Video Article

3D Organotypic Co-culture Model Supporting Medullary Thymic Epithelial Cell Proliferation, Differentiation and Promiscuous Gene Expression

Sheena Pinto*[1], Hans-Jürgen Stark*[2], Iris Martin[2], Petra Boukamp[2], Bruno Kyewski[1]

[1]Division of Developmental Immunology, German Cancer Research Center (DKFZ)

[2]Genetics of Skin Carcinogenesis, German Cancer Research Center (DKFZ)

*These authors contributed equally

Correspondence to: Sheena Pinto at s.pinto@dkfz.de, Hans-Jürgen Stark at hj.stark@dkfz-heidelberg.de

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Abstract

Intra-thymic T cell development requires an intricate three-dimensional meshwork composed of various stromal cells, *i.e.,* non-T cells. Thymocytes traverse this scaffold in a highly coordinated temporal and spatial order while sequentially passing obligatory check points, *i.e.,* T cell lineage commitment, followed by T cell receptor repertoire generation and selection prior to their export into the periphery. The two major resident cell types forming this scaffold are cortical (cTECs) and medullary thymic epithelial cells (mTECs). A key feature of mTECs is the so-called promiscuous expression of numerous tissue-restricted antigens. These tissue-restricted antigens are presented to immature thymocytes directly or indirectly by mTECs or thymic dendritic cells, respectively resulting in self-tolerance.

Suitable *in vitro* models emulating the developmental pathways and functions of cTECs and mTECs are currently lacking. This lack of adequate experimental models has for instance hampered the analysis of promiscuous gene expression, which is still poorly understood at the cellular and molecular level. We adapted a 3D organotypic co-culture model to culture *ex vivo* isolated mTECs. This model was originally devised to cultivate keratinocytes in such a way as to generate a skin equivalent *in vitro*. The 3D model preserved key functional features of mTEC biology: (i) proliferation and terminal differentiation of $CD80^{lo}$, Aire-negative into $CD80^{hi}$, Aire-positive mTECs, (ii) responsiveness to RANKL, and (iii) sustained expression of FoxN1, Aire and tissue-restricted genes in $CD80^{hi}$ mTECs.

Video Link

The video component of this article can be found at https://www.jove.com/video/52614/

Introduction

Developing thymocytes make up about 98 % of the thymus, while the remaining 2 % consists of a variety of cells that collectively compose the thymic stroma (*i.e.,* epithelial cells, dendritic cells, macrophages, B cells, fibroblasts, endothelial cells). The outer cortical epithelial cells (cTECs) procure immigration of pro-T cells from the bone marrow, T cell lineage induction in multipotent pre-T cells and positive selection of self-MHC restricted immature thymocytes. The inner medullary thymic epithelial cells (mTECs) are involved in tolerance induction of those thymocytes with a high-affinity TCR for self-peptide/MHC complexes by either inducing negative selection or their deviation into the T regulatory cell lineage. In the context of central tolerance induction, mTECs are unique in that they express a wide spectrum of tissue-restricted self-antigens (TRAs) thus mirroring the peripheral self. This phenomenon is called promiscuous gene expression (pGE)[1,2].

Most current studies on this fascinating cell type rely on *ex vivo* isolated cells, as various short-term 2D culture systems invariably resulted in the loss of pGE and key regulator molecules like MHC class II, FoxN1 and Aire within the first 2 days[3-6]. It remained however unclear, which particular components and features of the intact 3D meshwork of the thymus were missing in 2D models. The re-aggregation thymic organ culture (RTOC) has been so far the only 3D system that allows the study of T cell development, on the one hand, and stromal cell biology, on the other hand, in an intact thymic microenvironment[7]. Yet, RTOCs have certain limitations, *i.e.,* they already contain a complex mixture of cells, require the input of fetal stromal cells and endure a maximal culture period of 5 to 10 days.

The lack of reductionist *in vitro* culture systems has hampered the study of several aspects of T cell development and thymic organogenesis not least the molecular regulation of pGE and its relationship to the developmental biology of mTECs.

