Embryonic stem cell-derived tissues are immunogenic but their inherent immune privilege promotes the induction of tolerance

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Although human embryonic stem (ES) cells may one day provide a renewable source of tissues for cell replacement therapy (CRT), histoincompatibility remains a significant barrier to their clinical application. Current estimates suggest that surprisingly few cell lines may be required to facilitate rudimentary tissue matching. Nevertheless, the degree of disparity between donor and recipient that may prove acceptable, and the extent of matching that is therefore required, remain unknown. To address this issue using a mouse model of CRT, we have derived a panel of ES cell lines that differ from CBA/Ca recipients at defined genetic loci. Here, we show that even expression of minor histocompatibility (mH) antigens is sufficient to provoke acute rejection of tissues differentiated from ES cells. Nevertheless, despite their immunogenicity *in vivo*, transplantation tolerance may be readily established by using minimal host conditioning with nondepleting monoclonal antibodies specific for the T cell coreceptors, CD4 and CD8. This propensity for tolerance could be attributed to the paucity of professional antigen-presenting cells and the expression of transforming growth factor (TGF)-β_2. Together, these factors contribute to a state of acquired immune privilege that favors the polarization of infiltrating T cells toward a regulatory phenotype. Although the natural privileged status of ES cell-derived tissues is, therefore, insufficient to overcome even mH barriers, our findings suggest it may be harnessed effectively for the induction of dominant tolerance with minimal therapeutic intervention.

cell replacement therapy | acquired immune privilege | regulatory T cell

The first derivation of human embryonic stem (ES) cell lines in 1998 (1) proved a decisive turning point in biomedical science, offering a potentially limitless source of cell types and tissues for regenerative medicine. The differentiated products of human and other primate ES cells have been used successfully in animal models of diseases as diverse as myocardial infarction (2), ischemic-reperfusion injury (3), and Parkinson's disease (4), each requiring the administration of a single, purified cell type. Furthermore, advances in tissue engineering may facilitate the construction of functional organoids from the differentiated products of ES cells, raising the prospect of treating more complex diseases in the future. Nevertheless, rejection of the transplanted tissues remains the single greatest obstacle to cell replacement therapy (CRT) (5, 6), the immunological barriers potentially proving most profound after the implantation of composite tissues that necessarily provide a more diverse source of tissue-specific antigens than homogenous populations of cells. To assess whether rudimentary matching between donor and recipient might address the immunological barriers encountered in the clinic, Taylor and colleagues have estimated the magnitude of the bank of human ES cells required to make CRT accessible to a significant proportion of the population (7). Although their conclusion that 150 lines might be sufficient represents an achievable goal, this figure was based on matching only selected MHC loci, on the assumption that the judicious use of immune suppression might overcome residual immunogenicity. Given that the risks of long-term immune suppression may exceed those of many disease states amenable to CRT, such a premise may, however, be unfounded.

Although the need for more stringent matching of donor and recipient would inevitably increase the number of ES cell lines required, this trend may be partially tempered by the propensity for immune privilege reportedly enjoyed by ES cells and their differentiated progeny. Circumstantial evidence suggests that both mouse and human ES cells display immune privilege due, in part, to their low expression of MHC determinants (8–10). Nevertheless, the extent to which this may modulate alloreactivity *in vivo* remains controversial: Whereas Drukker and colleagues have demonstrated the unopposed acceptance of human ES cell-derived tissues in a trimera mouse model of CRT (11), others have found significant cellular infiltration of tissues differentiated from allogeneic mouse ES cells, leading ultimately to their rejection (6, 12, 13).

To define more precisely the extent of histoincompatibility that might be tolerated in the absence of immune suppression after the implantation of composite tissues rather than homogenous populations of cells, we have made use of CBA/Ca mice as recipients of CRT and derived a panel of ES cell lines from strains of mice with increasing levels of genetic disparity. Using so-called embryoid bodies (EB) as composite tissues for transplantation, we show that the differentiated progeny of ES cells are as susceptible to rejection as tissues from any conventional source. Nevertheless, their propensity for acquired immune privilege may be harnessed, through minimal host conditioning, to establish a profound state of transplantation tolerance in which a proportion of naïve, alloreactive T cells is polarized toward a regulatory phenotype.

