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TITLE:

Investigating Aortic Valve Calcification via Isolation and Culture of T Lymphocytes using Feeder Cells from Irradiated Buffy Coat

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KEYWORDS:
Aortic valve disease; aortic stenosis; T cells extraction; TAVI; buffy coat; flow cytometry analysis; adaptive immunity.

SUMMARY:
In this study, we describe the process of T lymphocyte isolation from fresh samples of calcified aortic valves and the analytical steps of T cell-cloning for the characterization of the adaptive leukocyte subsets by using flow cytometry analysis.

ABSTRACT:
Calcific aortic valve disease (CAVD), an active disease process ranging from mild thickening of the valve to severe calcification, is associated with high mortality, despite new therapeutic options such as transcatheter aortic valve replacement (TAVR).

The complete pathways that start with valve calcification and lead to severe aortic stenosis remain only partly understood. By providing a close representation of the aortic valve cells in vivo, the assaying of T lymphocytes from stenotic valve tissue could be an efficient way to clarify their role in the development of calcification. After surgical excision, the fresh aortic valve sample is dissected in small pieces and the T lymphocytes are cultured, cloned then analyzed using fluorescence activated cell sorting (FACS).

The staining procedure is simple and the stained tubes can also be fixed using 0.5% of paraformaldehyde and analyzed up to 15 days later. The results generated from the staining panel can be used to track changes in T cell concentrations over time in relation to intervention and could easily be further developed to assess activation states of specific T cell subtypes of interest. In this study, we show the isolation of T cells, performed on fresh calcified aortic valve samples and the steps of analyzing T cell clones using flow cytometry to further understand the role of adaptive immunity in CAVD pathophysiology.

INTRODUCTION:

Calcific aortic valve disease (CAVD) is one of the most common heart valve disorders, with a heavy impact on healthcare. The frequency of aortic valve replacement in the last years has increased dramatically and is expected to increase further, due to the growing elderly population[1].

The underlying pathophysiology of CAVD is only partially known and the current therapeutic strategies are limited to conservative measures or aortic valve replacement, either through surgical or percutaneous procedures. To date, no effective medical treatment can hinder or reverse CAVD progression and high mortality is associated with early symptom-onset, unless aortic valve replacement (AVR) is performed[2]. In patients with severe symptomatic aortic stenosis, the 3-year symptom-free survival was reported as low as 20%[3]. Transcatheter aortic valve replacement (TAVR) represents a new option, revolutionizing treatment for high-risk patients, especially among the elderly and has dramatically reduced the mortality, which was intrinsically high in this population[4-6]. Despite the promising results of TAVR, further research is necessary to understand CAVD pathophysiology to identify novel early therapeutic targets[7-9].

Previously thought to be a passive, degenerative process, CAVD is now recognized as an active progressive disease, characterized by an osteoblastic phenotype switch of the aortic valve interstitial cells[10]. This disease involves progressive mineralization, fibrocalcific changes and reduced motility of the aortic valve leaflets (sclerosis), which ultimately obstruct blood flow leading to narrowing (stenosis) of the aortic valve opening[11].

Inflammation is considered a key process in CAVD pathophysiology, similar to the process of vascular atherosclerosis. Endothelial injury enables the deposition and accumulation of lipid species, especially oxidized lipoproteins in the aortic valve[12]. These oxidized lipoproteins provoke an inflammatory response, as they are cytotoxic, with the inflammatory activity leading to mineralization. The role of innate and adaptive immunity in CAVD development and disease progression has been recently highlighted[13]. The activation and clonal expansion of specific

subsets of memory T cells have been documented in patients with CAVD and mineralized aortic valve leaflets, so that inflammatory processes are assumed to be involved at least in the development of CAVD and presumably in disease progression as well[14]. In fact, although antigen-presenting cells and macrophages are present in both the healthy and diseased valve, the presence of T lymphocytes is indicative of an aged and diseased aortic valve. This lymphocytic infiltrate along with an increase in neovascularization and metaplasia are characteristic histological signs of CAVD[15].