Owing to the close-relatedness of the structured organization of the epithelial cells of skin and thymus, we opted for a 3D organotypic culture (OTC) system that had been developed originally to emulate the differentiation of keratinocytes *in vitro* and thus create a dermal equivalent. The OTC system consists of an inert scaffold matrix overlaid with dermal fibroblasts that are trapped in a fibrin gel, onto which keratinocytes

are seeded[8,9]. Here, we replaced keratinocytes with purified mTECs. While keeping the basic features of this model, we optimized certain parameters.

In the adopted OTC model mTECs proliferated, underwent terminal differentiation and maintained mTEC identity and pGE, thus closely mimicking *in vivo* mTECs development[10]. This technical note provides a detailed protocol allowing the stepwise set-up of thymus OTCs.

Protocol

This study has been approved by the ethics committee of the Regierungspräsidium Karlsruhe. All animals were housed under specific pathogen-free conditions at the German Cancer Research Center (DKFZ). For all culture experiments mouse pups ranging from 1 to 7 days of age were used.

1. Isolation of mTECs from Thymus

NOTE: The following digestion steps were performed as described previously[1] under sterile conditions with some modifications as follows.

1. Decapitate the mouse pups and remove the thymus. Place the thymi on ice in a Petri plate containing RPMI 1640 medium (containing 5% FCS).
2. Cut the thymi into fine, small pieces and place in a round bottom tube with ~5-30 ml RPMI media and gently stir using a magnet for 10 min at RT.
3. Thereafter, decant the supernatant containing mainly thymocytes and digest the remaining tissue sequentially with one round of collagenase type IV (0.2 mg/ml and 57 U/ml final concentartion) for 15 min each at 37 °C, followed by collagenase/dispase (0.2 mg/ml and 1.2 U/ml final concentration) for 25 min each at 37 °C in a water bath with magnetic stirring until the thymi are completely digested. Use 1 ml enzyme per two to three thymi.
4. Agitate the tissue once every 7-10 min with a Pasteur pipette. Pool the collagenase/dispase fractions and filter through a 70 µm gauze.
5. Enrich the mTECs by magnetic cell sorting (MACS). Perform the purification of mTECs by magnetic cell sorting as described previously[11], and shown in **Figure 1**.
   NOTE: For magnetic sorting of mTECs we used the following antibodies: anti-CD80-PE (16-10A1, use at 1:100 dilution) and anti-EpCAM-bio (G8.8, use at 1:100 dilution)[12]. Immature and mature mTECs using MACS were defined as: $CD45^-$ $EpCAM^+$ $CD80^-$ and $CD45^-$ $CD80^+$ respectively.
6. After MACS purification (purity of immature mTECs = 83.1 ± 6.3 % and mature mTECs = 79.23 ± 3.42%), seed the mTECs onto the organotypic cultures as described below (Section 2.3).
7. Alternatively, sort mTECs by FACS (using 100 µm nozzle) after CD45 MACS depletion using the following antibodies: anti-CD45-PerCP (30-F11, use at 1:100 dilution), anti-Ly51-FITC (6C3, use at 1:100 dilution), anti-EpCAM-Alexa647 (G8.8, use at 1:500 dilution) and anti-CD80-PE (16-10A1, use at 1:100 dilution). Exclude dead cells using propidium iodide (1:5,000) (Day 4). Immature and mature mTECs using FACS were defined as: PI^- $CD45^-$ $Ly51^-EpCAM^+$ $CD80^-$ and PI^- $CD45^-$ $Ly51^-$ $EpCAM^+$ $CD80^+$, respectively.

2. 3D Organotypic Co-cultures (OTCs)

NOTE: The 3D-dermal constructs for organotypic cultivation of keratinocytes were prepared as described previously[9,13]. At all steps cells were incubated at 37 °C and 5% CO_2. The OTCs using mTECs were prepared with slight modifications as follows.