Results

Derivation of a Panel of Murine ES Cell Lines. No systematic study has so far been conducted to define the degree of genetic parity between donor and recipient required in the context of CRT. Such information cannot be inferred from studies of whole-organ transplantation because the properties of immune privilege reported for ES cells and their progeny may introduce some measure of leniency into the matching process. To address this issue, we made use of protocols established in one of our laboratories (14), to derive ES cell lines from strains of mice that

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Table 1. Details of ES cell lines used in this study

Cell line	Strain of origin	MHC haplotype	Gender	Disparity from female CBA/Ca	Ref.
ESF121	CBA/Ca	H-2^k	Female	None	14
ESF116	CBA/Ca	H-2^k	Male	Single mH	14
ESF191	B10.BR	H-2^k	Male	Multiple mH	–
ESF166	CB/K	H-2^k H-2K^b	Male	Single mH + MHC class I	–
ESF75	C57BL/6	H-2^b	Male	Fully allogeneic	14

differ at specific genetic loci from CBA/Ca recipients: When combined with lines generated previously, these ES cells form a comprehensive panel, representing increasing levels of immunological challenge. Table 1 shows details of the ES cell lines used, their gender, MHC haplotype, and the nature of their disparity from female CBA/Ca mice. All lines were capable of generating EB *in vitro* that formed teratomas after implantation under the kidney capsule of immune deficient CBA.RAG1$^{-/-}$ recipients. Histological analysis of teratomas typically revealed the differentiation of cell types and anatomical structures derived from each of the three embryonic germ layers [supporting information (SI) Fig. 6], consistent with the pluripotency of the parent cell lines. The integrity of selected lines was further investigated by their reintroduction into blastocysts from the PO (Pathology Oxford) strain of mouse: For ESF116, 8 of 19 pups were demonstrably chimeric, of which 2 showed germ-line competence, whereas 6 of 13 progeny proved to be chimeric after the introduction of ESF75, of which 1 offspring was capable of transmitting genes of the C57BL/6 background through the germ line.

Immunogenicity of Tissues Derived from ES Cells. To model the clinical process of CRT, we generated EB by culturing ES cells in suspension for 14 days and grafted them under the kidney capsule of recipient female CBA/Ca mice. EB derived from the syngeneic female ES cell line, ESF121, became well vascularized and had increased substantially in size by day 16 after transplantation (Fig. 1*A*). Histological analysis of serial sections revealed a wide variety of tissues, including cartilage, secretory epithelium, and neural tissue (Fig. 1*B*). By contrast, EB from ESF75, a fully allogeneic ES cell line, failed to grow or become vascularized (Fig. 1*A*, arrow), showing, instead, signs of extensive tissue damage and necrosis (Fig. 1*C*, arrows). Immunohistochemistry revealed a substantial inflammatory infiltrate including host T cells and macrophages (Fig. 1 *D* and *E*), which were present only in trace numbers within syngeneic EB (Fig. 1 *F* and *G*). Allogeneic EB grew well in CBA.RAG1$^{-/-}$ recipients (SI Fig. 7), confirming that their inability to engraft in normal mice is the result of an adaptive immune response, rather than intrinsic deficiencies in the donor cell line. Furthermore, the parenchymal cells of such EB expressed barely detectable levels of MHC class I (Fig. 1*H*), consistent with previous findings in the human (8): Nevertheless, far from securing their survival after implantation into immune competent mice, this level of expression was specifically up-regulated during inflammation (Fig. 1*I*), albeit to lower levels than seen in control sections of donor-strain liver (Fig. 1*J*), and induced vigorous rejection (Table 2).

To investigate whether rudimentary matching between donor and recipient might reduce the immunogenicity of ES cell-derived tissues, we used, as a source of EB, the cell lines ESF166 and ESF191, derived from CB/K and B10.BR mice, respectively. CB/K mice are genetically identical to CBA/Ca, with the exception of an additional MHC class I molecule, H-2K^b (15): The availability of ES cells from this strain therefore mimics the clinical scenario in which a near-perfect match between donor and recipient has been identified. By contrast, B10.BR mice share their entire MHC haplotype with CBA/Ca but differ in their expression of polymorphic proteins representing minor histocompatibility (mH) antigens (16). EB from ESF166, grafted under the kidney capsule of CBA/Ca recipients, were universally rejected, albeit with a tempo slower than that of fully allogeneic EB (Table 2). Surprisingly, EB derived from ESF191, which differed solely at multiple mH loci, were vigorously rejected at a rate similar to fully allogeneic EB (Table 2); indeed the pattern of rejection of all ES cell-derived tissues was indistinguishable from that of conventional skin grafts (Fig. 2), confirming that such tissues are fully immunogenic and succumb to rejection in precisely the same manner as tissues from alternative sources. Importantly, the inherent immune privileged status of ES cells and tissues differentiated from them (9, 10) was insufficient to prevent rejection across a multiple mH barrier, even when all MHC loci were shared, suggesting that no degree of matching between donor and recipient is likely to prevent rejection in the absence of immune intervention.

