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Central tolerance promoted by cell chimerism

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Historically, successful allotransplantation was only achieved by utilizing powerful immunosuppressive drugs that were exposing the patient to severe opportunistic infections. The thymus of the transplant recipient renders such therapy obligatory as it constitutively blocks self-reactive T cells while allowing alloreactive T cells to mature and populate the periphery. In 1992, a follow-up study revealed the presence of donor leukocytes in long-term transplant survivors. The stable persistence of recipient and donor leukocytes in the transplanted patient, referred to as "chimerism", was considered the reason why in some cases it was even possible to stop immunosuppressive treatment without damaging the transplanted organ. Unfortunately, it quickly became evident that stable, persistent allogeneic chimerism was not easily achievable by design. Recently, a novel approach has been identified to help address this clinical gap in knowledge: Cotransplantation of a donor graft with a thymic organoid populated with donor precursor cells generates stable, long-term chimerism in the recipient. In humanized mice, the implantation of thymic organoids, populated with human donor inducible pluripotent stem cell (iPSC)-derived thymic epithelial cells (TECs) and the same donor CD34+ bone marrow precursors, induces tolerance to human leukocyte antigen (HLA)-matched donor tissues/organs. This technology will allow successful allotransplantation of cells/organs even between Major Histocompatibility Complex (MHC)-noncompatible individuals and allow getting rid of immunosuppressive treatments reducing recipient morbidity.

tolerance | chimerism | thymic organoids

Five years ago, Thomas Starzl, MD died at the age of 91.

The thymus gland is the primary organ responsible for the generation of T cells necessary to effectively promote the adaptive immune response against invading pathogens, while also maintaining self-tolerance (1). Prior to maturation and migration to secondary lymphoid organs, developing T cells that originate in the bone marrow (BM) must undergo a rigorous selection process in the thymus to ensure their successful engagement with antigen (Ag), presented by Major Histocompatibility Complex (MHC) molecules at the surface of Ag-presenting cells (APCs) during a process known as positive selection. Additionally, those nascent T cells with high reactivity to the self-Ags are eliminated during the negative selection process (2). Thymic epithelial cells (TECs), the primary population of thymic stromal cells (TSCs), play critical roles in these "educational" processes (3). Recent studies have shown that TECs in the thymic medulla express almost the entire transcriptome of the body, that is, all the Ags that promote the establishment of immunological self-identity, thereby avoiding dangerous autoimmune reactions. Evidence that self-Ag expression in TECs helps define the self/nonself paradigm of the immune system is demonstrated by an autoimmune disease like type 1 diabetes being caused by the absence or the suboptimal presentation of certain self-Ags (e.g., insulin) in the thymus (4, 5).

The transplant of any cell/organ from one individual to another within the same species (allotransplant) will be rejected by the "effector" T cells of the recipient. These cells survive the critical negative selection process by displaying T cell receptors (TCRs) that are unable to recognize "self" Ags presented by recipient MHC molecules and thus are ready to attack foreign Ags. Here, "foreign" can be considered any peptide presented by cells carrying MHC molecules different from the recipient's ones. To protect the graft from this strong recipient immune response, an equally strong clinical intervention is needed to promote the success of the transplants.

Although matching donor and recipient MHC gene alleles as closely as possible is always the goal within allogeneic transplantation, donor pools do not always offer ideal organs for critically ill patients. Therefore, further intervention was needed to combat recipient effector T cell response. Powerful new drugs to cause immunosuppression were introduced as part of posttransplant maintenance therapy (6). This annihilation of recipient T cells, while permitting the survival of the allogeneic graft, results in the generalized weakening of the recipient immune system which, in turn, exposes the patient to severe opportunistic infections unrelated to the presence of the protected graft. In addition, these immunosuppressive agents were soon recognized to have severe side effects including an increased risk of death associated with cardiovascular complications and kidney disease. Furthermore, these powerful drugs were also found to be toxic to certain cells, such as the insulin-producing beta cells present in the pancreatic islets of Langerhans, limiting the utility of their transplants (7).

