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Construction of Thymus Organoids from Decellularized Thymus Scaffolds

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Summary

One of the hallmarks of modern medicine is the development of therapeutics that can modulate immune responses, especially the adaptive arm of immunity, for disease intervention and prevention. While tremendous progresses have been made in the past decades, manipulating the thymus, the primary lymphoid organ responsible for the development and education of T lymphocytes, remains a challenge. One of the major obstacles is the difficulty to reproduce its unique extracellular matrix (ECM) microenvironment that is essential for maintaining the function and survival of thymic epithelial cells (TECs), the predominant population of cells in the thymic stroma. Here, we described the construction of functional thymus organoids from decellularized thymus scaffolds repopulated with isolated TECs. Thymus decellularization was achieved by freeze/thaw cycles to induce intracellular ice crystal formation, followed by detergent-induced cell lysis. Cellular debris was removed with extensive wash. The decellularized thymus scaffolds can largely retain the 3-D extracellular matrix (ECM) microenvironment that can support the re-colonization of TECs. When transplanted into athymic nude mice, the reconstructed thymus organoids can effectively promote the homing of bone marrow-derived lymphocyte progenitors and support the development of a complex T cell repertoire. Bioengineering of thymus organoids can be a promising approach to rejuvenate/modulate the function of T-cell mediated adaptive immunity in regenerative medicine.

Keywords

Thymus; scaffold; tissue engineering; organoids; decellularization

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³When harvesting the thymus, it is critical that the thymus is not damaged. Any fissure or puncture will cause the thymus to collapse during the treatment, rendering it unusable for thymus organoid construction.

1. Introduction

As a pivotal immune organ in the adaptive immune system, the thymus is responsible for generating a diverse repertoire of T-cells that can effectively react to invading pathogens, while maintaining immune self-tolerance[1–3]. Paradoxically, the thymus glands in vertebrate animals begin to undergo a degenerative process termed “thymus involution”, the progressive reduction of tissue mass and function, at extremely young ages[4]. As early as the first year after birth, the stromal compartment of the human thymus begins to shrink about 3–5% per year until middle age and continues to decrease at an annual rate ~1% in the years followed[5, 6]. As the consequence of thymus deterioration, the newly generated T cells can no longer effectively replenish those lost in the periphery, resulting in constriction of the naïve T cell repertoire and expansion of the memory T cell pool[7, 8]. In addition to age-related thymus senescence, other pathological and environmental factors can also contribute to thymus involution. Inflammation caused by infectious pathogens such as viruses and bacteria can perturb the organization of the thymus microenvironments and accelerate the degeneration of thymic function[9, 10]. Chemotherapy and other cancer related treatments could cause irreversible damages to the thymus stroma, impeding timely recovery from immune deficiency. Thymus regeneration could be an effective means to rejuvenate the adaptive immune system and would have broad impacts in medicine[11–13].

While making up less than 0.5% of the total thymic cells, thymic epithelial cells (TECs) are the key population of residential stromal cells for the generation of T-cells[14–16]. After homing to the thymus, bone marrow-derived common lymphocyte progenitors follow a well-programmed, sequential order of steps (e.g. lineage restriction, somatic recombination of the T-cell receptor genes, and positive and negative selection), to differentiate into naïve T-cells[17]. TECs play essential regulatory roles in each of these steps; and proper cross talks between TECs and the developing thymocytes are critical to the development of a diverse and self-tolerant T-cell repertoire.

Unlike most of the epithelial cells making up the lining of tubular structures in organs and tissues, the endoderm-derived TECs are organized in three-dimensional (3-D) configurations. TECs cultured under 2-D conditions either undergo apoptosis or lose their molecular properties to support T cell development. Reproducing the 3-D thymic microenvironment by constructing artificial scaffolds with chemical or biological materials proves to be challenging as the composition and configuration of the extracellular matrix (ECM) provide not only the matrix support for the physical colonization of TECs, but also the necessary physiochemical signals to maintain their function[18]. Alternative to the chemical engineering approach, biological scaffolds have been successfully prepared from various organs (e.g. the heart, the lung, the liver and the kidney), and have been used to provide genuine ECM microenvironments for the parenchymal cells repopulated. Limited, but encouraging functional recoveries of the engineered organoids have been observed in preclinical studies[19, 20]. Taking advantage of the tissue engineering approach, we have reconstructed thymus organoids by repopulating decellularized thymus scaffolds with isolated TECs[21]. The thymus organoids can support T-cell development both *in vitro* and *in vivo*. When transplanted into athymic nude mice, the reconstructed thymus organoids enable the generation of a diverse T-cell repertoire that help to re-establish T-cell mediated

adaptive immune responses. Thus, bioengineering of thymus organoids can be an effective strategy to rejuvenate the adaptive immune system and

2. Materials

Equipments

- Dissecting scissors
- Curved forceps
- Cryotubes
- 5ml or 12ml round-bottom polystyrene tubes
- Pipets as needed
- Plate rocker
- 60mm and 100mm petri dish
- 50ml conical tube
- 1.5ml microcentrifuge tube
- 6-well tissue culture dish with transwell.