Tissues Derived from ES Cells Are More Susceptible to Tolerance Induction than Conventional Tissues. Given that the risks associated with immune suppression may not justify its long-term use for many diseases amenable to CRT, we next investigated whether the induction of transplantation tolerance might offer a viable form of immune intervention. Monoclonal antibodies (mAb) specific for CD4 and CD8 coreceptors have proven effective agents for establishing a robust form of transplantation tolerance across multiple mH barriers (17). Furthermore, when combined with costimulation blockade in the form of mAb to CD154, such a conditioning regime may also achieve tolerance across a full MHC disparity (18, 19). CBA/Ca mice were, therefore, treated with a mixture of nondepleting CD4 and CD8 mAb around the time of transplantation with EB from the various ES cell lines.

Twenty-eight days after grafting, CBA/Ca mice treated with mAb had accepted EB derived from multiple mH mismatched ($n = 5$), single MHC mismatched ($n = 5$), and even fully allogeneic ES cells ($n = 6$) (Table 2). The resulting tissues were equivalent in size and appearance to those arising from syngeneic EB and showed no signs of an inflammatory infiltrate or tissue damage. Significantly, EB from CB/K ES cells were fully accepted by five of six CBA/Ca recipients after administration of CD8 mAb alone (SI Fig. 8), whereas indefinite survival of CB/K skin grafts could not be achieved even when a combination of CD4 and CD8 mAb was used. Likewise, our ability to secure the survival of fully allogeneic EB without the need for additional blockade of costimulatory molecules such as CD154, suggests that, despite being fully immunogenic, ES cell-derived tissues may prove more amenable to tolerance induction than tissues from conventional sources.

ES Cell-Derived Tissues Provide a Microenvironment Conducive to Tolerance Induction. We reasoned that the propensity for tolerance induction of tissues derived from ES cells might be attributed to the absence of professional antigen presenting cells, the

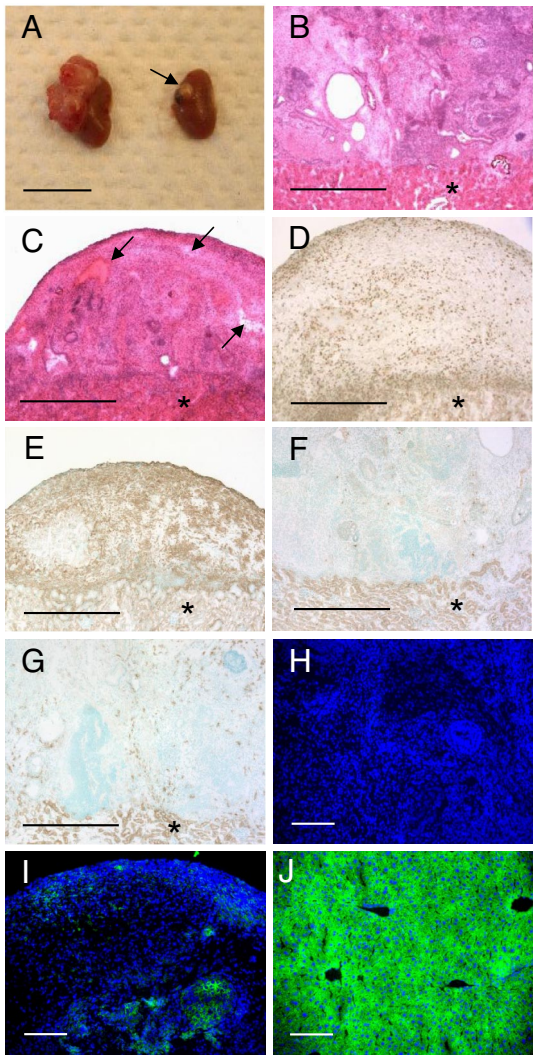

Fig. 1. ES cell-derived tissues are rapidly rejected by fully allogeneic recipients. (*A*) EB differentiated from ESF75 (C57BL/6) fail to engraft under the kidney capsule of female CBA/Ca recipients (arrow), compared with syngeneic EB from ESF121. (*B* and *C*) Histological analysis of serial sections from syngeneic (*B*) and allogeneic (*C*) EB 16 days after transplantation. The asterisk identifies tissue from the recipient kidney, and arrows denote areas of tissue damage within the engrafted EB. (*D–G*) Serial sections from allogeneic (*D* and *E*) and syngeneic (*F* and *G*) EB stained with mAb specific for the T cell marker CD3 (*D* and *F*) or the macrophage marker, F4/80 (*E* and *G*). All micrographs are fully representative of multiple recipients in each group ($n = 11$ for syngeneic, and $n = 12$ for allogeneic donor–recipient combinations). (*H–J*) Expression of the MHC class I determinant H-2K^b by EB from ESF75 grafted under the kidney capsule of immunodeficient CBA.RAG1$^{-/-}$ mice (*H*) or normal CBA/Ca recipients (*I*), compared with sections of liver from a mouse of the H-2^b haplotype, by way of a positive control (*J*). The distribution of H-2K^b is shown in green, and staining of nuclei by DAPI is overlaid in blue. (Scale bars: 1 cm in *A*, 500 μm in *B–G*, and 50 μm in *H–J*.)

