Video Article

Isolation of Myeloid Dendritic Cells and Epithelial Cells from Human Thymus

Christina Stoeckle[1,2], Ioanna A. Rota[1], Eva Tolosa[3], Christoph Haller[4], Arthur Melms[5], Eleni Adamopoulou[1]

[1]Department of General Neurology, Hertie Institute for Clinical Brain Research

[2]Institute of Pharmacology, University of Bern

[3]Department of Immunology, University Medical Center Hamburg-Eppendorf

[4]Department of Thoracic and Cardiovascular Surgery, University Clinic Tuebingen

[5]Department of Neurology, University Hospital Erlangen

Correspondence to: Eleni Adamopoulou at eleni.adamopoulou@uni-tuebingen.de

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Abstract

In this protocol we provide a method to isolate dendritic cells (DC) and epithelial cells (TEC) from the human thymus. DC and TEC are the major antigen presenting cell (APC) types found in a normal thymus and it is well established that they play distinct roles during thymic selection. These cells are localized in distinct microenvironments in the thymus and each APC type makes up only a minor population of cells. To further understand the biology of these cell types, characterization of these cell populations is highly desirable but due to their low frequency, isolation of any of these cell types requires an efficient and reproducible procedure. This protocol details a method to obtain cells suitable for characterization of diverse cellular properties. Thymic tissue is mechanically disrupted and after different steps of enzymatic digestion, the resulting cell suspension is enriched using a Percoll density centrifugation step. For isolation of myeloid DC ($CD11c^{+}$), cells from the low-density fraction (LDF) are immunoselected by magnetic cell sorting. Enrichment of TEC populations (mTEC, cTEC) is achieved by depletion of hematopoietic ($CD45^{hi}$) cells from the low-density Percoll cell fraction allowing their subsequent isolation via fluorescence activated cell sorting (FACS) using specific cell markers. The isolated cells can be used for different downstream applications.

Video Link

The video component of this article can be found at http://www.jove.com/video/50951/

Introduction

The thymus is the organ in which T cell development occurs. Its relative and absolute size decreases with age when it becomes successively replaced by fat although thymic activity can still be detected in the old age. Its importance for the immune response was demonstrated in the early 1960s[1].

The T cell repertoire is shaped through the interaction of T cell receptors with peptide-MHC complexes on different kinds of thymic APC, which provide survival or death cues to developing T cells, resulting in a functional and largely self-tolerant T cell repertoire[2].

Approximately 98% of the cells in the human thymus are developing T cells referred to as thymocytes. The remaining 2% consist of a number of different cell types, including a variety of TEC (cortical, medullary, subcapsular), myeloid and plasmacytoid DC (mDC, pDC), macrophages, B cells, mature re-circulating T cells, granulocytes, fibroblasts, endothelial cells and very rare epithelial cells with an expression phenotype resembling that of cells from other tissues such as muscle, neurons and respiratory epithelium (**Figure 1**). Of these, TEC and DC are the major APC types found in a normal thymus. In recent years, purification of these APC types for culture and molecular profiling has gained more and more interest. Due to their low frequency, isolation of any of these cell types for detailed analysis requires an efficient, reproducible and cost-effective procedure. The method presented here is a modification from previously published studies[3,4].

As with any other tissue, cell extraction from the thymus can be achieved by enzymatically disaggregating the cell-cell and cell-matrix interaction networks, in order to obtain a suspension of single cells. There are certain parameters like good dissociation efficiency, cell yield, cell viability and retention of cell surface markers that are crucial and need to be optimized for the successful isolation of these rare cell populations.

In this protocol, isolation of DC and TEC subsets is performed by making a single-cell suspension of the tissue by mechanical disruption and enzymatic digestion. We use Collagenase A from *Clostridium histolyticum*, which has a balanced ratio of different enzyme activities, to break down the native collagen that holds the tissue together. DNase I is included in the enzyme solution to reduce cell aggregation due to free DNA from dead cells (thymocytes are very sensitive).We also provide an alternative approach to the typical enzymatic tissue digestion involving mechanical and enzymatic tissue treatment assisted by a tissue dissociator. The single cell suspension is then subjected to a single Percoll

density centrifugation for enrichment of low density fraction (LDF) of cells. From this fraction of cells, DC can be isolated by staining for DC-surface markers (*i.e.* $CD11c^+$) and using magnetic separation or fluorescence-activated cell sorting (FACS). Unlike the lymphoid cells comprising the vast majority of cells in the thymus, TEC do not express CD45 at high levels, but are positive for the epithelial cell adhesion molecule EpCAM. cTEC can be distinguished from medullary TEC by the expression of a yet undefined antigen recognized by the CDR-2 (cortical dendritic reticulocyte-2) antibody[4,5] and somewhat lower EpCAM expression. The differential co-expression of EpCAM and CDR2 allows the efficient isolation of these TEC subsets via high-speed cell sorting[6].

The protocol presented here is optimized for human thymic tissue. The duration of the procedure depends on the amount of tissue and the ability of the experimenter as well as the speed of the cell sorter, if FACS sorting is used. Normally, the protocol for the isolation of DC can be completed within 5-6 hr and for the isolation of TEC in 8-10 hr. The isolation of DC and TEC subsets from thymic tissue is time sensitive. The faster the isolation procedure, the better the condition of the cells. Finally, the isolated cells can be used for further investigations like comparative studies of mRNA and protein expression, PCR experiments, protein isolation, molecular profiling (*i.e.* transcriptomics, micro RNA analysis) as well as cell culture[6].

Ethics Statement

In order to be able to work with human thymus tissue the researcher needs to obtain approval from the local ethics committee or responsible authorities as well as an informed written consent of the donor (or usually his or her parents, since tissue is usually obtained from underage children). Furthermore, all human tissues should be handled as being potentially infectious and appropriate measures should be taken, such as working with gloves, *etc.*

Protocol

1. Preparation of Tools, Enzyme Solutions, and Buffers

Perform the following preparative steps prior to beginning the protocol.