We hypothesize the existence of an interaction between the aortic valve interstitial cells and the activation of the immune system, which potentially triggers the initiation of a chronic inflammatory process in the aortic valve. The assaying of T cells from stenotic aortic valve tissue could be an efficient way to clarify their role in calcification development, as it can provide a close representation of the aortic valve cells in vivo. In the present work, using aortic valve tissue, we isolate T-lymphocytes, culture and clone them, and subsequently characterize them using fluorescence-activated cell sorting (FACS). Fresh aortic valve samples were excised from CAVD patients who received surgical valve replacement for severe aortic stenosis. After the surgical excision, the fresh valve sample was dissected in small pieces and the T cells were cultured, cloned then analyzed using flow cytometry. The staining procedure is simple and the stained tubes can be fixed using 0.5% of paraformaldehyde and analyzed up to 15 days later. The data generated from the staining panel can be used to track changes in T lymphocyte distribution over time in relation to intervention and could easily be further developed to assess activation states of specific T cell subsets of interest.

The extraction of calcified tissue, the isolation of leukocytes from calcified tissue and particularly the use of flow cytometry on this type of tissue can be challenging, due to issues such as autofluorescence. Few publications exist with protocols for this specific purpose[16-18]. Herein we present a protocol designed specifically for the direct isolation and culture of T lymphocyte from human aortic valve samples. Clonal expansion of lymphocytes is a hallmark of adaptive immunity. Studying this process in vitro provides insightful information on the level of lymphocyte heterogeneity[19]. After a three-week incubation period, the T cell clones are ready to be explanted, as an adequate amount of T cells from each clone was obtained, so as to allow the phenotypic and functional study. Subsequently the phenotype of T clones is studied by cytofluorometry.

This immunological protocol is an adaptation of a method previously developed by Amedei et al. for T cell isolation and characterization from human tissue, especially designed for calcified human tissue, such as in CAVD[20-22]. The protocol here for the isolation of PBMCs (peripheral blood mononuclear cells) using irradiated buffy coat describes an effective way to obtain feeder cells (FC), specifically adjusted for the cloning phase of T lymphocytes isolated from valve interstitial cells. The feeder layer consists of in growth arrested cells, which are still viable and bioactive. The role of feeder cells is important to support in vitro survival and growth of T lymphocytes isolated from valve interstitial cells[23]. In order to avoid feeder cell proliferation in culture, these cells must undergo a growth arrest. This can be achieved in two ways: through physical methods such as irradiation, or through treatment with cytotoxic chemicals, such as mitomycin C (MMC), an

antitumoral antibiotic that can be applied directly to the culture surface[24]. Here we show feeder cell growth arrest achieved through cell irradiation.

This method presents an efficient, cost-effective way to isolate and characterize T cells from aortic valve tissue, contributing to broadening the spectrum of immunological methods for exploring CAVD pathophysiology.

PROTOCOL:

The study was conducted according to the Statute of the Charité for Ensuring Good Scientific Practice and the legal guidelines and provisions on privacy and ethics were respected. The Ethics Committee approved all human experiments and the privacy and anonymity of the patients were maintained in accordance with the rules reported on the Ethic Form.

NOTE: For the protocol described below fresh human stenotic valve samples were used.

1. Reagent preparation

1.1. Prepare the enriched RPMI complete medium by adding in RPMI 1640 Medium: Non-essential amino acids; Sodium Pyruvate, L- Glutamine, ß-Mercaptoethanol and Penicillin-Streptomycin (Pen/Strep). Filter it before use. Store at +4 °C and use within two months.

1.2. Prepare the cell culture medium (CCM) for the cloning phase using the enriched RPMI 1640 complete medium added heat-inactivated Fetal Bovine Serum (FBS), HB basal medium, 50 U of Interleukin 2 (IL-2) and Human Serum (HS). Filter and use it within the day. Do not store.

1.3. Prepare the cell culture medium for the refeeding phase using the enriched RPMI 1640 complete medium with added FBS, HB basal medium, 30 U of IL-2 and HS. Filter and use it within the day. Do not store.