1. Preparation of Human Fibroblasts
   NOTE: The human dermal fibroblasts were obtained from explant cultures of de-epidermised dermis as described previously[9].
   1. In brief, cut strips of human skin (~5 cm length) and treat with thermolysin (0.5 mg/ml in saline with 10 mM Hepes pH 7.4) O/N at 4 °C.
   2. Thereafter, separate the epidermis from the dermis using forceps.
   3. Finely cut the dermis into small pieces, place in a 10 cm Petri plate and allow to dry for 1-2 hr under a sterile airflow. Supplement the explants regularly with DMEM containing 20 % FBS.
   4. Split the out-growing fibroblasts when confluent (usually around 3 weeks) using 0.1 % trypsin making sure that the explants continue to adhere to the plate for further consecutive rounds of outgrowth.
   5. Expand the fibroblasts from the same explants for up to 3 times in DMEM with 10 % FBS and cryo-preserve them[14]. Use the same batch of fibroblasts for each experimental series.
      NOTE: The fibroblasts were not irradiated for the set-up of the dermal equivalents.

2. Preparation of the Scaffold
   1. Cut the 0.4-0.6 mm thick viscose, nonwoven fibrous material (product details in supporting excel sheet) into well demarcated circles using a sharp 11 mm diameter metal puncher to exactly fit into 12 well-filter inserts. Then place it into the 12- well filter insert (polyester capillary pore membrane, 3 µm pore size) as a scaffold. Place the complete filter setup into a sterile 12-well plate.
   2. Prepare the fibrin gel using a fibrin glue-kit for surgery consisting of a combination of fibrinogen and thrombin. Pre-dilute the fibrinogen- as well as the thrombin-component of the kit to 8 mg/ml and 10 units/ml, respectively. For a single well of a 12-well plate proceed as follows.
      1. Dilute 100 µl fibrinogen (8 mg/ml) with 100 µl phosphate buffered saline (PBS) without Ca_2^+ and Mg_2^+, pH 7.0. Dilute 100 µl thrombin (10 units/ml) with 100 µl FCS containing 270,000 fibroblasts.
      2. Dispense 200 µl of the thrombin containing fibroblasts cell-mix onto the scaffold, to which add 200 µl fibrinogen (1:1 mixture), resulting in a final concentration of fibrinogen of 2 mg/ml and of thrombin of 2.5 units/ml. Mix well and distribute evenly over the whole area of the scaffold by gentle pipetting (Day 1).

NOTE: After 30 min at 37 °C a clot enclosing the fibroblasts will have formed, filling completely the internal spaces of the scaffold and forming a smooth upper surface.

