Journal of Visualized Experiments

A Three-dimensional Thymic Culture System to Generate Murine Induced Pluripotent Stem Cell derived Tumor Antigen-Specific Thymic Emigrants

--Manuscript Draft--

Article Type:	Invited Methods Article - JoVE Produced Video
Manuscript Number:	JoVE58672R2
Full Title:	A Three-dimensional Thymic Culture System to Generate Murine Induced Pluripotent Stem Cell derived Tumor Antigen-Specific Thymic Emigrants
Keywords:	stem cell; Induced Pluripotent Stem Cell; Immunology; Adoptive Cell Transfer; T Cell Differentiation; Tumor Antigen Specificity; 3D culture; Fetal Thymic Organ Culture
Corresponding Author:	R Dr. Vizcardo National Institutes of Health Bethesda, MD UNITED STATES
Corresponding Author's Institution:	National Institutes of Health
Corresponding Author E-Mail:	raul.vizcardosakoda@nih.gov
Order of Authors:	Raul Vizcardo
	Rafiqul SM Islam
	Takuya Maeda
	Naritaka Tamaoki
	Meghan L. Good
	Nicholas D. Klemen
	Marta Bosch-Marce
	Li Jia
	Mikhael J. Kruhlak
	Nicholas P. Restifo
Additional Information:	
Question	**Response**
Please indicate whether this article will be Standard Access or Open Access.	Open Access (US$4,200)
Please indicate the **city, state/province, and country** where this article will be **filmed**. Please do not use abbreviations.	Bethesda, MD 20892, USA

Xiaoyan Cao, Ph.D.
Review Associate
JoVE

June 25, 2018

Dear Dr. Xiaoyan Cao,

Thank you for sending us the reviewers comments for our manuscript. Please find enclosed our revised version entitled: “A 3D Thymic Culture System to Generate Mouse iPSC-Derived Thymic Emigrants with Tumor Antigen-Specificity “, which we would like to submit for publication as a video produced by JoVE.

As requested, all comments are answered in a separate word file and the corrections are included in the updated manuscript.

Please address all correspondence to:

Raul Vizcardo, Ph.D.
National Institutes of Health
National Cancer Institute
10 Center Drive
Building 10-CRC, Rm 3-5700
Bethesda, MD 20892
Email address: vizcardo@nih.gov
Tel: (240) 760-7638

We look forward to hearing from you at your earliest convenience.

Yours sincerely,

TITLE:

A Three-dimensional Thymic Culture System to Generate Murine Induced Pluripotent Stem Cell-derived Tumor Antigen-Specific Thymic Emigrants

AUTHORS AND AFFILIATIONS:

Raul Vizcardo[1,2,*], SM Rafiqul Islam[1,2,*], Takuya Maeda[1,2], Naritaka Tamaoki[1,2], Meghan L. Good[1,2], Nicholas D. Klemen[1,2], Marta Bosch-Marce[1,2], Li Jia[1], Michael J. Kruhlak[3], Nicholas P. Restifo[1,2]

[1]Surgery Branch, National Cancer Institute, NIH, Bethesda, MD, USA
[2]Center for Cell-Based Therapy, National Cancer Institute, NIH, Bethesda, MD, USA
[3]Laboratory of Cancer Biology and Genetics, Center for Cancer Research, National Cancer Institute, Bethesda, MD, USA

*These authors contributed equally.

Corresponding Author:

Raul Vizcardo (vizcardo@nih.gov)

Email Addresses of Co-authors:

SM Rafiqul Islam (rafiqul.islam@nih.gov)
Takuya Maeda (takuya.maeda@nih.gov)
Naritaka Tamaoki (naritaka.tamaoki@nih.gov)
Meghan L. Good (meghan.good@nih.gov)
Nicholas D. Klemen (nicholas.klemen@yale.edu)
Marta Bosch-Marce (marta.bosch-marce@nih.gov)
Li Jia (li.jia2@nih.gov)
Michael J. Kruhlak (kruhlakm@mail.nih.gov)
Nicholas P. Restifo (restifon@mail.nih.gov)

KEYWORDS:

Stem cell, induced pluripotent stem cell, immunology, adoptive cell transfer, T cell differentiation, tumor antigen specificity, 3D culture, fetal thymic organ culture

SUMMARY:

This article describes a novel method to generate tumor antigen-specific induced pluripotent stem cell-derived thymic emigrants (iTE) by a three-dimensional (3D) thymic culture system. iTE are a homogenous subset of T cells closely related to naïve T cells with the capacity for proliferation, memory formation, and tumor suppression.

ABSTRACT:

The inheritance of pre-rearranged T cell receptors (TCRs) and their epigenetic rejuvenation make induced pluripotent stem cell (iPSC)-derived T cells a promising source for adoptive T cell therapy (ACT). However, classical *in vitro* methods for producing regenerated T cells from iPSC result in

either innate-like or terminally differentiated T cells, which are phenotypically and functionally distinct from naïve T cells. Recently, a novel three-dimensional (3D) thymic culture system was developed to generate a homogenous subset of CD8αβ$^+$ antigen-specific T cells with a naïve T cell-like functional phenotype, including the capacity for proliferation, memory formation, and tumor suppression *in vivo*. This protocol avoids aberrant developmental fates, allowing for the generation of clinically relevant iPSC-derived T cells, designated as iPSC-derived thymic emigrants (iTE), while also providing a potent tool to elucidate the subsequent functions necessary for T cell maturation after thymic selection.

INTRODUCTION:

Adoptive T cell therapy (ACT) can be an effective treatment for some patients with advanced cancer. Unfortunately, many patients do not experience tumor regression, and transferred cells fail to persist after infusion. This may be due to the quality of the infused T cells. An ACT mouse model showed that compared to naïve or less differentiated central memory T cells, terminally differentiated effector cells are less potent due to poor *in vivo* persistence[1], an observation also supported by clinical data[2,3].

In an effort to improve the efficacy of current ACT, T cell-derived induced pluripotent stem cells (T-iPSC) have been studied extensively[4,5]. When T cells are reprogrammed into T-iPSC and re-differentiated into T cells, the rearranged configuration of TCR genes is inherited by T-iPSC, and subsequently the re-differentiated T cells. Therefore, the capacity of T-iPSC to undergo unlimited *in vitro* expansion permits the efficient reproduction of immature T cells carrying the neoantigen-specific T cell receptors (TCR) when such cells are engineered from tumor antigen-specific T cells[6,7]. However, the precise method for differentiation of T-iPSC into mature T cells, which would allow the production of cancer antigen-specific T cells with a less differentiated phenotype and better anti-tumor potency, remains to be elucidated.

T-iPSC differentiation employing the co-culture of OP9 murine stromal cells over-expressing human Notch ligand DLL1 is a well-established method to produce T cells *in vitro*[6,7]. In mice and humans, this co-culture system can consistently differentiate iPSC, thereby recapitulating developmental events from the blastocyst stage until the immature T cell lineage stage[6,7]. Despite these biotechnological advances, the physiological differentiation after the CD4$^+$CD8$^+$ double positive (DP) stage is still difficult to achieve. One of the reasons is that *in vivo* CD4$^+$CD8$^-$ and CD4$^-$CD8$^+$ single positive (SP) T cells are generated in the thymus, an organ responsible for the maturation and selection of T cells that have foreign antigen-specificity but not auto-reactivity[8]. These selective processes are defined as positive and negative selection, respectively. However, most of the molecular mechanisms necessary to mature T cells in the thymus are still not fully understood, making it difficult to reconstruct this process *in vitro*. In an attempt to overcome this physiological hurdle, several groups have stimulated the TCR complex using anti-CD3 antibodies or agonist peptides. These *in vitro* techniques generate cell products which express key T cell markers, like CD3, CD8αβ, TCRαβ, and CD62L, while still retaining tumor antigen-specificity. Unfortunately, T cells generated by these extrathymic methods constitute a broad heterogenous population of cells characterized by incomplete positive selection, innate-like features, TCR non-specific killing, inability for memory formation, and non-persistent anti-tumor effects *in vivo*[8-11].

These abnormalities have raised concerns that such cells might trigger a variety of side effects, including lymphoma and both skin and bone abnormalities, if used for therapeutic applications[12-14].

To recreate the physiological signals missing in current *in vitro* differentiation systems, tumor antigen-specific T-iPSC were differentiated using a harvested thymus. The classical fetal thymus organ culture (FTOC) system, which was designed to study the intra-thymic development of T cells, was improved by using a 3D culture system which successfully produced T cells that completed thymic education. These post-thymic T cells, which were designated as iPSC-derived thymic emigrants (iTE), exhibited naïve-like properties[15]. iTE showed proliferation, memory formation, and adequate anti-tumor effects in a mouse model against established B16 melanoma tumors. This article describes in detail the protocol of this novel FTOC system using a 3D culture system **(Figure 1)**.

PROTOCOL:

All the animal experiments were approved by the Institutional Animal Care and Use Committees of the National Cancer Institute (NCI) and performed in accordance with NIH guidelines.

1. Preparation of OP9/DLL1 Cells for Co-culture with iPSC

1.1. Culture OP9/DLL1 cells in OP9 media (α-minimum essential medium [α-MEM] + 20% non-heat inactivated fetal bovine serum [FBS] + 1x penicillin-streptomycin + ascorbic acid [50 ng/mL] and mono-thioglycerol [100 nM]) at 37 °C. When OP9/DLL1 cells reach 80-95% confluency, wash once with 1x magnesium, calcium, and phenol red free phosphate buffered saline (hereafter referred to as PBS).

1.2. Add 4 mL of 0.05% trypsin and incubate for 5 min at 37 °C. Then add 4 mL of OP9 media, dissociate the cell layer by pipetting to make a single cell suspension.