In 1992, a 2 to 22 y posttransplant follow-up study of 25 liver transplant patients, managed with azathioprine- or

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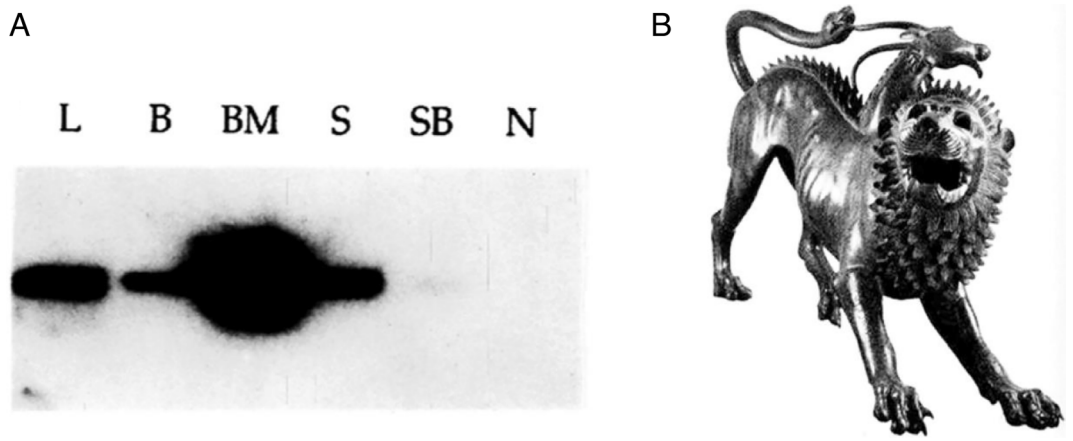

Fig. 1. Artistic representation of a chimera from Greek mythology and evidence of an immunologic chimera in a patient with Gaucher disease. In (*A*), genomic DNA was extracted from the recipient's tissues and amplified with HLA-DR beta-chain "generic" oligonucleotide primers to determine the subgroup of the donor's alleles. "Specific" primers were then used to amplify the alleles selectively. The alleles were identified by hybridizing the amplified DNA to radiolabeled allele specific probes. HLA-DR1 was only present in the donor. After HLADR1- specific amplification of DNA from the liver (L), blood (B), bone marrow (BM), skin (S), and small bowel (SB) of the recipient, the DNA was separated by electrophoresis on an agarose gel and then analyzed by Southern blotting. The denatured DNA present on the nylon membrane was hybridized to a labeled HLA-DR1 (donor)-specific oligonucleotide probe. For liver DNA, the quantity analyzed was reduced to 1% of the other samples. Although lower in intensity, the signal from donor DNA in the small bowel was clearly positive in the original film, although this can be seen here only faintly. The negative control was a reaction run without DNA (last lane; N = negative control). In (*B*), Khimaira, also spelled in the Latin "Chimera," was a powerful beast with the head of a male lion, the body of a goat, and, as a tail, a serpent. The legend states that she was slain by Bellerophon with a golden arrow, while he rode on the back of Pegasus, a horse with wings. The Chimera of Arezzo is conserved at the Archaeologic Museum in Florence, Italy. [Adapted From T. E. Starzl et al. (9)]

cyclosporine-based immunosuppression, revealed long-term survival of donor leukocytes (8, 9). Using cytochemical and PCR-based techniques (10), living immunocompetent cells from the donor, recognized by their respective MHC gene alleles nonmatching those of the recipient, were found to be present in all 25 patients studied, in locations that included skin, lymph nodes, heart, lungs, spleen, intestine, kidneys, BM, and thymus (Fig. 1*A*) (9). Donor leukocytes were also found in long-term surviving kidney transplant recipients, although, at any given site, they were decreased as compared to donor cells found in the liver transplant recipients (11). The heavier load of potential migratory leukocytes within transplanted livers was postulated to be the basis for the "hepatic tolerogenicity" that allows the liver to induce its own acceptance more readily than other transplanted organs. This was also postulated to be the basis by which donors' livers shield concomitantly transplanted organs from rejection and even resist the attack of preformed antibodies (12).