1. Tools

   Clean, dry and autoclave the following tools and keep them in sterile packaging until use.
   1. Small sharp scissors with either curved or straight tips for cutting the thymus tissue. Small curved forceps with serrated tips for handling the tissue.
   2. 50 ml Oak Ridge Centrifuge Tubes, PC, for the Percoll-density centrifugation step.

2. Enzyme solutions

   Prepare the Collagenase/DNase I enzyme mix (2 mg Collagenase/ml and 0.1 mg DNase I/ml) as follows:
   1. Dissolve 500 mg of lyophilized Collagenase A (Roche) in 250 ml of RPMI 1640 plain (no FCS) to obtain a 2 mg/ml Collagenase solution. Collagenase is quite hard to dissolve, so it is recommended to leave it some time on a roller-shaker at RT.
   2. Dissolve 100 mg DNase I (Roche) in 10 ml of sterile distilled water. 2.5 ml aliquots can be stored at -20 °C. Add 2.5 ml of the 10 mg DNase I solution in the 2 mg Collagenase A solution.
   3. Sterile filter the Collagenase/DNase mix using a Stericup filter unit (0.22 µm). Store in 10-20 ml aliquots at -20 °C until use.

3. Buffers and Solutions
   1. 1.5 M NaCl stock solution: Dissolve NaCl in distilled sterile water to a final concentration of 1.5 M. Filter the solution through a 0.22 µm filter and store at RT.
   2. 10x MACS buffer: 1x PBS (Ca^{2+}/Mg^{2+}-free) containing 5% BSA and 20 mM EDTA. Sterile filter the solution with a 0.22 µm filter and store at 4 °C. Prior to use prepare a 1x solution by diluting the 10x stock to 1x in cold sterile 1x PBS.
   3. FACS Buffer: 1x PBS (Ca^{2+}/Mg^{2+}-free) containing 1% BSA and 0.02% NaN_3.
   4. FACS Buffer for cell sorting: 1x PBS (Ca^{2+}/Mg^{2+}-free) containing 2% BSA. Sterilize using a 0.22 µm syringe filter and store at 4 °C.

2. Preparation of the Tissue

The processing of the tissue should be performed using sterile reagents and work in a laminar flow cabinet. Before the procedure begins, prepare the following reagents and equipment:

1. 1. Warm the following to RT: RPMI 1640 complete medium (RPMI 1640, 10% Fetal Calf Serum, 1% pen/strep), RPMI 1640 plain, Ca^{2+}/Mg^{2+}-free PBS and 2x Collagenase/DNase enzyme solution.
   2. Warm thermal incubator with rotation unit to 37 °C and cool fixed angle rotor centrifuge to 4 °C.
   3. Prepare a box with ice.

Note: the duration of this step depends on the condition and size of the tissue. Approximately 20-30 min is reasonable time needed for a medium size piece of tissue in good condition (~5 cm in width).

2. Place tissue in a Petri dish containing sterile PBS and rinse off any residual blood.

1. Add fresh PBS to prevent tissue from drying and using forceps and scissors clean the tissue from blood clots, connective and fat tissue and any part that does not look healthy.
2. Take care to remove necrotic tissue that will increase the amount of debris.
3. After cleaning, weigh tissue for reference.
4. Cut the tissue in approximately 1 cm^3 pieces/blocks. Add enough PBS to cover the tissue.

Note: For reproducibility of results, tissue should be cut into pieces of uniform size.

3. Using the back of a sterile syringe (10-20 ml) apply pressure (not too vigorous or prolonged) onto the thymic tissue pieces. This procedure will remove the bulk of thymocytes, thus reducing the tissue volume to be digested later. Perform this step on ice and **work fast**.
4. The solution will become visibly cloudy as thymocytes are released. Stir the dish gently and then with the help of a 5 ml pipette or a glass aspirator remove the supernatant containing the thymocytes taking care not to accidentally aspirate the tissue pieces.

Tip: A sterile cell culture scraper can be used to concentrate all tissue pieces at one side of the dish then tilt the dish (45° angle) and aspirate the supernatant. Replace with fresh PBS and repeat this procedure until the supernatant is relatively transparent. Perform last wash with RPMI plain.

5. Mince tissue pieces using sharp scissors (as finely as possible, fragments should be at least 2-4 mm). The fragments should be small enough to enter a 5-ml pipette.

3. Preparation of Single Cell Suspension from Thymus Tissue

Here, two alternative approaches for this step are described. The first approach describes a typical enzymatic tissue digestion (section 3.1) while the second involves mechanical and enzymatic tissue treatment assisted by a tissue dissociator (section 3.2).

This protocol is optimized for the processing of tissue samples weighing 5 g. For larger or smaller tissue samples, adjust enzyme volumes accordingly.

3.1 Typical enzymatic tissue digestion to obtain a single cell suspension

1. Place mashed tissue into 50 ml tubes with 10 ml collagenase/DNase solution per 5 g of tissue. Add RPMI plain to give a total volume of 20 ml.

Note: in a 50 ml tube digest up to 10 g of tissue. Adjust enzyme volume accordingly.

2. Incubate the tissue suspension for 40 min at 37 °C under slow rotation in a thermal incubator.

Note: the incubation steps can be performed either in a thermal incubator with a rotation unit, or in a 37 °C bacterial incubator with a shaker.

3. The enzyme solution will become cloudy as cells are released into it. At the end of the digestion, centrifuge tube at 110 x g for 2 min to sediment tissue fragments.
4. Collect supernatant from this digestion round. Pellet the cell suspension for 10 min at 400 x g. Aspirate supernatant and resuspend cells in 10-50 ml of RPMI complete depending on the size of the cell pellet.
5. Keep cell suspension in 37 °C incubator with the cap loose.
6. If larger cell numbers are desired, the digestion step can be repeated with the remaining tissue fragments and the first and the second digest pooled. Also, if TEC should be isolated, two rounds of digestions should be performed.
7. Dilute an aliquot of the cell suspension in trypan blue (1:10-1:100) and count viable cells using a hemocytometer.
8. Keep cells at 37 °C in the incubator until ready to proceed to the Percoll density centrifugation (section 4). Subsequent isolation of DC subsets (section 5) will be performed from these cells.