1.3. Transfer the cell suspension into a 50 mL conical tube through a 100 µm cell strainer. Centrifuge at 300 x g for 5 min at 4 °C, aspirate the supernatant, and resuspend in 12 mL of OP9 media.

1.4. Plate 2 mL of OP9/DLL1 cell suspension onto a new 10 cm cell-culture Petri dish and add additional 8 mL of OP9 media. Repeat passage every 2 - 3 days.

NOTE: The quality of the FBS and culture conditions are critical to maintain the expansion of OP9/DLL1 cells without losing their ability to support iPSC differentiation. Therefore, it is recommended to pre-evaluate the lot of FBS and passage consistently at 80% confluency to prevent cell differentiation and senescence. It is also important to make enough frozen stock of OP9/DLL1 cells and thaw a new stock every 4 - 6 weeks.

2. *In Vitro* Differentiation of iPSC into Immature T Cells

2.1. On day 0, begin iPSC co-culture on OP9/DLL1 confluent dishes.

2.1.1 Harvest iPSC as a single cell suspension by trypsinization (5 min in 0.05% trypsin at 37 °C), collect the cells, and centrifuge at 300 x g for 5 min at 4 °C.

2.1.2 Aspirate the supernatant and resuspend cells at 1.0 x 10^5 iPSC per 10 mL of OP9 media. Plate 1.0 x 10^5 iPSC onto a confluent OP9/DLL1 10 cm dish.

NOTE: OP9/DLL1 10 cm dishes are used for iPSC differentiation when they reach 90-100% confluency. Differences in confluency can affect the efficiency of iPSC differentiation.

2.2. On day 3, aspirate old media and replace with 10 mL of fresh OP9 media.

2.3. On day 6, passage cells.

2.3.1. Wash each 10 cm confluent OP9 dish with 10 mL of PBS. Add 3 mL of 0.05% trypsin per dish and incubate for 3 - 5 min at room temperature (RT).

2.3.2. Add 4 mL of OP9 media and collect cells by gentle pipetting. Pass cells through a 100 µm cell strainer and centrifuge at 300 x g for 5 min at 4 °C. Discard supernatant.

2.3.3. Resuspend cells in 10 mL of differentiation media (OP9 media with 5 ng/mL mouse Flt3 ligand [FLT3L] and 5 ng/mL mouse IL-7) and plate cell suspension onto a new 10 cm OP9/DLL1 confluent dish.

2.4. On day 9, aspirate old media and replace with 10 mL of fresh differentiation media.

2.5. On day 11 when cardiomyocytes are observed in iPSC colonies, mechanically detach non-adherent cells by pipetting and filter through a 100 µm cell strainer. Spin at 300 x g for 5 min at 4 °C.

2.5.1. Aspirate the supernatant and resuspend in 24 mL of differentiation media. Plate iPSC into a confluent OP9/DLL1 6-well plate (4 mL/well).

2.6. On day 15, collect all non-adherent cells and filter through a 40 µm cell strainer.

2.6.1. Spin at 300 x g for 5 min at 4 °C.

2.6.2. Continue passaging non-adherent cells every 3 - 4 days by repeating step 2.5.1.

3. 3D Thymic Organ Culture to Generate iTE

3.1. Harvest mouse fetal thymic lobes and deploy of endogenous lymphocytes by deoxyguanosine (dGUO) treatment as previously described[16].

3.2. On day 7 of dGUO treatment, take four new 10 cm dishes and fill each with 20 mL of complete media (Rosswell Park Memorial Institute Media 1640 [RPMI 1640] + 10% FBS + 1x L-alanyl-L-glutamine + 1x sodium pyruvate + 1x minimum essential medium with non-essential amino acids (MEM-NEAA) + 1x penicillin-streptomycin + [1:1000] 2-mercapto ethanol).

3.3. Transfer all nitrocellulose membranes with thymic lobes into one 10 cm dish. Detach the individual lobes from the membrane with forceps, allowing them to be submerged in media. Discard the membranes. Incubate for 1 h at RT.

3.4. Transfer the thymic lobes to a new 10 cm dish with complete media and incubate for 1 h at RT. Repeat this step 2 more times.

3.5. Using forceps, fix the thymic lobes to the dish (one at a time), and with the other hand make a 100 - 200 µm deep incision in the center and extending half the diameter of the lobe to facilitate T cell progenitor migration into the lobe.

3.6. Transfer the thymic lobes to a new 10 cm dish filled with complete differentiation media (complete media + 5 ng/mL mouse IL-7 + 5 ng/mL mouse FLT3L + 5 ng/mL SCF).

3.7. Optionally, if using 3D culture plates with lower and upper level grids, fill both grids with sterile PBS to prevent the evaporation and drying of the hanging drops.

3.8. Transfer 30 µL of complete media containing one dGuo-treated thymic lobe from step 3.6 into each well of 3D culture plate.

3.9. Collect non-adherent T lineage cells (iPSC-derived immature T cells) from OP9/DLL1 co-culture (days 16-21) (step 2.6.2) and resuspend at 2 - 5 x 10^3 T lineage cells per 20 µL media.

3.10. Add 20 µL of T lineage cell suspension to each thymic lobe in the 3D culture plate. Incubate overnight at 37 °C with 5% CO_2.

3.11. Set the P200 pipet to 30 µL and aspirate the media after pipetting several times from each well to remove all the cells surrounding the thymic lobes. Discard media and add 30 µL of complete media. Repeat this procedure 5 - 7 times to remove any extra immature T cells which does not migrate into the lobes. Change 25 - 30 µL of media daily thereafter.

3.12. Confirm the formation of a halo of iPSC-derived thymic emigrants (iTE) around the lobes beginning on day 4 - 5 by light microscopy.

3.13. Collect iTE daily by pipetting media without lobe disruption. Change media every day and continue collection up to approximately 12 days.

3.14. Harvested iTE are ready to use for molecular analyses (**Figure 2, Figure 3, Figure 4,** and **Figure 5**) or *in vivo* transplantation experiments.

4. Preparation of Antigen Presenting Cells (APC)

4.1. Sacrifice a C57BL/6 mouse by cervical dislocation and place onto a lab soaker mat as described above.

4.2. Remove the spleen and place it onto a 100 µm cell strainer. Compress the spleen onto the strainer using a 12 mL syringe plunger to make a single cell suspension.

4.3. Transfer the cell suspension through a sterile 40 µm cell strainer. Centrifuge the suspension at 300 x g for 5 min at 4 °C to pellet the cells.

4.4. Aspirate the supernatant and resuspend the cell pellet in 2 mL of ammonium-chloride-potassium (ACK) lysis buffer to exclude red blood cells (RBC). Incubate for 5 min at RT.

4.5. Quench the ACK lysis buffer by adding 10 mL of PBS. Pellet the cells by centrifugation at 300 x g for 5 min at 4 °C.

4.6. Aspirate the supernatant and resuspend the cell pellet in 10 mL of complete media and transfer to a 10 cm sterile Petri dish.

4.7. Irradiate splenocytes with 3500 rad using an irradiation device (γ-radiation) to prevent cell proliferation.

4.8. Immediately return the irradiated cells to a 37 °C incubator and culture overnight.

4.9. Use irradiated cells as APC or freeze in cell banker.

5. Pulsing APC with Antigen

5.1. Count live irradiated APC using a Neubauer hemocytometer and trypan blue dye. Incubate APC with peptides (hgp100) or nucleoprotein for 30 min at 37 °C.

5.2. Wash APC twice with 10 mL of PBS to remove any extra peptide.

5.3. Count iTE and mix with APC in a 1:1 ratio in complete media with 100 IU IL-2 and 5 ng/mL IL-7. Aliquot 100 µL of the mixture of cells (total concentration: 1 x 10^6 cells/mL) into each well of an ultra-low attachment U bottom 96 well plate and culture for 48 h at 37 °C.

5.4. After 48 h, transfer cells to a new plate using a multichannel pipette and passage every 2 - 3 days thereafter.

5.5. On day 3, analyze the cytokine secretion profile by staining the cells with intracellular antibody and analyze by flow cytometry (**Figure 3**).

5.5.1. Add 0.67 µL/mL of protein transport inhibitor (*e.g.*, GolgiStop) and incubate at 37 °C for 6 h to enhance the intracellular accumulation of cytokines. Wash with 10 mL of PBS.

5.5.2. Resuspend cells in 3 mL of cold (4 °C) PBS and slowly add 1 mL of cold 4% paraformaldehyde (PFA) solution.

5.5.3. After 10 min, spin down cells at 300 x g for 5 min at 4 °C, discard supernatant and wash with 10 mL of PBS.

5.5.4. Resuspend cells in 1 mL PBS + 1% FBS + 0.1% nonionic surfactant, and place in 4 °C for 10-15 min.

5.5.5. Add antibodies, protect samples from light and place in 4 °C for 30 min.

5.5.6. Spin down cells at 300 x g for 5 min at 4 °C, discard supernatant, and wash with 10 mL of PBS.

5.5.7. Spin down cells at 300 x g for 5 min at 4 °C and resuspend cells in 1 mL of PBS. Cells are ready to be analyzed in a flow cytometer.

REPRESENTATIVE RESULTS:

Co-cultured fetal thymuses were sectioned to analyze whether iPSC-derived T lineage cells can migrate into the thymic lobes. Unseeded control lobes had a tissue architecture characterized by an astrocyte-like thymic epithelial web[17], deployed of endogenous $CD3^+$ cells. On the other hand, thymic lobes seeded with iPSC-derived immature T cells were repopulated with $CD3^+$ mononuclear cells, indicating migration of iPSC-derived immature T cells into the lobes (**Figure 2A**).