This protracted simultaneous presence of recipient and donor immunocompetent cells in the transplanted recipients was then called "cellular chimerism" (Fig. 1*B*) (13). The ability to reduce or sometimes even completely stop the maintenance immunosuppressive treatment in some patients, without damaging the transplanted graft, was thereafter attributed to the presence of chimerism or even "microchimerism" when only a few donor cells were found still present in the recipient. Something, facilitated by a strong immunosuppressive regimen, appeared to have changed in the graft, the recipient, or both. Cellular chimerism appeared to be at least an invariable finding, if not an essential condition for successful long-term graft survival once the immunosuppressive treatment was interrupted.

Yet, the questions of whether, how, and when tolerance took place and the immunological process(es) of cell-to-cell interaction involved in this induction of "transplant tolerance" remained unknown. Even if it was postulated that the leukocytes present in the transplanted organ had not reached terminal differentiation as previously assumed and were capable of immediate migration and cell division, subsequent survival and renewal of these cells should depend on chronic stimulation of both donor and recipient cell populations. This would result in some form of reciprocal regulation that would facilitate a final "acceptance" of each other. Cellular interactions in both directions of host versus graft (HVG) and graft versus host (GVH) were hypothesized to occur, resulting in "mutual natural immunosuppression". This hypothesis seemed to be supported by the data showing that the load of migratory immune cells in the liver accounted for the immunological advantages of the hepatic graft, with the understanding that organs with a smaller leukocyte component such as the kidney and heart must have similar inherent, although less tolerogenic, potential.

This consideration suggested a possible strategy of intravenously infusing donor BM, donor blood, or other haemato-lymphoid cells at the same time of, or shortly after, whole organ transplantation to provide an artificial augmentation of the normal posttransplant cell migration from the graft into the recipient periphery (14). Although this therapeutic intervention did not cause any appreciable harm to the recipient, the benefits associated with the procedure were not easy to define in terms of graft survival. Unfortunately, even with additional infusion of donor BM cells and the best available immunosuppression, it became evident that systemic allogeneic chimerism was not always achievable by design. It was, in fact, impossible to determine the conditions necessary to avoid a donor/recipient cell imbalance to prevent allograft rejection. Cell quality, cell quantity, time of administration, and prolongation of chimeric status were all considered variables that supported the "feeling" of improved results but did not offer any unquestionable and tangible parameter to quantify this sense of improvement (15). Although the proposed hypothesis of "mutual natural

immunosuppression" was quite suggestive, little support was found within the general understanding of the physiologic activity of the immune system (16), even invoking the possible activity of "facilitating" and/or "vetoing" cells (17, 18). Nevertheless, the presence of immunologic chimerism certainly continued to be considered the conditio sine qua non for protracted graft survival.

Currently, the most utilized immunosuppressive approaches are aimed at the elimination or suppression of the donor-reactive T cells themselves. The immunosuppression cannot be interrupted as long as the recipient thymus continues to allow alloreactive T cells to mature and eventually populate the periphery. Thus, to better promote the establishment of a graft-supportive chimerism, we proposed to not focus on the peripheral T cell but rather on the enclave where the alloreactive T cells mature: the thymus.

Using the thymus as a mechanism to better understand central immune tolerance is a concept that has been studied by numerous groups all trying to move the field of organ transplantation forward. Methods like inducing donor-specific unresponsiveness through intrathymic transplantation of islets of Langerhans (19), introducing allogeneic Ags into the thymus via alloantigen-expressing viral vectors (20, 21), or transplanting B6 mouse thymus fragments into the lymph nodes of Balb/C nude mice to support thymopoiesis and induce immune tolerance of B6 mouse grafts (22) have all been tested. However, a majority of these approaches predominantly affect APCs, especially thymic dendritic cells. This is potentially problematic given that APCs like thymic dendritic cells are replenished by fresh recipient cells migrating into the thymus just a few weeks posttransplant, a fast enough turnover to compromise the long-term efficacy of these treatments. Unlike these thymic APCs, the TECs of the thymic stroma, which are so critical to establishing central tolerance, rely heavily on self-renewal to maintain homeostatic conditions. However, due to its spatial and architectural needs, the TEC population is uniquely challenging to culture in vitro, making attempts to induce central tolerance that much more difficult.

