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# Embryonic Stem Cells, Derived Either after In Vitro Fertilization or Nuclear Transfer, Prolong Survival of Semiallogeneic Heart Transplants

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Tolerance induction toward allogeneic organ grafts represents one of the major aims of transplantation medicine. Stem cells are promising candidates for promoting donor-specific tolerance. In this study, we investigated the immunomodulatory properties of murine embryonic stem cells (ESCs), obtained either by in vitro fertilization (IVF-ESCs) or by nuclear transfer (NT-ESCs), in heart transplant mouse models. IVF-ESCs did not prolong the survival of fully allogeneic cardiac transplants but significantly prolonged the survival of semiallogeneic hearts from the same ESC donor strain for >100 d in 44% of the animals. However, 28% of transplanted animals infused with IVF-ESCs experienced development of a teratoma. NT-ESCs similarly prolonged semiallogeneic heart graft survival (>100 d in 40% of the animals) but were less teratogenic. By in vitro studies, IVF-ESC and NT-ESC immunoregulation was mediated both by cell contact-dependent mechanisms and by the release of soluble factors. By adding specific inhibitors, we identified PGE<sub>2</sub> as a soluble mediator of ESC immunoregulation. Expansion of regulatory T cells was found in lymphoid organs and in the grafts of IVF-ESC- and NT-ESC-tolerized mice. Our study demonstrates that both IVF-ESCs and NT-ESCs modulate recipient immune response toward tolerance to solid organ transplantation, and that NT-ESCs exhibit a lower tendency for teratoma formation. Because NT-ESCs are obtained by NT of a somatic cell from living individuals into an enucleated oocyte, they could represent a source of donor-derived stem cells to induce tolerance to solid organ allograft. *The Journal of Immunology*, 2011, 186: 4164–4174.

Organ transplantation is now firmly established as the therapy of choice for end-stage organ failure. Improvements in immunosuppressive drugs have led to out-

standing short-term patient and graft survival rates. Despite this short-term success, the long-term kidney allograft survival has not shown a similar rate of improvement. Chronic allograft nephropathy remains the most common cause of late allograft loss, whereas cardiovascular disease remains the leading cause of death post-transplantation (1–6). Therefore, to improve transplantation outcomes, it is critical to continue the development of novel strategies to prevent acute and chronic graft rejection, whereas lessening the need for lifelong immunosuppression, and to promote the development of a state of donor-specific tolerance.

Induction of mixed chimerism by donor-derived hematopoietic stem cell transplantation in conjunction with myeloablative conditioning has been the first and, so far, the sole successful approach for donor-specific tolerance to organ transplantation in humans (7–9). However, even if successful, this myeloablative protocol is so far too difficult and complex to be broadly applicable. Efforts are therefore under way to discover novel strategies for the induction of tolerance possibly based on donor stem cell infusion without the need of preconditioning procedures. In this context, embryonic stem cells (ESCs) have been proposed as an alternative source of donor stem cells to induce immune tolerance in the transplantation setting (10). ESCs derived from the inner cell mass (ICM) of the preimplantation blastocyst have the potential to generate all tissues and are capable of self-renewal and unlimited proliferation (11–13). Several reports showed that ESCs possess important immunoprivileged properties. ESCs and their derivatives were shown to express very low levels of MHC class I (MHC-I) Ags, to lack expression of MHC class II (MHC-II) and costimulatory molecules, to bypass immune recognition by natural NK cells, and to inhibit T cell proliferation induced by third-party APCs (10,

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Abbreviations used in this article: AP, alkaline phosphatase; ESC, embryonic stem cell; FasL, Fas ligand; HPF, high-power field; ICM, inner cell mass; IVF, in vitro fertilization; MHC-I, MHC class I; MHC-II, MHC class II; NT, nuclear transfer; *Oct-4*, *Octamer-4*; PI, propidium iodide; RESC, rat ESC-like cell; SSEA-1, stage-specific embryonic Ag-1; Treg, regulatory T cell.

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14–17). Nevertheless, the extent to which these immunological features may modulate alloreactivity *in vivo* in the context of solid organ transplantation remains controversial.

A preliminary report suggested that the intraportal infusion of undifferentiated rat ESC-like cells (RESCs) induced donor-specific tolerance to a subsequent heart transplant without the need for host preconditioning or immunosuppression (10). A subsequent study, however, has found that injection of true ICM-derived ESCs failed to induce tolerance to nonvascularized cardiac and skin allografts in mice (17). Whether these discrepancies may depend on the different nature of the ESCs used (ESC-like cells of probable extra ICM origin versus ICM-derived ESCs) or may depend on different models of acute rejection to organ transplantation (vascularized heart transplant versus the more severe nonvascularized heart and skin grafts) remains to be established.

The three main aims of this study were: 1) to explore the possibility to induce long-term graft acceptance by intraportal infusion of donor mouse ESCs obtained after *in vitro* fertilization (IVF-ESCs), in models of vascularized heart transplant in mice; 2) to explore the possibility to induce long-term graft acceptance using donor murine ESCs obtained by somatic cell nuclear transfer (NT-ESCs), thus opening a clinical perspective to obtain donor-specific ESCs; and 3) to clarify the mechanism(s) by which IVF-ESCs and NT-ESCs induce graft acceptance.

## Materials and Methods

### Mice

Male and female inbred C57BL/6 (B6, H-2<sup>b</sup>), C57BL/6xC3H F1 (B6C3, H-2<sup>b/k</sup>), BALB/c (H-2<sup>d</sup>), C3H (C3H, H-2<sup>k</sup>), DBA/2NcrL (DBA/2, H-2<sup>d</sup>) mice were from Charles River (Charles River, Calco, Italy). B6.Cg-Foxp3<sup>tm2(EGFP)<sup>Tch</sup>/J</sup> mice (Foxp3-GFP, H-2<sup>b</sup>) coexpressing GFP and the regulatory T cell (Treg)-specific transcription factor Foxp3 were from Jackson Laboratory (Sacramento, CA). Animal care and treatments were conducted in conformity with the institutional guidelines and international laws and policies.

### Establishment of ESC lines

After IVF of oocytes (B6C3F1) with spermatozoa (B6C3F1) (18), a murine ESC line was derived (BGPV-IVF1). ESCs were isolated from the ICM of a single blastocyst and expanded on mitomycin C (2 mg/ml; Sigma, St. Louis, MO) mitotically inactivated mouse embryonic fibroblast feeder layer, STO-SNL2 cell line (ATCC CRL-2225, LGC Promochem; ATCC Europe, Middlesex, U.K.) in complete ESC medium: knockout DMEM supplemented with 20% ESC qualified FBS (ES-FBS), 2mM L-glutamine, 1% MEM/nonessential amino acids, 0.5% penicillin-streptomycin (all from Invitrogen, Carlsbad, CA), 0.1 mM 2-ME (Sigma), and 500 U/ml ESGRO-LIF (Chemicon International, Temecula, CA). IVF-ESCs were cultured for >40 passages. For experiments, IVF-ESCs were used at passages 25–30. By PCR analysis of the trophectoderm, we verified the male sex of the IVF-ESCs by amplifying the Y-linked gene (*Sry*, not shown).

An ESC line was derived by NT (NT-ESCs) by transferring nuclei obtained from cumulus cells of B6C3F1 mice into oocytes obtained from B6C3F1 (named BGPV-NT1) as previously reported (18). NT-ESCs were isolated from the ICM of a single blastocyst and expanded on mitomycin C-treated STO-SNL2 in complete ESC medium as reported for ESCs obtained by IVF. NT-ESCs were maintained in culture for >30 passages. For experiments, NT-ESCs were used at passages 24–30. Before administration to mice, IVF-ESCs and NT-ESCs were cultured for two passages on bovine gelatin-coated dishes to remove the feeder layer.

### Genotyping

The H2<sup>b</sup> × H2<sup>k</sup> genotype was verified by PCR with two sets of primers, p1 (5'-AGTTTGGCCAATTGGCAAGC-3') and p2 (5'-CCACCTTGCAAGT-CATAAATG-3'), specific for mouse H2-I/Aαb, that amplified a DNA fragment of 789 bp; p3 (5'-CTGGATGCTTCCTGAGTTTGCT-3') and p4 (5'-GGAACACAGTCGCTTG AGGAG-3'), specific for mouse H2-I/Aαk, amplified a DNA fragment of 584 bp. Thermal cycling condition was as follows: 10 min at 94°C, then samples were cycled 32 times at 94°C for 30 s, 55°C for 30 s, and 72°C for 30 s. PCR products were run on agarose gel and visualized by ethidium bromide staining.

### Immunofluorescence microscopy

Immunofluorescence on IVF-ESCs and NT-ESCs was performed using mouse anti-stage-specific embryonic Ag-1 (SSEA-1) mAb (Chemicon) followed by FITC-goat anti-mouse IgG (Jackson ImmunoResearch Europe, Suffolk, U.K.). Images were acquired using inverted confocal laser microscopy (LSM 510 meta; Zeiss, Jena, Germany). To visualize alkaline phosphatase (AP) activity, we stained IVF-ESCs and NT-ESCs using a Sigma kit according to the manufacturer's protocol (Sigma-Aldrich).