T cells that migrated into and matured within the thymic microenvironment subsequently egressed as iTE. To test their phenotypic characterization, flow cytometric analysis of C57BL6 thymocytes, Pmel iPSC-derived immature T cells (extrathymic), and cells that egressed from thymic lobes (iTE) was performed. Extrathymic T cells on OP9/DLL1 showed $CD4^+CD8^+$ (DP) T cells and CD8αSP T cells without expression of the positive selection marker MHC-I, whereas iTE had a clear population of CD8αSP MHC-I^+ T cell phenotype, indicating their successful passage through positive selection prior to egressing from the thymic lobes. iTE consistently express MHC-I and CD62L, which are markers associated with high proliferative competency, cytokine production, peripheral survival, and lymphoid homing[18-20]. This phenotype is consistent with M2 SP thymocytes that are the most mature population of single positive T cells in the thymus[20], which suggests that iTE have transitioned through a normal thymic developmental program (**Figure 3**). To monitor the efficiency of iTE generation, cells that had egressed from individual thymic lobes were isolated. On day 7, thymic lobes generated an average of 1×10^3 live CD8SP

CD45.1$^+$ CD3$^+$ iTE per day (**Figure 3B**). A similar rate of iTE production is observed from day 6 to day 12 of 3D thymic co-culture.

Antigen-dependent activation and secretion of cytokines were analyzed to observe the functional properties of thymically educated iPSC-derived immature T cells. In the presence of an irrelevant peptide (nucleoprotein), Pmel-iTE did not release significant amounts of TNF-α, IL-2, or IFN-γ. When stimulated with the cognate peptide for Pmel T cells (hgp100), Pmel-iTE released robust amounts of TNF-α and IL-2, while also producing low amounts of IFN-γ (**Figure 4**), indicating that thymically educated iTE can recognize their cognate peptide and secrete effector cytokines with a profile resembling that of natural recent thymic emigrants (RTE).

To examine the transcriptional differences between iPSC-derived T lineage cells differentiated on OP9/DLL1 with or without thymic education (*i.e.*, iTE *versus* extrathymic T cells), RNA-seq analysis was performed on these two populations and compared to that of DP T lineage cells differentiated using OP9/DLL1 (DP) and primary naïve CD8$^+$ Pmel T cells. The expression of 102 genes which play crucial roles in T cell ontogeny, thymocyte activation, and memory formation were analyzed[15,20-22]. A principal component analysis of those four studied populations demonstrated that extrathymically generated DP and CD8SP T cells clustered together, while iTE clustered closer to naïve T cells (**Figure 5**). Collectively, these data demonstrate that iTE have a phenotype closer to naïve T cells than do T lineage cells generated by extrathymic methods.

FIGURE LEGENDS:

Figure 1: **Schematic overview of the differentiation of iPSC to iTE using OP9/DLL1 and 3D thymic culture.** The protocol involves three separate differentiation steps; (**Left**) from iPSC cells to hematopoietic lineage cells on OP9/DLL1 (day 0 to 6), (**Middle**) from hematopoietic lineage cells to immature T cells on OP9/DLL1 with cytokines (day 6 to 16 - 21), and (**Right**) from immature T cells (day 16 - 21) to iTE using a 3D thymic culture system.

Figure 2: Immuno-histochemistry of thymic lobes seeded with iPSC-derived immature T cells. Top: H&E staining of a thymic lobe with and without seeding of iPSC-derived immature T cells. From second top to bottom: confocal images of the sectioned lobes stained with DAPI (nucleus), CD3 (T cell), and merge. Scale bars, 100 μm.

Figure 3: iTE show a post-thymic T cell phenotype. (**A**) FACS analyses of thymocytes, extrathymic T cells (OP9/DLL1 co-culture system) and Pmel-iTE. Live cells were gated on congenic CD45$^+$. CD8 SP populations were further analyzed for CD62L and MHC-I expression. (**B**) Average number of CD8SP CD45.1 iTE produced overnight per lobe 7 days after pre-seeding. Data were collected from 12 independent experiments.

Figure 4: iTE produce various cytokines by antigen-specific stimulation. FACS analyses of intra-cellular production of cytokines by iTE. iTE were co-cultured with APCs pre-loaded with irrelevant (nucleoprotein) or cognate (hgp100) peptide for three days. The numbers shown in upper right quadrants indicate the percentages of iTE producing cytokine.

Figure 5: Whole-transcriptome analysis reveals a shift in iTE gene expression toward a naïve CD8$^+$ T cell program. Principle component analysis (PCA) of RNA-seq data from DP, extrathymic CD8 SP, iTE, and naïve T cells. (Analysis of 102 genes related to thymic differentiation using public database GSE105110)[15].

DISCUSSION:

Using T-iPSC to regenerate tumor antigen-specific T cells may overcome many of the current obstacles of ACT by generating young cells with improved persistence. Although several methods using the OP9/DLL1 co-culture system have been reported to generate CD8 SP cells[6,7,10,13] that express CD8 molecules and tumor antigen-specific TCRs, global gene expression patterns and functional analysis show that these extrathymically regenerated CD8 SP cells are different from naïve T cells (**Figure 4**). Here, we describe a 3D thymic culture system that can generate iPSC-derived thymic emigrants (iTE) with high fidelity and homogeneity from murine T-iPSC. iTE resemble naïve T cells in global gene expression pattern and in functionality, such as memory formation and *in vivo* anti-tumor effect against established tumor[15].

The classical FTOC system is a way to recapitulate thymic selection *in vitro*. It has been used for studying intra-thymic development of thymocytes[23], and there are a few reports of FTOC being used to generate RTE[24]. However, the FTOC system has several limitations. To deal with the lack of oxygen in an artificial organ culture, several groups have used either a semi-dry membrane based culture[23], or high oxygen submersion culture systems[25]. However, no current methods can constantly generate a homogenous population of post-thymic T cells. To overcome the limitations of the classical FTOC system, we designed a 3D thymic culture system that provides technical improvements over conventional methods[15]. For example, using our 3D thymic culture method, maximal oxygen exchange and the absence of surface-lobe mechanical stress keep the thymic lobes in a more physiological environment. Additionally, long term culture permits mature T cells to egress naturally from the thymic lobes. Finally, real time observation and micro-manipulation enable media exchange and a constant collection of iTE without physically disturbing the thymic lobes. Thus, the 3D thymic culture method provides significant technical improvements as well as an avenue to study thymically selected naïve T-cells that was not previously available.

There are several key points for the successful generation of iTE using this 3D thymic culture system. The quality of the FBS and culture conditions is critical to maintain the expansion of OP9/DLL1 cells without losing their ability to support iPSC differentiation. Therefore, we recommend pre-evaluation of the FBS lot as well as consistently passaging at 80% confluency to prevent cell differentiation and senescence. Additionally, a confluent OP9/DLL1 culture is required for *in vitro* differentiation of iPSC into immature T cells, as differences in confluency can affect their efficiency. Finally, the embryonic age of thymic lobes is crucial for the generation of iTE. We recommend using E14.5 - 15.5 thymic lobes.

As with any new protocol, this method has limitations and is subject to improvement. The culture technique presented here generates approximately 1000 iTE per thymic lobe per day for a period

of two weeks. Increased iTE generation may be possible with further modifications, including optimization of oxygen concentration, media volume, and type of 3D culture plate. Addition or removal of cytokines, as well as changes in cytokine concentration, may also contribute to improved iTE yield.

Given that the 3D thymic culture system presented here can generate thymic emigrants in a completely *ex vivo* system, this technique can be applied to a variety of immunological and adoptive cell transfer research projects including, but not limited to T cell differentiation, post-thymic T cell maturation, and generation of antigen-specific T cells from hematopoietic progenitor or stem cells. Although this method is not directly applicable to human samples, iTE and the 3D thymic culture system hold great potential for elucidating the molecular mechanisms of positive and negative selection and may facilitate the creation of a culture system that enables the generation of clinically relevant tumor antigen-specific naïve-like T cells for ACT.

ACKNOWLEDGMENTS:

We thank Hiroshi Kawamoto and Kyoko Masuda for kindly providing the OP9/DLL1 cell line. We thank Alan B. Hoofring and Erina Z. He for graphical assistance. This research was supported by the Intramural Research Program of the US National Cancer Institute (ZIA BC010763) and the Cancer Moonshot program for the Center for Cell-based Therapy at the NCI, NIH. The work was also supported by the Milstein Family Foundation.

DISCLOSURES:

Authors Raul Vizcardo, Nicholas D. Klemen, and Nicholas P. Restifo are inventors on pending international patent application PCT/US2017/65986, filed December 13, 2017, entitled “Methods of Preparing an Isolated or Purified Population of Thymic Emigrant Cells and Methods of Treatment Using the Same.”

REFERENCES:

1 Gattinoni, L. *et al.* Wnt signaling arrests effector T cell differentiation and generates CD8+ memory stem cells. *Nature Medicine.* **15** (7), 808-813, doi:10.1038/nm.1982, (2009).

2 Rosenberg, S. A. *et al.* Durable complete responses in heavily pretreated patients with metastatic melanoma using T-cell transfer immunotherapy. *Clinical Cancer Research.* **17** (13), 4550-4557, doi:10.1158/1078-0432.CCR-11-0116, (2011).

3 Singh, N., Perazzelli, J., Grupp, S. A., Barrett, D. M. Early memory phenotypes drive T cell proliferation in patients with pediatric malignancies. *Science Translational Medicine.* **8** (320), 320ra323, doi:10.1126/scitranslmed.aad5222, (2016).

4 Crompton, J. G., Clever, D., Vizcardo, R., Rao, M., Restifo, N. P. Reprogramming antitumor immunity. *Trends of Immunology.* **35** (4), 178-185, doi:10.1016/j.it.2014.02.003, (2014).

