regulatory cells) were determined. Compared with controls, mice receiving CCL21-cultured islets showed almost 10-fold fewer activated T-cells (as shown by the absence of CD4$^+$CD25$^+$ cells) in the DLN (0.88 ± 0.1 vs. 8.10 ± 2.0%, $P < 0.01$) (Fig. 5*D*). A reduced number of effector cells defined as CD4$^+$CD44^{hi} were also recovered in the spleen of recipients of CCL21-cultured islets ($P = 0.02$) (data not shown). No differences were noted in the percentage of CD4$^+$CD25$^+$Foxp3$^+$ cells (T regulatory cells) in the DLN or spleen of both groups (data not shown). To compare the capacity to generate an alloresponse between mice that received CCL21-cultured or control islets, we measured their allostimulatory capacity in an MLR assay. BALB/c irradiated splenocytes were used to stimulate splenocytes extracted from primed C57BL/6 mice that received either CCL21-cultured islets or control islets. As shown in Fig. 5*E*, the control group exhibited a higher T-cell proliferative response ($P < 0.01$). To confirm that the direct allorecognition pathway was affected by the CCL21 treatment, as an add-back experiment we adoptively transferred BALB/c DC extracted with CD11c$^+$ microbeads into C57BL/6 recipients immediately following islet transplantation. Indeed, dDC adoptive transfer completely abrogated the protective effect of CCL21-culture treatment on islet allograft survival; all mice rejected within 10 days following islet transplantation (MST 9 days, Fig. 5*C*). Finally, CCL21-treated and untreated islet allografts were harvested at day 7 post-transplantation. Compared with the control group, CCL21-treated islets show significantly less infiltrates and well-maintained insulin staining (Fig. 5*F* and *G*, respectively).