Solutions to prepare: (Note 1)

- Washing buffer: 1x PBS, 0.5% BSA, 2mM EDTA
- 0.5% SDS: sodium dodecyl sulfate, ddH₂O (for mice)
- 0.1% SDS: sodium dodecyl sulfate, ddH₂O
- MgSO₄/CaCl₂/Triton-X100 buffer: dH₂O, 5mM MgSO₄, 5mM CaCl₂, 1% Triton-X100
- 1x PBS
- RPMI-10: RPMI-1640, 10% FBS, 1% Penicillin/Streptomycin, 1% L-glutamine, 1% NEAA, 0.5% HEPES, 1% 2-mercaptoethanol.
- 60% w/v iodixanol

3. Methods

3.1 Mouse thymus decellularization

1. Harvest thymi from euthanized, appropriately aged mice (Note 2–4).
2. Freeze the thymi in cryotubes at –80°C. Thymi can be stored at –80°C until future use.

¹All solutions must be filtered sterile.

²The size of the thymus gland varies with age. 2–6 week-old mice provide a thymus scaffold with a suitable size for injections.

⁴It is not necessary to remove all the connective tissue or blood clots from the thymus after harvesting. It will not affect the decellularization steps.

3. Thaw frozen thymi in 30°C water bath for 20 minutes.
4. Freeze the thymi at –80°C in a styrofoam box for 20 minutes.
5. Repeat steps 3) and 4) twice. It is possible to stop the procedure at this step and store the thymi at –80°C until proceeding further.
6. Thaw thymi in 30°C water bath for 20 minutes.
7. Under sterile conditions, transfer the thymic samples to 5ml round-bottom polystyrene tubes with freshly prepared 3 ml 0.5% SDS solution. One thymus per tube is recommended to ensure thorough permeation of reagents (Note 5).
8. Place the tubes on a tube rocker set at moderate speed (~12–20 cycles per minute) at room temperature. Check the clarities of the thymic samples every hour and change the SDS solution every 1.5 – 2 hour for 2–3 times. The thymus will become transparent at the end of the procedure (Note 6, 7).
9. Transfer the thymus to a fresh 5ml flow tube with 3ml of 0.1% SDS and rock at 4°C overnight.
10. Transfer the decellularized thymus scaffolds to fresh tubes with 3ml Triton buffer (Note 8). Rock the tubes on the rocker at 4°C for 15 minutes. Repeat the washing step twice. Use fresh tubes each time.
11. Wash the thymus scaffolds in new tubes with 2ml of 1x PBS at 4°C for 30 minutes. Repeat the washing step two more times.
12. Store the thymus scaffolds in washing buffer at 4°C until use (Note 9).

3.2 Thymic cell isolation

1. Harvest thymus from euthanized mice and place in flow tubes with 3ml of washing buffer (Note 10).
2. Prepare the digesting solution and keep in a 37°C water bath until use.
3. In a 60mm petri dish with 6ml RPMI-1640, tear the thymi into small fragments (about 1mm) with 28G insulin syringes.
4. Rinse the thymic fragments briefly by pipetting up and down twice and discard the supernatant with a 5ml glass pipet (Note 11).

⁵It is advisable to perform the procedures under the laminar flow hood when handling thymi, in order to keep the thymus scaffolds sterile for cell culture purposes.

⁶Changing the solution after 1 hour is recommended for the 1st cycle, in order to remove most of the cellular components released.

⁷Depending on the size of thymus, decellularization with 0.5% SDS solution can be switched to 0.1% SDS once the thymus is clear, followed by an overnight incubation with 0.1% SDS.

⁸Thymi should be mostly transparent before replacing the solution to Triton buffer. It is fundamental to carefully verify the condition of the thymus to ensure the removal of debris from the thymus; in the next step, Triton X-100 in the washing buffer will re-nature the ECM proteins that are still left in the scaffold.