active expression of genes associated with immune privilege, or a combination of the two. To investigate the extent to which donor dendritic cells (DC) might contribute to rejection by the direct presentation of alloantigen to host T cells, we mechanically dissociated EB after 14 days in culture and analyzed the expression of DC-associated markers by flow cytometry, using mature DC differentiated from bone marrow progenitors (bmDC) for comparison. Whereas bmDC stained strongly for all markers (Fig. 3 *A–E*), the differentiated progeny of mouse ES cells expressed low levels of MHC class I molecules and no

Table 2. Survival of ES cell-derived grafts in female CBA/Ca mice with or without treatment with mAb specific for CD4 and CD8

	Proportion (%) of grafts surviving			
Cell line	Day 16	Day 21	Day 28	Day 28 with CD4/CD8 mAb
ESF121	6/6 (100)	ND	11/11 (100)	ND
ESF191	0/12 (0)	ND	ND	5/5 (100)
ESF166	5/7 (71)	1/8 (12.5)	0/12 (0)	8/8 (100)
ESF75	0/7 (0)	0/14 (0.)	ND	6/6 (100)

ND, not determined.

detectable MHC class II (Fig. 3 *F* and *G*) in accordance with previous reports for human ES cells (8). Furthermore, the absence of staining for CD11c and CD80 (Fig. 3 *H* and *I*) is consistent with a paucity of endogenous DC: although low levels of CD86 could be detected above background staining with isotype controls (Fig. 3*J*), its equivalent expression by undifferentiated ES cells (N.J.R. and P.J.F., unpublished observations) suggests it may be a vestige of the differentiation process.

To determine whether the absence of DC has functional relevance in our model, we treated male CBA/Ca recipients with mAb specific for CD4 and/or CD8 and grafted them with EB from the CB/K ES cell line ESF166. Whereas conditioning secured 100% survival of EB, the i.v. administration of mature DC differentiated from CB/K bone marrow provoked their prompt rejection: Indeed, as few as 5×10^5 cells per mouse proved sufficient to antagonize the induction of tolerance (Table 3). Our results imply, therefore, that the absence of direct presentation of alloantigen by endogenous DC removes a powerful stimulus for rejection. Nevertheless, irrespective of the presence of donor DC, the recognized capacity of the indirect pathway to provoke allograft rejection (20), suggests that additional mechanisms may operate to favor tolerance induction in this system.

To address this issue, we investigated a number of genes implicated in immune privilege and compared their expression by EB and skin grafts using quantitative PCR (qPCR). Fig. 4 shows the pattern of expression of selected genes from the 87 immunologically relevant genes included in the Taqman array (SI Table 4). The antiinflammatory cytokines IL-10 and transforming growth factor (TGF) β_1 and β_2 are shown, together with the enzymes indoleamine 2,3-dioxygenase (Indo) and arginase 1 and 2, thought to foster immune privilege by virtue of their capacity to deplete the essential amino acids tryptophan and arginine from the local microenvironment (21, 22). Whereas expression of the catalytic enzymes arginase 1 and 2 and of TGF-β_1 was largely unremarkable (Fig. 4 *A–C*), IL-10 and Indo appeared more strongly correlated with rejection of EB than their survival (Fig. 4 *D* and *E*), suggesting that their expression is unlikely to contribute to their privileged status. In contrast,

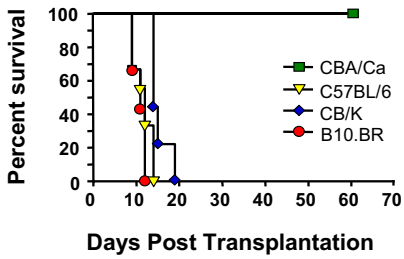

Fig. 2. Kinetics of the survival of skin grafts from each of the strains of mouse from which ES cell lines were derived, after transplantation to female CBA/Ca recipients.