### Flow cytometric analysis

IVF-ESC and NT-ESC surface phenotypic analysis was performed by flow cytometer FACSort or FACSAria (BD Bioscience, San Jose, CA). Cells were incubated with FITC-conjugated mouse anti-mouse H-2K<sup>b</sup> mAb (MHC-I) or R-PE-conjugated rat anti-mouse I-A/I-E mAb (MHC-II) (BD Bioscience). To block nonspecific binding, we performed 30-min preincubation with 5% rat serum. Negative controls were performed using isotype Abs. TCR Vβs expression in CD4<sup>+</sup> T cells was evaluated by labeling splenocytes with PE-Cy7 anti-mouse CD4 (BD Bioscience) and PE anti-mouse Vβ8.1/8.2 (BioLegend, San Diego, CA) or PE Vβ5.1/5.2 (BD Bioscience) or PE Vβ11 (BioLegend). The evaluation of CD4<sup>+</sup> Treg in Foxp3-GFP mice was performed by labeling splenocytes with PE-Cy7 anti-mouse CD4 and determining GFP<sup>+</sup> cells in the CD4<sup>+</sup> T cell population.

### Octamer-4 expression

Total RNA was obtained from IVF-ESCs and NT-ESCs by TRIzol extraction (Invitrogen). RNA was treated with DNase and reverse transcribed to cDNA by Superscript II (Invitrogen). mRNA expression was evaluated by quantitative real-time PCR (TaqMan ABI Prism 5700 Sequence Detection System; Applied Biosystems, Foster City, CA) with SYBR Green PCR core reagents, using specific primers: *Octamer-4* (*Oct-4*; forward: 5'-CCTGG-GCGTTCTCTTTGGA-3'; reverse: 5'-GGCCGACGCTTACACATGTT-3'). GAPDH mRNA expression was analyzed as a housekeeping gene to assess the overall cDNA content. The  $\Delta\Delta C_t$  equation was used to compare the mRNA expression in each sample with the expression in mouse fibroblasts taken as reference unit = 1 (calibrator).

### Cytogenetic analysis

ESCs from IVF and NT, at the same passage used for *in vivo* experiments, were harvested for the analysis of nuclei and mitoses, by conventional methods. Slides were scored for the identification of chromosomal abnormalities and for karyotype reconstruction after staining with Giemsa.

### Engraftment of donor IVF-ESCs in recipient tissues

To evaluate IVF-ESC engraftment, we isolated genomic DNA by the proteinase K/phenol/chloroform method from liver, thymus, spleen, lung, lymph nodes, kidney, small bowel, bone marrow, and heart. Genomic DNA from blood was isolated by a Nucleon BACC2 kit (GE Healthcare, Piscataway, NJ). Donor IVF-ESC levels were evaluated by real-time quantitative PCR using primers and fluorescent probe specific for *Sry* on the Y chromosome, as previously described (19): 300 nM forward primer (5'-GGT TAA AGT GCC ACA GAG GAG TTA TAC-3'), 300 nM reverse primer (5'-TCC CAT TGC AGC AGG TTG TA-3'), 200 nM probe (FAM 5'-TTG CAG CGT GAA GTT GCC TCA ACA AA-3'), and 100 ng of each DNA sample in triplicate were used. By comparison with standard curve samples, this method allows specific determination of male DNA into female DNA up to a dilution of 1:5000 (0.02%), corresponding to the presence of three male cells in the PCR reaction. No signal was observed with female DNA alone or without DNA in PCR reaction (data not shown).

Donor NT-ESC level was evaluated after infusion in semiallogeneic mice (B6 strain) by real-time quantitative PCR using primers and fluorescent probe specific for k haplotype: 300 nM forward primer (5'-CTG GAT GCT TCC TGA GTT TGC T-3'), 300 nM reverse primer (5'-TGT TTT CCT TGA GCT ATG TTT TGC A-3'), 200 nM probe (FAM 5'-AAC TGA GAA GAT TTG AGC CCC AAG GTG G-3'), and 100 ng of each DNA sample in triplicate. By comparison with standard curve samples, this method allows specific determination of k haplotype DNA into b haplotype DNA until a dilution of 1:5000 (0.02%), corresponding to the presence of three target cells in the PCR reaction. No signal was observed with b haplotype DNA alone or without DNA in PCR (data not shown). Real-time PCR was performed on a TaqMan ABI Prism 5700 Sequence Detection System (Applied Biosystems) with TaqMan Universal Master Mix. Thermal cycling conditions were as follows: 2 min at 50°C, 10 min at 95°C, then samples were cycled 45 times at 95°C for 15 s and 60°C for 60 s.

To further evaluate cell engraftment in liver and thymus, we labeled IVF-ESCs and NT-ESCs with PKH26 according to the manufacturer's protocol



(Red Fluorescence Cell Linker Kit; Sigma-Aldrich). Livers and thymi were fixed in paraformaldehyde, impregnated with sucrose, and rapidly frozen. Tissues were then sectioned on a cryostat, fixed with acetone, and stained with DAPI (1  $\mu\text{g}/\text{ml}$ ; Sigma-Aldrich). For each tissue, 50–60 randomly selected high-power fields (HPFs) were analyzed and PKH2<sup>+</sup> cells in each HPF were counted.

### Histology and immunohistochemistry

Fragments of livers were fixed in 10% neutral formalin, embedded in paraffin, sectioned, and stained with H&E for routine histological analysis. In situ intragraft CD4<sup>+</sup> T cells were analyzed by immunofluorescence technique, as previously described (20). Sections of transplanted hearts were incubated with Alexa 647-conjugated anti-mouse CD4 mAb (0.5  $\mu\text{g}/\text{ml}$ ; BioLegend) and rat anti-mouse Foxp3 mAb (20  $\mu\text{g}/\text{ml}$ ; Alexis Biochemicals, San Diego, CA) followed by Cy3-conjugated goat anti-rat IgG Ab (6  $\mu\text{g}/\text{ml}$ ; Jackson ImmunoResearch Laboratories). The number of total single- and/or double-positive cells was counted in at least 10 randomly selected HPFs. For each animal, the total CD4<sup>+</sup> cell counts and the percentage of CD4<sup>+</sup>Foxp3<sup>+</sup> on CD4<sup>+</sup> were calculated.

### Heterotopic heart transplant

Donor hearts were transplanted into the abdomen of recipients (21). Mice were anesthetized by inhalation of isoflurane (Abbott, Abbott Park, IL). Donor hearts were perfused with chilled, heparinized saline via the inferior vena cava and harvested after ligation of the superior vena cava and pulmonary veins. The aorta and pulmonary artery of donor hearts were anastomosed to the abdominal aorta and inferior vena cava of recipient mice, respectively, using microsurgical technique. Ischemic time during the surgical procedure was routinely 30 min. Graft survival was followed by palpation at least three times per week. Rejection was defined by complete cessation of palpable contraction confirmed by direct visualization.

### Ex vivo MLR

Spleen cell suspensions were obtained by passing the spleen through a 70- $\mu\text{m}$ -cell stainless-steel strainer, and the erythrocytes were depleted by hypotonic lysis. Cells were resuspended in complete RPMI 1640 supplemented with 10% heat-inactivated FCS and antibiotics (Invitrogen). MLRs were performed by incubating responder splenocytes with 4000-rad irradiated splenocytes from donor, third-party, or syngeneic (control combination) mice. Cell proliferation was determined after 72-h culture in 96-well plates and pulsing the cells with [<sup>3</sup>H]thymidine during the last 14–16 h. The radioactivity incorporated was then measured by liquid scintillation counting (Beckman). Proliferative response was expressed as  $\Delta\text{cpm}$  by subtracting the cpm recorded in control syngeneic combination from the cpm of allogeneic combinations.

ELISPOT assay was performed after 48-h MLR incubation using BD ELISPOT mouse IFN- $\gamma$  reagents (BD Bioscience), as previously described (20). The resulting spots were counted on a computer-assisted Immunospot image analyzer (Aelvis EliSpot Scanner system). Results are the mean value of spots per 125,000 recipient splenocytes.

### Adoptive cell transfer

Spleen cells were isolated from tolerant mice (>100 d), diluted in PBS, and injected into the tail vein of naive mice the day before heart transplantation. No immunosuppressive drugs were given to the animals.

### In vitro studies

Either IVF-ESCs or NT-ESCs were plated on gelatin-coated 12-well plates ( $1 \times 10^6$  cells), and after adhesion, cells were washed and exposed to medium alone or to B6 splenocytes ( $1 \times 10^6$  cells), cocultured in MLR with  $1 \times 10^6$  syngeneic (B6), allogeneic (B6C3), or third-party (BALB/c) stimulator cells prepared as described earlier. Cocultures were conducted either in direct cell contact or in transwell system where MLRs were conducted in the upper chamber, whereas IVF-ESCs or NT-ESCs were cultivated in the lower compartment of a Transwell plate with a 0.4- $\mu\text{m}$  pore size membrane (BD Falcon). After 48-h incubation aliquots of splenocytes were transferred either to a 96-well plate and incubated for an additional 12 h in the presence of tritiated [<sup>3</sup>H]thymidine for proliferation assay, or to ELISPOT plates for the assessment of IFN- $\gamma$  spots (BD Bioscience). Results are the mean value of spots per 250,000 recipient splenocytes. The following inhibitors were added during MLR: anti-mouse TGF- $\beta$  Ab (R&D Systems), anti-mouse IL-10 Ab (R&D Systems), 1-methyl-DL-tryptophan (Sigma), N<sup>G</sup>-nitro-L-arginine (Sigma), and indomethacin (Sigma). Apoptosis of T cells was evaluated in responder cells cultured for 6 h with IVF-ESCs or NT-ESCs by labeling with PE-Cy7 anti-mouse CD3 (BD

and then with FITC Annexin V/Dead Cell Apoptosis Kit (Molecular Probes). Fas ligand (FasL) expression (Santa Cruz Biotechnology, Santa Cruz, CA) on IVF-ESCs and on NT-ESCs were evaluated by FACS analysis after 6-h incubation.