Unlike the usual 2-D, polystyrene in vitro setting that most cells culture within, TECs depend on a properly configured 3-D extracellular matrix (ECM) for their maturation, survival, and proliferation. Previous studies have demonstrated that thymus fragments that were cultured in 3-D scaffolds, which were constructed with biocompatible inorganic materials, can support the development of CD34+ hematopoietic progenitor cells into CD3+ T cells. It was also seen that TECs injected into thymus scaffolds could preserve their characteristic molecular properties for up to 8 wk in an in vitro setting. This finding verified the importance of not only the 3-D architecture necessary for TEC survival but also the need for an intact ECM to support TEC development, both of which can be found in a decellularized thymus free of all its own cells. It was also found that BM progenitors coinjected with TECs into the decellularized thymus scaffold can differentiate into CD4+CD8+ double-positive (DP) thymocytes as well as CD4+CD8− or CD4−CD8+ single-positive (SP) thymocytes. This suggests that it may be possible, with further optimization, to use the decellularized thymus scaffolds as suitable in vitro culture microenvironments to study not only T cell development but also TEC biology as well (23).

One other advantage to using the 3-D scaffold system is that it can easily be utilized in in vivo studies as well. Transplanting 3-D scaffolds composed of cocultures of BM progenitors and TECs into mice offers a number of different avenues to study thymus functionality and all that falls within the mechanisms of action of the thymus. When transplanted into athymic nude mice, 3-D thymus scaffolds promoted the homing of lymphocyte progenitors and supported thymopoiesis. Next-generation-sequencing-spectra analysis for investigating TCR β-chain amplicons revealed a broad spectra of Vβ gene families, indicating that the newly formed T cell repertoire, trained on the TECs within the transplanted thymus scaffold, was highly diversified. More importantly, tolerance to entirely MHC-mismatched skin allografts was achieved through transplanting thymus scaffolds constructed with TECs either coexpressing syngeneic and allogeneic MHCs or by injecting mixtures of TECs isolated from both an MHC-discordant donor and the recipient mouse into the 3-D scaffold, generating a "hybrid" thymus (Fig. 2) (23).

However, it is well known that treatment approaches that work perfectly in the mouse rarely give the same results in humans (24). Thus, to test the ability of the murine tolerance protocol to provide similar results once human cells are utilized, human embryonic stem cells (hESCs) were subjected to a well-tested differentiation protocol (25, 26). Also in our hands, human thymic epithelial progenitor cells (hTEPCs) were successfully differentiated, as proven by the expression of genes specific to TEC development (e.g., *FOXN1* and *EYA1*), epithelial lineage (e.g., *KRT18*), as well as TEC function (e.g., *PRSS16*, *AIRE,* and *ACKR4*) when examined with RT-qPCR. The functionality of the tissue-engineered thymus organoids in their ability to support T-lymphopoiesis was also examined. Cells were isolated from in vitro cultured thymus organoids, stained with antibodies specific to developing thymocytes, and characterized with flow cytometry. Thymus organoids were not only able to support the survival of the injected hematopoietic lineage cells (derived from the CD34+ HSCs) but also able to promote differentiation of new thymocytes.