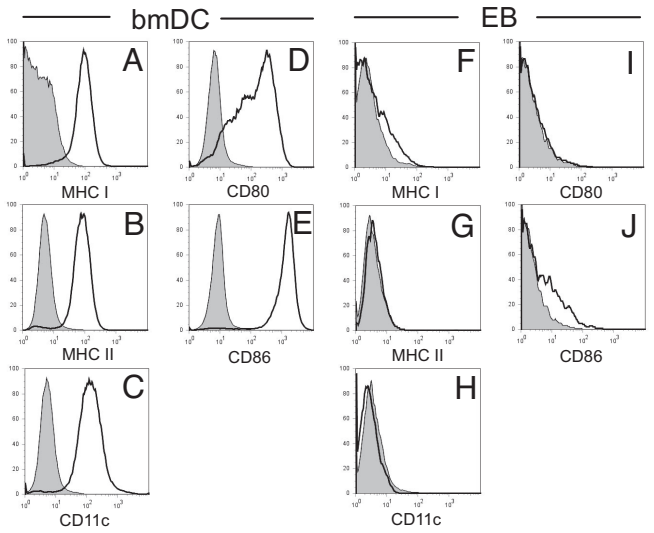

Fig. 3. EB are devoid of professional antigen presenting cells at the time of transplantation. EB were dissociated into a single-cell suspension at day 14 of culture and were analyzed for the surface expression of DC markers by flow cytometry. Mature bmDC (*A–E*) and cells derived from EB (*F–J*) were stained for expression of MHC I (*A* and *F*), MHC II (*B* and *G*), CD11c (*C* and *H*), CD80 (*D* and *I*), and CD86 (*E* and *J*). Shaded histograms denote background staining using control, isotype-matched antibodies.

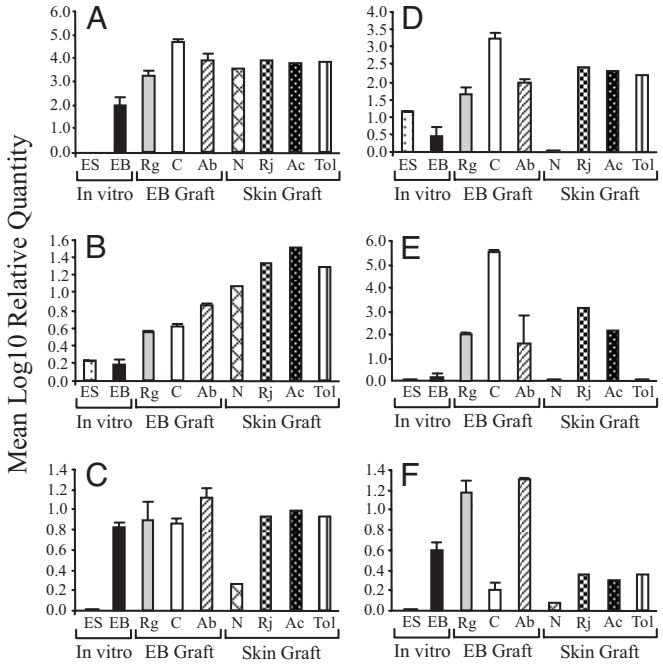

Fig. 4. Quantitative PCR showing the relative expression of genes associated with immune privilege by ESF166 (ES; dotted bars) and by differentiated EB either *in vitro* (EB; black bars) or after their implantation under the kidney capsule of CBA.RAG1$^{-/-}$ mice (Rg; gray bars), CBA/Ca recipients (C; white bars) or CBA/Ca mice treated with mAb specific for CD4 and CD8 (Ab; hatched bars). For comparison, expression of the relevant genes is displayed for normal skin (N; diamond bars), or skin grafts that are either in the process of being rejected (Rj; checkered bars), or have been accepted spontaneously by syngeneic mice (Ac; stippled bars), or allogeneic recipients after the induction of tolerance by using mAb (Tol; striped bars). Expression of the following genes was assessed: *Arginase 1* (*A*), *Arginase 2* (*B*), *Tgfb1* (*C*), *Il-10* (*D*), *Indo* (*E*), and *Tgfb2* (*F*). All values were normalized to expression levels of the housekeeping gene *Hprt*.

TGF-β_2, known to be strongly implicated in acquired immune privilege in the eye (23), was expressed upon differentiation of ES cells into EB *in vitro*, as described (24) and was further up-regulated after their long-term survival *in vivo* (Fig. 4*F*). Although these data cannot distinguish cause and effect, the expression of TGF-β_2 was found to be minimal in skin grafts, whether tolerated or rejecting, suggesting that ES cell-derived tissues are qualitatively different from tissues derived from conventional sources with respect to their expression of this immunomodulatory cytokine. Although we are unable to attribute the up-regulation of TGF-β_2 solely to parenchymal cells of the graft, mRNA levels were found to correlate inversely with the presence of an inflammatory infiltrate, suggesting that recipient leukocytes are not the principal source.