3. Co-culture with mTECs
    1. For pre-culture, submerse the organ cultures in DMEM with 10 % FBS, 50 µg/ml L-ascorbic acid and 1 ng/ml TGF-β1 with a medium change every other day for 4-5 days.
    2. On the day of mTEC seeding, replace the medium by rFAD medium (1:1 DMEM + DMEM/F12) with 10% FBS, 10^{-10} M cholera toxin, 0.4 mg/ml hydrocortisone, 50 µg/ml L-ascorbic acid, RANK ligand (0.1 µg/ml) and 500 units/ml of Aprotinin, thus preventing precocious fibrinolysis by serine proteases secreted by fibroblasts.
    3. 7-8 hr later, set-up the co-cultures by seeding 250,000 mTECs (either complete mTECs, or $CD80^{lo}$ and $CD80^{hi}$ subsets), in a volume of 100 µl per well on top of the fibroblast scaffold. Count the cells using a Neubauer chamber (Day 4).
    NOTE: At all times the thymus 3D organotypic cultures are submersed in media unlike skin OTCs which are air-lifted.
    4. After 24 hr incubation, supply the cultures with medium (total medium exchange) as mentioned above (step 2.3.2) now containing reduced amounts of Aprotinin, 250 units/ml (Day 5).
    5. In order to assess the proliferative activity of mTECs, add EdU (6.7 µM/ml, *i.e.,* 10 µM/well) to OTCs for 4 hr before termination of the cultures. Perform the staining of OTC cryo-sections as described in the EdU Imaging Kit combined with co-staining of keratin 14. Determine the proliferative indices of mTECs, by either counting the $K14^{+}$ EdU^{+} cells in two sections of each culture specimen or by flow cytometry (EdU flow cytometry, Section 1.7 but instead of Ly51-FITC use CDR1-PB[15] at a 1:100 dilution and 2.3.6.4).
    6. Following 4-7 days of co-culture, terminate the OTCs and process for RNA isolation, cryo-sectioning or FACS analysis (Day 8-11).
        1. Terminate the cultures using a forceps, separating the scaffold/dermal equivalent from the filter of the well insert.
        2. For cryo-sectioning, embed the entire OTC in OCT compound and freeze in liquid nitrogen vapor before cryo-sectioning. Prepare 5-7 µm thick OTC sections using a cryostat and store at -20 °C until use. For immuno-histochemistry of cryo-sectioned OTCs use anti-keratin 14 (AF64, use at 1:1,000 dilution), and anti-vimentin (GP53, use at 1:100 dilution) antibodies. Perform the indirect immuno-histochemistry staining using respective secondary antibodies.
        3. For RNA isolation, add 1 ml Denaturing solution (containing phenol and guanidinium thiocyanate) in a screw cap of a 2 ml RNase free tube. Cut the entire OTC into pieces with a scalpel and add into the tube containing denaturing solution. Mechanically shred the OTCs with FastPrep instrument twice for 30 sec at a speed of 6.0, place in between on ice for 2 min (the sample can be stored at -80 °C after this point). If frozen at -80 °C, thaw on ice. Centrifuge the tubes at 11,500-13,000 rpm for 10 min at 4 °C. Transfer the supernatant to a fresh tube, incubate for 5 min at RT and follow RNA isolation protocols using Acid guanidinium thiocyanate-phenol-chloroform extraction as described by the manufacturer.
        4. For FACS analysis, remove the scaffold and separate the membrane from the fibrin/fibroblast/mTEC gel. Finely cut the gel with a scalpel and digest it in a FACS tube for ~20 min or until completely digested with 2 ml of collagenase/dispase at 37 °C in a water bath with magnetic stirring. Agitate the enzyme solution with a Pasteur pipette once every 5 min. After complete digestion, filter the cell suspension through a 70 µm filter; stain the single cell suspension using the antibodies as described in 1.7 and analyze by flow cytometry.

Representative Results

We adopted a 3D organotypic co-culture model (3D OTC) which had been originally developed for *in vitro* long term culture of keratinocytes[9]. MACS-enriched mTECs (see MACS enrichment scheme **Figure 1**) were seeded onto a scaffold comprising of a fibrin gel and entrapped fibroblasts. The fibroblasts provide the essential extracellular matrix (ECM) supporting mTECs *in vitro*. MTECs were cultivated in OTCs for 4-14 days in the presence of RANKL in submerged cultures unlike keratinocytes, which are air-exposed mimicking their *in vivo* environment (see OTC set-up scheme **Figure 2**).

Both mTEC subsets analyzed here (*i.e.,* $CD80^{lo}$ and $CD80^{hi}$ mTECs) survived during the entire culture period of up to 14 days. MTECs were identified by keratin 14 expression and were easily distinguishable from vimentin-positive fibroblasts (**Figure 3A**, **3B**). Interestingly, immature and mature mTECs grew in different patterns in culture. The $CD80^{lo}$ mTECs typically formed bi-layers (in close contact with fibroblasts), while $CD80^{hi}$ mTECs tended to form compact cell aggregates delimited by fibroblasts. These patterns were highly reproducible.

The mTEC subsets not only survived but also proliferated under 3D OTC conditions as assessed by EdU incorporation (**Figure 3C**, **3D**). Interestingly, the $CD80^{lo}$ mTECs proliferated at a higher rate in the presence of RANKL, while the reverse was true for $CD80^{hi}$ mTECs[10].

Additionally, $CD80^{lo}$ mTECs differentiated into $CD80^{hi}$ mTECs in the presence of RANKL within 4 days of culture, as indicated by the strong up-regulation of CD80. The differentiated mTECs also maintained the expression of Aire, FoxN1 (gene and protein) as well as promiscuously expressed Aire-independent and -dependent TRAs[10].