Note: thymic tissues can vary in their composition, especially with age, which affects the digestion efficiency and the generation of a single-cell suspension.

9. For the subsequent isolation of thymic epithelial cells (TEC) a third round of enzymatic digestion is required. Resuspend tissue remnants in 10 ml of fresh Collagenase/DNase solution plus 10 ml of RPMI plain and add Trypsin/EDTA (1:50 from 2.5% stock) in the solution.
10. Incubate at 37 °C for 40 min with gentle rotation. During the last 10 - 15 min of the incubation add FCS (1:5) to neutralize the activity of Trypsin.
11. Centrifuge at 110 x g for 2 min at RT to sediment the tissue fragments.

Note: The tissue may not be completely digested after these three rounds of digestions. Age and overall condition of the tissue are two parameters that influence the dissociation efficiency of the thymus tissue.

12. Collect the cell supernatant and discard the sedimented tissue fragments.
13. Centrifuge the cell supernatant at 400 x g for 10 min. Aspirate supernatant and resuspend cell pellet in RPMI complete.

Tip: Cell pellets are quite loose, so take care when discarding/aspirating supernatant.

14. Count viable cells using a hemocytometer. Place the cells in a 37 °C incubator until proceeding to Percoll separation (section 4). Subsequent pre-enrichment of TEC cells by depletion of $CD45^{hi}$ cells will be performed from these cells (section 6).

3.2 Mechanical and enzymatic tissue treatment assisted by a tissue dissociator

Using the tissue dissociator approach the tissue digestion steps can be reduced to half of the time needed with the approach described above (section 3.1). The following protocol is a modified version of that used to dissociate mouse thymus tissue for the isolation of TEC[7,8].

1. Transfer 5 g of minced tissue pieces (step 2.5) into a C Tube containing 10 ml of Collagenase/DNase solution.
2. Adapt C tube onto the tissue dissociator and run program m_spleen_02 (10 sec). Repeat this program 4-5x (40-50 sec).
3. Incubate with gentle rotation for 20 min at 37 °C.
4. Centrifuge at 110 x g for 2 min at RT.
5. Collect supernatant and proceed as in step 3.1.4. Add 10 ml of fresh Collagenase/DNase solution.
6. Adapt C tube onto the tissue dissociator and run program m_spleen_01 (56 sec).
7. Incubate at 37 °C for 20 min with gentle rotation.
8. Centrifuge at 110 x g for 2 min at RT.
9. Collect cell supernatant and pellet the cell suspension for 10 min at 400 x g. Proceed as in step 3.1.4.
10. As described in 3.1.9 a third round of digestion is needed for the subsequent isolation of thymic epithelial cells (TEC). For this, resuspend tissue remnants in 10 ml of fresh Collagenase/DNase solution plus Trypsin/EDTA (1:50 dilution of stock 2.5%)
11. Incubate at 37 °C for another 20 min with gentle rotation. Add FCS (1:5) and incubate for further 10 min.

Note: Using this approach, after this last digestion step the tissue is usually completely dissociated and no undigested tissue fragments are observed.

12. Proceed as in steps 3.1.11-3.1.14.

4. Enrichment of LDF of Cells Using Percoll-density Separation

After enzymatic digestion of the tissue, total thymic single cell suspensions are subjected to a single Percoll density centrifugation step to enrich for the LDF cells. The LDF of cells is highly enriched for APC. Both DC and TEC are detected in this fraction of cells.

1. Use single cell suspension obtained from the 1st and 2nd digests for mDC isolation (pooled cells, see step 3.1.8) or 3rd digest (for TEC isolation) (3.1.14). Centrifuge single cell suspension obtained from each digestion step at 400 x g for 10 min.
2. Prepare a Percoll solution with a final density of 1.07 g/ml during this wash step (see example in the Table below).

Preparation of **Percoll solution** to a final density of 1.07 g/ml **(ρ = 1.07)**				
Tube No.	1	2	3	4
Undiluted Percoll (ρ = 1.130) (ml)	2.96	5.92	8.88	11.84
1.5 M NaCl (ml)	0.6	1.20	1.8	2.4
distilled H_2O (ml)	2.44	4.88	7.32	9.76
Volume of final working dilution	6 ml	12 ml	18 ml	24 ml

3. The number of Percoll tubes depends on the cell numbers determined in steps 3.1.8 and/or 3.1.14. Combine the sterile solutions and mix thoroughly. For optimal isolation, 0.6-1 x 10^9 cells can be loaded per tube of Percoll. Combine the sterile solutions and mix thoroughly.

Tip: Percoll is light sensitive and in addition it should be kept cold. Prepare first the mixture containing the NaCl and H_2O (RT) and add undiluted Percoll last, just before resuspending the cells in the Percoll solution.

Note: Percoll density might vary between different suppliers and batches! If the density of the undiluted Percoll solution is not equal to 1.130 g/ml, use the instructions supplied by the manufacturer to calculate the exact amounts of Percoll and H_2O required to get a final density of 1.07 g/ml.