5 Takahashi, K., Yamanaka, S. Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. *Cell.* **126** (4), 663-676, doi:10.1016/j.cell.2006.07.024, (2006).

6 Vizcardo, R. *et al.* Regeneration of human tumor antigen-specific T cells from iPSCs derived from mature CD8(+) T cells. *Cell Stem Cell.* **12** (1), 31-36, doi:10.1016/j.stem.2012.12.006, (2013).

7 Nishimura, T. *et al.* Generation of rejuvenated antigen-specific T cells by reprogramming to pluripotency and redifferentiation. *Cell Stem Cell.* **12** (1), 114-126, doi:10.1016/j.stem.2012.11.002, (2013).
8 Takada, K., Kondo, K., Takahama, Y. Generation of Peptides That Promote Positive Selection in the Thymus. *Journal of Immunology.* **198** (6), 2215-2222, doi:10.4049/jimmunol.1601862, (2017).
9 Yamagata, T., Mathis, D., Benoist, C. Self-reactivity in thymic double-positive cells commits cells to a CD8 alpha alpha lineage with characteristics of innate immune cells. *Nature Immunology.* **5** (6), 597-605, doi:10.1038/ni1070, (2004).
10 Themeli, M. *et al.* Generation of tumor-targeted human T lymphocytes from induced pluripotent stem cells for cancer therapy. *Nature Biotechnology.* **31** (10), 928-933, doi:10.1038/nbt.2678, (2013).
11 Serwold, T., Hochedlinger, K., Inlay, M. A., Jaenisch, R., Weissman, I. L. Early TCR expression and aberrant T cell development in mice with endogenous prerearranged T cell receptor genes. *Journal of Immunology.* **179** (2), 928-938 (2007).
12 Lei, F. *et al.* In vivo programming of tumor antigen-specific T lymphocytes from pluripotent stem cells to promote cancer immunosurveillance. *Cancer Research.* **71** (14), 4742-4747, doi:10.1158/0008-5472.CAN-11-0359, (2011).
13 Maeda, T. *et al.* Regeneration of CD8alphabeta T Cells from T-cell-Derived iPSC Imparts Potent Tumor Antigen-Specific Cytotoxicity. *Cancer Research.* **76** (23), 6839-6850, doi:10.1158/0008-5472.CAN-16-1149, (2016).
14 Serwold, T. *et al.* T-cell receptor-driven lymphomagenesis in mice derived from a reprogrammed T cell. *Proceedings of the National Academy of Sciences of the United States of America.* **107** (44), 18939-18943, doi:10.1073/pnas.1013230107, (2010).
15 Vizcardo, R. *et al.* Generation of Tumor Antigen-Specific iPSC-Derived Thymic Emigrants Using a 3D Thymic Culture System. *Cell Reports.* **22** (12), 3175-3190, doi:10.1016/j.celrep.2018.02.087, (2018).
16 Jenkinson, W., Jenkinson, E., Anderson, G. Preparation of 2-dGuo-treated thymus organ cultures. *Journal of Visualized Experiments.* (18), doi:10.3791/906, (2008).
17 Hamazaki, Y., Sekai, M., Minato, N. Medullary thymic epithelial stem cells: role in thymic epithelial cell maintenance and thymic involution. *Immunological Reviews.* **271** (1), 38-55, doi:10.1111/imr.12412, (2016).
18 Vivier, E. *et al.* Innate or adaptive immunity? The example of natural killer cells. *Science.* **331** (6013), 44-49, doi:10.1126/science.1198687, (2011).
19 Rosen, S. D. Ligands for L-selectin: homing, inflammation, and beyond. *Annual Review of Immunology.* **22** 129-156, doi:10.1146/annurev.immunol.21.090501.080131, (2004).
20 Hogquist, K. A., Xing, Y., Hsu, F. C., Shapiro, V. S. T Cell Adolescence: Maturation Events Beyond Positive Selection. *Journal of Immunology.* **195** (4), 1351-1357, doi:10.4049/jimmunol.1501050, (2015).
21 Best, J. A. *et al.* Transcriptional insights into the CD8(+) T cell response to infection and memory T cell formation. *Nature Immunology.* **14** (4), 404-412, doi:10.1038/ni.2536, (2013).
22 Schmitz, I., Clayton, L. K., Reinherz, E. L. Gene expression analysis of thymocyte selection *in vivo*. *International Immunology.* **15** (10), 1237-1248 (2003).

23 Nitta, T., Ohigashi, I., Takahama, Y. The development of T lymphocytes in fetal thymus organ culture. *Methods in Molecular Biology.* **946**, 85-102, doi:10.1007/978-1-62703-128-8_6, (2013).
24 Ueno, T. *et al.* Role for CCR7 ligands in the emigration of newly generated T lymphocytes from the neonatal thymus. *Immunity.* **16** (2), 205-218 (2002).
25 Watanabe, Y., Katsura, Y. Development of T cell receptor alpha beta-bearing T cells in the submersion organ culture of murine fetal thymus at high oxygen concentration. *European Journal of Immunology.* **23** (1), 200-205, doi:10.1002/eji.1830230131, (1993).

Figure 1

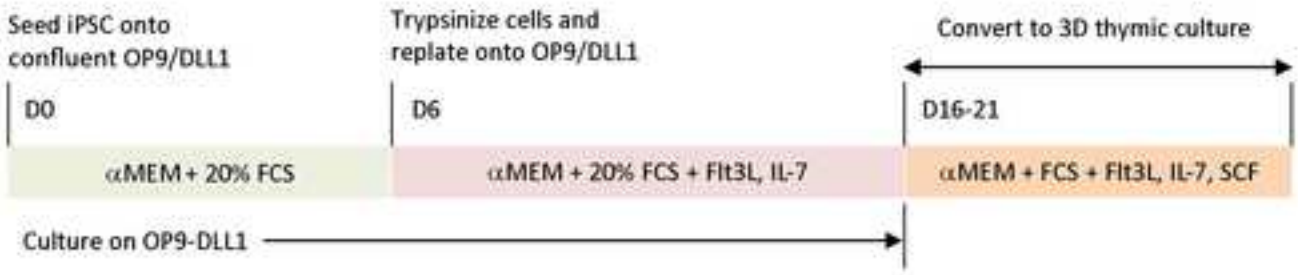


Figure 2

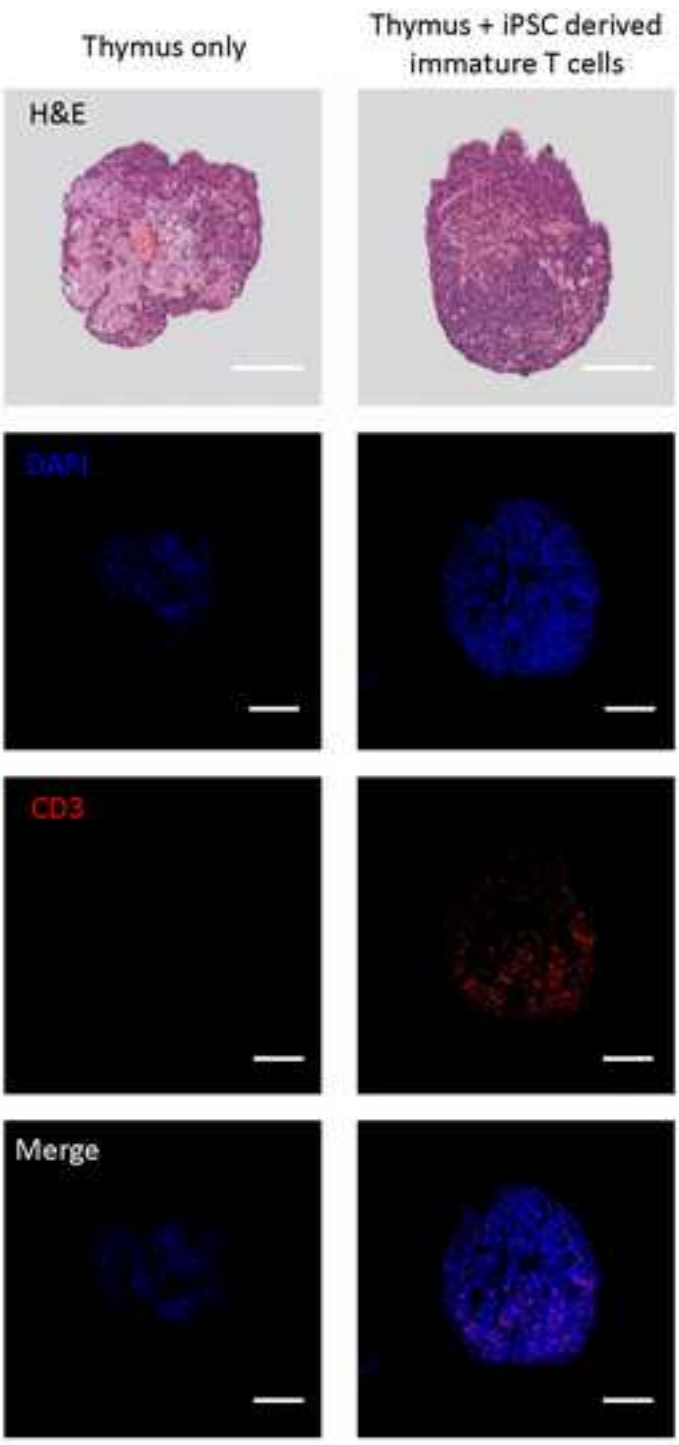
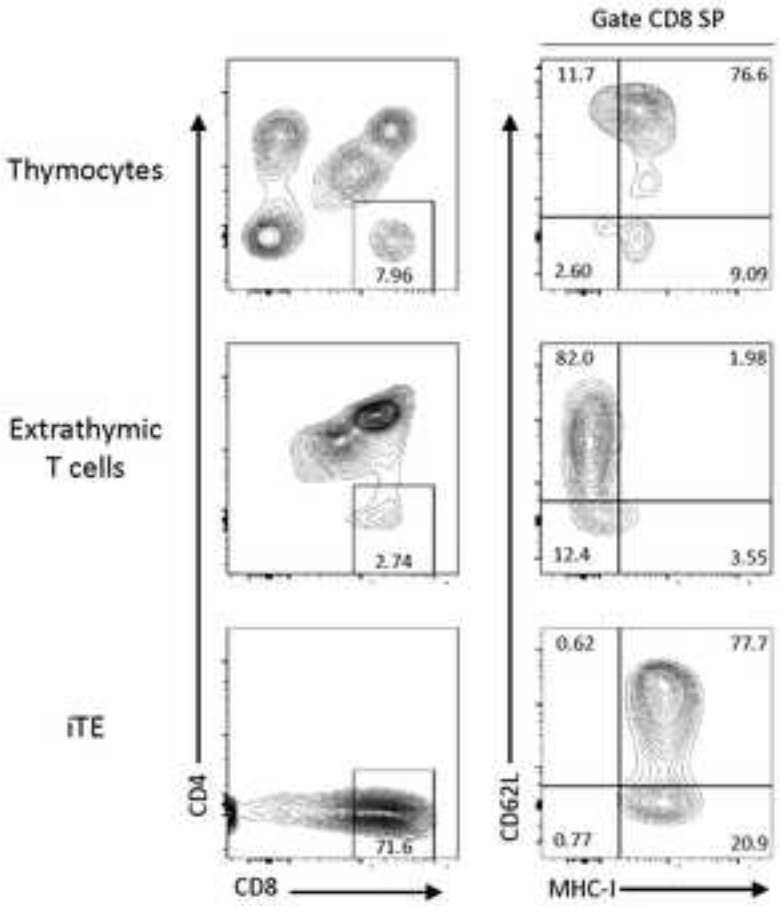