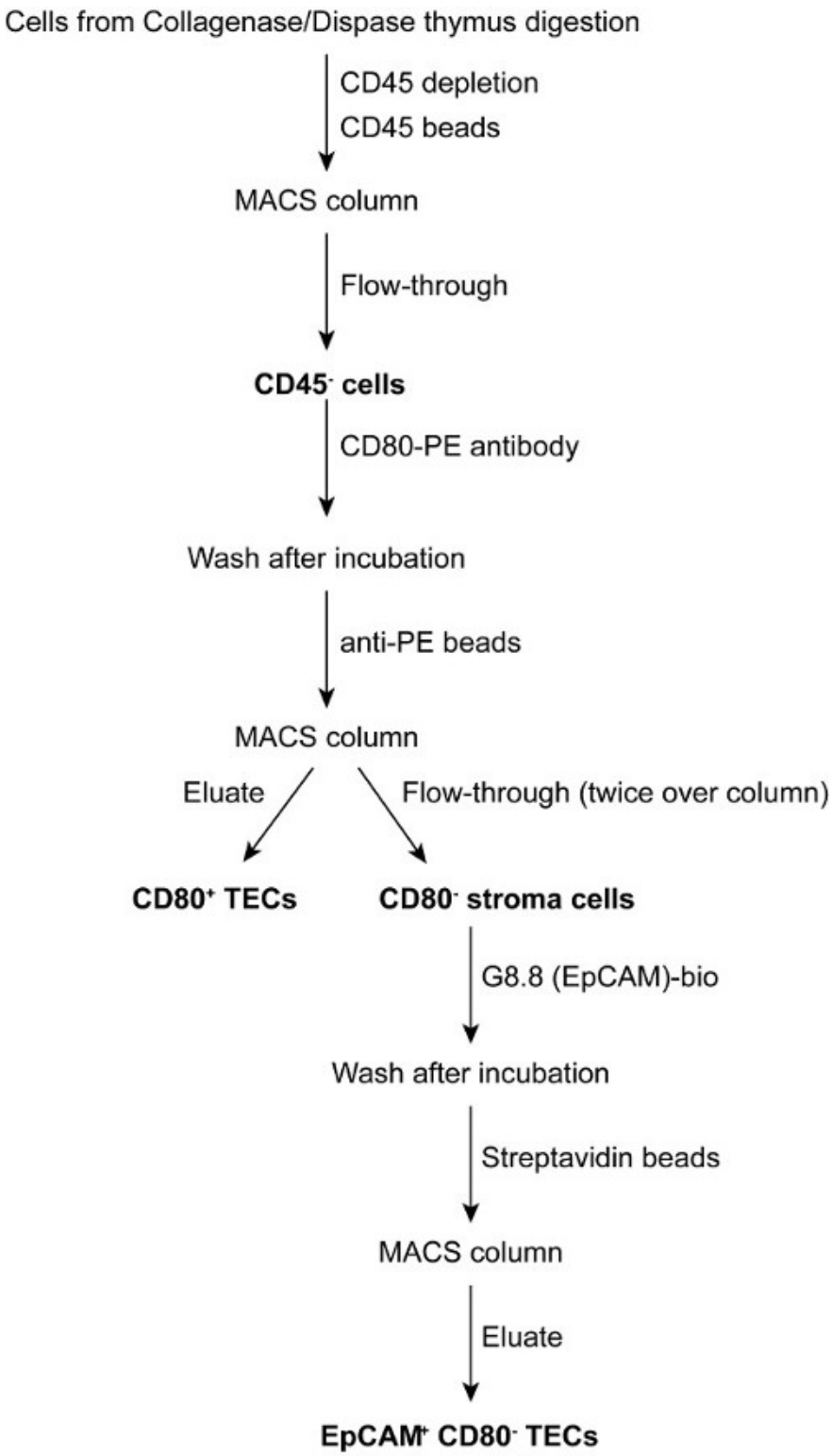

Figure 1. MACS enrichment of TECs. After filtering, mTECs were enriched from the thymus single cell suspension using magnetic cell sorting (MACS). First, the hematopoietic cell lineages were depleted using anti-CD45 Microbeads. The $CD45^-$ cells were then incubated with anti-CD80 PE antibody, followed by anti-PE Microbeads. The eluate containing $CD80^+$ -mature mTECs was directly cultured on the OTCs, while the flow-through contained $CD80^-$ immature mTECs and other stromal cells. MTECs were further enriched using anti-EpCAM-bio antibody and streptavidin Microbeads. Please click here to view a larger version of this figure.