1.4. Prepare the wash buffer containing 250 mL of RPMI and 5 mL Pen/Strep. Store at +4 °C, use within two months.

2. Human T lymphocyte isolation and culture into T-25 flasks

2.1. Place the stenotic valve sample into a sterile Petri dish filled with Wash Buffer, ensuring that the entire valve sample is covered with it. Let stand for 15 minutes.

2.2. Cut the stenotic valve in small pieces using a scalpel and let it stand again for 10 minutes.

2.3. Prepare the cell culture medium with 50 U of IL-2, as previously described, and filter it.

2.4. Add the CCM inside the T25 flasks and using a sterile plastic plier, place the valve pieces inside the flasks.

2.5. Store the flasks in a vertical position inside a CO_2 incubator for one week, taking care to observe the flask's status under the microscope. T cell clones should be visible approximately three days after the culture step; for better observation it is advisable to place the flasks in horizontal position for 20 min before the microscopic analysis.

NOTE: The quantity of cell culture medium depends on the amount of sample present and on how many T25 flasks will be used. Considering that each piece of valve requires 10 mL of CCM inside a flask, 25 mL of CCM in a T25 flask is appropriate for 3 pieces of valve.

3. Cloning phase

NOTE: For the cloning phase of T cells, it is necessary to start with the isolation of feeder cells from an irradiated buffy coat (BC), following the PBMCs protocol.

3.1. Open the BC bag under the laminar flow hood using the bag spike with needle-free valve and transfer 50 mL of blood in a T150 flask. Add 70 mL of PBS to dilute blood inside the flask.

3.2. Take four 50 mL conical tubes and place 20 mL of density gradient medium in each and carefully layer 30 mL of blood over it.

3.3. Centrifuge for 25 min at 800 x g and 20 °C with the brake off.

3.4. Carefully aspirate the supernatant and discard it as waste. Collect the PBMCs ring in a new 50ml conical tube adding inside the PBS solution up to a volume of 50ml.

3.5. Centrifuge for 10 min at 400 x g and 20 °C.

3.6. Discard the supernatant and suspend the pellet by adding inside the PBS solution up to a volume of 50 mL. Repeat the centrifugation phase for 10 min at 400 x g and 20 °C.

3.7. Discard the supernatant and suspend the FCs in 10 mL of cell culture medium.

3.8. Take a new collection tube and dilute the FCs with PBS 1:10 by adding inside 9.9 mL of PBS and 100 µL from the tube with FCs and cell culture medium. Rinse well with a pipette and take 7 µL from this dilution and count the cells under the microscope.

3.9. Prepare the cell culture medium for the cloning phase: 70 mL with 50 U of IL-2, and then divide it in two 50 mL conical tubes, each tube will contain 25 mL of cell culture medium, as describe below:

- Tube 1: 25 mL of CCM + FCs + 0.6% of phytohemagglutinin (PHA)
- Tube 2: 25 mL of CCM + T lymphocytes

NOTE: The aim of the PMBCs method is to obtain 2 x 10^6 cells for each mL, so the 25 mL of CCM

prepared (Tube 1) needs to be calculated accordingly. The general formula used is: Number of counted cells x 10^6: 1 mL = CCM x 10^6: X

4. T Lymphocytes culture in multiwell plates

4.1. Using the electronic pipette, collect all the cell culture medium inside the flasks and transfer it into a 50 mL conical tube. The empty flasks can be thrown away.

4.2. Pellet the cells by centrifugation for 10 min at 400 x g and 20 °C.

4.3. Discard the supernatant, suspend the pellet in 50 mL of PBS and collect the cells by centrifugation for 10 min at 400 x g and 20 °C. Repeat this step twice.

4.4. Discard the supernatant and suspend the pellet in 1 mL of CCM. Take 7 µL from this dilution and count the cells under the microscope.

NOTE: The number of counted cells needs to be multiplied for the volume of the chamber and for dilution applied.