⁹Successfully decellularized thymus scaffolds are bulbous. Scaffolds will retain this property for about 1–2 months at 4°C. Scaffolds should be transferred to a culture medium for 30 minutes or more before cell injections.

¹⁰It is recommended that 3–4 thymi are processed in one batch, to obtain enough number of TECs for thymus scaffolds.

¹¹Glass pipet is necessary to avoid the attachment of thymic fragments on the pipet wall. Fragments can easily stick to plastic pipets, causing the loss of materials.

5. Add 6–7ml of fresh RPMI-1640 to the dish. Rinse the thymic fragments again with a glass pipet and discard the solution, leaving the fragments settled on the bottom of the dish (Note 12).
6. Transfer the thymic fragments into a 5ml round-bottom polystyrene tube with 3ml digesting solution (Note 13).
7. Incubate the thymic fragments on a rocker by gentle agitation at 37°C for 6 min.
8. After the incubation, aspirate the supernatant and transfer to a 50 ml conical tube with 10ml of washing buffer. Set aside on ice.
9. In the polystyrene tube with the thymic fragments, add another 3 ml of digesting solution and repeat the incubation at 37°C for 6 minutes.
10. During the 2nd digesting step, centrifuge the 50 ml tube with the supernatant with from the 1st digestion. Discard the supernatant, resuspend the cells in 10 ml washing buffer and keep on ice.
11. After the 2nd digestion is over, let the fragments settle to the bottom by gravity and transfer the supernatant to the tube in 10).
12. Add 3 ml digesting solution to the thymic fragments and incubate at 37°C for 6 minutes. After the incubation, pipet up and down 5 times to further break down the thymic fragments (Note 14).
13. Centrifuge the 50 ml tube containing all of the digested thymic fragments and digesting solution. Discard the supernatant, resuspend in 10ml of washing buffer and filter through 100µm strainer. Count the cells as necessary. Keep the cells on ice until further use.

3.3 TEC enrichment

1. Prepare 21% gradient medium solution from 60% w/v iodixanol.
2. Centrifuge the thymic cells and resuspend in 2.5ml washing buffer.
3. Add 20ml RPMI-1640 to the cell suspension. (Note 15)
4. Placing the tip of the pipette with 12ml of 21% gradient medium solution at the bottom of the tube. Let the density gradient solution drain from the pipette by gravity (Note 16).
5. Centrifuge at 600x g for 20 minutes at room temperature with decelerating brake off.

¹²Tilting the petri dish helps the fragments to sink to the bottom. When discarding the solution, it is better to aspirate slowly from the surface or from where fragments are not floating.

¹³Use 1–1.5ml of solution at a time to rinse off all the fragments from the surface of the petri dish.

¹⁴Fat tissue and connective tissue may remain undigested. These are distinguishable because they will often float at the surface, and can be transferred together with the rest of the solution.

¹⁵The red color in RPMI-1640 is help to distinguish the two layers.

¹⁶Add the gradient density solution as gentle as possible. It is important to form 2 distinct layers at this step. Any vigorous mixing will result in an obscure boundary after the centrifugation.

6. Transfer the top layer and the interface to a new 50 ml tube. These layers will include the enriched TECs (Note 17).
7. Wash the TECs by adding washing buffer to 40 ml and centrifuge at 400x g for 6 minutes.
8. Repeat the washing step twice (Note 18).
9. Resuspend the cells in 1 or 2ml of washing buffer, depending on the size of the pellet. Count the cells.

3.4 TEC isolation by FACS

1. Resuspend the cells in washing buffer at the concentration of 1×10^7 cells/100ul.
2. Add 2ul anti-CD16/CD32 antibody per 1×10^6 cells and incubate at 4°C for 10 minutes.
3. Add anti-CD45 and anti-EpCAM antibodies and incubate at 4°C for more than 20 minutes.
4. Wash the cells with 2ml of washing solution and centrifuge.
5. Resuspend the cells in 2 ml 1xPBS (Note 19).
6. Filter the cells through 100µm strainer.
7. Set the cell suspension for sorting. Select the CD45-G8.8+ population and sort into washing buffer by FACS. It is important to include not only the lymphocyte population but also bigger, more complicated cells in the SSC/FSC panel.
8. Keep the cells on ice until use.