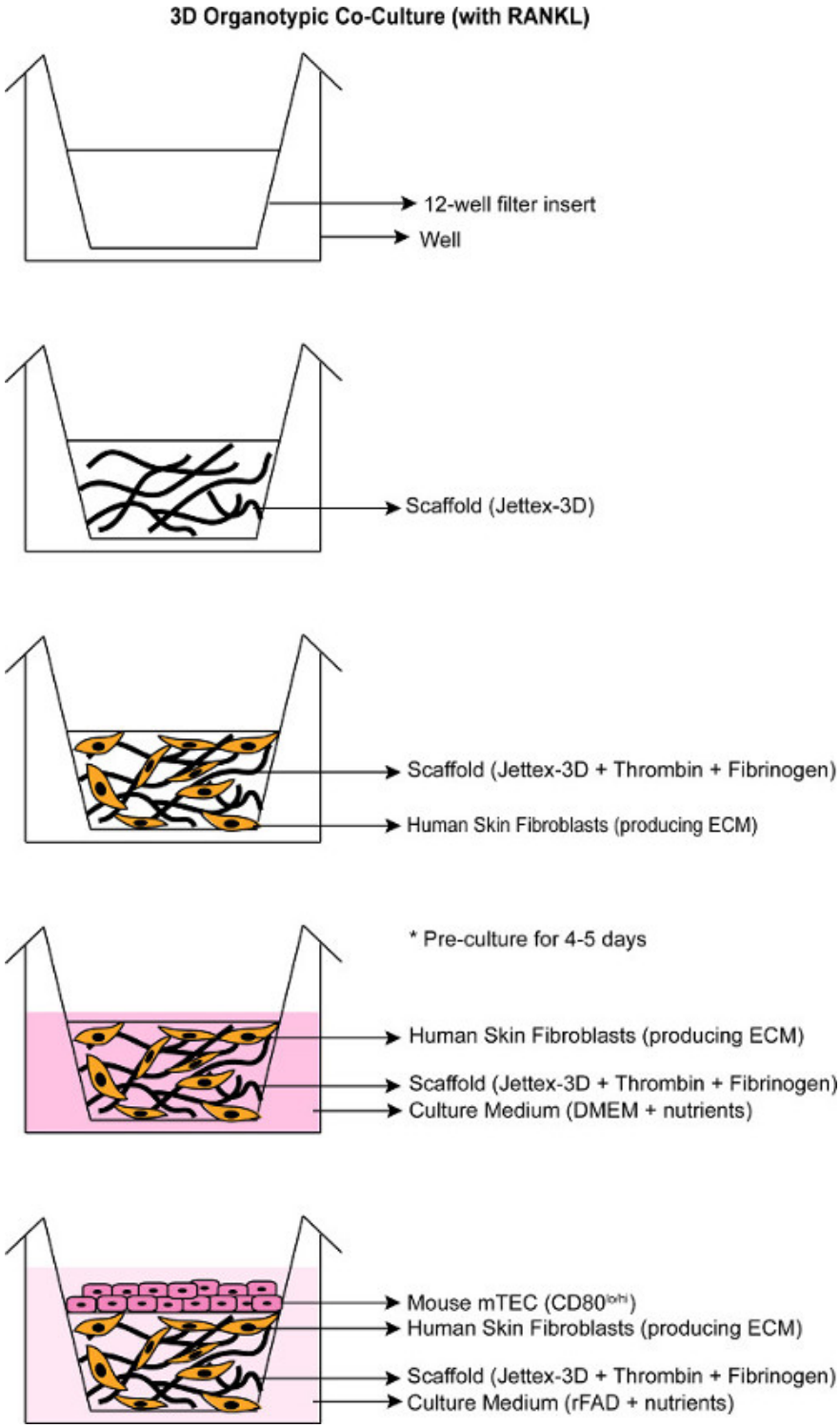

Figure 2. Scheme for the stepwise setup of the 3D OTCs. A scaffold matrix was placed into a 12-well filter insert. The dermal fibroblasts from explanted skin (270,000 cells/OTC well) were inoculated into a fibrin gel (consisting of 1:1 ratio of fibrinogen and thrombin). These dermal equivalents were sustained for 4-5 days with DMEM + nutrients until mTECs (250,000 cells/OTC well) were seeded on top and cultured with medium enriched with different nutrient (rFAD + nutrients).