TGF-β_2 Is a Likely Contributor to the Induction of Dominant Tolerance. To determine whether TGF-β_2 mRNA is translated into the functionally active cytokine and to investigate the role it may play in the induction of tolerance, we made use of T cell receptor (TCR) transgenic mice as recipients of CRT. A1.RAG1$^{-/-}$ mice express an MHC class II-restricted TCR specific for residues 479–493 of Dby, a male-specific mH antigen, encoded on the Y chromosome (25). In common with other mouse strains on a RAG1$^{-/-}$ genetic background, A1.RAG1$^{-/-}$ mice are entirely devoid of CD4$^+$Foxp3$^+$ regulatory T (Treg) cells that may actively inhibit allograft rejection (26): Consequently, female A1.RAG1$^{-/-}$ mice readily reject male skin grafts. Strikingly, however, EB differentiated from the male ES cell line ESF116, survived indefinitely after transplantation under the kidney capsule of female A1.RAG1$^{-/-}$ mice, even in the absence of conditioning with mAb. Far from being rejected, EB became vascularized and formed teratomas containing a wide variety of differentiated tissues (Fig. 5*A*), despite a significant T cell infiltrate (Fig. 5*B*).

Table 3. Capacity of mature DC to prevent the induction of tolerance to ES cell-derived tissues

Treatment regime	Grafts surviving at day 28 (%)
No treatment	3/8 (37.5)
CD8 mAb	5/5 (100)
CD8 mAb + 5×10^5 bmDC	0/4 (0)
CD8 mAb + 2×10^6 bmDC	0/5 (0)

We investigated the potential role played by TGF-β_2 in the acceptance of male EB by staining serial sections with polyclonal antisera specific for phospho-smad2/3, an essential component of the TGF-β signaling pathway. Fig. 5*B* shows almost ubiquitous nuclear staining of cells that was readily inhibited by a smad2 blocking peptide, consistent with an environment replete with functionally active TGF-β_2. Significantly, immunohistochemistry revealed the presence of infiltrating CD4$^+$ T cells that had up-regulated Foxp3, indicative of a regulatory phenotype (Fig. 5*C*). That their conversion to Treg cells had occurred *in situ* in response to the local production of TGF-β_2 was confirmed by using, as recipients, A1.RAG1$^{-/-}$ females that had been crossed with dnTGFβRII.RAG1$^{-/-}$ mice, so as to introduce a dominant-negative receptor for TGF-β. Fig. 5*D* shows that, despite readily infiltrating male EB, CD4$^+$ T cells incapable of responding to TGF-β_2, failed to acquire the phenotype of Treg cells by up-regulating Foxp3. Staining for smad2/3 confirmed the presence of functionally active TGF-β_2, which was confined to the implanted ES cell-derived tissues, the recipient kidney showing no staining above background (Fig. 5*D*, asterisk).

To determine whether the microenvironment alone was sufficient to secure conversion to a Treg phenotype or whether