3.5 Isolation of progenitor cells from the bone marrow

1. Harvest the bones from euthanized mice. Larger bones such as femur and tibia are more feasible to work with and the bone marrow can be collected more efficiently from these bones. Remove the muscles and connective tissues as much as possible with sharp scissors, and store in washing buffer.
2. Using 2 forceps and a sterile gauze, scrape off the remaining tissues from the bones on a sterile 100mm petri dish. Transfer the bones into a 60mm petri dish with washing buffer.
3. Hold a bone with the forceps. Fill a 28G insulin syringe with washing buffer, insert the needle into one end of the bone and flush out the bone marrow with washing buffer (Note 18). Work from both sides of the bone. Repeat the washing

¹⁷The interface is visible as an opaque ring, if the cell number in the layer is high. In some cases, the “ring” may not be distinguishable first and the boundary between the top and the bottom layer might be blurry after the spin. However, it will become clearer when the interface is removed.

¹⁸Cutting the tip of the bone will help for needle insertion into the bone, which is necessary to flush out the bone marrow. The practice will not negatively affect the amount progenitor cells obtained.

¹⁹Bone marrow is clearly visible when it is pushed out of the bone; and the bones will appear more white than pink afterwards.

step with the washing buffer to ensure that most of the bone marrow cells are flushed (Note 19).

4. Repeat the procedure to collect bone marrow from all of the bones.
5. Break down the bone marrow by passing the clumps through a 21-gauge needle on a 5ml syringe.
6. Pass the cells through a 40µm strainer into a 50 ml conical tube.
7. Adjust the total volume with washing buffer to 30 ml and centrifuge.
8. Remove the supernatant, and resuspend the cell pellet with 5ml of red blood cell lysis buffer. Incubate in the dark at room temperature for 5 minutes. Add 25ml of washing buffer and centrifuge.
9. Resuspend the cells in 10ml of washing buffer and count the cells.
10. Select the lineage negative cells with commercial kit (e.g. Miltenyi Biotec lineage cell depletion kit), following manufacturer's suggested protocol.
11. Collect the lineage negative cells. Resuspend the cells in washing buffer at 1×10^7 /ml and keep on ice until use.

3.6 Construction of thymus organoids

1. Under sterile condition, transfer the thymus scaffolds from washing buffer to a complete media at least 30 minutes prior to the cell injection. Keep at room temperature.
2. Mix the TECs and the lineage negative cells from the bone marrow at 1:1 ratio.
3. Centrifuge and collect in a 1.5ml microcentrifuge tube.
4. Centrifuge again and resuspend in the complete medium at the concentration of 20µl per 1×10^6 cells per scaffold (Note 20).
5. Fill the 28-gauge insulin needle with the cell suspension.
6. Take the scaffold out of the medium and place on a 12mm petri dish. Under the dissection microscope, gently pinch the scaffold with fine forceps and puncture the scaffold with the syringe needle.
7. Gently infuse the cell suspension in the scaffold, and slowly pull out the needle. If there are any unnoticed ruptures in the scaffold, the cells will start coming out and this phenomenon can be observed under the dissection microscope.
8. In a 6-well transwell dish, add 2 ml of pre-warmed complete medium in the bottom well. Place the reconstructed thymus scaffold onto the upper dish of the 6-well transwell and add 50–100ul of complete medium onto the scaffold (Note 21).

²⁰We typically limit the injection volume to 20µl per scaffold, to prevent overloading.

²¹Up to 4 scaffolds can be cultured together in the same 6-well insert.

9. Incubate at 37°C with 5% CO₂ until use.
10. If it is to be cultured for a long term, change half of the medium in the bottom well every other day. Note that the reconstructed scaffolds will start to shrink after 2–4 days of incubation.