In the clinical setting, donors generally are not embryos and adult thymi could have already undergone physiologic senescence, thus reducing the number of possible available TEPCs. To address these challenges, hTEPCs were generated from human inducible pluripotent stem cells (iPSCs) (27, 28). The culturing method for hTEPC generation was based on the encapsulation of iPSCs in alginate spheres, to create a 3-D microenvironment to support stem cell differentiation (Fig. 3) (29). Once decapsulated, the cells were tested for the expression of TEC markers. These hTEPCs expressed genes specific to TEC development (e.g., *HOXA3* and *EYA1*), epithelial lineage (e.g., *KRT8* and *KRT18*), as well as TEC function (e.g., *PRSS16*, *CD74,* and *ACKR4*) when examined with RT-qPCR and single-cell mRNA sequencing. Additionally, they showed expression of specific mTEC (*AIRE* and *CSN2*) and cTEC (*CD205*) lineage markers, indicating the necessary heterogeneity needed to mimic the cellularly complex and spatially regulated thymus gland. At the protein level, surface expression of EpCAM, a widely used marker for defining the TEC population in the thymus, was detected in 70 to 90% of hTEPCs, whereas CD45+, the marker for hematopoietic lineage cells, was largely absent (30).

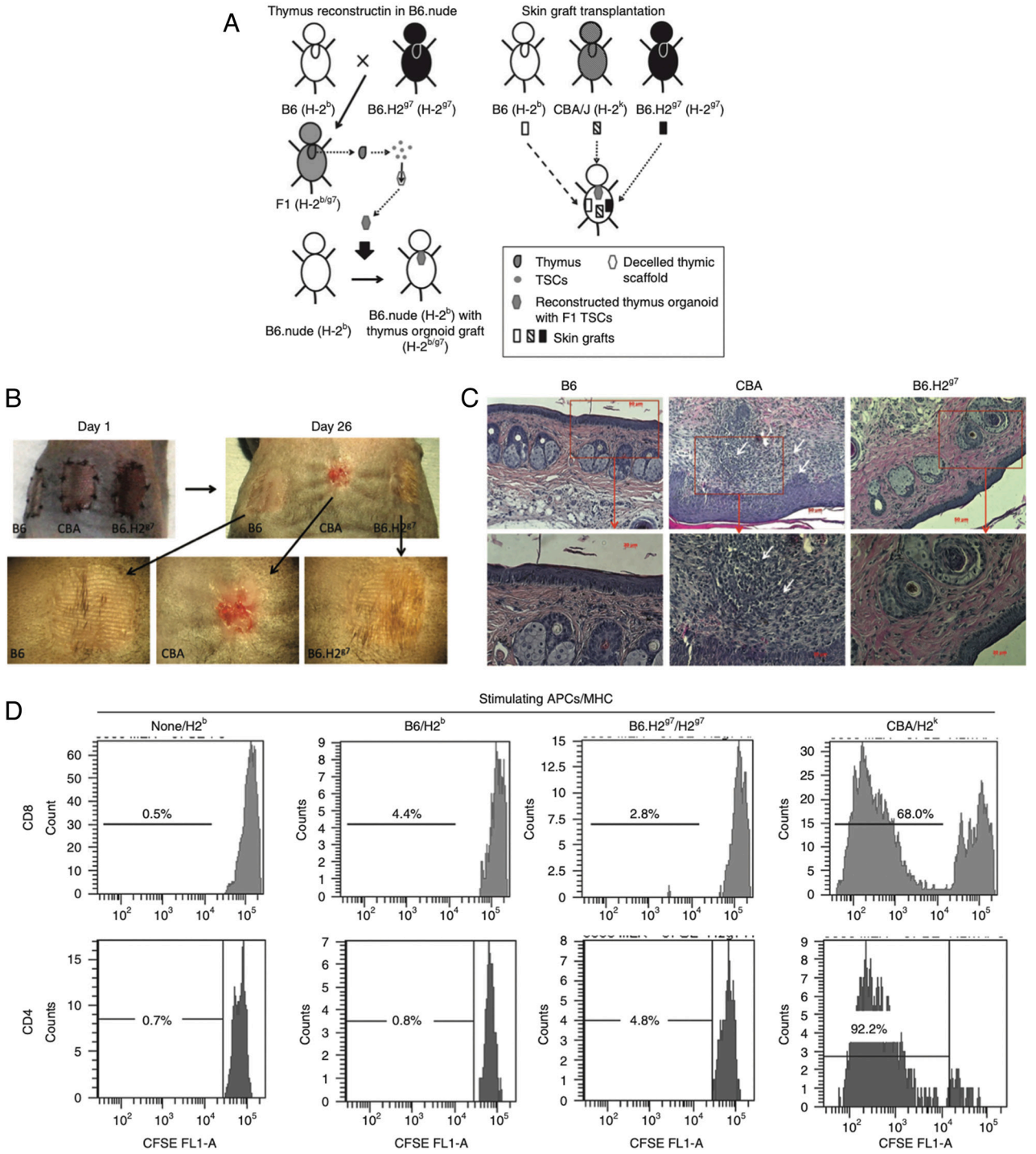

Fig. 2. Using decellularized 3-D thymus scaffolds to establish donor-specific immune tolerance in mice. (*A*) Schematic mapping of donor and recipient animals and their genetic backgrounds to investigate the induction of immune tolerance skin allografts after 3-D thymus scaffold transplantation. Thymus scaffolds were constructed with thymic cells harvested from B6 (H-2b) and congenic B6.H2g7 (H-2g7) mice and transplanted to athymic B6.nude mice to generate Tot. B6.nude recipients. At 12 to 16 wk postthymus transplantation, skin grafts harvested from wild-type (WT), B6 (syngeneic), congenic B6.H2g7 (allogeneic), and CBA/J (H-2k, third party allogeneic) mice were transplanted to the recipients, and both graft tolerance and animal survival were monitored. (*B*) Representative images of syngeneic (B6) and allogeneic (CBA and B6.H2g7) skin grafts of Tot.B6.nude recipients (n = 4) at early