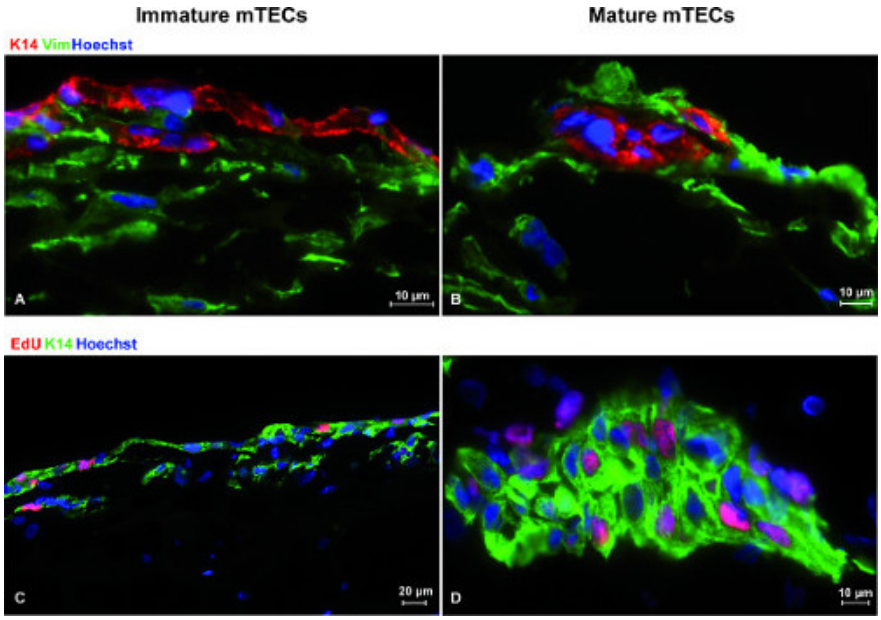

Figure 3. Growth patterns and proliferation of mTECs within the OTCs. The enriched CD80^{lo} mTECs tend to grow as a bi-layer in close contact with the fibroblasts (**A**), whereas the differentiated CD80^{hi} mTECs grow as cell aggregates or tight clusters (**B**). OTCs were labeled on day 4 of culture with anti-keratin 14 (red) and anti-vimentin antibodies (green) along with nuclear staining (Hoechst, blue). To evaluate the proliferation of mTECs, the OTCs were pulsed with EdU (6.7 µM/ml, *i.e.,* 10 µM/well) for 4 hr prior to termination of the cultures. The OTC cryo-sections were then stained with anti-keratin 14 (green), and EdU-Click-iT reaction mixture (magenta) along with nuclear staining (Hoechst, blue). Representative images depicting proliferating EdU^{+} CD80^{lo} mTECs (**C**) and EdU^{+} CD80^{hi} mTECs (**D**) are shown. Please click here to view a larger version of this figure.