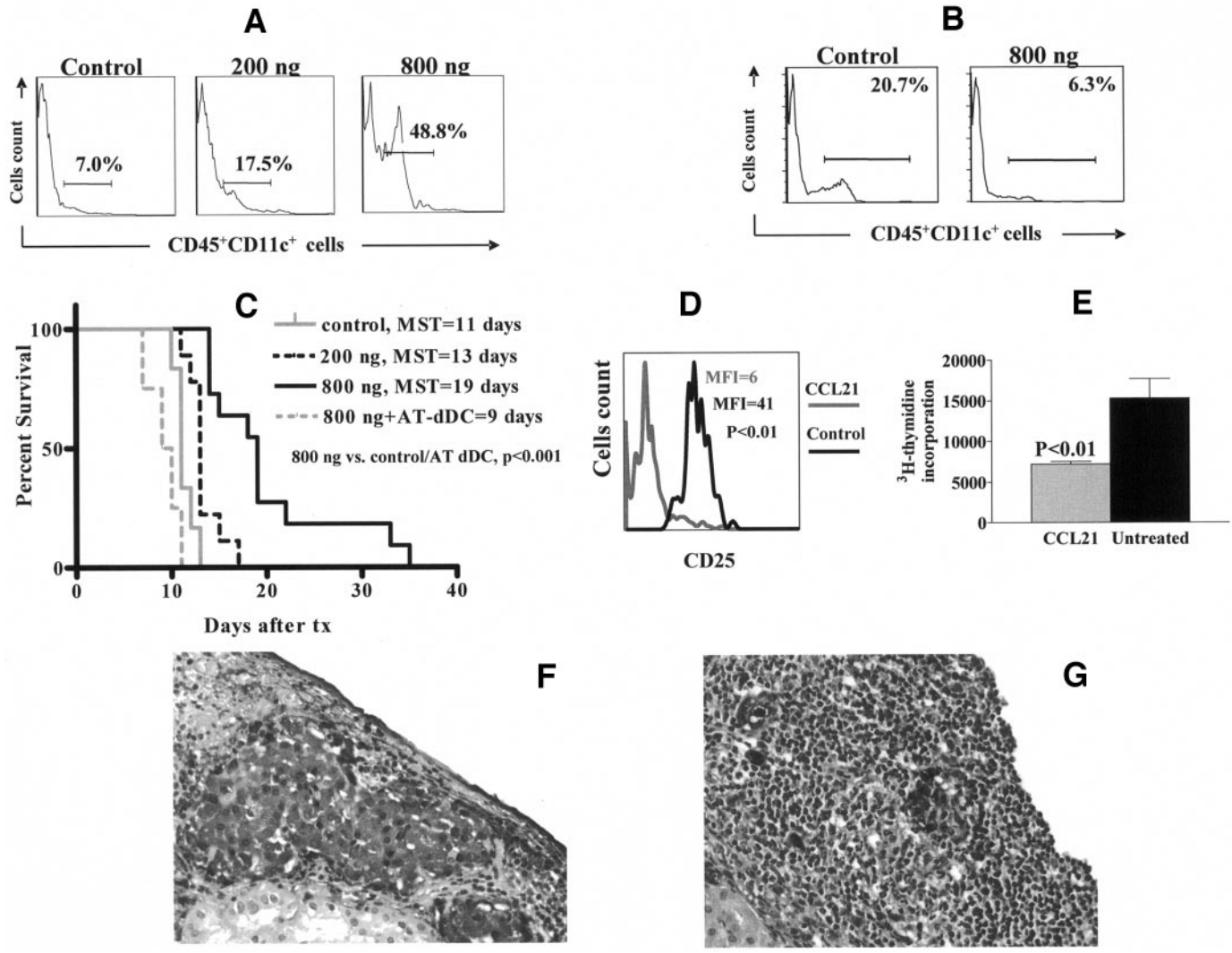

FIG. 5. An increase in the percentage of $CD11c^+CD45^+$ cells was noted in the medium of CCL21-cultured islets after 24 h of culture (*A*) ($P < 0.01$), with a parallel decrease of $CD11c^+CD45^+$ cells recovered from islets (*B*) ($P < 0.01$). Prolongation of islet allograft survival was observed when using CCL21-cultured islets in allogeneic islet transplantation (*C*) ($P < 0.001$). An abrogation of prolonged islet allograft survival occurred when WT-dDC were adoptively transferred (*C*). A reduction in activated T-cells ($CD4^+CD25^+$ cells) in the draining lymph nodes (*D*) ($P < 0.01$) and a lower proliferative response (measured by MLR) were noted in the recipients of CCL21-cultured islet donors compared with mice that received control islets (*E*) (both experiments at day 7 after islet transplantation, $P < 0.01$). Islet grafts harvested at day 7 after transplantation of CCL21-cultured islets appeared less infiltrated by mononuclear cells and showed a well-maintained insulin staining (*F*) compared with control islets (*G*).

DISCUSSION

DCs are migratory antigen-presenting cells that migrate to the lymphoid tissue DLN and spleen and present antigens to recipient T-cells, a central event in generating an alloresponse (1–3). Allorecognition occurs via both "direct" and "indirect" pathways. In direct allorecognition, recipient T-cells recognize intact allogeneic MHC molecules on donor antigen-presenting cells (i.e., tissue resident DCs/passenger leukocytes) (4,5). Larsen et al. (6) demonstrated that donor-derived DCs migrate from transplanted heart allografts into recipient lymphoid tissue. Similar results were demonstrated in a mouse model of heart transplantation, in which the efflux of dDCs into recipient spleens as well as the presence of rDCs in the grafts were noted (19). Due to a dearth of suitable models, examining the specific contribution of dDCs and rDCs in transplantation has been a difficult task. No data are yet available on the distinct characteristics of dDCs and rDCs or on the role of specific chemokine receptors important in the migration of DCs in islet transplantation. Using DTR-GFP-DC mice (which have GFP linked to CD11c) allowed us to explore the differential features of dDCs and rDCs, both of which are central in the generation and regulation of alloimmune responses. These features include DC trafficking, maturation, and proliferation and their linkage with the temporal expression of chemokine expression. Our data demonstrate that dDCs first quickly migrate to lymphoid tissue such as spleen instead of DLN. Compared with rDCs, dDCs exhibit a higher level of maturity at earlier time points posttransplantation and also show differential levels of chemokine receptor expression.