Figure 3

A



B

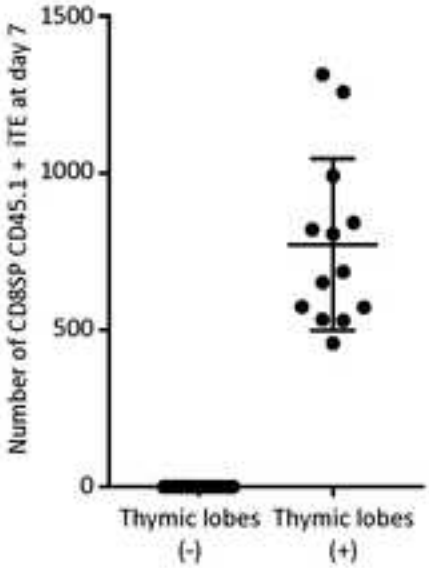


Figure 4

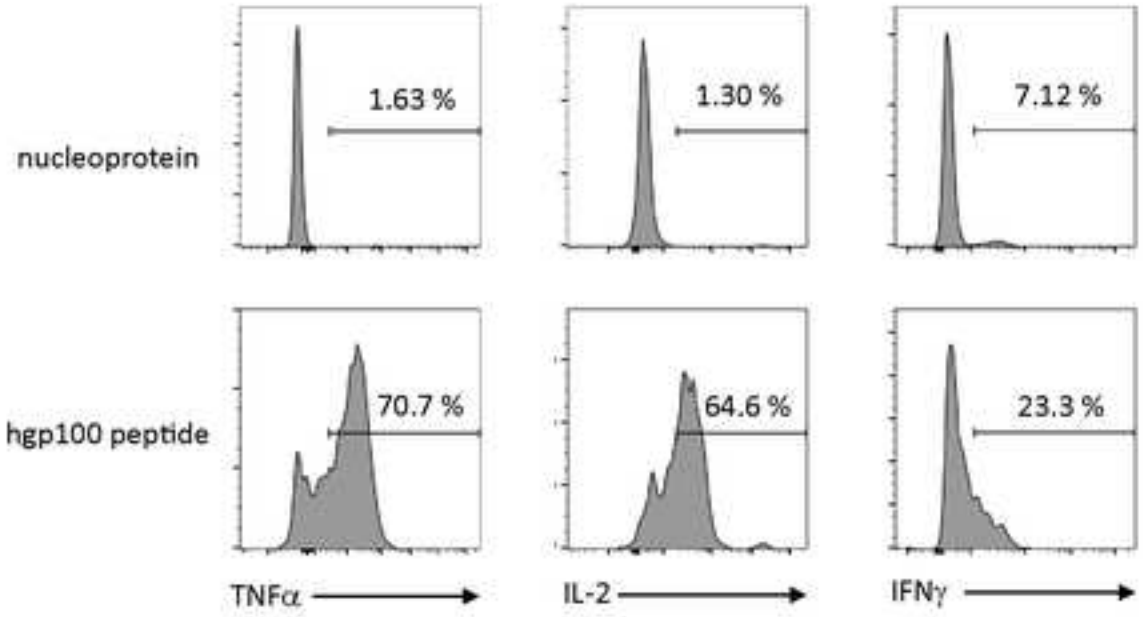


Figure 5

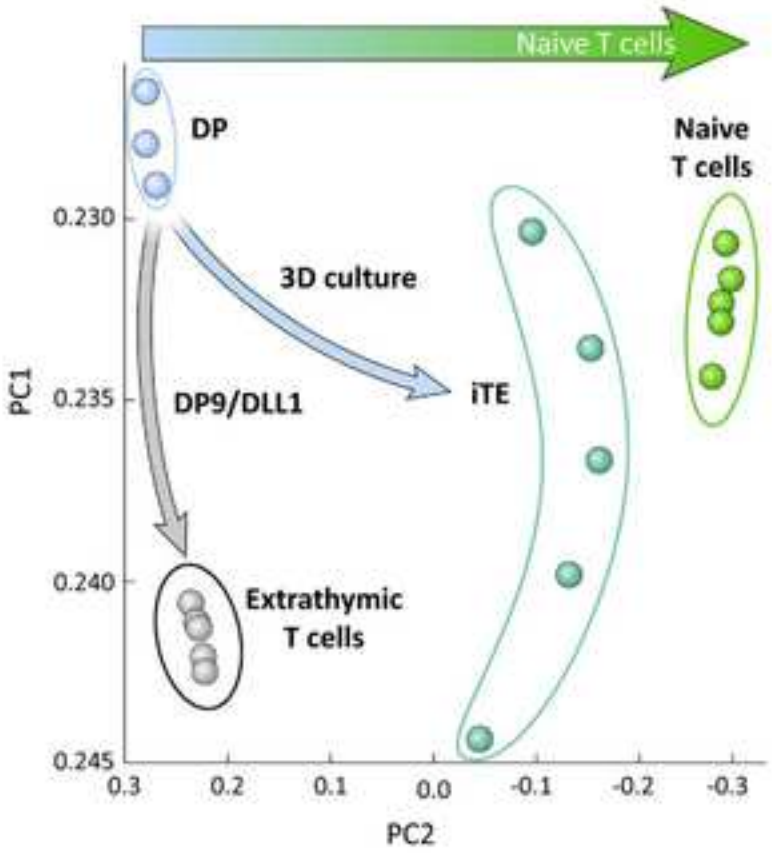


Table of Materials

Reagents/Materials	Sources	Catalog Number/RRID
Chemicals, Peptides and Recombinant Proteins		
2-deoxyguanosine	Sigma-Aldrich	312693-72-4
2-Mercaptoethanol (1000X)	Thermo Fisher Scientific	21985-023
ACK Lysing Buffer	Gibco	A1049201
Ascorbic acid	Sigma-Aldrich	A8960
Blasticidin	Thermo Fisher Scientific	R21001
FBS	Gemini	100-500
Flt-3 ligand	R&D Systems	427-FL
GlutaMAX (100X)	Thermo Fisher Scientific	35050-061
hgp100	Genscript	282077-1, KVPRNQDWL
Interleukin-2	R&D Systems	402-ML
Interleukin-7	R&D Systems	407-ML
MEM Non-Essential Amino Acids Solution	Gibco	11140050
MEM powder	Gibco	61100061
Monothioglycerol	Sigma-Aldrich	M-6145
Nucleoprotein	Global Peptides	ASNENMETM
Penicillin/streptomycin	Thermo Fisher Scientific	15140-122
Phosphate buffered saline pH 7.4 (1x)	Thermo Fisher Scientific	10010-023
Puromycin	Thermo Fisher Scientific	A1113803
RPMI 1640	Gibco	11875093
Sodium Pyruvate	Thermo Fisher Scientific	11360-070
Stem Cell Factor (SCF)	R&D Systems	455-MC
Stemfactor LIF, Mouse Recombinant	STEMGENT	03-0011-100
Trypsin-EDTA (0.05%), phenol red	Thermo Fisher Scientific	25300-062
Cell Culture Vessels and others		
10 cm dish	Corning, Inc.	353003
12ML Syringe	Covidien Monoject	22-652-090
6 well plate	Corning/Coster	3516