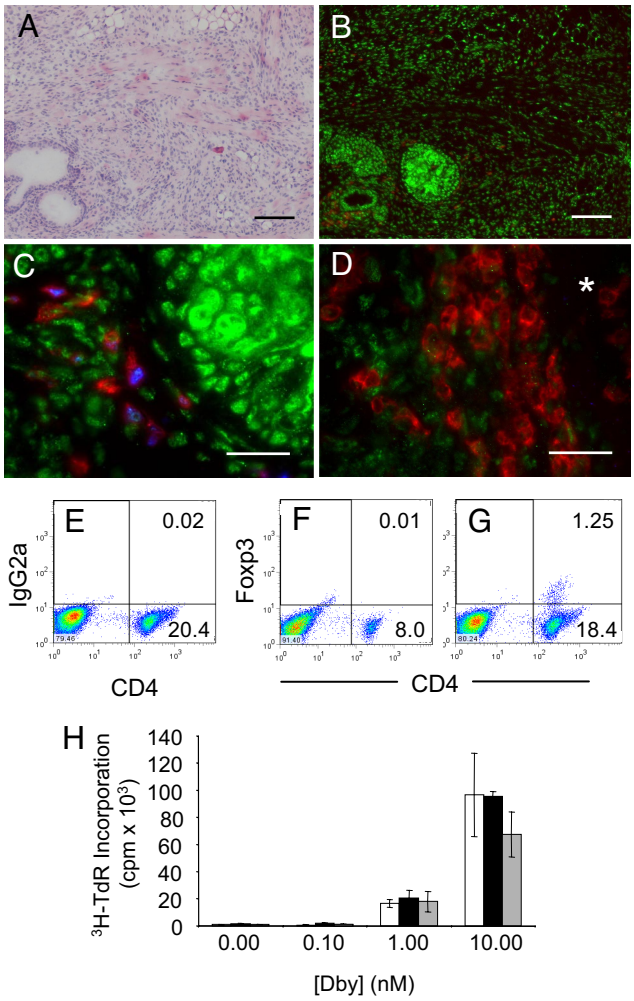

Fig. 5. Tissues derived from ES cells provide a microenvironment conducive to the induction of CD4$^+$Foxp3$^+$ Treg cells. (*A–D*) Serial sections of teratomas derived from the male ES cell line ESF116 after implantation under the kidney capsule of female A1.RAG1$^{-/-}$ mice (*A–C*) or female A1.RAG1$^{-/-}$ × dnTGFβRII.RAG1$^{-/-}$ mice (*D*). Sections are stained with hematoxylin and eosin (*A*), CD4 (red) versus phospho-smad 2/3 (green) (*B–D*), and with the distribution of Foxp3 overlaid in blue (*C* and *D*), showing that expression of Foxp3 is restricted to T cells capable of responding to TGF-β_2 within the local microenvironment. The asterisk identifies the recipient kidney. (Scale bars: 100 μm in *A* and *B* and 25 μm in *C* and *D*). (*E–G*) Flow cytometric analysis of cells purified from female (*F*) and male (*G*) EB stained for CD4 and Foxp3, compared with isotype controls (*E*). (*H*) Dose-dependent proliferative responses of splenic T cells from naïve mice (white bars) or mice receiving female (black bars) or male EB (gray bars) stimulated with Dby peptide.

antigen recognition was also required, we extracted infiltrating CD4$^+$ cells from male EB spontaneously accepted by A1.RAG1$^{-/-}$ mice. Flow-cytometric analysis revealed that ≈6% of T cells were Foxp3$^+$, the equivalent cells purified from female grafts showing no evidence of Foxp3 expression (Fig. 5 *E–G*). These results provide unequivocal evidence that the recognition of specific antigen in the presence of TGF-β_2 is responsible for the polarization of alloreactive T cells toward a regulatory phenotype. Interestingly, Foxp3$^+$ cells appeared to accumulate within accepted male EB, as described for male skin grafts (27), but were far less prevalent within the lymphoid tissues, consistent with local rather than systemic tolerance. This was supported by proliferation assays in which the response to Dby (479–493) of T cells from the spleens of mice receiving male EB was not significantly different from those of control T cells from naïve mice or the recipients of female EB (Fig. 5*H*). These findings provide a physiological basis for the immune privilege widely reported among ES cells and their differentiated progeny and suggest that CD4 and CD8 mAb, known to promote regulation *in vivo* (26), may harness this natural immune privilege to secure long-term survival of composite tissues, even across a full MHC barrier.

Discussion

Prospects for the use of ES cells for the treatment of chronic and degenerative diseases in man have recently received support from estimates of the number of notional lines that would be required to make CRT accessible to a significant proportion of the population (7). The suggestion that much of the benefit of tissue typing could be realized from as few as 150 clinically approved lines, was, however, based on matching of selected MHC loci. Indeed, "beneficial" matches were considered to share HLA-A, -B and -DR alleles alone, on the assumption that residual mismatches between donor and recipient might be mitigated by standard immune suppression. Given the ethical constraints surrounding the protracted use of immune suppression for chronic disease states amenable to CRT, the level of matching required may, in reality, prove rather greater than anticipated. Conversely, the immune privileged status enjoyed by ES cells and their differentiated progeny (9, 10) may help to temper aggressive alloreactivity, permitting some level of histoincompatibility to be accommodated. The uniqueness of this context therefore suggests the need for a systematic study to be performed to define more rigorously the degree of disparity that may be tolerated after CRT.

Our own studies add weight to the notion of immune privilege by showing that differentiation of mouse ES cells is associated with the up-regulation of TGF-β_2 (Fig. 4*F*), whose antiinflammatory properties have been strongly implicated in acquired immune privilege encountered in the anterior chamber of the eye (23). We show here that male EB, grafted under the kidney capsule of female A1.RAG1$^{-/-}$ mice, survive indefinitely, despite the prevalence of T cells specific for the male mH antigen and their evident reactivity to Dby *in vitro* (Fig. 5*H*). Although the resulting teratomas show a significant T cell infiltrate, the integrity of the tissues appears unaffected. Critically, ≈6% of infiltrating T cells adopt a regulatory phenotype, typified by up-regulation of the Foxp3 transcription factor (Fig. 5 *E–G*). The conclusion that polarization toward this phenotype depends on TGF-β_2 within the local microenvironment is supported by the observation that smad2 is actively phosphorylated and translocated to the nucleus of T cells expressing Foxp3 (Fig. 5*C*). Furthermore, A1.RAG1$^{-/-}$ mice, engineered to express a dominant-negative form of the TGF-β receptor within the T cell compartment, fail to show any Foxp3 expression (Fig. 5*D*). Although these results confirm the direct effect of TGF-β_2 on infiltrating T cells, various studies have also demonstrated how DC, cultured in TGF-β_2, are able to polarize naïve T cells toward regulatory function, thereby antagonizing adaptive immune responses (28, 29). It is conceivable, therefore, that recipient DC, infiltrating the graft, are actively influenced by the local microenvironment, allowing them to present alloantigen, acquired *in situ*, in a tolerogenic manner. Paradoxically, therefore, although composite tissues may have been anticipated to represent a greater immunological challenge than the purified cell types used routinely in animal models of disease, our results suggest that they may still be readily accepted by virtue of their capacity to create an immunologically privileged environment, conducive to the establishment of a repertoire of Treg cells.