4. Resuspend up to 1 x 10^9 cells in 6 ml of the prepared Percoll solution (ρ = 1.07), mix sufficiently to get a homogenous suspension, and transfer to a 50 ml Oak Ridge polycarbonate screw cap centrifuge tube.
5. Carefully layer 30 ml of RPMI complete per tube with a pipette on top of the Percoll solution/cell suspension. Load the medium slowly, so that it remains on the top of the suspension and take care not to disturb the layer.
6. Weigh the tubes to make sure that they have equal weights so that they are balanced during centrifugation. If they are not, carefully add more medium to the lighter tube (under sterile conditions).
7. Carefully transfer the tubes to a pre-cooled centrifuge with a **fixed angle** rotor, and spin at 3,500 x g for 35 min, at 4 °C **with the brake off**. Make sure that the temperature of the centrifuge in not higher than 4 °C.
8. Remove tubes from the centrifuge and carefully collect the enriched APC, found at the interphase between Percoll and medium (low-density fraction) from each tube using a sterile Pasteur pipette. Transfer cells into a 50 ml tube containing cold RPMI complete. Fill up the tube in order to dilute out the remaining Percoll.
9. Centrifuge the cell suspension for 10 min at 300 x g at 4 °C. Discard supernatant and repeat wash.
10. Resuspend the cell pellet in medium and determine the cell number (using trypan blue and a hemocytometer). In our experience, the percentage of the LDF cells is about 2-20% of the total single cell suspension obtained after enzyme digestion. A typical yield of low-density enriched cells (from a young thymus, range 1 day-2 years) is 1x10^8 cells per 1 x 10^9, representing a 10% of total single cell suspension cells.

Note: At this stage, you may observe that cells aggregated in the suspension (especially with samples from children of older age). Cell clumps are indication of cell death and result from the release of DNA from the dying cells that can stick cells together. In such a case, add DNase I into the sample (50 µg/ml) and incubate it for up to 20 min at RT (gently invert every 5 min) to digest the free DNA molecules.

5. Isolation of Thymic mDC

After APC enrichment (via Percoll separation), mDC can be efficiently isolated from the LDF following the first and second round of enzymatic digestions. The following protocol is a modified version of magnetic cell separation for the isolation of mDC ($CD11c^+$).

1. Centrifuge APC enriched cells isolated from the single cell suspension obtained from the pooled first and second enzymatic digestion at 300 x g for 10 min at 4 °C and resuspend cell pellet in 100 µl MACS buffer per 10^7 cells.
2. Add 5 µl of CD11c-PE antibody per 10^7 cells.

Note: Titration experiments are recommended for optimal results.

3. Mix well and incubate for 15 min at 4 °C, protected from light.
4. Wash cells by adding 1-2 ml of MACS buffer per 10^7 cells and centrifuge at 300 x g for 10 min.
5. Aspirate supernatant completely and resuspend up to 10^7 cells in 80 µl of MACS buffer.
6. Add 20 µl anti-PE microbeads per 10^7 cells.
7. Mix well (**Do Not Vortex**) and incubate for 15 min at 4 °C protected from light.
8. Repeat as in step 5.4.
9. Resuspend up to 10^8 cells in 500 µl of buffer.
10. If necessary, filter cell suspension using a 40 µm cell strainer to remove debris and cell aggregates that may obstruct the flow of the column.
11. Use a LS column since thymus cell suspensions flow better through the LS columns. Prepare LS column following manufacturer's instructions. Pipette cell suspension slowly into the column avoiding generating bubbles. Use one column for every 1 x 10^8 cells.
12. Wash column following the manufacturer's instructions. Remove column from magnet and place it in a sterile 12 ml round bottom polypropylene tube. Add 3 ml MACS buffer onto the column and collect magnetically-labeled cells by inserting and firmly pushing the plunger into the column.
13. Collect the eluted fraction representing the $CD11c^+$ mDC and centrifuge at 400 x g for 6 min.
14. Determine cell number and confirm purity of the selected cell population by flow cytometric analysis. Suggested markers include CD45, CD11c, and HLA-DR.

Note: $CD11c^+$mDC may also be isolated using a FACS sorter.

6. Enrichment of $CD45^{lo/neg}$ Cells for Cell Sorting of TEC

For isolation of TEC, cells can be pre-enriched by depletion of $CD45^{hi}$ cells using CD45 microbeads, in order to speed up their isolation through cell sorting.

Use single cell suspension obtained from the 3rd digestion step (3.1.14) and subsequently separated by Percoll centrifugation.

1. Centrifuge cell suspension at 300 x g for 10 min at 4 °C and resuspend cell pellet in 80 µl MACS buffer per 10^7cells.
2. Use CD45 microbeads at 1/3 of the recommended amount (6.7 µl) per 10^7cells.

Note: By using a lower amount of the microbeads we pre-enrich cells for both $CD45^{lo}$ and $CD45^{neg}$ cells. Titration is recommended for optimal results.

3. Mix well by gently flicking the tube (**Do Not Vortex**) and incubate for 15 min at 4 °C.
4. Wash unbound microbeads by adding 10 ml of MACS buffer and centrifuge for 10 min at 300 x g at 4 °C. Aspirate supernatant completely.
5. Resuspend up to 10^8 cells in 500 µl of buffer.
6. If necessary, filter cell suspension using a 70 µm cell strainer to remove debris and cell aggregates that may obstruct the flow of the column.
7. Use a LS column since thymus cell suspensions flow better through the LS columns. Prepare LS column following manufacturer's instructions. Pipette cell suspension slowly into the column avoiding generating bubbles. Use one column for every 10^8 cells.
8. Collect unlabeled cells (flow-through and washes) containing the $CD45^{lo/neg}$ cell fraction and wash column following manufacturer's instructions.
9. Centrifuge at 300 x g for 10 min at 4 °C and resuspend cell pellet in sterile FACS buffer in order to determine cell number and proceed to staining for cell sorting.