### Statistical analysis

Data are reported as mean  $\pm$  SD. Survival data were compared using the log-rank test. All other data were analyzed by ANOVA. Differences with a  $p$  value <0.05 were considered significant.

## Results

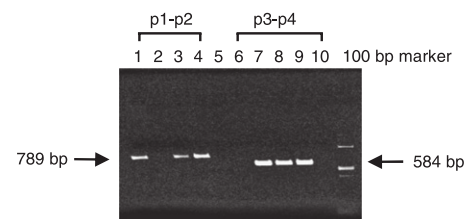
### Characterization of murine ESCs obtained by IVF

We derived by IVF a male B6C3 ESC line (IVF-ESCs) with H2<sup>b</sup>  $\times$  H2<sup>k</sup> genotype (Fig. 1). Cells had typical morphology of ESCs with well-defined, rounded colonies (Fig. 2A). IVF-ESCs were positive for AP activity (Fig. 2B) as assessed by specific fast blue staining and for SSEA-1 expression as evidenced by immunofluorescence microscopy (Fig. 2C). Finally, we showed, by real-time PCR, that IVF-ESCs highly expressed the transcription factor *Oct-4* (Fig. 2D). By contrast, mouse embryonic fibroblasts, STO-SNL2, used as feeder layer, expressed very low levels of *Oct-4* (Fig. 2D) and did not show AP activity or SSEA-1 immunostaining (not shown). In addition, IVF-ESCs were negative for MHC-I and -II Ags (Fig. 2E, 2F). IVF-ESCs cultured for >40 passages retained ESC-specific markers. The karyotype of IVF-ESCs was checked at passage 25, and most of the cells were diploid with few cells showing tetraploidy or chromosomal loss (data not shown).

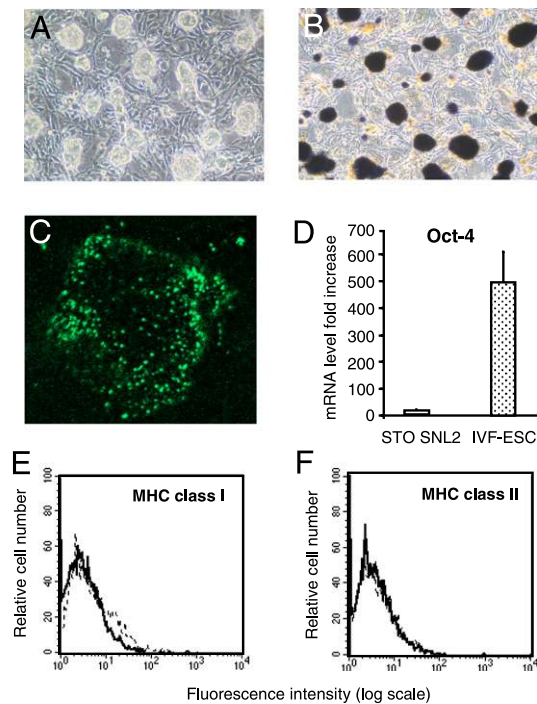
To confirm that cells retained pluripotency, we infused  $1 \times 10^6$  IVF-ESCs into the portal vein of syngeneic B6C3 mice. Recipient livers documented rare, small, teratoma-like structures in animals sacrificed 7 d after IVF-ESC administration ( $n = 3$ ; Fig. 3A), whereas livers from animals killed 21 d after cell infusion ( $n = 3$ ) appeared abnormally enlarged with multiple ectopic lobes. Typical teratoma structures, with tissues derived from endoderm (gut epithelial), mesoderm (adipose, smooth muscle, bone cartilage), and ectoderm (neural epithelium) (Fig. 3B), consistent with the pluripotent nature of IVF-ESC line, were found. Animals sacrificed 45 d after cell administration ( $n = 3$ ) exhibited a liver entirely occupied by teratoma (not shown).

### In vivo tissue distribution of IVF-ESCs

To assess the extent and distribution of IVF-ESC engraftment into recipient mice, we also analyzed tissues from B6C3 female mice, infused with  $1 \times 10^6$  syngeneic male IVF-ESCs via the portal vein, for the presence of donor DNA using a quantitative real-time PCR specific for a Y-linked sequence. IVF-ESCs were found in the recipient liver as soon as 24 h after injection ( $n = 3$ ;  $0.35 \pm 0.17\%$  of donor DNA) and then progressively augmented as



**FIGURE 1.** Real-time PCR analysis of IVF-ESC genotype. Primers p1 (5'-AGTTTGGCCAATTGGCAAGC-3') and p2 (5'-CCACCTTGCAGT-CATAAATG-3'), specific for mouse H2-I/Al<sup>b</sup>, amplified a DNA fragment of 789 bp; p3 (5'-CTGGATGCTTCTCTGAGTTTGTCT-3') and p4 (5'-GGAA-CACAGTCGCTTGAG GAG-3'), specific for mouse H2-I/Al<sup>k</sup>, amplified a DNA fragment of 584 bp. Lanes 1 and 6, C57 (H-2<sup>b</sup>) mice; lanes 2 and 7, C3H (H-2<sup>k</sup>) mice; lanes 3 and 8, IVF-ESCs; lanes 4 and 9, C57  $\times$  C3H (H-2<sup>b</sup>  $\times$  H-2<sup>k</sup>) mice; lane 10, no template control.



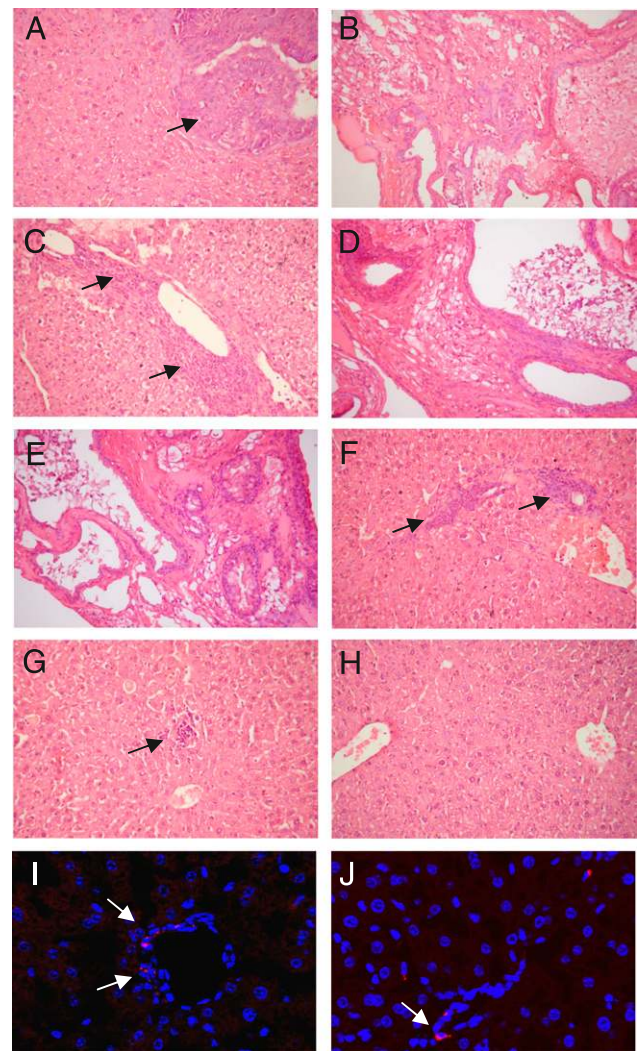
**FIGURE 2.** Characterization of IVF-ESCs. *A*, Contrast-phase microscopy analysis of IVF-ESC colonies at passage 25 cultured on inactivated fibroblast feeder layer STO-SNL2. *B*, AP activity of IVF-ESCs evidenced by specific histochemical fast blue staining. *C*, Immunofluorescence of stage-specific Ag-1, SSEA-1 (green) on IVF-ESCs. Original magnification  $\times 400$ . *D*, Real-time PCR analysis of the transcription factor *Oct-4* expression on IVF-ESCs and STO-SNL2 feeder layer. Data are reported as mean  $\pm$  SD. *E* and *F*, Representative flow cytometric analysis of MHC-I and -II Ags on IVF-ESC cultures and respective isotype controls (dashed lines).

documented by the increasing frequency of donor DNA ( $1.48 \pm 1.06$ ,  $2.54 \pm 1.76$ ,  $91.29 \pm 17.71$ , and  $\geq 97\%$ , at 4, 7, 21, and 45 d, respectively;  $n = 3$  for each time point). There was no or very low engraftment in other somatic or lymphoid organs. Analysis of blood, spleen, bone marrow, lung, lymph nodes, kidney, heart, small bowel, and thymus showed absence or very low levels of donor DNA ( $<0.3\%$ ) at each time point analyzed.