Reaggregation thymic organ cultures **(RTOC)**	***Organotypic co-cultures*** **(OTC)**
Useful system to study cellular interactions that can be readily monitored and manipulated using a largely intact 3D thymic architecture; develops into a properly organized structure upon grafting.	Useful system to study development and differentiation of enriched/ purified thymic epithelial cells in an artificial 3D meshwork generated by human skin fibroblasts entrapped in a fibrin scaffold.
Well established for the study of the development of thymic epithelial cells and thymocytes using inhibitors or modifiers, which penetrate the capsule.	Amenable to manipulate thymus epithelial development or to study thymocyte development in co-culture with TECs.
Since the system is air-lifted, the membrane pore size (0.8 µm) is crucial when supplying factors to the RTOC: larger molecules may not penetrate through the membrane and/or capsule.	Culture is submerged in media, which facilitates uptake of substances/ factors (successfully tested using morpholino oligos).
Adult and post-natal TECs or thymocytes can be spiked into fetal RTOCs.	Post-natal TECs grow better than adult TECs; fetal TECs not yet tested.
Require embryonic E14-14.5 thymi (optimal) to allow for reaggregation and integration of the added desired population; difficult to control the fetal thymic (acceptor) cell composition; RTOCs require cells from either fluorescently labelled or congenic mice as donor or acceptor cells.	Possible to study single cells/clones; only “contaminants” are human skin fibroblasts required to provide ECM to support the TECs.
Imaging limited to penetration depth of live two-photon microscopy; amenable to FACS analysis or sorting; gene expression studies require FACS sorting of the desired cell type after culture.	Fully accessible to imaging; suitable for FACS analysis, immuno-histochemistry, and gene expression studies.

Table 1. Comparison between two 3D thymic culture systems.

Discussion

Alongside RTOCs, the 3D OTCs have been by far superior in terms of TEC differentiation and pGE maintenance/induction (**Table 1**) compared to other (i) ‘simplified 3D cultures’ using - fibroblasts alone without the scaffold; (ii) 2D systems using - fibroblasts/feeder cells co-cultured with TECs[10], (iii) 3T3-J2 cells wherein TEC clones develop, but pGE is lost, (iv) matrigel or (v) ECM components (unpublished data). PGE was maintained for up to 7 days in the 3D OTCs, 4 days being the optimum time-point thereafter, pGE starts to decline. Other morphological TEC features were maintained for up to 14 days. Intriguingly, the OTCs supported the end-stage of mTEC differentiation seen by the occasionally formed Hassall-like structures[10].

The thymi used for OTCs were derived from young post-natal mice as they have a higher propensity to survive in culture compared to adult thymi. TECs derived from embryonic thymi have not yet been tested in OTCs. More so, after the long digestion period (not using trypsin due to

cleavage of certain epitopes) the cells appeared more viable using MACS rather than FACS sorting cells. Using a two-step positive enrichment protocol on MACS columns (anti-PE beads) of $CD80^+$ mTECs additionally improved TEC purity.

For the OTC setup it is essential to make sure that the viscose, nonwoven fibrous material is cut into sharp, well demarcated circles without loose fragments or serrated ends as stated above that exactly fit into 12 well-culture inserts. Alternatively, scaffolds such as BEMCOT- viscose wiper M-3 (http://www.bemliese.com) can also be used. The well size for the OTC is also critical and should be tested, if plate formats other than 6-well and 12-well formats should be used.

The fibrin gel should be prepared such that the fibrinogen and thrombin components do not come into contact with each other before being set onto the OTC, make sure to exchange the pipette tip. Mix the two components thoroughly in the plate over the scaffold to form a smooth upper surface. Always prepare a test gel alongside in a well without scaffold to check for proper clot formation and to have a good estimate of the time required for clotting. Make sure that the fibrin gel in the OTC has completely clotted before supplying the cultures with media.

Upon termination of the cultures the seeded mTECs can be easily retrieved by enzymatic digestion (collagenase/dispase) of the fibrin gel, which can be used for characterization of cultured mTECs *e.g.,* by flow cytometry or PCR.

The 3D OTCs described herein has the potential to study or manipulate several TEC parameters namely: 1) to study and/or manipulate (via siRNA, morpholino oligos) developmental pathways and functions of mTECs; 2) to study the role of mTECs in T cell development; 3) to culture cTECs; 4) to test the progenitor potential of thymic derived stem cells; 5) to culture human TECs and stem cells; 6) to perform single TEC clonal assays. The 3D OTCs represent an elaborate culture technique emulating part of the *in vivo* thymus microenvironment. It should be emphasized that any additional assays applied to TECs in the current OTC setup will need to be thoroughly tested and optimized separately.

Disclosures

The authors declare no financial or commercial conflict of interest.

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