7. Stain Cells for Fluorescence Activated Cell Sorting

1. Perform FcR blocking prior to cell staining, in order to reduce non-specific antibody binding. Incubate cell suspension with pooled human immunoglobulins solution for 15 min at RT. These reagents are commercially available (*i.e.* Gammunex 10% solution).
2. Wash cells by adding cold sterile FACS buffer. Centrifuge at 400 x g for 6 min at 4 °C.
3. Discard supernatant and resuspend cells in cold sterile FACS buffer. Prepare 5 samples according to the following example:

Sample	Sample type	Antibody	Cell Number	Total volume
1. unstained	control		1×10^6	50 µl
2.scc*-Pacific Blue	control	CD45-Pacific Blue	1×10^6	50 µl
3. scc-APC	control	CD3-APC	1×10^6	50 µl
4.scc-Alexa 488	control	CD8-Alexa 488	1×10^6	50 µl
5. Cell sorting	analyte	CD45-Pacific Blue EpCAM-APC CDR2-Alexa 488	10×10^6 - 50×10^6	500 µl 10×10^6 cells/100 µl

*scc; single color control

4. Incubate cells with antibodies for 30 min, on ice in the dark.
5. Wash twice with FACs buffer by centrifuging cells at 400 x g for 6 min at 4 °C.
6. Adjust the cell concentration of the sample to be sorted to 1 $x10^7$ cells/ml (or to the concentration recommended by the cell sorting facility) using sterile FACS buffer for cell sorting (*i.e.* without NaN_3).
7. Pass cell sample through a 70 µm cell strainer to remove any cell clumps that may clog the cytometer during sorting.
8. Resuspend control samples in 200-400 µl of buffer. Keep tubes on ice and protected from light.
9. Prepare collection tubes with medium.

Notes:

- Perform titration of the antibodies for optimal staining.
- In one sample for sorting, up to 50 x 10^6 cells can be stained in a total reaction volume of 500 µl.
- Perform staining of the sample to be sorted in 5 ml Falcon Polystyrene round bottom tubes. The single color controls can be stained in separate wells of a 96-well plate.
- Since TEC subsets are rare populations, it is advisable to use another positive marker (of high frequency) for each fluorochrome to facilitate determination of proper FACS settings and to make sure you have proper compensation if the choice of fluorochromes requires compensation. Cells from the $CD45^+$ fraction can be stained with markers such as CD45, CD3 or CD8 for the different fluorochromes and after staining unstained $CD45^+$ cells can be added to the sample.

8. Isolation of TEC by Fluorescence Activated Cell Sorting

TEC subsets can be sorted from the $CD45^{lo/neg}$ fraction as $EpCAM^{hi}CDR2^-$ (mTEC) or $EpCAM^{lo}$ $CDR2^+$ (cTEC).

1. Run the sample with the unstained cells and adjust the forward and side scatter in order to place the population of interest in scale. Adjust the voltage on each detector so that the cells are visible but located in the far left hand portion of the histogram.
2. Run each single stained sample and adjust compensation for each color.
3. After adjusting the voltage and compensation for the unstained and single stained controls run the sample to be sorted and use gating tools to define the population of interest. Use a wide forward and side scatter gate to ensure inclusion of all stromal cell sizes, while excluding cell debris.
4. On the dot plot with CD45-Pacific Blue versus Forward Scatter gate on the $CD45^{low}$ and $CD45^{neg}$ cells.
5. Apply this gate to an EpCAM versus CDR2 dot plot and determine by gating the populations to be sorted.
6. Once gates have been determined, select the gates containing the populations of interest and start sorting.
7. Once sorted cells have been collected, centrifuge the cell suspension, and resuspend cells in medium or buffer of choice depending the intended downstream application.

Note: During sorting in order to prevent cells from sticking to the sides of the collection tube, the tube can be pre-coated by filling it up with 50% FCS in PBS and incubated at RT for 30 min. Discard the coating solution before adding collection media.

Representative Results

As starting material in this protocol we use thymus tissue removed from children undergoing corrective cardiovascular surgery (Department of Thoracic and Cardiovascular Surgery, University Clinic Tuebingen) obtained after informed consent and under institutional guidelines. This discarded material can vary greatly in size from 2-30 g or more. The number of mDC and TEC subsets (cTEC and mTEC) that are obtained depends on the size as well as the age of the thymus tissue sample used for isolation.

Figure 2 shows a rather large piece of tissue (~9 cm) obtained from a six year old child. An important first preparative step in this protocol is the cleaning of the tissue from undesirable parts (indicated by white arrows).

Thymus tissue was treated as outlined in sections 2 and 3.1 of the protocol to obtain a single cell suspension which was separated using a single Percoll density centrifugation step While the single cell suspension contains approximately 3% of HLA-DR+ cells (**Figure 3A**), after Percoll centrifugation this percentage is increased in the low-density fraction (LDF), containing large-sized cells, to 15-40% while the high-density fraction (HDF) that consists mainly of uniform small-sized thymocytes contains virtually no such cells (**Figure 3B**). The fraction of interest (LDF), containing enriched APC was subsequently collected and washed to collect cells for use in subsequent isolation steps.

While thymic mDC can be identified by the expression of a number of surface markers, including CD11c9,10, pDC express markers such as BDCA-2, BDCA-4, CD45RA and CD123[11,12]. After enrichment of the LDF cells, both cell types constitute 2-10% of this fraction, allowing their efficient isolation also in larger numbers, either by magnetic cell separation or flow sorting of $CD11c^+$ or $BDCA\text{-}4^+$ cells[6].

Figure 4 shows the efficient isolation of $CD11c^+$ cells using the modified magnetic cell separation protocol described in section 5. After isolation, the purity of the $CD11c^+$ DC was 93% as established by immunostaining of isolated cells. On average, the recovery of isolated $CD11c^+$DC is 5 x 10^5-5 x 10^6 mDC per 10^9 total thymic cells. **Table 1** shows data obtained from several individual thymus samples of different age and tissue weight with a range of input single cell suspensions as well as total LDF numbers and the subsequent $CD11c^+$ yield and purity.

In the APC enriched fraction, only approximately 0.5% of the cells are $CD45^{lo}EpCAM^+$ or $CD45^{neg}EpCAM^+$. cTEC and mTEC can be sorted from the trypsin-digested $CD45^{lo/neg}$ enriched stromal cells as $EpCAM^{lo}CDR2^+$ and $EpCAM^{hi}CDR2^-$, respectively as shown in **Figure 5**.