To evaluate whether IVF-ESCs could engraft into MHC-incompatible recipient mice,  $1 \times 10^6$  B6C3 male IVF-ESCs were administered into the portal vein of female B6 mice. Recipient mice ( $n = 3$  for each time point) were killed at 4, 7, 21, and 45 d after cell infusion for donor DNA evaluation. In the liver, the presence of low levels of donor DNA was detected at 4 and 7 d postinfusion ( $0.18 \pm 0.16$  and  $0.38 \pm 0.55\%$ , respectively); thereafter, the percentage of donor DNA decreased ( $0.1 \pm 0.05\%$  at 21 d and  $0.03 \pm 0.02\%$  at 45 d after cell infusion). Additional B6 mice ( $n = 3$ ) receiving male B6C3 IVF-ESCs at the dose of  $0.5 \times 10^6$  into the portal vein exhibited, 7 d after infusion, lower levels of donor DNA in the liver ( $0.08 \pm 0.02\%$ ). Donor DNA was absent in all the other organs analyzed (blood, thymus, spleen, bone marrow, lymph nodes, lung, heart, kidney, small bowel) with both doses of IVF-ESCs, indicating that they did not migrate outside the liver. Thus, the capability of IVF-ESCs to engraft into an MHC-incompatible recipient was very low and restricted to the site of injection.

#### Liver histology and teratoma formation after infusion of allogeneic IVF-ESCs in naive mice

At macroscopic examination, most livers from B6C3 IVF-ESC-infused B6 mice appeared normal with the exception of several



**FIGURE 3.** Histological analysis of livers from IVF-ESC- and NT-ESC-infused mice. H&E staining of livers from mice infused with IVF-ESCs (*A–D*) or NT-ESCs (*E–G*) or from untreated mice (*H*). Livers from IVF-ESC-infused syngeneic B6C3 mice showed small rare teratoma (arrow) at 7 d after infusion (*A*) and more extended teratoma at 21 d (*B*). Livers from semi-allogeneic B6 mice infused with B6C3 IVF-ESCs showed areas of inflammation at 7 d (arrows, *C*) and small teratoma at 21 d (*D*). Liver from a B6C3 mouse receiving syngeneic NT-ESCs and sacrificed 21 d after infusion showed teratoma (*E*). Undifferentiated cell and infiltrates (arrows) were observed in liver from a B6 mouse infused with semi-allogeneic NT-ESCs and sacrificed 7 d after infusion (*F*). Liver from a long-term surviving mouse (graft survival  $> 100$  d) after NT-ESC infusion showing areas with undifferentiated cells (arrow) (*G*). Liver from an untreated mouse (*H*). *A–H*, Original magnification  $\times 200$ . Representative images of PKH26<sup>+</sup> IVF-ESCs (*I*, arrows) and NT-ESCs (*J*, arrow) in recipient livers 21 d after intraportal cell infusion. *I* and *J*, Original magnification  $\times 400$ .

white spots on the Glisson's capsule, particularly evident at 4 and 7 d after infusion, that at histological examination showed the presence of adipose tissue with areas of inflammation and necrosis. These signs, together with the presence of inflammatory infiltrates (Fig. 3*C*), suggested ongoing rejection of semi-allogeneic IVF-ESCs. Within liver parenchyma, areas containing IVF-ESC-like undifferentiated cells were found at each time point analyzed. These areas might represent engrafted donor cells not detected by real-time PCR because of the random sampling or the detection limit of the assay. To better deal with allogeneic IVF-ESC engraftment, we infused additional B6 mice ( $n = 8$ ) with



PKH26-labeled IVF-ESCs. Four mice were sacrificed at 7 d and the other four mice at 21 d after cell infusion. Approximately six cells per HPF were detected in livers 7 d after IVF-ESC infusion ( $6 \pm 1$  cells/HPF). At day 21 after infusion, despite the number of engrafted IVF-ESCs halved ( $3 \pm 1.3$  cells/HPF), focal aggregates of IVF-ESCs were present in the liver (Fig. 3I). We also identified small, teratoma-like structures in livers of 33% (4/12) of animals killed from 7–45 d postinfusion (Fig. 3D). No PKH26-labeled cell was detected in thymus of infused mice at either 7 or 21 d after cell infusion.

#### Splenocytes from IVF-ESC-injected mice are hyporesponsive in ex vivo MLR

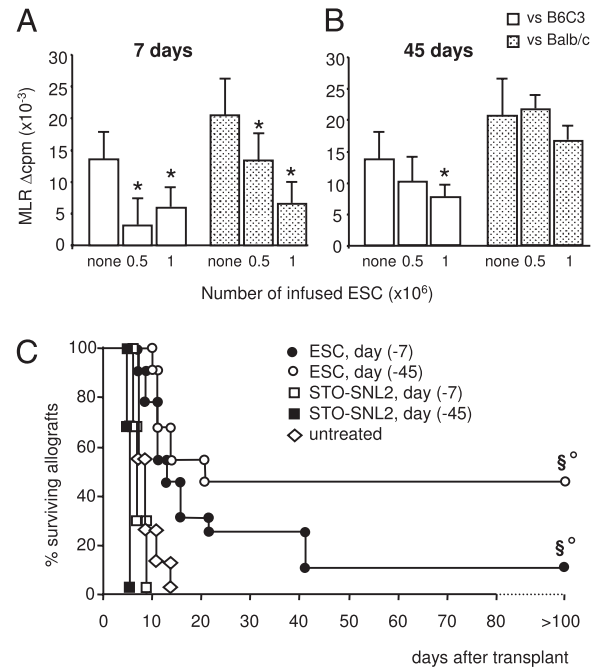
To determine whether IVF-ESCs had immunomodulatory effects in vivo, the proliferative response of splenocytes isolated from B6 mice sacrificed 7 d after either  $0.5 \times 10^6$  or  $1 \times 10^6$  B6C3 IVF-ESC infusion ( $n = 3$  each) toward donor B6C3 or third-party BALB/c Ags was assessed in MLR. Splenocytes from B6 mice infused with  $1 \times 10^6$  B6C3 IVF-ESCs showed around 60% reduction of the proliferative response toward B6C3 Ags ( $p < 0.05$  versus noninfused mice) (Fig. 4A). T cell hyporesponsiveness was not specific for donor Ags, because the proliferative response toward third-party BALB/c Ags was reduced as well. Similar results were obtained with splenocytes from mice receiving  $0.5 \times 10^6$  IVF-ESCs ( $p < 0.05$  versus noninfused mice) (Fig. 4A). Of note, the infusion of IVF-ESCs in syngeneic B6C3 recipients also resulted in a nonspecific T cell hyporesponsiveness. Indeed, splenocytes isolated from IVF-ESC-infused B6C3 mice showed a reduced proliferative response against BALB/c alloantigens in ex vivo MLR (Supplemental Fig. 1).

To evaluate whether the state of immune hyporesponsiveness in IVF-ESC-treated animals was long lasting, we repeated the same experiments in mice sacrificed 45 d after IVF-ESC infusion. Splenocytes from B6 animals that had received  $1 \times 10^6$  B6C3 IVF-ESCs showed donor-specific hyporesponsiveness ( $p < 0.05$  versus noninfused mice; Fig. 4B) and a normal response toward BALB/c alloantigens (Fig. 4B). At this time point, the proliferation of splenocytes from mice injected with  $0.5 \times 10^6$  B6C3 IVF-ESCs was not statistically different compared with that of splenocytes from noninfused mice (Fig. 4B).

These results indicate that IVF-ESC administration dose-dependently downregulates donor-specific T cell response, possibly setting the basis for allograft acceptance.

#### Donor-specific IVF-ESCs prolong the survival of a semiallogeneic heart transplant

We sought to address the question of whether ESC pretreatment had tolerogenic properties in a murine model of heart transplantation, using the semiallogeneic combination of B6C3 donors (the same ESC donor strain) in B6 recipients. Based on the results obtained from the MLR assay, we used the IVF-ESCs dose of  $1 \times 10^6$  cells. Seven ( $n = 9$ ) or 45 d ( $n = 9$ ) after B6C3 IVF-ESC infusion via the portal vein, mice underwent heterotopic transplantation of B6C3 heart. As negative control, additional mice received the same amount of embryonic fibroblasts (STO-SNL2) and were transplanted 7 or 45 d after cell infusion ( $n = 3$  each). All the untreated B6 recipients ( $n = 7$ ) rejected B6C3 heart within 13 d (Fig. 4C). Mice receiving IVF-ESCs either 7 or 45 d before transplant experienced a significant prolongation of graft survival as compared with untreated mice ( $p < 0.05$ ) and with mice receiving embryonic fibroblasts at the same time point ( $p < 0.01$ ). Indefinite graft survival (>100 d) was achieved in 10 and 44% of recipient mice receiving IVF-ESCs 7 and 45 d before transplantation, respectively (Fig. 4C). No chimerism was found in the thymus of



**FIGURE 4.** B6C3 IVF-ESC infusion induces ex vivo MLR hyporesponsiveness and prolongs the survival of B6C3 heart transplants. Recipient B6 mice receiving  $1 \times 10^6$  ( $n = 3$ ) or  $0.5 \times 10^6$  ( $n = 3$ ) IVF-ESC infusion or left untreated (none,  $n = 3$ ) were sacrificed 7 (A) or 45 d (B) after B6C3 IVF-ESC infusion. Splenocytes were isolated and cultured with 4000 rad-irradiated B6C3 (white bars), BALB/c (dotted bars), or syngeneic B6 splenocytes. MLR proliferative results are expressed as  $\Delta\text{cpm} \times 10^{-3}$  by subtracting cpm of syngeneic MLR from allogeneic combination. Data are reported as mean  $\pm$  SD; \* $p < 0.05$  versus none. C, Survival time of B6C3 cardiac grafts in B6 mice receiving either IVF-ESCs (day 7,  $n = 9$ ; day 45,  $n = 9$ ) or STO-SNL2 (day 7,  $n = 3$ ; day 45,  $n = 3$ ) or left untreated ( $n = 7$ ). <sup>§</sup> $p < 0.01$  versus STO-SNL2 at the corresponding time, <sup>°</sup> $p < 0.05$  versus untreated.