4.5. Proceed to dilute the cells counted from 10^5 to 10^3 in cell culture medium and then take 500 µL from 10^3 and put it inside the Tube 2.

4.6. Pour the 25 mL cell culture medium of Tube 1 inside a 100 mm x 15 mm plastic Petri dish.

4.7. Take four 96-U bottom multiwell plates and using a multichannel pipette set to 100 µL seed FCs in each well.

4.8. Take the Tube 2 and pour the cell culture medium inside a Petri dish. Using a multichannel pipette set to 100 µL, seed T cells in each well.

4.9. Store the multiwell plates inside a CO_2 incubator for one week.

5. First refeeding phase

5.1. Using an irradiated buffy coat bag, follow the PBMCs method to obtain FCs as described in the previous method.

5.2. Prepare the suitable quantity of CCM without PHA and filter it.

5.3. Using a multichannel pipette, remove 100 µL from each well, ensuring not to touch the bottom of the well.

5.4. Add 100 µL of FCs in all the wells as a feeding layer to provide cell culture nutrients and change the medium.

NOTE: For each multiwell it is advised to consider 6 mL of CCM, so the recommended quantity for 4 multiwells is about 30 mL. PHA (0.4% of the total CCM quantity) needs to be added to the CCM once every two weeks.

6. Second refeeding phase

6.1. Repeat the refeeding phase following the passage described above. 0.4% PHA needs to be added to the cell culture medium.

7. First splitting phase from 1 well/sample to 2 wells/sample

7.1. Check all the four multiwells under the microscope and go ahead splitting those wells with T cell clones (TCCs).

7.2. Follow the PBMCs protocol to isolate feeder cells from an irradiated buffy coat bag.

7.3. Prepare the cell culture medium with 30 U of IL-2. Add the FCs and take a new 96-well multiwell plate with U bottom.

7.4. Using a pipette set to 100 µL, rinse well inside the wells of the selected samples and divide the 200 µL of volume contained in each well into 2 new wells of the new multiwell plate, in order to have 100 µL of volume for each of the new 2 wells.

7.5. Add 100 µL of FCs inside all the new wells. Each well must contain a total volume of 200 µL.

7.6. Set the pipette at 100 µL and add 100 µL into an additional two wells, containing only FCs to serve as a control.

NOTE: Use light microscopy to observe all multiwell plates in order to determine which wells have successfully grown TCC. To make the selection of clones easier, a comparison can be made to the control (the two wells containing only FCs), with the clone appearing darker and larger compared to the control. The wells to be selected are those with a large, clear, round clone, with lymphocytes densely packed together. If no TCCs are present after two weeks of refeeding, it is recommended to wait one more week and perform an additional refeeding phase.

8. Second splitting phase from 2 wells/sample to 4 wells/sample

8.1. Set the pipette to 100 µL, rinse well inside the two wells of each sample and seed two new wells of 100 µL for each one.

8.2. Prepare the cell culture medium with 30 U of IL-2 and follow the PBMCs technique to extract the FCs from the buffy coat bag and place the FCs inside the CCM.

8.3. Set the pipette to 100 µL and seed the FCs in all the wells. Store the multiwells in a CO_2 incubator for one week.

9. Third splitting phase from 4 wells/sample to 8 wells/sample:

9.1. Using a multichannel pipette set to 100 µL, rinse well inside all four wells of each sample and seed 100 µL into each of the four new wells in a new 96-well plate.

9.2. Prepare the cell culture medium and follow the PBMCs technique to extract the FCs from an irradiated buffy coat bag.

9.3. Place the FCs inside the CCM. Set the pipette to 100 µL and seed the FCs in all the wells. Store the multiwells in a CO_2 incubator for one week

10. Cytofluorimetric analysis

10.1. Take the multiwell plate with the oldest date from the CO_2 incubator and identify the collection tubes with the sample numbers to analyze.

10.2. Using a multichannel pipette set to 200 µL with 2 tips, thoroughly rinse inside two wells of the same sample and put both inside the same collection tube. Repeat this step for all the samples.