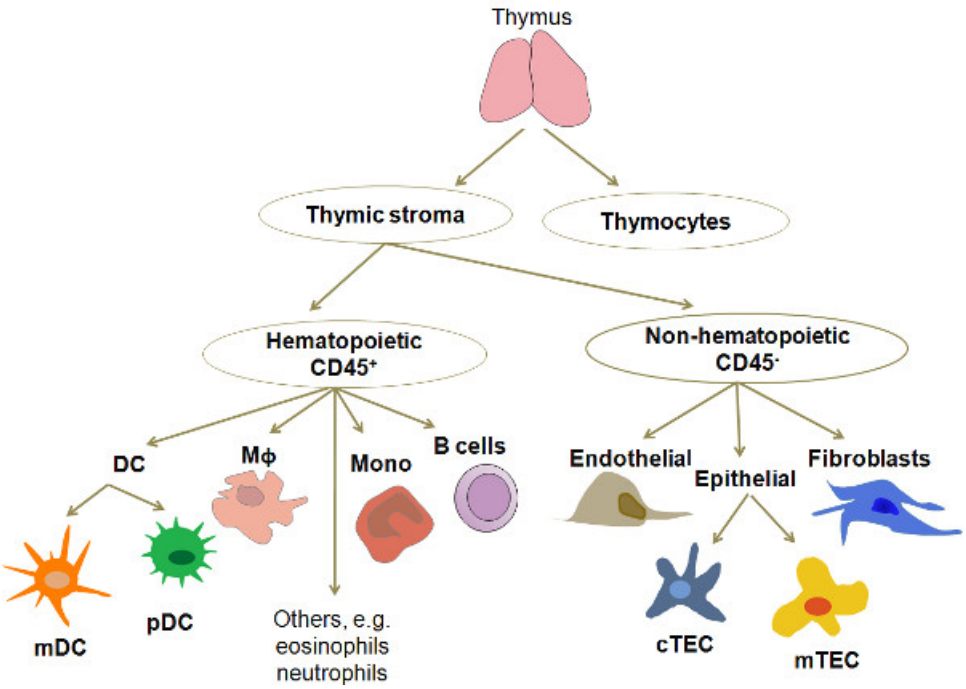

Figure 1. A simplified diagram of the cellular organization and composition of the human thymus. The thymus consists of thymocytes, at different maturation stages, and a heterogeneous cellular network referred to as the thymic stroma forming the thymic environment. The main cell types of the thymic stroma are mDC and pDC, epithelial cells (divided into two main categories according to their localization within the lobule: cortical, and medullary) and macrophages. *Abbreviations:* DC, dendritic cells; mDC, myeloid dendritic cells; pDC, plasmacytoid dendritic cells; Mφ, macrophage; mono, monocytes; cTEC, cortical epithelial cells; mTEC, medullary epithelial cells.

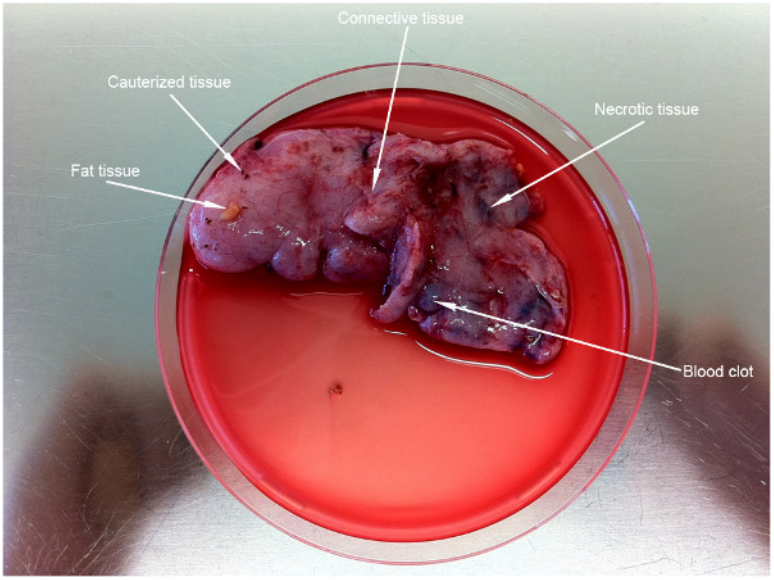

Figure 2. First preparative step of the tissue for single-cell preparation. Arrows point to the undesirable tissue parts that need to be discarded prior to the execution of the protocol.