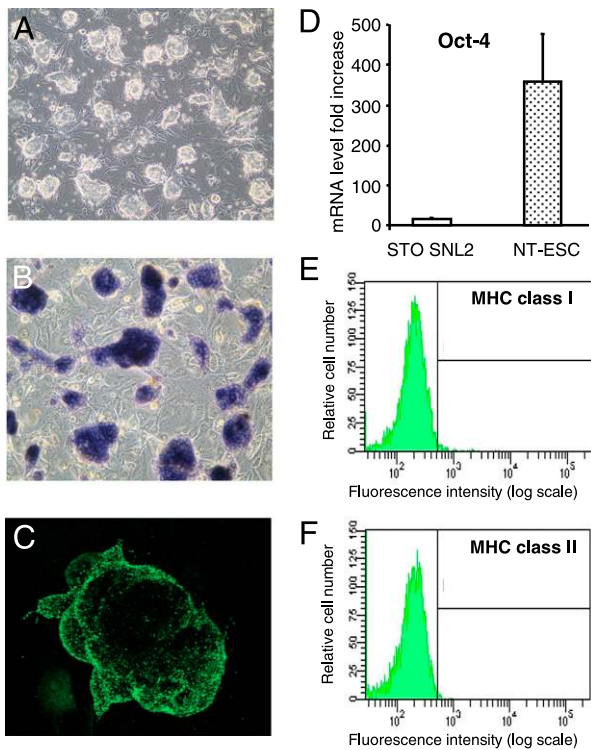
mice accepting long-term graft analyzed at sacrifice at >100 d after transplant.

#### IVF-ESCs do not prolong fully allogeneic cardiac allograft survival

We then investigated whether infusion of B6C3 IVF-ESCs ( $H-2^{b,k}$ ) was able to prolong a B6C3 ( $H-2^{b,k}$ ) cardiac allograft survival when infused into fully allogeneic recipients. DBA/2 mice ( $H-2^d$ ,  $n = 7$ ) were infused with IVF-ESCs ( $1 \times 10^6$ , portal vein) and then transplanted with a B6C3 ( $H-2^{b,k}$ ) heart 45 d after cell infusion. No prolongation of graft survival was observed, and recipient mice rejected the fully allogeneic grafts within 8 d.

#### Characterization of murine ESCs obtained by NT

In the perspective of a clinical use of donor-specific pluripotent stem cells, we derived ESCs by NT (NT-ESCs) and repeated the experiments described earlier. Cells showed well-defined NT-ESC colonies at phase-contrast microscopy (Fig. 5A). NT-ESCs were positive for AP, SSEA-1, and the transcription factor *Oct-4* (Fig. 5B–D), negative for MHC-I and -II Ags (Fig. 5E, 5F), and maintained pluripotent-specific markers at any analyzed passages (up to 30). NT-ESCs showed normal diploid chromosomal set except for few cells exhibiting tetraploidy or chromosomal loss (data not shown). As done for IVF-ESCs, we first verified the pluripotency of NT-ESCs by analyzing the capability of these cells to form teratomas in a syngeneic environment. Twenty-one days after intraportal infusion of  $1 \times 10^6$  NT-ESCs, macroscopically visible teratomas were found in the livers of all infused mice ( $n = 3$ ) (Fig. 3E).



**FIGURE 5.** Characterization of NT-ESCs. *A*, Morphology of NT-ESC colonies at passage 24 cultured on inactivated fibroblast feeder layer STO-SNL2. *B*, Fast blue staining for AP activity on NT-ESCs. *C*, Confocal microscopy analysis of stage-specific Ag-1, SSEA-1 (green) on NT-ESCs. Original magnification  $\times 400$ . *D*, Real-time PCR analysis of the transcription factor *Oct-4* expression on NT-ESCs and STO-SNL2 feeder layer. Data are reported as mean  $\pm$  SD. *E* and *F*, Flow cytometric analysis of MHC-I and -II Ags on NT-ESCs.

*In vivo tissue distribution of NT-ESCs*

Seven days after intraportal infusion of  $1 \times 10^6$  B6C3 NT-ESCs in semiallogeneic B6 mice, a very low level of donor DNA (0.14  $\pm$  0.06%;  $n = 3$ ) was observed in the liver, which further decreased at 45 d (0.03  $\pm$  0.03%;  $n = 3$ ). All the other organs (blood, thymus, spleen, bone marrow, lymph nodes, lung heart, kidney, small bowel) were negative for the presence of donor DNA, indicating that, like IVF-ESCs, NT-ESCs did not migrate outside the organ of injection.

Results from liver histology (Fig. 3*F*) were similar to those obtained in IVF-ESC-infused mice with respect to the presence of inflammation, necrosis, and areas containing undifferentiated cells, but teratomas were observed in a lower percentage of NT-ESC-treated mice (12% NT-ESCs versus 33% IVF-ESCs).

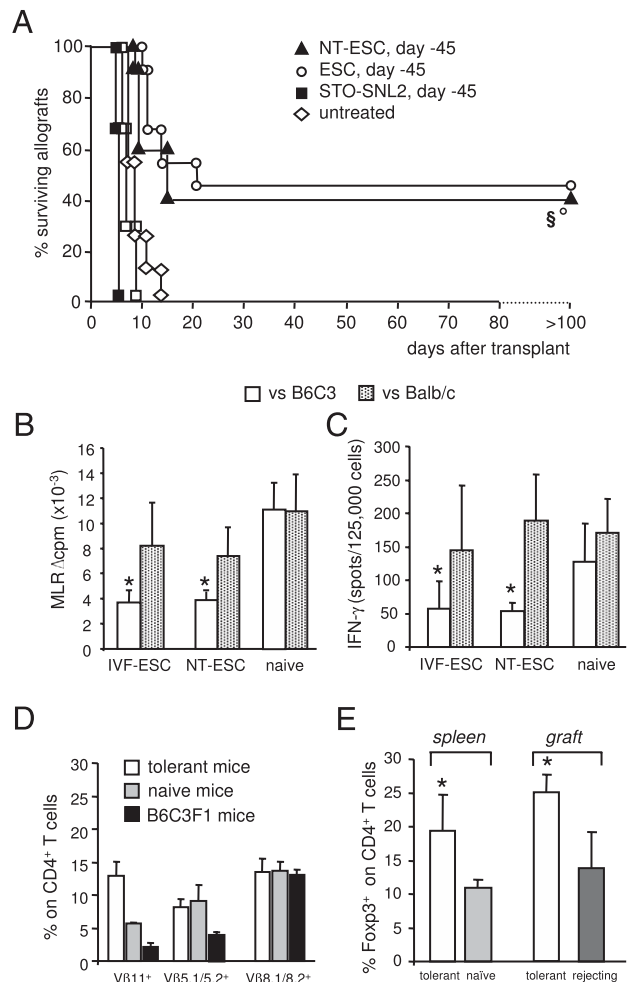
As described for IVF-ESCs, additional B6 mice ( $n = 8$ ), infused with PKH26-labeled NT-ESCs, were sacrificed at 7 ( $n = 4$ ) and 21 d ( $n = 4$ ) after cell infusion. Approximately 3 NT-ESCs/HPF were detected in recipient liver 7 d after infusion (2.6  $\pm$  0.4 cells/HPF). At day 21, few NT-ESCs were still detectable in focal aggregates (0.8  $\pm$  0.3 cells/HPF) (Fig. 3*L*). No PKH26-labeled cells were detected in thymus of infused mice at either 7 or 21 d after cell infusion.

*Donor-specific NT-ESCs prolonged the survival of a semiallogeneic heart transplant*

One million B6C3 NT-ESCs were intraportally given to B6 mice 45 d before B6C3 heart transplantation ( $n = 10$ ), to assess whether ESCs obtained by NT shared the same tolerogenic properties of IVF-ESCs. Long-term allograft survival (>100 d) was observed in

4 of 10 transplanted mice (40%;  $p < 0.01$  versus STO-SNL2 and  $p < 0.05$  versus untreated; Fig. 6*A*), indicating that, similar to the infusion of IVF-ESCs, the infusion of NT-ESCs induced graft acceptance ( $p = \text{NS}$  versus IVF-ESC-infused mice).