NOTE: The FCs samples will not be analyzed, as they only serve as controls.

11. Antibody Staining Panel Preparation for cytofluorimetric analysis

11.1. Add 1 mL of PBS solution for each tube and centrifuge for 10 min at 400 x g and 20 °C.

11.2. After the centrifugation phase discard the supernatant.

NOTE: The antibody staining panel can be found in **Table 1**. Each single antibody concentration calculated is about 2 µL/sample. It is recommended to briefly spin the antibody tubes before use. To protect the fluorophore-conjugated antibodies from light, all steps should be performed in the dark.

11.3. Prepare the antibody mix inside a 1.5 mL tube and vortex it.

11.4. Add the antibodies inside the samples and leave samples to incubate in the dark at room temperature for 15 min.

11.5. Add 1 mL of PBS in each sample and centrifuge for 10 min at 400 x g and 20 °C.

11.6. Discard the supernatant and suspend the pellet with 500 µL of PBS solution.

11.7. Proceed with the cytofluorimetric analysis (FACS analysis, **Table 1**).

NOTE: The stained samples can be fixed using 0.5% paraformaldehyde (PFA) diluted in PBS solution and analyzed up to 15 days later.

CAUTION: PFA is toxic and must be handled carefully.

[Place the Table 1. Here]

REPRESENTATIVE RESULTS:

We used a simple and cost-effective method to characterize the leukocyte population of fresh aortic valve samples derived from human patients with severe aortic valve stenosis (refer to protocol). The method for isolating PBMCs is a vital step in obtaining feeder cells, which are used in every step of the experiment (cloning, refeeding and splitting phases) and enable the detection and characterization of infiltrating leukocytes in aortic valve samples. The key steps of this method are shown in **Figure 1**.

[Place the Figure 1 here]

After two weeks of incubation, we successfully cloned and grew a T cell population, as shown in **Figure 2**.

[Place the Figure 2 here]

Figure 3 illustrates the gating scheme utilized for the analysis of T cell subpopulations in patients with CAVD. As shown from the result of the FACS analysis, there are more CD4+ T-cells (43.032%) than CD8+ T-cells (1.079%).

[Place the Figure 3 here]

To show that the same T cell markers are present in both native valve samples and cloned samples, we performed FACS analysis without the cloning phase, as illustrated in **Figure 4**.

[Place the Figure 4 here]

The entire workflow is summarized in **Figure 5**, starting from the dissection of the human aortic valve and leading up to the FACS analysis. All steps are required for the analysis of one aortic valve sample. Phase 1 shows the lymphocyte isolation from stenotic aortic valve tissue. The valve samples need to be cut and placed in a Petri dish filled with wash buffer. The valve pieces can be placed inside a T25 flask filled with CCM and stored in a CO_2 incubator for one week. Phase 2 shows the Cloning phase, which consists of two parts: 1) FCs isolation from irradiated buffy coat bag and 2) T cells cloning phase and culminates with the cell culture in a 96-wells multiwell plate,

which is stored in a CO_2 incubator for one week. Phases 3 and 4 consist of the refeeding phase using FCs from irradiated buffy coat; this phase must be repeated for two consecutive weeks. Phases 4, 5 and 6 show the splitting phase of T cell clones; the first splitting phase is from 1 well/sample to two wells for each sample in a new multiwell plate; the second splitting is from 2 wells /sample to 4 wells for each in the same multiwell plate; the third and last splitting phase is from 4 wells/sample to 8 in a new multiwell plate. Phase 7 is the final phase, where the samples are analyzed using FACS analysis.

[Place the Figure 5. Here]

From the preliminary results (**Figure 2**) we can conclude that lymphocytes, along with CD45+ leukocytes are present in calcified aortic valves, thus indicating that calcific aortic valve disease is linked with activation of the immune system and inflammatory activity.

Table 1. Antibody staining panel to detect the T lymphocytes population in calcification aortic valve disease.