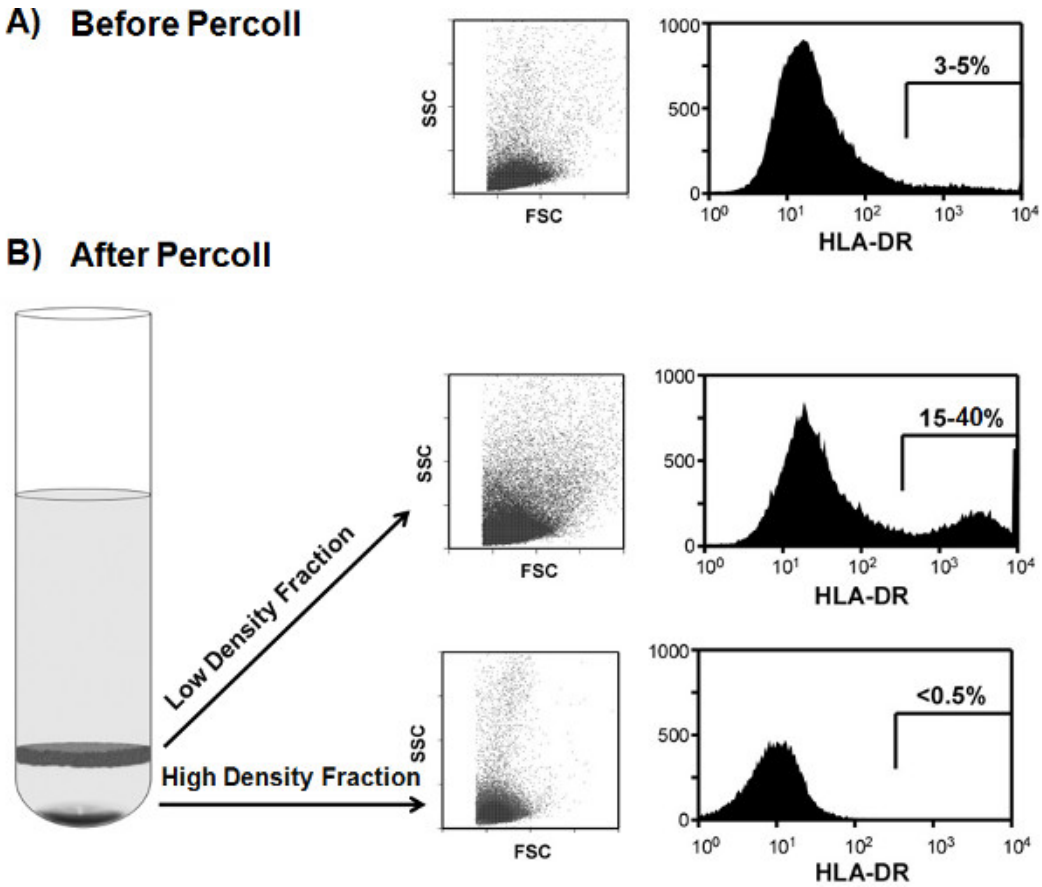

Figure 3. Purification of thymic APC. FACS analysis of different stages of the purification procedure. Single cell suspensions of thymus tissue were obtained by mechanical disruption and serial enzymatic digestions and APC enriched (LDF cells) by Percoll-density separation. Cells before (**A**) and after (**B**) the Percoll-density separation were stained with HLA-DR-PE antibody to check for APC enrichment. Percentage range of HLA-DR+ cells typically observed is indicated.

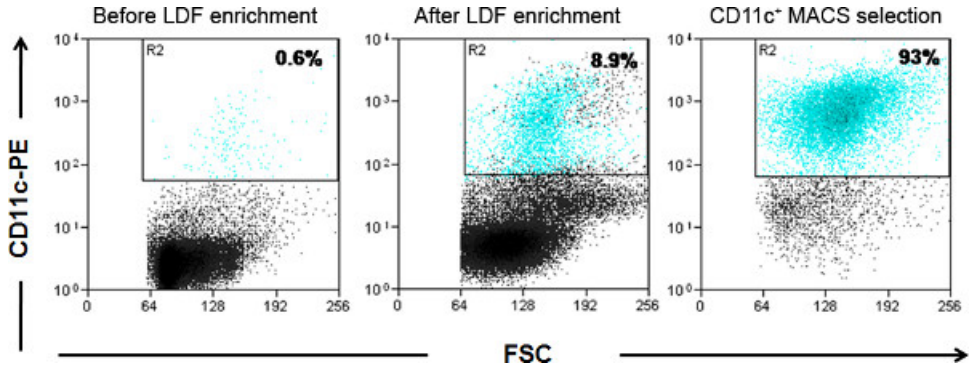

Figure 4. Isolation of CD11c$^+$ mDC. Thymus tissue was digested using Collagenase A/DNase I to create a single cell suspension. CD11c$^+$mDC represent only a minor population of the single cell suspension (0.6%). After enrichment of the LDF cells via Percoll-density separation the percentage of CD11c$^+$ cells increases to ~9%. The LDF cells were labeled with CD11c-PE antibody and subsequently isolated using magnetic beads (anti-PE). Reanalysis of the CD11c$^+$ population directly after magnetic cell separation by FACS indicated 93% purity. Click here to view larger figure.