We then investigated whether infusion of B6C3 NT-ESCs (H-2<sup>b,k</sup>) was able to prolong a B6C3 (H-2<sup>b,k</sup>) cardiac allograft survival when infused into fully allogeneic recipients. DBA/2 mice (H-2<sup>d</sup>,  $n = 7$ ) were infused with NT-ESCs ( $1 \times 10^6$ , portal vein) and then transplanted with a B6C3 (H-2<sup>b,k</sup>) heart 45 d after cell infusion. As observed with IVF-ESCs, no prolongation of graft survival was



**FIGURE 6.** Cardiac allograft survival after NT-ESC infusion, and phenotypic and functional analysis of T cells from long-term surviving mice. *A*, Survival time of B6C3 cardiac grafts in B6 mice receiving NT-ESCs ( $n = 10$ ), IVF-ESCs ( $n = 9$ ), or STO-SNL2 ( $n = 3$ ) 45 d before transplantation or left untreated ( $n = 7$ ).  $^{\S}p < 0.01$  versus STO-SNL2,  $^{\circ}p < 0.05$  versus untreated. *B*, MLR proliferative response (as  $\Delta \text{cpm} \times 10^{-3}$ ) and (*C*) frequency of IFN- $\gamma$ -producing splenocytes from long-term graft surviving mice after IVF-ESC (MLR:  $n = 5$ ; IFN- $\gamma$  ELISPOT:  $n = 4$ ) or NT-ESC infusion (MLR:  $n = 5$ ; IFN- $\gamma$  ELISPOT:  $n = 4$ ), or from untreated B6 mice (naive,  $n = 6$ ) after exposure to donor B6C3 (white bars) and third-party BALB/c (dashed bars) irradiated splenocytes. *D*, FACS analysis of CD4<sup>+</sup> T cells expressing V $\beta$ 11, V $\beta$ 5.1/5.2, and V $\beta$ 8.1/8.2 from long-term graft surviving mice (pooled data from two IVF-ESC- and two NT-ESC-infused mice, white bars), from naive B6 mice ( $n = 3$ , gray bars), and from donor B6C3 F1 mice ( $n = 3$ , black bars). *E*, Percentage of GFP<sup>+</sup> (Foxp3<sup>+</sup>) on CD4<sup>+</sup> T cell population in the spleen ( $n = 4$ , left) and in the graft ( $n = 3$ , right) from long-term graft surviving Foxp3-GFP mice after IVF-ESC or NT-ESC infusion. Data are reported as mean  $\pm$  SD.  $^*p < 0.05$  versus naive or untreated rejecting mice.

found and recipient mice acutely rejected fully allogeneic grafts within 8 d post-transplant.

*IVF-ESC, but not NT-ESC, infusion is associated with liver teratoma in heart-transplanted mice*

Histological analysis of livers from transplanted mice preinfused with IVF-ESCs revealed the presence of different sized teratoma in 28% of animals (5/18, 1 rejecting the graft and 4 long-term surviving mice). By contrast, no teratoma formation was observed in the livers of 10 transplanted mice infused with NT-ESCs, although several groups of undifferentiated cells were present (Fig. 3G).

*IVF-ESC- and NT-ESC-induced graft acceptance was associated with donor-specific T cell hyporesponsiveness and Treg expansion*

We next investigated the mechanism underlying the long-term allograft survival in mice receiving either IVF-ESCs or NT-ESCs. In MLR experiments, splenocytes isolated from mice infused with either IVF-ESCs or NT-ESCs ( $n = 4-6$ ) and accepting a long-term heart allograft showed an antidonor B6C3 hyporesponsiveness both in MLR proliferation and in ELISPOT assay for IFN- $\gamma$ , whereas the responses toward third-party BALB/c Ags were similar to those of splenocytes from naive mice (Fig. 6B, 6C).

To evaluate the possibility that donor-reactive T cell clones could have been deleted in the thymus of ESC-tolerized mice (10), we took advantage from our donor-recipient strain combination. It allows for assessment of clonal deletion of antidonor MHC thymocytes by tracking the total T cell population bearing V $\beta$ 5.1/2 $^+$  and V $\beta$ 11 $^+$  TCR (22, 23). Indeed, B6 mice are H2-IE $\alpha^k$  null and possess ~10–20% of TCR V $\beta$ 11 $^+$  and V $\beta$ 5.1 $^+$ /5.2 $^+$  T cells. C3 strain mice, and hence also our donor B6C3 F1 mice, express I-E complexes (formed by E $\alpha^k$  and E $\beta^b$  endogenous chains) that typically present retrovirally encoded endogenous superantigen MIs. When I-E complexes are expressed, T cells bearing V $\beta$  regions to which the MIs proteins bind (V $\beta$ 5.1/5.2 and V $\beta$ 11 in this case) die during intrathymic maturation, resulting in a measurable peripheral loss of TCR V $\beta$ 5.1/5.2 $^+$  and V $\beta$ 11 $^+$  lymphocytes (22, 23) (Fig. 6D). Thus, we stained splenocytes from long-term surviving, ESC-infused mice and from naive untransplanted mice with mAbs anti-TCR V $\beta$ 5.1/5.2 and anti-V $\beta$ 11, and as control, with anti-V $\beta$ 8.1/8.2, and analyzed by FACS. Results showed no differences in the percentage of TCR V $\beta$ 5.1/5.2 $^+$ , V $\beta$ 11 $^+$ , and V $\beta$ 8.1/8.2 $^+$  on the CD4 $^+$  T cell population between the two groups of mice (Fig. 6D), excluding that ESC induced a thymic-dependent clonal deletion of donor-reactive T cells in tolerant mice. This finding fits with engraftment results showing the absence of ESCs in the recipient thymus.

To establish the role of Tregs in this model, we analyzed the percentage of GFP $^+$ (Foxp3 $^+$ ) cells within the splenic CD4 $^+$  T cell population in Foxp3-GFP-tolerant mice ( $n = 4$ ). We observed an increased percentage of GFP $^+$ (Foxp3 $^+$ ) CD4 $^+$  T cells in the spleen of tolerant mice as compared with naive untransplanted mice (Fig. 6E).

We next performed adoptive transfer experiments (20). Splenocytes ( $40 \times 10^6$ ) obtained from long-term surviving transplanted B6 mice (>100 d post-transplant) tolerized by IVF-ESC ( $n = 3$ ) or NT-ESC infusion ( $n = 3$ ) were i.v. administered to naive B6 mice 1 d before a B6C3 heart transplantation. No prolongation of cardiac allograft was observed and rejection occurred within 20 d. However, the transfer of greater amounts of splenocytes ( $100 \times 10^6$ ) from mice tolerized by IVF-ESC or NT-ESC was able to significantly prolong the survival of a B6C3 heart when infused into naive mice (>30 d;  $n = 2$ ). Next, we performed

immunohistochemical analysis of cardiac allograft tissues to document Treg recruitment at the graft site. Approximately 25% of CD4 $^+$  T cells in heart allografts taken from ESC-treated mice at >60 d post-transplant expressed Foxp3 as compared with 14% of those infiltrating rejected heart from untreated mice (Fig. 6E).

Altogether, these data indicate that ESC-induced tolerance is associated with a reduction of antidonor IFN- $\gamma$ -producing effector T cells and with Treg expansion in lymphoid organs and in the graft.

*IVF-ESCs and NT-ESCs induce profound T cell hyporesponsiveness in vitro through either cell-contact-dependent mechanisms or the release of soluble factors*