Cell strainer 100um	Fisher Scientific	22-363-549
Cell strainer 40um	Fisher Scientific	22-363-547
Forceps	DUMONT	0108-5PO
Lab soaker mat	Versi-Dry	Cat. EF2175CX 74018-00
Membrane filters (0.8 μm, 47diam)	Whatman	WHA7408004 ALDRICH
Perfecta3D Hanging Drop Plate	Sigma-Aldrich	HDP1096
U Bottom 96 well plate	Corning/Coster	3799
Experimental Cell lines		
CD3-iPSC	Vizcardo et al., Cell Report 2018	N/A
MEF-iPSC	Vizcardo et al., Cell Report 2018	N/A
Mouse Embryonic Fibroblasts (MEF)	ATCC	SCRC-1040; RRID:MGI:5007926
OP9/N-DLL1	Riken Bioresource center	Cat# RCB2927; RRID:CVCL_B220
Pmel-iPSC	Vizcardo et al., Cell Report 2018	N/A
Experimental mouse models		
B6.SJL-*Ptprc* ^{a} *Pepc* ^{b} /BoyCrCrl	Charles River	Strain Code 564; RRID:IMSR_CRL:564
C57BL/6N	NCI/Charles River	N/A
Pmel-1 mice	Overwijk et al.	J Exp Med 198(4):569-80
Antibodies		
Anti-αTCR	Biolegend	109202; RRID:AB_313425
Anti-CD3	abcam	ab11089; RRID:AB_369097
Anti-CD4	BD Biosciences	553730; RRID:AB_395014
Anti-CD44	BD Biosciences	559250; RRID:AB_398661
Anti-CD45.1	BD Biosciences	553775; RRID:AB_395043
Anti-CD45.2	BD Biosciences	553772; RRID:AB_395041
Anti-CD62L	BD Biosciences	560516; RRID:AB_1645257
Anti-CD69	BD Biosciences	552879; RRID:AB_394508
Anti-CD8α	BD Biosciences	557959; RRID:AB_396959
Anti-CD8β	BD Biosciences	550798; RRID:AB_393887
Anti-H-2Kb	BD Biosciences	553570; RRID:AB_394928

Anti-IFN-γ	BD Biosciences	557998; RRID:AB_396979
Anti-IL-2	BD Biosciences	554428; RRID:AB_395386
Anti-TCRβ	Thermo Fisher Scientific	35-5961-81; RRID:AB_469741
Anti-TCRVβ13	BD Biosciences	553204; RRID:AB_394706
Anti-TNFα	BD Biosciences	557644; RRID:AB_396761

jove VISUALIZED JOURNAL OF EXPERIMENTS
1 Alewife Center #200
Cambridge, MA 02140
tel. 617.945.9051
www.jove.com

ARTICLE AND VIDEO LICENSE AGREEMENT

Title of Article: 3D Thymic Culture System: A method for the Generation of Tumor Antigen-Specific iPSC-derived Thymic Emigrants

Author(s): Raul Vizcardo, SM Rafiqul Islam, Takuya Maeda, Naritaka Tamaoki, Marta Bosch-Marce, Meghan L. Good, Li Jia, Nicholas J. Klemen, Michael J. Kruhlak, Nicholas P. Restifo

Item 1: The Author elects to have the Materials be made available (as described at http://www.jove.com/publish) via:

☐ Standard Access ☒ Open Access

Item 2: Please select one of the following items:

☐ The Author is **NOT** a United States government employee.

☒ The Author is a United States government employee and the Materials were prepared in the course of his or her duties as a United States government employee.

☐ The Author is a United States government employee but the Materials were NOT prepared in the course of his or her duties as a United States government employee.

ARTICLE AND VIDEO LICENSE AGREEMENT

1. **Defined Terms.** As used in this Article and Video License Agreement, the following terms shall have the following meanings: "**Agreement**" means this Article and Video License Agreement; "**Article**" means the article specified on the last page of this Agreement, including any associated materials such as texts, figures, tables, artwork, abstracts, or summaries contained therein; "**Author**" means the author who is a signatory to this Agreement; "**Collective Work**" means a work, such as a periodical issue, anthology or encyclopedia, in which the Materials in their entirety in unmodified form, along with a number of other contributions, constituting separate and independent works in themselves, are assembled into a collective whole; "**CRC License**" means the Creative Commons Attribution-Non Commercial-No Derivs 3.0 Unported Agreement, the terms and conditions of which can be found at: http://creativecommons.org/licenses/by-nc-nd/3.0/legalcode; "**Derivative Work**" means a work based upon the Materials or upon the Materials and other pre-existing works, such as a translation, musical arrangement, dramatization, fictionalization, motion picture version, sound recording, art reproduction, abridgment, condensation, or any other form in which the Materials may be recast, transformed, or adapted; "**Institution**" means the institution, listed on the last page of this Agreement, by which the Author was employed at the time of the creation of the Materials; "**JoVE**" means MyJove Corporation, a Massachusetts corporation and the publisher of The Journal of Visualized Experiments; "**Materials**" means the Article and / or the Video; "**Parties**" means the Author and JoVE; "**Video**" means any video(s) made by the Author, alone or in conjunction with any other parties, or by JoVE or its affiliates or agents, individually or in collaboration with the Author or any other parties, incorporating all or any portion of the Article, and in which the Author may or may not appear.

2. **Background.** The Author, who is the author of the Article, in order to ensure the dissemination and protection of the Article, desires to have the JoVE publish the Article and create and transmit videos based on the Article. In furtherance of such goals, the Parties desire to memorialize in this Agreement the respective rights of each Party in and to the Article and the Video.

3. **Grant of Rights in Article.** In consideration of JoVE agreeing to publish the Article, the Author hereby grants to JoVE, subject to **Sections 4** and **7** below, the exclusive, royalty-free, perpetual (for the full term of copyright in the Article, including any extensions thereto) license (a) to publish, reproduce, distribute, display and store the Article in all forms, formats and media whether now known or hereafter developed (including without limitation in print, digital and electronic form) throughout the world, (b) to translate the Article into other languages, create adaptations, summaries or extracts of the Article or other Derivative Works (including, without limitation, the Video) or Collective Works based on all or any portion of the Article and exercise all of the rights set forth in (a) above in such translations, adaptations, summaries, extracts, Derivative Works or Collective Works and(c) to license others to do any or all of the above. The foregoing rights may be exercised in all media and formats, whether now known or hereafter devised, and include the right to make such modifications as are technically necessary to exercise the rights in other media and formats. If the "Open Access" box has been checked in **Item 1** above, JoVE and the Author hereby grant to the public all such rights in the Article as provided in, but subject to all limitations and requirements set forth in, the CRC License.

ARTICLE AND VIDEO LICENSE AGREEMENT

4. **Retention of Rights in Article.** Notwithstanding the exclusive license granted to JoVE in **Section 3** above, the Author shall, with respect to the Article, retain the non-exclusive right to use all or part of the Article for the non-commercial purpose of giving lectures, presentations or teaching classes, and to post a copy of the Article on the Institution's website or the Author's personal website, in each case provided that a link to the Article on the JoVE website is provided and notice of JoVE's copyright in the Article is included. All non-copyright intellectual property rights in and to the Article, such as patent rights, shall remain with the Author.

5. **Grant of Rights in Video – Standard Access.** This **Section 5** applies if the "Standard Access" box has been checked in **Item 1** above or if no box has been checked in **Item 1** above. In consideration of JoVE agreeing to produce, display or otherwise assist with the Video, the Author hereby acknowledges and agrees that, Subject to **Section 7** below, JoVE is and shall be the sole and exclusive owner of all rights of any nature, including, without limitation, all copyrights, in and to the Video. To the extent that, by law, the Author is deemed, now or at any time in the future, to have any rights of any nature in or to the Video, the Author hereby disclaims all such rights and transfers all such rights to JoVE.

6. **Grant of Rights in Video – Open Access.** This **Section 6** applies only if the "Open Access" box has been checked in **Item 1** above. In consideration of JoVE agreeing to produce, display or otherwise assist with the Video, the Author hereby grants to JoVE, subject to **Section 7** below, the exclusive, royalty-free, perpetual (for the full term of copyright in the Article, including any extensions thereto) license (a) to publish, reproduce, distribute, display and store the Video in all forms, formats and media whether now known or hereafter developed (including without limitation in print, digital and electronic form) throughout the world, (b) to translate the Video into other languages, create adaptations, summaries or extracts of the Video or other Derivative Works or Collective Works based on all or any portion of the Video and exercise all of the rights set forth in (a) above in such translations, adaptations, summaries, extracts, Derivative Works or Collective Works and (c) to license others to do any or all of the above. The foregoing rights may be exercised in all media and formats, whether now known or hereafter devised, and include the right to make such modifications as are technically necessary to exercise the rights in other media and formats. For any Video to which this **Section 6** is applicable, JoVE and the Author hereby grant to the public all such rights in the Video as provided in, but subject to all limitations and requirements set forth in, the CRC License.

7. **Government Employees.** If the Author is a United States government employee and the Article was prepared in the course of his or her duties as a United States government employee, as indicated in **Item 2** above, and any of the licenses or grants granted by the Author hereunder exceed the scope of the 17 U.S.C. 403, then the rights granted hereunder shall be limited to the maximum rights permitted under such statute. In such case, all provisions contained herein that are not in conflict with such statute shall remain in full force and effect, and all provisions contained herein that do so conflict shall be deemed to be amended so as to provide to JoVE the maximum rights permissible within such statute.

8. **Protection of the Work.** The Author(s) authorize JoVE to take steps in the Author(s) name and on their behalf if JoVE believes some third party could be infringing or might infringe the copyright of either the Author's Article and/or Video.

9. **Likeness, Privacy, Personality.** The Author hereby grants JoVE the right to use the Author's name, voice, likeness, picture, photograph, image, biography and performance in any way, commercial or otherwise, in connection with the Materials and the sale, promotion and distribution thereof. The Author hereby waives any and all rights he or she may have, relating to his or her appearance in the Video or otherwise relating to the Materials, under all applicable privacy, likeness, personality or similar laws.