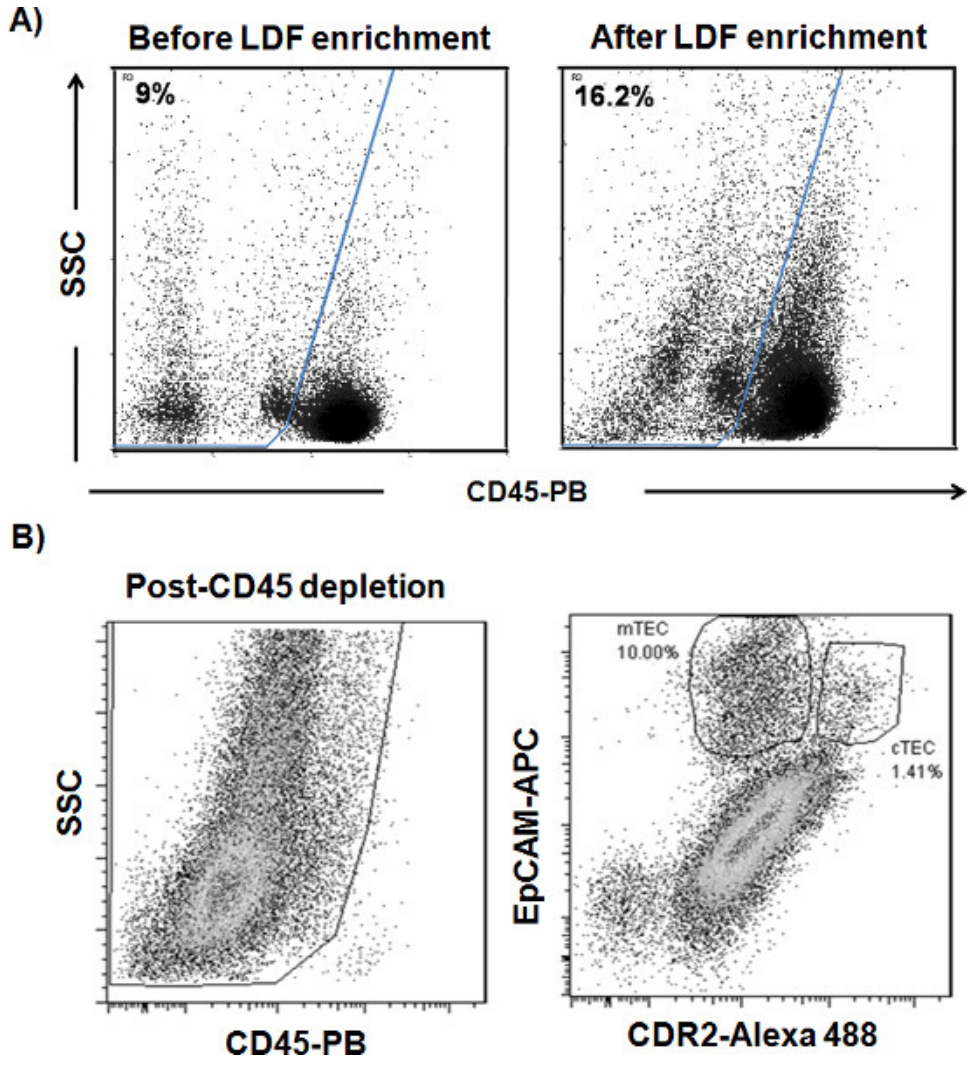

Figure 5. Enrichment of CD45$^{lo/neg}$ cells and subsequent sorting of TEC subsets. A) Representative dot plots showing percentages of CD45 negative/low thymic stromal cells in total single cell suspensions before and after Percoll density separation (9% and 16.2%, respectively). **B**) The LDF cells was depleted of CD45^{hi} cells using magnetic beads and the TEC subsets sorted from the depleted fraction as EpCAM^{hi}CDR2$^-$ (mTEC) or EpCAM^{lo}CDR2$^+$ (cTEC).

CD11c$^+$ mDC frequencies, cell yields and purities of different donors							
Donor	**Age**	**Tissue weight**	**Total cells per sample**	**LDF cells per sample**	**CD11c$^+$ %**	**CD11c$^+$isolated per sample**	**CD11c$^+$ purity**
1	5 days	7 g	1.45 x 10^9	9.5 x10^7	12.6	2.4 x 10^6	89%
2	3 years	15 g	2 x 10^9	6.5 x10^7	4.3	1.9 x 10^6	81%
3	21 days	9 g	2.6 x 10^9	22 x10^7	10.3	3.6 x 10^6	93%
4	6 years	13 g	1.4 x 10^9	25.4 x10^7	4.2	5.7 x 10^6	82%
5	5 days	6.5 g	1.3 x 10^9	16.2 x10^7	6	2.9 x 10^6	91%
6	39 days	3 g	0.6 x 10^9	8 x10^7	3.4	1.3 x 10^6	80%

Table 1. Numbers of total single cell suspension cells released after two rounds of digestion as well as yields of LDF cells after Percoll separation are shown for isolations performed in thymus samples of six individuals of different age and tissue weight. Frequencies of mDC were determined by flow cytometry based on CD11c expression in total LDF cells (APC enriched).Yield and purity of CD11c$^+$ cells after magnetic bead separation are shown for each individual donor.

Discussion

The protocol described here is a modification of the protocol published by Gotter *et al*[4]. Critical steps in the protocol are the condition and initial preparation of the tissue as well as the Percoll density separation. We strongly recommend to process the tissue as soon as possible after collection. It is important to work fast but thoroughly when cleaning and cutting the tissue. During the thymocyte wash described in step 2.3, it is crucial to find the right balance when applying pressure with the back of the syringe plunger on the tissue pieces as too vigorous and prolonged pressure can damage the stromal cells. Layering the medium on top of the Percoll solution/cell suspension without causing perturbations may also require some practice. After centrifugation, it is important to collect all the low density fraction cells as quickly as possible from the Percoll layer with a minimum amount of Percoll solution.

The overall cell yields and relative APC numbers can be very variable (see **Table 1**) and depend on the donor (especially with respect to age) and the preparative skills of the experimenter. For a brief overview, cell suspensions can be stained with HLA-DR and/or other markers following different steps of the protocol as desired. All antibodies used for phenotypic analysis should be titrated to achieve optimal results.

Due to the relatively low abundance of APC types in the thymus compared to thymoctyes, larger pieces of thymus might be required if higher numbers of isolated cells are desired.

If a tissue dissociator is available in the lab, this will considerably speed up tissue processing, but both variations described here work equally well. A relatively frequent problem might be clumping of the cells, especially during the steps between the Percoll separation and MACS/FACS sorting. In this case, a short DNase I digestion as described in the protocol will usually solve the problem. Since thymus tissue is successively replaced by fat tissue during aging, thymi from older donors might contain some fat, which will swim on top of the tube after centrifuging the single cell suspensions obtained from the different digestion steps. In such a case, fat should be removed with a pipette before continuing the protocol. For optimal results as well as economy reasons we advice to titrate not only the antibodies but also the anti-PE microbeads (sections 5 and 6). For isolation of TEC from mouse thymus, alternative enzyme mixtures have been described to enhance TEC yield[8,13], but we have not tested them with human tissue.

In principle, other thymic APC and stromal cells like B cells and pDC can also be isolated from the LDF cells (following collagenase/DNase digestion and Percoll) by FACS/MACS sorting if the respective markers are known. For example, for pDC, BDCA-2, BDCA-4 and CD123 have been described as markers[6,14]. If analysis or isolation of thymocyte subsets is desired, we recommend using the cells released by squeezing (step 2.3), since they are quite pure and have undergone minimal manipulation.

Disclosures

No conflict of interest declared.

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