and late timepoints. *Lower* panels show images of the grafts at higher magnification at day 26. (*C*) Representative histological images (H&E) of syngeneic (B6), allogeneic (B6. H2g7), and third-party allogeneic (CBA) skin grafts harvested from Tot.B6.nude recipients (n = 4) at 26 d post skin transplant. Red boxes denote areas of higher magnification shown in the *Lower* panels. White arrows show areas with lymphocytic infiltration. (*D*) T cells enriched from Tot.B6.nude (n = 3) were labeled with carboxyfluorescein diacetate succinimidyl ester (CFSE) and cocultured with T cell depleted syngeneic (B6), allogeneic (B6.H2g7), and third-party allogeneic (CBA) APCs. Representative flow cytometry images are presented with both CD8+ (*Top*) and CD4+ (*Bottom*) lineages. [From: Y. Fan et al. (23)].

To further promote the maturation of the "new" hTEPCs into functional TECs, hTEPCs were mixed with human CD34+ hematopoietic stem cells (HSC) isolated from umbilical cord blood, which is notoriously rich in precursor CD34+ cells. This cell mixture was injected into decellularized thymus scaffolds and cultured in the top chamber of a 12-well transwell plate in vitro. Expression of genes specific to maturation and function of TECs was examined with RT-qPCR. Significant

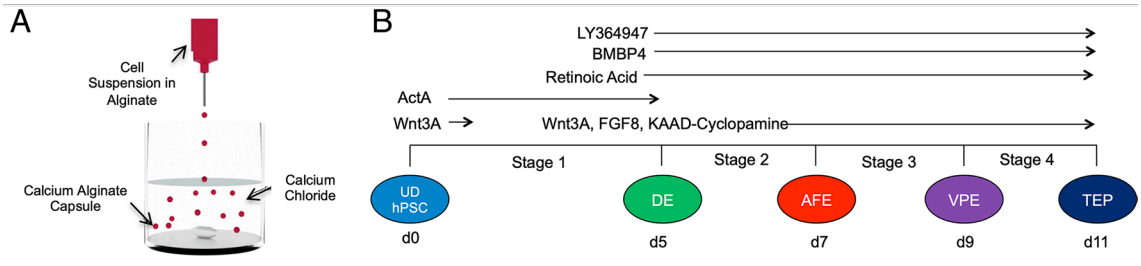

Fig. 3. Schematics of hESC/iPSC alginate encapsulation for hTEPC differentiation (*Left*) and of the directed differentiation protocol to thymic epithelial progenitors under alginate encapsulation (*Right*). [Adapted from: A. Zeleniak et al. (30)].

increases in expression of *MHC Class II* and *CD74*, both critical for the self-Ag presentation function of the TECs, were observed after extensive in vitro culture. The functionality of the tissue-engineered thymus organoids in their ability to support T-lymphopoiesis was also examined. Cells were isolated from in vitro cultured thymus organoids, stained with antibodies specific to developing thymocytes, and characterized with flow cytometry. Thymus organoids were not only able to support the survival of the injected hematopoietic lineage cells (derived from the CD34+ HSCs) but also able to promote differentiation of new thymocytes.