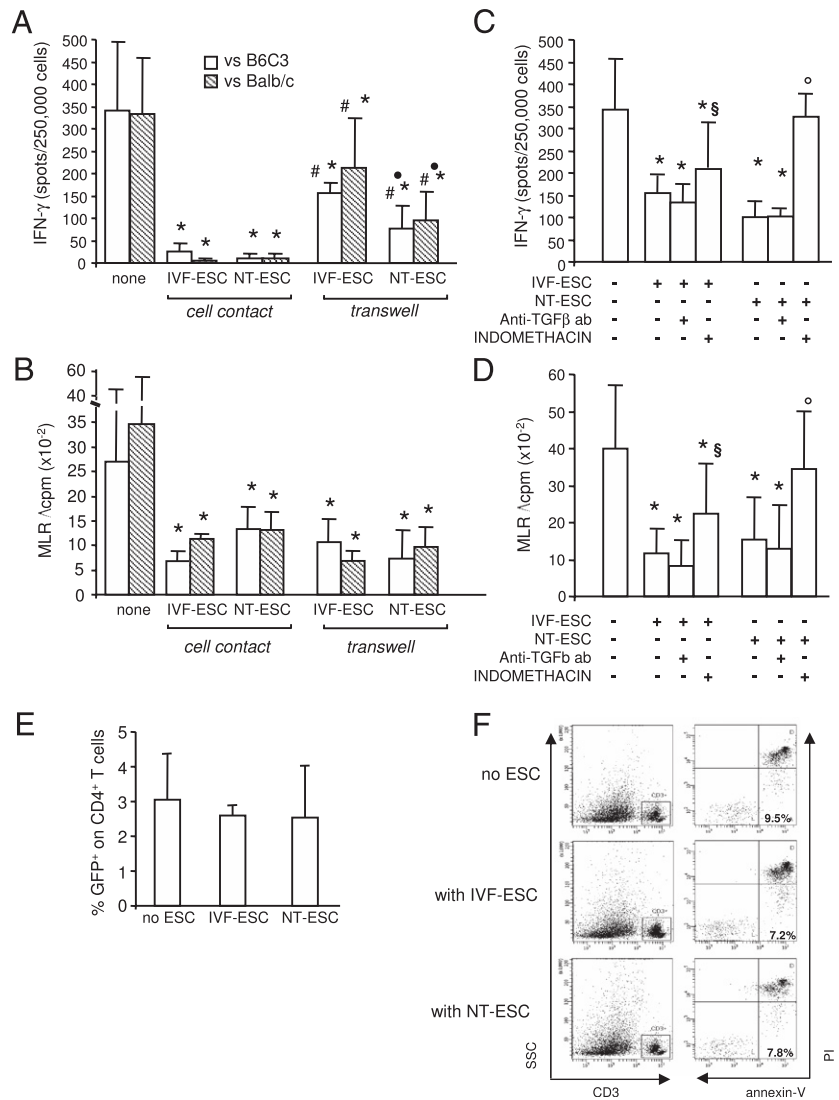
To further investigate the mechanisms by which IVF-ESCs and NT-ESCs inhibit T cell alloreactivity, we stimulated B6 splenocytes, isolated from naive mice, in MLR by B6C3 stimulator cells in the presence of either IVF-ESCs or NT-ESCs. The frequency of IFN- $\gamma$ -producing splenocytes (Fig. 7A) was drastically reduced when IVF-ESCs or NT-ESCs were added to the cultures. When the same cocultures were performed in a transwell system, to physically separate ESCs from the MLR culture, we still observed a significant reduction of the frequency of IFN- $\gamma$ -producing splenocytes (Fig. 7A), even though the effect was less pronounced than that observed in direct cell contact cocultures with either IVF-ESCs or NT-ESCs (Fig. 7A). These findings indicate that both cell-contact-dependent mechanisms and soluble factors are involved in IVF-ESC- and NT-ESC-mediated reduction of IFN- $\gamma$ -producing cells in vitro. By comparing the effect of IVF-ESCs with that of NT-ESCs, we found a similar suppressive effect on IFN- $\gamma$  spots in cell contact experiments, whereas NT-ESCs appeared to be more potent than IVF-ESCs when added in transwell (Fig. 7A). Regarding the effect of IVF-ESCs and NT-ESCs on splenocyte proliferative response in MLR, we found that the two ESC lines inhibited thymidine incorporation in responder splenocytes to the same degree (Fig. 7B). In addition, we found a comparable effect when either IVF-ESCs or NT-ESCs were added in cell contact or in a transwell system (Fig. 7B), indicating that the antiproliferative activity of IVF-ESCs and NT-ESCs is mainly mediated by soluble factors.

We also tested the effect of IVF-ESCs and NT-ESCs added in an MLR with B6 splenocytes as responders and irradiated third-party BALB/c splenocytes as stimulators, and we found inhibitory effects comparable with those observed when ESC donor-specific splenocytes were used as stimulator cells (Fig. 7A, 7B).

Finally, we performed mechanistic experiments to explore the role of a number of soluble mediators in mediating the suppressive effect of IVF-ESCs and NT-ESCs on IFN- $\gamma$  spots and proliferation. Because previous studies have suggested a role for TGF- $\beta$  (24, 25), IL-10 (25), IDO (26), NO (27, 28), and PGE $_2$  (29) as mediators of stem cell immunosuppression in vitro, we added their specific inhibitors in our in vitro transwell experiments. We found that neither an anti-TGF- $\beta$  mAb (5  $\mu$ g/ml; Fig. 7C, 7D), nor an anti-IL-10 mAb (2.5  $\mu$ g/ml) nor 1-methyl tryptophane (IDO inhibitor, 0.5 mM), nor nitroarginine (NO inhibitor, 2 mM, not shown) was able to revert IVF-ESC and NT-ESC inhibition of the frequency of IFN- $\gamma$ -producing splenocytes and splenocyte proliferation in response to B6C3 Ags. However, we found that indomethacin (inhibitor of PGE $_2$  synthesis, 40  $\mu$ M) reverted both IVF-ESC- and NT-ESC-mediated immunosuppression of IFN- $\gamma$  spots (Fig. 7C) and splenocyte proliferation (Fig. 7D). Intriguingly, indomethacin partially reverted the inhibition of IFN- $\gamma$  spots and proliferation mediated by IVF-ESCs, whereas it completely reverted the inhibition mediated by NT-ESCs (Fig. 7C, 7D).



**FIGURE 7.** IVF-ESCs and NT-ESCs induce profound T cell hyporesponsiveness in vitro. *A*, Frequency of IFN- $\gamma$ -producing splenocytes and MLR proliferative response (*B*) of splenocytes from naive B6 mice in response to B6C3 (white bars) or to BALB/c stimulator cells (dashed bars) in the absence (none) or the presence of IVF-ESCs and NT-ESCs in direct cell contact cultures or in transwell system. Effect of anti-TGF- $\beta$  (5  $\mu$ g/ml) and indomethacin (40  $\mu$ M) on the IVF-ESCs and NT-ESCs mediated inhibition of the frequency of IFN- $\gamma$ -producing splenocytes (*C*) and on MLR proliferative response (*D*) against B6C3 stimulator cells in transwell experiments. Data are mean  $\pm$  SD of three to six independent experiments. \* $p$  < 0.05 versus none, # $p$  < 0.05 versus respective cell contact,  $\bullet$   $p$  < 0.05 versus IVF-ESCs in transwell,  $\S$   $p$  < 0.05 versus IVF-ESCs,  $\circ$   $p$  < 0.05 versus NT-ESCs. *E*, Percentage of GFP<sup>+</sup> (Foxp3<sup>+</sup>) on responder CD4<sup>+</sup> T cells after MLR culture against B6C3 stimulator cells in the absence (no ESCs) or presence of either IVF-ESCs or NT-ESCs. Data are mean  $\pm$  SD of three independent experiments. *F*, Representative dot plots of Annexin V and PI staining (right panels) by gated CD3<sup>+</sup> T cells (left panels) after MLR culture against B6C3 stimulator cells in the absence (no ESCs) or presence of either IVF-ESCs or NT-ESCs. Numbers in outlined areas indicate percentage of Annexin V<sup>+</sup> PI<sup>-</sup> apoptotic CD3<sup>+</sup> T cells. Data are representative of three independent experiments.



To evaluate whether IVF-ESCs or NT-ESCs expand Tregs in vitro, we used splenocytes from Foxp3-GFP mice as responder cells in MLR against B6C3 stimulator cells in the presence of either IVF-ESCs or NT-ESCs (in direct cell contact culture). No in vitro expansion of GFP<sup>+</sup> CD4<sup>+</sup> T cells was observed at the end of coculture MLR, indicating that regulatory Foxp3<sup>+</sup> T cells are not involved in ESC immunosuppression in vitro (Fig. 7E).

Finally, to assess whether IVF-ESCs or NT-ESCs induce T cell apoptosis, we stained CD3<sup>+</sup> T cells, collected after 6-h coculture in MLR with IVF-ESCs or NT-ESCs, with FITC Annexin V/Dead Cell Apoptosis Kit. We found that neither IVF-ESCs nor NT-ESCs induced CD3<sup>+</sup> T cell apoptosis in our experimental conditions (MLR: 9  $\pm$  0.7; MLR+IVF-ESCs: 6.6  $\pm$  0.92; MLR+NT-ESCs: 9.8  $\pm$  2.8% Annexin V<sup>+</sup> propidium iodide<sup>-</sup> apoptotic cells on CD3<sup>+</sup> T cells; the  $p$  value was not significant) (Fig. 7F). By cytofluorometry, we also observed that IVF-ESCs and NT-ESCs, either in resting conditions or after in vitro exposure to stimulated splenocytes, did not express FasL (data not shown).

**Discussion**

In this study, we found that infusion of ESCs, produced from a blastocyst developed following IVF, IVF-ESCs, is effective in prolonging the survival of semiallogeneic vascularized heart transplants from the same ESC donor strain in unconditioned recipient mice. More importantly, we found that ESCs obtained by

NT of the donor somatic cell nucleus into an enucleated oocyte, NT-ESCs, share with IVF-ESCs the same capability of prolonging a semiallogeneic cardiac allograft survival.

For both ESC lines, the mechanism of immunomodulation relies on their capability to induce donor-specific T cell hyporesponsiveness either by contact-dependent mechanisms or by the release of soluble factors. By adding specific inhibitors, we found that PGE<sub>2</sub> is a mediator of ESC-induced immunosuppression in vitro.