Current theory proposes that while dDCs (direct pathway) are potent initiators of early acute rejection, the indirect pathway (rDCs) is responsible for late acute/chronic rejection (24–27). We have shown that dDCs display much greater proliferative activity than rDCs and that ischemia and tissue preparation could in part contrib-

ute to this higher dDC activity. Our data suggest that oxidative stress and tissue injury could increase the level of expression of DC costimulatory molecules and CCR7, through which dDCs may acquire superior mobility and allostimulatory capacity. Results of our studies may explain in part the mechanisms behind the excessive immunogenicity of islet allografts, in which panels of tissue injuries (due to ischemia and tissue preparation) are more likely to occur than in a transplant setting such as the heart. We also detected long-lasting dDCs in spleens of islet allograft recipients >1 month following transplant. It is surprising to find these cells weeks after allograft rejection, as it has been commonly thought that the dDC half-life is much shorter (34). The question of how dDCs can escape recipient immune surveillance is unclear to us. We can however hypothesize that they do so through acquiring a tolerogenic phenotype by failing to provide a second signal, but this is a subject of future studies.

One of the important aspects of our study was to abrogate the effect of direct allorecognition. The importance of direct allorecognition is well established, as stimulation with DCs has been shown to be very potent in stimulating primary allogeneic MLRs and depletion of donor DCs can prolong islet and thyroid allograft survival (35,36). We used a novel DT-based system that allows the inducible in vivo ablation of DCs (8,9). We show that DCs can be depleted in islet allografts with DT treatment of donors. Using these mice as donors, however, only minimally prolonged islet allograft survival. This could be due to the subsequent repopulation or expansion of dDCs within a few days of depletion and after transplantation. We also report that islets can be cultured with CCL21 to increase the efflux of DCs from islets. Our data show that this strategy leads to prolongation of islet allograft survival. A reduction in T-cell activation and in the potency of the alloresponse was noted in recipients that received CCL21-cultured islets compared with the control group. Although all grafts eventually were rejected, we should note that this prolongation was achieved with no immunosuppression in the recipients and that this outcome is comparable with the prolongation investigators have reported when using considerable immunosuppression (37). We view this as a potential adjunct therapeutic approach to combine with immunosuppressive or tolerogenic strategies to promote islet cell engraftment in the future.

During maturation, DCs upregulate surface expression of the chemokine receptor CCR7. CCL21, the ligand for CCR7, which was used in our in vitro assay, is constitutively expressed at high levels in lymph nodes (38). Given that CCL21 exhibits chemotactic activity mostly for mature DCs, it is plausible that immature DCs or DC progenitors remained in the graft even after the CCL21-based depletion (38,39). These remaining DCs could mature following transplantation and mount a delayed alloimmune response, leading to eventual graft rejection. The differential survival outcomes observed between DT depletion and CCL21 strategies could be the fact that with CCL21 treatment we were able to selectively deplete $CCR7^{high}$ DCs, which are most likely responsible for mounting an allogeneic response. Further studies are necessary to optimize culture conditions to achieve more robust prolongation; possible adjustments include using higher concentrations of CCL21 and/or repetitive treatment as well as using multiple key chemokines/ligands in culture. The latter requires precise study of chemokine receptors expressed on dDCs in addition to CCR7. With improvement, this method could easily be translated to clinical practice. Common immunosuppressive agents used in islet cell transplantation are specifically associated with serious islet toxicity, and a method such as ours may allow clinicians to use significantly lower doses of immunosuppression to promote tolerance. In mice, a prolongation of islet allograft survival was reported not only by immunosuppression strategies, but also by anti-inflammatory treatments (e.g., the use of oxygen free radical scavengers and heme oxygenase-1 induction) (40–41). Tissue pretreatment such as culturing at reduced temperatures or treating with anti–class II antibodies, with the goal of eliminating antigen-presenting cells within endocrine tissues, has also resulted in prolongation of allograft survival in immunocompetent recipients (13–17). Of note, these strategies nonspecifically targeted all antigen-presenting cells. Our work is unique because our approach specifically deals with DCs and can potentially lead to greater manipulation and selective transformation of the islet DC population by using specific chemokines to induce selectively the efflux of allogeneic DCs so that tolerogenic DCs remain. The efficacy of such a strategy will be tested in NOD recipients in our future studies.

We believe these results could easily be translated into clinical transplantation and could have a tremendous impact on the outcome of human islet cell transplantation, which requires further improvement (42–45).

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