The function of the tissue-engineered thymus organoids was also demonstrated in vivo, when human thymus organoids were transplanted under the kidney capsules of *NOD.scid.IL2rg*^{null} (NSG) immunosuppressed mice. Our model, known as the hu.Thor humanized mouse model (for mice humanized by thymus organoid transplantation), was able to reject teratomas derived from the allogeneic CC1 iPS cell line while those derived from syngeneic Y1 iPS cells showed robust growth, reflecting both an established functional T cell repertoire and an evident tolerance toward syngeneic tissues (30).

One aspect still needing investigation within this model is related to the fate of the memory T cells (Tmem). These Tmem cells are in theory already present in the recipient before the transplant of the thymic organoid is performed. Clinically, these Tmem cells have been shown to be resistant to the conventional immunosuppressive treatment routinely performed before any transplant. Thus, their presence may challenge the efficiency of the establishment of peripheral tolerance in the recipient. In terms of showing resistance to the de novo generated Tmems in our hu.Thor mice, since we didn't observe any GVH or HVG once the initial immunosuppression was withheld, we hypothesized that if any Tmem leaves the BM (or any of the lymph nodes in which it resides), the activity of the T regulatory cells, which we demonstrated can be generated by the thymic organoids (30), may protect against the possible Tmems' mediated aggression against the graft by controlling or even eliminating them. While our hypothesis is focused on the Treg compartment, we cannot exclude that this peripheral tolerance could be accomplished via other peripheral regulatory pathways that include myeloid-derived suppressor cells (MDSCs), regulatory dendritic cells (DCregs), and regulatory B cells (Bregs), but more detailed research will be needed to elucidate these mechanisms.

Ideally, in a not-too-distant future, if this protocol is tested in human transplant trials, organoids can be generated by making iPSC-derived TECs dedifferentiated from skin fibroblasts of any available donor providing transplant tissue. Combining those iPSC-derived TECs with CD34+ HSCs isolated from the same donor BM, these cells can be injected into the decellularized thymic scaffold, perhaps isolated from an animal donor or artificially generated with a 3-D printer. This newly generated patient-specific organoid will be transplanted into the recipient, ready to promote tolerance of the matching graft that was transplanted into the patient. At this point in time, the initial immunosuppressive regimen, necessary to protect the graft during the time needed to generate the TECs from the donor iPSCs, can be tapered. In time, the patients not only will develop and maintain an effective immune response offered by educated T cells ready to react against foreign Ags but will also generate a tolerogenic immune chimerism, permitting the preservation of both recipient and donor cells/tissues without the need for lengthy immunosuppression.

The potential of these thymic organoids, once implementable in humans, is evident in the realm of allogeneic tissue and organ transplantation since there will not be any need for a protracted conventional immunosuppressive treatment. Furthermore, the successful transplantation of organs between MHC-noncompatible individuals would become possible, thus extending this treatment to a much larger population of patients in need. These results confirm what Thomas Starzl's said concluding a famous talk in 1982:" History tells us that procedures that were inconceivable yesterday, and are barely achievable today, often become routine tomorrow!"

Data, Materials, and Software Availability. Any original research work quoted or referred to herein, where the data were obtained by the laboratories of the authors is peer-reviewed and published (see references). Data have been deposited in (Nature Methods) (https://doi.org/10.1038/s41592-022-01583-3). Previously published data were used for this work [T. E. Starzl et al. (9), Y. Fan et al. (23), and A. Zeleniak et al. (30)].

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