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References

1. Klein L, Kyewski B, Allen PM, Hogquist KA. Positive and negative selection of the T cell repertoire: what thymocytes see (and don't see). *Nat Rev Immunol*. 2014; 14(6):377–91. DOI: 10.1038/nri3667 [PubMed: 24830344]
2. Fan Y, Gualtierotti G, Tajima A, Grupillo M, Coppola A, He J, et al. Compromised central tolerance of ICA69 induces multiple organ autoimmunity. *Journal of autoimmunity*. 2014; 53:10–25. DOI: 10.1016/j.jaut.2014.07.001 [PubMed: 25088457]
3. Fan Y, Rudert WA, Grupillo M, He J, Sisino G, Trucco M. Thymus-specific deletion of insulin induces autoimmune diabetes. *The EMBO journal*. 2009; 28(18):2812–24. DOI: 10.1038/emboj.2009.212 [PubMed: 19680229]
4. Boehm T, Swann JB. Thymus involution and regeneration: two sides of the same coin? *Nat Rev Immunol*. 2013; 13(11):831–8. DOI: 10.1038/nri3534 [PubMed: 24052146]
5. Bodey B, Bodey B Jr, Siegel SE, Kaiser HE. Involution of the mammalian thymus, one of the leading regulators of aging. *In vivo*. 1997; 11(5):421–40. [PubMed: 9427047]
6. Goronzy JJ, Fang F, Cavanagh MM, Qi Q, Weyand CM. Naive T cell maintenance and function in human aging. *Journal of immunology*. 2015; 194(9):4073–80. DOI: 10.4049/jimmunol.1500046
7. Nikolich-Zugich J, Rudd BD. Immune memory and aging: an infinite or finite resource? *Current opinion in immunology*. 2010; 22(4):535–40. DOI: 10.1016/j.coi.2010.06.011 [PubMed: 20674320]
8. Palmer DB. The effect of age on thymic function. *Frontiers in immunology*. 2013; 4:316.doi: 10.3389/fimmu.2013.00316 [PubMed: 24109481]
9. Borges M, Barreira-Silva P, Florido M, Jordan MB, Correia-Neves M, Appelberg R. Molecular and cellular mechanisms of *Mycobacterium avium*-induced thymic atrophy. *Journal of immunology*. 2012; 189(7):3600–8. DOI: 10.4049/jimmunol.1201525
10. Ye P, Kirschner DE, Kourtis AP. The thymus during HIV disease: role in pathogenesis and in immune recovery. *Current HIV research*. 2004; 2(2):177–83. [PubMed: 15078181]
11. Black S, De Gregorio E, Rappuoli R. Developing vaccines for an aging population. *Science translational medicine*. 2015; 7(281):281ps8.doi: 10.1126/scitranslmed.aaa0722
12. Di Stefano B, Graf T. Hi-TEC reprogramming for organ regeneration. *Nature cell biology*. 2014; 16(9):824–5. DOI: 10.1038/ncb3032 [PubMed: 25174818]
13. Bredenkamp N, Nowell CS, Blackburn CC. Regeneration of the aged thymus by a single transcription factor. *Development (Cambridge, England)*. 2014; 141(8):1627–37. DOI: 10.1242/dev.103614
14. Takahama Y. Journey through the thymus: stromal guides for T-cell development and selection. *Nat Rev Immunol*. 2006; 6(2):127–35. DOI: 10.1038/nri1781 [PubMed: 16491137]
15. Ohigashi I, Kozai M, Takahama Y. Development and developmental potential of cortical thymic epithelial cells. *Immunol Rev*. 2016; 271(1):10–22. DOI: 10.1111/imr.12404 [PubMed: 27088904]
16. Anderson G, Takahama Y. Thymic epithelial cells: working class heroes for T cell development and repertoire selection. *Trends Immunol*. 2012; 33(6):256–63. DOI: 10.1016/j.it.2012.03.005 [PubMed: 22591984]
17. Starr TK, Jameson SC, Hogquist KA. Positive and negative selection of T cells. *Annual review of immunology*. 2003; 21:139–76. DOI: 10.1146/annurev.immunol.21.120601.141107

18. Seach N, Mattesich M, Abberton K, Matsuda K, Tilkorn DJ, Rophael J, et al. Vascularized tissue engineering mouse chamber model supports thymopoiesis of ectopic thymus tissue grafts. *Tissue engineering Part C, Methods*. 2010; 16(3):543–51. DOI: 10.1089/ten.TEC.2009.0135 [PubMed: 19715386]
19. Baptista PM, Orlando G, Mirmalek-Sani SH, Siddiqui M, Atala A, Soker S. Whole organ decellularization - a tool for bioscaffold fabrication and organ bioengineering. *Conference proceedings : Annual International Conference of the IEEE Engineering in Medicine and Biology Society IEEE Engineering in Medicine and Biology Society Conference*. 2009; 2009:6526–9. DOI: 10.1109/IEMBS.2009.5333145
20. Booth C, Soker T, Baptista P, Ross CL, Soker S, Farooq U, et al. Liver bioengineering: current status and future perspectives. *World journal of gastroenterology : WJG*. 2012; 18(47):6926–34. DOI: 10.3748/wjg.v18.i47.6926 [PubMed: 23322990]
21. Fan Y, Tajima A, Goh SK, Geng X, Gualtierotti G, Grupillo M, et al. Bioengineering thymus organoids to restore thymic function and induce donor-specific immune tolerance to allografts. *Molecular therapy : the journal of the American Society of Gene Therapy*. 2015; doi: 10.1038/mt.2015.77