Conflicting results on the capability of ESCs to modulate alloreactivity in the context of solid organ transplantation have been reported. Intraportal infusion of RESCs induced long-term graft acceptance to heart transplants in rats (10). Cells that have been used in the earlier experiments were not true ESCs, at least by standard accepted definitions. Indeed, these cells did not express *Oct-4* (30), showed a mosaic expression of SSEA-1 (10), and derived from the outgrowth of cells from the whole blastocyst, suggesting a probable extra ICM origin of RESC. When pluripotent undifferentiated bona fide mouse ESCs, as demonstrated by uniform expression of *Oct-4* and SSEA-1 and teratogenicity, were used, they failed to induce tolerance to neonatal nonvascularized cardiac allografts (17) or to skin transplant (24). In these latter studies, however, the immunomodulatory properties of ESCs were tested in the most stringent models of rejection: s.c. transplant of neonatal hearts (17) and skin transplants (24) in a fully MHC

mismatched strain combination. Thus, whether these discrepancies may depend on the different nature of ESCs used (probable extra ICM derived ESC-like cells versus ESCs derived from ICM) or on different models of acute rejection remains to be established.

In this study, intraportal infusion of murine undifferentiated IVF-ESCs in unconditioned mice induces long-term graft acceptance to a semiallogeneic vascularized heart transplant from the same IVF-ESC donor strain. This success might be considered as a proof of concept of the procedure, because of the obvious clinical impracticability to have ESCs and solid organ graft from the same donor. One way to circumvent this problem is to produce ESCs using the NT approach. Experiments in several animal species documented that the developmental program of adult somatic cell can be reversed to an embryonic phenotype (31) obtaining cells with nearly equivalent molecular signature and differentiation potential (32). In this article, we document that NT-ESCs, expressing similarly to IVF-ESCs pluripotency markers *SSEA-1*, *Oct-4*, and *AP*, and able to form teratomas when infused in syngeneic environment, prolonged the survival of a semiallogeneic heart transplant from the same NT-ESC donor strain. Moreover, the efficacy of tolerance induction was comparable with that obtained with IVF-ESCs. This achievement has groundbreaking implications for cell therapy research considering that NT-ESCs might be created from a solid organ donor-specific source. The tolerogenic potential of ESC, however, is not strong enough to control the immune response elicited by a fully allogeneic allograft. It would be interesting in future studies to investigate whether the combination of ESC with low doses of immunosuppressants, used in clinical practice, given transiently during the first days after transplantation, could synergize with ESC in inducing fully allogeneic graft tolerance.

Several evidences suggested that ESCs are immunologically inert: they express low levels of MHC-I molecules and lack MHC-II and costimulatory molecule expression (10, 14–17). Such low immunostimulating phenotype could confer them the capability to escape immunorecognition and survive in an allogeneic environment without need of host conditioning. In support of this hypothesis are studies on human ESCs that demonstrated the unopposed acceptance of human ESCs in xenogenic and allogeneic settings (10, 14–17). In contrast, others have found that in vivo differentiating ESCs upregulate MHC molecules that are associated with significant inflammatory cell infiltrate and ultimately rejection of differentiated allogeneic ESCs (33, 34). In this article, we documented that unstimulated IVF-ESCs and NT-ESCs did not express MHC-I and -II Ags, but their intraportal infusion into semiallogeneic mice was associated with focal small areas of inflammatory infiltrates and necrosis in the recipient liver, suggesting an ongoing immune reaction of the host against infused cells. Nevertheless, few IVF-ESCs and NT-ESCs survived long term as documented by the low, but persistent, chimerism and by development of teratomas in the liver of semiallogeneic recipient mice. Consistent with our findings are published data reporting successful engraftment after s.c. and i.v. administration of ESCs in unmanipulated mice provided that a sufficient number of cells were delivered (24). In another study, a partial ESC engraftment in the liver with hepatic teratoma formation after intraportal infusion of murine ESCs in immunocompetent mice (17) was observed. At variance, administration of ESCs in the myocardium of allogeneic mouse recipients resulted in a robust inflammatory response (34–36) that paralleled the differentiation of the implanted cells and their upregulation of MHC genes (34, 36). These findings could indicate that the site of ESC injection is a major determinant for ESC engraftment in an allogeneic host, and that the liver may be the ideal inoculation site for successful cell engraftment.

In the published report of intraportal infusion of RESCs (10), these cells, after successful engraftment in the liver of the allogeneic recipient, migrated into the recipient thymus where they mediated clonal deletion of alloreactive T cells. Moreover, this process ultimately resulted in the induction of mixed chimerism and tolerance to a subsequent cardiac transplantation from the same ESC donor strain (10). In our study, IVF-ESC and NT-ESC liver engraftment in the semiallogeneic condition was not associated with any detectable chimerism in thymus or in blood.

In addition, analysis of T cells bearing TCR V $\beta$ s specific for superantigens expressed by donor MHC showed a normal peripheral T cell repertoire in tolerant mice. Thus, failure of finding either donor cells in the recipient thymus or a peripheral loss of donor-reactive T cell clones would exclude a role of central clonal deletion in our model of tolerance.

Also, mechanisms of peripheral apoptosis have been suggested to mediate the immunosuppressive properties of ESCs. Bonde and Zavazava (37) showed that murine ESCs expressed FasL Ags and, when allowed to extensively engraft recipient lymphoid organs in sublethally irradiated mice, induced deletion of alloreactive T cells by Fas-induced apoptosis in recipient T cells. However, published data about the expression of FasL on ESCs are contradictory, and other studies provided evidence that ESCs lack the intrinsic ability to synthesize a functional FasL protein (38, 39). Accordingly, we found that our ESC lines did not constitutively express FasL and did not induce T cell apoptosis, at least in these experimental settings.

Despite that, we clearly documented that IVF-ESCs exerted an immunomodulatory action in vivo as shown by profound hyporesponsiveness of splenocytes isolated from recipient mice after IVF-ESC administration. The infusion of IVF-ESCs resulted in early nonspecific T cell hyporesponsiveness toward alloantigens and, later, in persistent hyporesponsiveness toward ESC donor-specific Ags. The transplantation of a heart from the same ESC donor strain at this time point resulted in long-term graft acceptance. However, we were not able to test the donor specificity of ESC infusion because of the lack of a suitable model of semi-allogeneic third-party cardiac allograft model.

The long-term survival of semiallogeneic heart transplants was associated with reduced IFN- $\gamma$ -producing effector T cells against donor Ags and with Treg expansion in the periphery and in the graft. Accumulation of Tregs in the graft has been proposed as the mechanism of tolerance induction mediated by ESC-derived hematopoietic stem cells (40). However, by in vitro studies, we excluded that ESCs are able per se to induce Treg expansion, but they did so in vivo in the context of allotransplantation, possibly as a consequence of their capability to suppress effector T cells, thus creating a facilitating environment for Treg expansion.

By in vitro studies, we found that both IVF-ESCs and NT-ESCs potentially inhibited the frequency of IFN- $\gamma$ -producing cells and T cell proliferation in MLR either when cultured in direct cell contact or, to a lesser extent, in a transwell system, indicating that IVF-ESCs and NT-ESCs exert their immunomodulatory action by cell-contact-dependent mechanisms or through the release of soluble mediators. Experiments with rodent ESCs have suggested a role of TGF- $\beta$  (24) or TGF- $\beta$ <sub>2</sub> (33) in the immunomodulatory action of ESCs. In our setting, the addition of an anti-TGF- $\beta$  Ab did not influence ESC-induced immunosuppression, whereas indomethacin was able to revert it. Thus, we found a role for PGE<sub>2</sub> as a soluble mediator that partially accounts for immunomodulatory activity of ESCs. A recent article (41) demonstrated that *cox-2*, the gene controlling the pathway that leads to the production of PGE<sub>2</sub>, is constitutively expressed in resting and growing mouse embryo stem cells, and PGE<sub>2</sub> protects embryo stem cells from

apoptosis, indicating its fundamental role in physiological development of embryos (41). In this article, we suggest that, through PGE<sub>2</sub> release, ESCs could also exert immunomodulatory properties, a mechanism proposed also for mesenchymal stem-cell-mediated immunomodulatory activities (29).

The high likelihood to have teratoma hampers the clinical applications of ESCs. Also, in our study, infusion of ESCs in semiallogeneic animals was associated with teratoma formation. However, NT-ESCs formed teratomas in a lower percentage of allogeneic animals compared with IVF-ESCs. Findings that NT-ESCs were less likely to form teratomas as compared with IVF-ESCs may be taken to indicate that NT might have induced epigenetic modification of genes involved in tumor degeneration, finally representing a major advantage over IVF-ESCs.

Although teratoma degeneration was beyond the scope of our study, it is worth noting that the mice that experienced long-term graft survival are not necessarily the same mice showing teratoma formation, suggesting that the IVF-ESC degeneration in teratoma is regulated by immune mechanisms other than those involved in the response to allogeneic cells.

In summary, our findings prove that ESCs represent a good research tool for possible therapeutic applications in solid organ transplantation because of their capability, when given alone, to induce tolerance to semiallogeneic solid organ allograft. More importantly, we have shown that the same immunomodulation and tolerance induction can be achieved using ESCs obtained from somatic NT. These findings bring near, in principle, a clinical applicability by using embryonic-derived stem cells and solid organ grafts from the same donor. Induced pluripotent stem cells (42) share with NT-ESCs the possibility to derive pluripotent cells directly from cells of the organ donor. Whether induced pluripotent stem cells possess the same tolerogenic properties of NT-ESCs will be worth investigating.

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## Disclosures

The authors have no financial conflicts of interest.

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