10. **Author Warranties.** The Author represents and warrants that the Article is original, that it has not been published, that the copyright interest is owned by the Author (or, if more than one author is listed at the beginning of this Agreement, by such authors collectively) and has not been assigned, licensed, or otherwise transferred to any other party. The Author represents and warrants that the author(s) listed at the top of this Agreement are the only authors of the Materials. If more than one author is listed at the top of this Agreement and if any such author has not entered into a separate Article and Video License Agreement with JoVE relating to the Materials, the Author represents and warrants that the Author has been authorized by each of the other such authors to execute this Agreement on his or her behalf and to bind him or her with respect to the terms of this Agreement as if each of them had been a party hereto as an Author. The Author warrants that the use, reproduction, distribution, public or private performance or display, and/or modification of all or any portion of the Materials does not and will not violate, infringe and/or misappropriate the patent, trademark, intellectual property or other rights of any third party. The Author represents and warrants that it has and will continue to comply with all government, institutional and other regulations, including, without limitation all institutional, laboratory, hospital, ethical, human and animal treatment, privacy, and all other rules, regulations, laws, procedures or guidelines, applicable to the Materials, and that all research involving human and animal subjects has been approved by the Author's relevant institutional review board.

11. **JoVE Discretion.** If the Author requests the assistance of JoVE in producing the Video in the Author's facility, the Author shall ensure that the presence of JoVE employees, agents or independent contractors is in accordance with the relevant regulations of the Author's institution. If more than one author is listed at the beginning of this Agreement, JoVE may, in its sole

jove JOURNAL OF VISUALIZED EXPERIMENTS
1 Alewife Center #200
Cambridge, MA 02140
tel. 617.945.9051
www.jove.com

ARTICLE AND VIDEO LICENSE AGREEMENT

discretion, elect not take any action with respect to the Article until such time as it has received complete, executed Article and Video License Agreements from each such author. JoVE reserves the right, in its absolute and sole discretion and without giving any reason therefore, to accept or decline any work submitted to JoVE. JoVE and its employees, agents and independent contractors shall have full, unfettered access to the facilities of the Author or of the Author's institution as necessary to make the Video, whether actually published or not. JoVE has sole discretion as to the method of making and publishing the Materials, including, without limitation, to all decisions regarding editing, lighting, filming, timing of publication, if any, length, quality, content and the like.

12. **Indemnification.** The Author agrees to indemnify JoVE and/or its successors and assigns from and against any and all claims, costs, and expenses, including attorney's fees, arising out of any breach of any warranty or other representations contained herein. The Author further agrees to indemnify and hold harmless JoVE from and against any and all claims, costs, and expenses, including attorney's fees, resulting from the breach by the Author of any representation or warranty contained herein or from allegations or instances of violation of intellectual property rights, damage to the Author's or the Author's institution's facilities, fraud, libel, defamation, research, equipment, experiments, property damage, personal injury, violations of institutional, laboratory, hospital, ethical, human and animal treatment, privacy or other rules, regulations, laws, procedures or guidelines, liabilities and other losses or damages related in any way to the submission of work to JoVE, making of videos by JoVE, or publication in JoVE or elsewhere by JoVE. The Author shall be responsible for, and shall hold JoVE harmless from, damages caused by lack of sterilization, lack of cleanliness or by contamination due to the making of a video by JoVE its employees, agents or independent contractors. All sterilization, cleanliness or decontamination procedures shall be solely the responsibility of the Author and shall be undertaken at the Author's expense. All indemnifications provided herein shall include JoVE's attorney's fees and costs related to said losses or damages. Such indemnification and holding harmless shall include such losses or damages incurred by, or in connection with, acts or omissions of JoVE, its employees, agents or independent contractors.

13. **Fees.** To cover the cost incurred for publication, JoVE must receive payment before production and publication the Materials. Payment is due in 21 days of invoice. Should the Materials not be published due to an editorial or production decision, these funds will be returned to the Author. Withdrawal by the Author of any submitted Materials after final peer review approval will result in a US$1,200 fee to cover pre-production expenses incurred by JoVE. If payment is not received by the completion of filming, production and publication of the Materials will be suspended until payment is received.

14. **Transfer, Governing Law.** This Agreement may be assigned by JoVE and shall inure to the benefits of any of JoVE's successors and assignees. This Agreement shall be governed and construed by the internal laws of the Commonwealth of Massachusetts without giving effect to any conflict of law provision thereunder. This Agreement may be executed in counterparts, each of which shall be deemed an original, but all of which together shall be deemed to me one and the same agreement. A signed copy of this Agreement delivered by facsimile, e-mail or other means of electronic transmission shall be deemed to have the same legal effect as delivery of an original signed copy of this Agreement.

A signed copy of this document must be sent with all new submissions. Only one Agreement is required per submission.

CORRESPONDING AUTHOR

Name:	Raul Vizcardo
Department:	Center for Cell-based Therapy
Institution:	National Institutes of Health, National Cancer Institute
Title:	Team Leader
Signature:	
Date:	6/25/2018

Please submit a **signed** and **dated** copy of this license by one of the following three methods:

1. Upload an electronic version on the JoVE submission site
2. Fax the document to +1.866.381.2236
3. Mail the document to JoVE / Attn: JoVE Editorial / 1 Alewife Center #200 / Cambridge, MA 02140

You are being carbon copied ("cc:'d") on an e-mail "To" "R Dr. Vizcardo" raul.vizcardosakoda@nih.gov
CC: rafiqul.islam@nih.gov, takuya.maeda@nih.gov, naritaka.tamaoki@nih.gov, marta.bosch-marce@nih.gov, meghan.good@nih.gov, li.jia2@nih.gov, nicholas.klemen@yale.edu, mk396p@nih.gov, restifon@nih.gov

Dear Dr. Vizcardo,

Your manuscript, JoVE58672 3D Thymic Culture System: A Method for the Generation of Tumor Antigen-Specific iPSC-Derived Thymic Emigrants, has been editorially and peer reviewed, and the following comments need to be addressed. Note that editorial comments address both requirements for video production and formatting of the article for publication. Please track the changes within the manuscript to identify all of the edits.

Please note that the manuscript has been modified to include line numbers and minor formatting changes. The updated manuscript is attached and please use this updated version for future revisions.

After revising and uploading your submission, please also upload a separate rebuttal document that addresses each of the editorial and peer review comments individually. Please submit each figure as a vector image file to ensure high resolution throughout production: (.svg, .eps, .ai). If submitting as a .tif or .psd, please ensure that the image is 1920 pixels x 1080 pixels or 300 dpi.

Your revision is due by **Aug 03, 2018**.

To submit a revision, go to the JoVE submission site and log in as an author. You will find your submission under the heading "Submission Needing Revision".

Best,

Peer Review,
Peer Review
JoVE
617.674.1888
Follow us: Facebook | Twitter | LinkedIn
About JoVE

_____

Editorial comments:
Changes to be made by the Author(s) regarding the manuscript:
1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. The JoVE editor will not copy-edit your manuscript and any errors in the submitted revision may be present in the published version.
Done
2. Please upload each Figure individually to your Editorial Manager account as a .png, .tiff, .svg, .eps, .psd, or .ai file.
Done
3. Figure 2: Please explain the bottom two panels in the figure legend.
Done
4. Figure 4 lines 294: Should be upper right, not lower right.

Done

5. Please revise the title to be more concise.

Done

6. Please provide an email address for each author.

Email addresses for all of the authors have been included.

7. Please rephrase the Long Abstract to more clearly state the goal of the protocol.

The abstract was rephrased.

8. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials and Reagents. For example: GemCell, Invitrogen, Gibco, R&D Systems, Sigma Aldrich, Whatman, Dumont, etc.

We removed all commercial language.

9. Please place the ethics statement before your numbered protocol steps, indicating that the protocol follows the animal care guidelines of your institution.

Done

10. Please revise the protocol text to avoid the use of any personal pronouns (e.g., "we", "you", "our" etc.).

We fixed any personal pronouns in the protocol.

11. Please revise the protocol (lines 94-101, lines 116-117, lines 124-125, 3.1.1, 3.1.2, 4.14-4.16, etc.) to contain only action items that direct the reader to do something (e.g., “Do this,” “Ensure that,” etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as “could be,” “should be,” and “would be” throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a “Note.” Please include all safety procedures and use of hoods, etc. Please move the discussion about the protocol to the Discussion.

Done.

12. Please add more details to your protocol steps. There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol. Please ensure you answer the “how” question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action.

13. Line 94: Please specify culture conditions.

Corrected.

14. 1.1.6: What is the diameter of the petri-dish?

Corrected.

15. 2.3.1: What volume of PBS is used to wash?

Corrected.

16. 5.7: Please describe how this is done. How irradiation is set up? What is the distance between splenocytes and the irradiation source?

Since the technical specifications of laboratory radiators can vary widely, we preferred to mention only the total amount of irradiation exposure to the mice.

17. 6.1: What is used to count?

It was counted using a Neubauer hemocytometer and Trypan Blue. It is now specified in the text.

18. Please include single-line spaces between all paragraphs, headings, steps, etc.

Done.

19. After you have made all the recommended changes to your protocol (listed above), please highlight 2.75 pages or less of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol.