To investigate the extent of genetic disparity between donor and recipient that might be accommodated by this inherent tendency for immune privilege, we made use of ES cell lines differing from CBA/Ca recipients at defined genetic loci (Table

1). Importantly, in the absence of immune intervention, even tissues that differed solely at multiple mH loci were vigorously rejected (Table 2), strongly implying that neither the low levels of MHC expression evident among ES cell-derived tissues (Fig. 3 *F* and *G*) nor the secretion of TGF-β_2 is sufficient to secure their immunological acceptance. Nevertheless, a short course of treatment with mAb specific for CD4 and/or CD8 permitted the indefinite survival of all donor tissues, even those differentiated from fully allogeneic ES cells, which closely mimics the likely clinical scenario encountered during CRT in humans. The comparative ease with which transplantation tolerance may be established in this model, may be due partly to the capacity for CD4 and CD8 mAb to solicit regulation (26), thereby synergizing with the same tendency of the transplanted tissues to induce Treg cells through the privileged microenvironment they create. Furthermore, the evident lack of endogenous DC, capable of presenting alloantigen directly to the recipient T cell repertoire (Fig. 3), may remove an acute stimulus for rejection, providing a critical window of opportunity during which Treg cells may be deployed.

In summary, our results from a mouse model of CRT, strongly suggest that the success of matching tissues between donor and recipient by establishing a bank of human ES cells is likely to critically depend on additional strategies for avoiding rejection. Given the attendant risks associated with the protracted use of immune suppression, the evident susceptibility of ES cell-derived tissues to the induction of transplantation tolerance may offer an attractive alternative.

Materials and Methods

ES Cell Derivation. All mice were bred and maintained at the University of Oxford, and experimental work was carried out under Home Office regulations with local ethical committee approval. ES cell lines were derived from delayed-implanting blastocysts as described (14). EB were generated for transplantation as detailed in *SI Methods*.

Transplantation. Skin grafting was performed as described (30). Transplantation of EB was carried out as detailed (31). Briefly, two to four EB were grafted under the kidney capsule, and groups of mice were killed for analysis at specified time points. Acceptance of the grafted tissues was defined as an increase in diameter to >5 mm, vascularization, and the lack of leukocyte infiltrate and tissue damage, revealed by histology.

Immunohistochemistry. Explanted tissues were embedded and frozen in OCT compound (Sakura Finetek). Immunohistochemistry and immunofluorescence staining was performed on sections 6-μm thick, as detailed in *SI Methods*.

Tolerance Induction. Nondepleting mAb specific for CD4 (clone YTS 177.9.6.1) and CD8 (clone YTS 105.18.10) were administered i.p. (1 mg) on days 0, 2, and 4 after transplantation.

Flow Cytometry. ES cells and EB were harvested by using an enzyme-free dissociation buffer (GIBCO), and the latter were passed through a 21-gauge needle. DC were generated from bone marrow as described (32) and, where indicated, matured with 1 μg/ml of lipopolysaccharide from *Escherichia coli* serotype 0127:B8 for the final 18 h of culture. Graft-infiltrating leukocytes were prepared as detailed in *SI Methods*. Samples were stained for 30 min at 4°C with 7-AAD (Sigma) to exclude dead cells, the indicated mAb (BD Biosciences), or isotype-matched control mAb (Serotec). The MHC II mAb (clone OX6) was prepared in-house. Foxp3-specific mAb (eBiosciences) was used according to manufacturer's instructions.

Quantitative PCR. Expression of the indicated genes was determined by quantitative PCR (qPCR) by using the Taqman low-density array (Applied Biosystems) according to the manufacture's instructions and Taqman validated gene expression assays, as detailed in *SI Methods*.

T Cell Proliferation Assay. To assess antigen-specific T cell responsiveness, splenocytes from grafted or naïve A1.RAG1$^{-/-}$ mice were cocultured with immature bmDC and Dby peptide, as detailed in *SI Methods*.

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