Highlighted.
20. Please highlight complete sentences (not parts of sentences). Please ensure that the highlighted part of the step includes at least one action that is written in imperative tense. Please do not highlight any steps describing anesthetization and euthanasia.
Done.
21. Please include all relevant details that are required to perform the step in the highlighting. For example: If step 2.5 is highlighted for filming and the details of how to perform the step are given in steps 2.5.1 and 2.5.2, then the sub-steps where the details are provided must be highlighted.
Done.
22. Please include at least one paragraph of text to explain the Representative Results in the context of the technique you have described, e.g., how do these results show the technique, suggestions about how to analyze the outcome, etc. The paragraph text should refer to all of the figures. However for figures showing the experimental set-up, please reference them in the Protocol. Data from both successful and sub-optimal experiments can be included.
The updated manuscript now includes a results section.
23. As we are a methods journal, please revise the Discussion to explicitly cover the following in detail in 3-6 paragraphs with citations:
a) Critical steps within the protocol
b) Any modifications and troubleshooting of the technique
c) Any limitations of the technique
d) The significance with respect to existing methods
e) Any future applications of the technique
The updated manuscript covers all the mentioned topics.
24. References: Please do not abbreviate journal titles.
Done.
25. Table of Equipment and Materials: Please provide lot numbers and RRIDs of antibodies, if available.
Done.

Reviewers' comments:

Reviewer #1:

Minor Concerns:
This is a useful protocol description that provides welcome detail to the3D culture system the authors described in a Cell Reports article published a few months ago. I have some minor concerns that could be addressed:
1. in line 134, is the IL-7 at 5ng/mL also? Please clarify.

Yes, cells were cultured with 5ng/mL FLT3L and 5 ng/mL IL-7 from Day 10. This has been corrected in the text.

2. In line 160, is this d0 or d1 of the 16 day developmental period? Please clarify.

Given that mice pairing will not occur until the room is dark, Day 1 pregnancy stage is counted as e0.5. Therefore, fetal thymic lobes harvested on Day16 are e15.5 mice embryos. These lobes were depleted of endogenous thymocytes by deoxy-guanosine treatment for an additional 7 days.

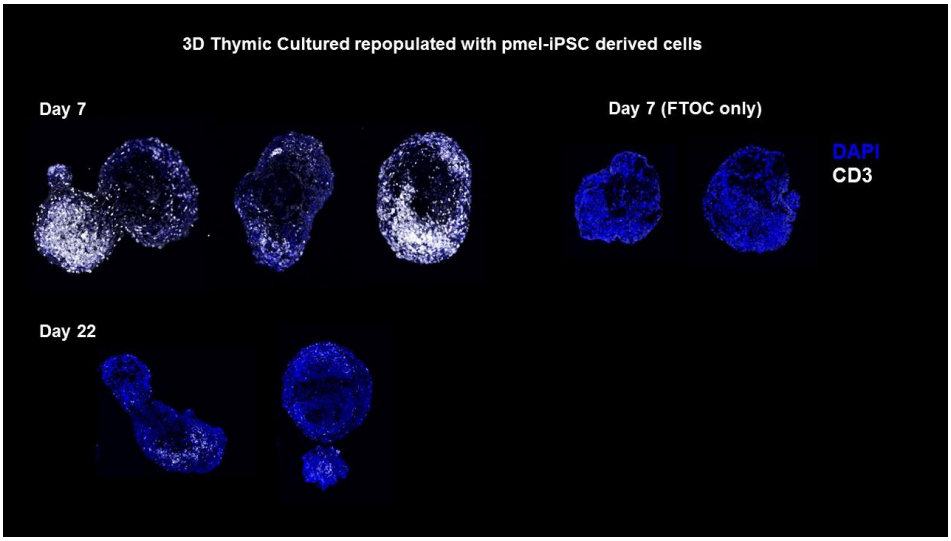
3. in lines 169-170, please expand on the intracellular cytokine staining protocol. When are the cells stains relative to their last stimulation with antigen? Is golgi-stop or golgi-plug used to amplify staining? How are the positive versus negative markers set? How were the samples in Figure 4 pre-gated? The legend to Figure 4 mentions "quadrants", but histograms are shown.

Thank you for the advice. iTE that exit the fetal thymic lobes are collected daily, therefore they are not pre-stimulated. iTE were stained 72 hrs after first stimulation. BD GolgiStop was used and incubated for 6 hrs to enhance the staining. Cells were gated on congenically marked, Live/Dead excluded CD3+CD8+ T cells. Bulk cells without antibody staining were used for negative controls (not shown). We added these changes to the main text.

4. Please show or mention the numbers of cells collected at each stage. It is crucial for the reader to understand just how many cells it is possible to generate with this system. This is an important point to discern just how useful this technique is likely to be.

Thymic lobes co-cultured with 2x10^3 bulk iPSC-derived T cell lineage for 6 days generated an average of 0.77±0.27x10^3 iTE overnight. These data are now presented as a distribution plot of 12 independent experiments (Figure 3B). As detailed in our recent Cell Reports published article, the 3D thymic culture system produced a daily halo of iTE from days 6-12. After day 12, T cell production varies from lobe to lobe, with some lobes still producing iTE at day 21 (See Panel A).

Panel A for editorial use only: We have observed iTE produced in the third week. To assess production of $CD3^+$ cells, we have attached a confocal image of 3D thymic cultures that we photographed 22 days after seeding with Pmel-iPSC.



Reviewer #2:

Manuscript Summary:
The JoVe manuscript by Vizcardo et al describes a readapted system using a multistep culture to obtain naïve T cells from iPS cells. The system as described involves a short term culture on OP9 delta like 1

without lymphoid cytokines, followed by a culture with lymphoid cytokines where T lineage cells are obtained and finally a organ culture in thymic lobes. This appears to be the crucial step of the method and it would just be useful to get more information on this phase of the methodology

Major Concerns:It would be useful to know for how long are these lobes efficient in generating naïve T cells, in other words, a time course and how many T cells are in general generated. Is the number of cells variable in different cultures. How reliable is the method? If the T cells coming out of several independent lobes are analyzed how much do they vary?

All these informations are also important to evaluate how much can this procedure be applied to different experimental situations

In general, differentiation experiments using induced pluripotent stem cells are characterized by inherent variability in yield and heterogeneity of their cell products. Our protocol was optimized to reduce the variability of the output in several steps. We included a more detailed description of the cell yield in Reviewer #1's comment number 4. Briefly, we generate up to 1x10^6 cells of T cell lineage from 1x10^6 starting iPSC. A single addition of 2x10^3 immature T cells into a single fetal thymic lobe produces a daily yield of 0.5-1x10^3 iTE between days 6-12. After day 12, we observe individual lobe variability in cell production, with some lobes still producing a low number of immature T cells even at Day 21 (See Panel A above).

Reviewer #3:

Manuscript Summary:
This manuscript represents a method to develop iPSC-derived T cells that are potentially useful to study anti-tumor immunity or other means to for adoptive T cell therapy to modify immune responses. Overall the method is well described. The included data support the feasibility of the approach

Major Concerns: None

Minor Concerns:
It would be useful for some discussion concerning the cells yields one might expect.
Thank you for your constructive comment. Please see our response to Reviewers #1 and #2 regarding this comment.

Reviewer #4:

Manuscript Summary:
The authors of this paper describe a new method to generate pluripotent stem cell (iPSC) derived T cells, using a 3D thymic organ culture system.
Successful reprograming of iPSC cells into antigen specific T cells in-vitro holds huge therapeutic potential to drive successful adoptive T cell therapy against advanced cancers. The classically used in-vitro OP9/DLL1 culture system, however, it is severely limited in its ability to successfully support T cell development, due to its inability to provide essential signals needed to drive differentiation of T cells past the DP stage in development. The authors, therefore, recreate the thymic environment, using a novel 3D thymic organ culture system, providing the necessary signals to support successful T cell

development from iPSC, and thus generating a CD8 T cell repertoire that is more successful after adoptive transfer.
Authors describe an interesting, novel method, which could have wide applications to understanding T cells development and improve adoptive T cell transfers, by enhancing in-vitro, antigen specific T cell generation. Overall the work is executed to a high standard and the method is detailed and easy to follow. No results provided support the claims that iTE generated in this culture system can proliferate, form memory populations and have anti-tumor properties, however this is referenced in the authors recent Cell Reports paper.

Minor Concerns:
Overall the protocol is very descriptive and easy to follow. General comments:

1. Section 3 'Mouse fetal organ isolation' has already been published on Jove; Jenkinson, W., Jenkinson, E., Anderson, G. Preparation of 2-dGuo-Treated Thymus Organ Cultures. J. Vis. Exp. (2008). This section, therefore, should be referenced, and may not be need.

We agree with this comment and have revised our manuscript.

2. Section 5 and 6 'preparation and pulsing of APC' is confusing. How are the authors ensuring a pure population of APC, and an accurate count of APC, on a total irradiated spleen sample that is not enriched or cell sorted. Clarification of why and how this is achieved is needed in this section. If this APC population is not pure, could it be affecting the results shown in figure 4?

As the reviewer highlights, the population of APC is not pure. We used whole irradiated live splenocytes depleted of red blood cells by ACK lysis. The APC origin can be excluded in the FACS because we are using a congenic marker. Moreover, since only the group co-cultured with hgp-100 peptide has a considerable increment of cells expressing TNFa, IL-2 and IFNg, we can conclude that iTE are antigen specific.

3. Overall more description is need for the results. No results section is provided, and figure legends have very little information. For example, what is Pmel-iTE? How was the RNA seq performed, on what populations?

We have included a Results section in the updated manuscript.

4. In the discussion authors refer to the system as long-term culture. Authors, therefore, should clarify how long these cultures systems can be kept in-vitro producing iTE.

The 3D thymic culture is able to generate a constant output of iTE between 6-12 days. After this, lobe-to-lobe production starts to become variable and the overall cell output decreases gradually. Some lobes are able to continue producing iTE even 21 days after co-culture.

5. Introduction needs more references. Long paragraphs with very few references.

Thank you for the advice.

6. This method is performed only with mouse material, so this could be included in the title.

We agree and have updated the title. Thank you for all the constructive comments.