Figure 1. FCs isolation from buffy coat. (**A**) Blood layered over density gradient medium (**B**) White blood cells ring obtained after centrifugation (**C**) White blood cells collected and resuspended in PBS solution (**D**) White cells observed under light microscopy

Figure 2. T cell clone after two weeks of incubation observed using light microscopy.

Figure 3. Gating scheme utilized for T cell-subpopulation analysis. (**A)** A gate has been applied to identify the specific T cell population in a stenotic valve. (**B)** CD14 negative lymphocytes and CD3 positive lymphocytes. (**C)** CD3 lymphocytes are gated to distinguish CD4+ T cells from CD8+ T cells.

Figure 4. FACS analysis results of two valve samples. We compared the T cells obtained from a native valve sample and a cloned sample, validating that the T cells found in the native valve share specific markers of the T cells in the final cloned product. (**A**) FACS results of a native valve sample, without the cloning phase. (**B**) FACS results of the valve sample analysed after the cloning phase.

Figure 5. CAVD Experimental workflow.

DISCUSSION:

Here we present a method to characterize T lymphocyte subpopulations isolated from stenotic aortic valve samples, using flow cytometry. This method requires the use of irradiated buffy coat to isolate the PBMCs. The radiation frequency to which the buffy coat bags must be subjected is 9000 Rad/90 Gray (Gy) and it represents a crucial step to halt the proliferation of the feeder cells. The role of the cells isolated from the buffy coat bags is to act only as feeder cells and provide nutrients for the T cells isolated from the valves. The use of an unirradiaded buffy coat bag would promote proliferation of PBMCs in culture, as has been noted in the past[25]. A radiation frequency

below 90 Gy showed PBMCs proliferation in culture, so we recommend using exactly 90 Gy of radiation. Of note, it is advisable to use the buffy coat irradiated on the same day or at most the next day, keeping it on a device that keeps it in constant agitation. Another crucial step of this method could be represented by the lymphocyte cloning phase, which could be affected by artifacts; to avoid this event we perform the FACS on fresh aortic valve samples. The cloning phase of T lymphocytes has the advantage of obtaining a larger number of T clones (an average of 15 T cells clones for each valve) to be analyzed phenotypically and functionally, than the number obtained from the analysis of one fresh sample. The cloning technique has been used by this research group for many years and depending on the type of tissue analyzed, the lymphocyte profile was different[21,26,27]. The first passage of the method concerns the T cell isolation from a stenotic aortic valve. All the steps described must be performed under a laminar flow hood in sterile conditions and it is strongly recommended to disinfect all materials before use. The time required to obtain results was 6 weeks and the average of T lymphocyte cells obtained from the cloning phase was 20 x 10^6 cells. The monitoring phase of the multiwell plates is very important and must not be overlooked. A change in the color of the medium to yellow could represent bacterial contamination, in which case all the instruments used need to be sterilized and the multiwell plates thrown away.

Before FACS analysis it is important to establish a suitable gate, to verify that there is no specific overlap between the antibody channels. This enables the optimal separation between positive and negative gates. The use of the same lots of antibodies for all samples involved in the study is recommended to obtain homogeneous result. It is also necessary to protect the fluorophore-conjugated antibodies from light, performing all steps with the light off.

This method is effective in yielding results with high reproducibility and is not expensive. A limitation of this method is the small sample size, due to the limited availability of the human valve samples, as well as the lack of a control group.

The preliminary results support the role of adaptive immunity as a crucial element in the development and progression of calcific aortic valve disease. All the patients enrolled in this study had a diagnosis of severe symptomatic calcific aortic stenosis, with an average age of 70, mostly male. The existence of T cell populations in the aortic valves analyzed provides evidence for the inflammatory activity of the diseased aortic valve as a checkpoint of immune cell activation. A point of future interest could be to analyze the functionality of CAVD-infiltrating T cells and characterize T cell specificity as previously reported in similar diseases, such as in atherosclerosis[28-30].

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DISCLOSURES:

The authors have nothing to disclose.

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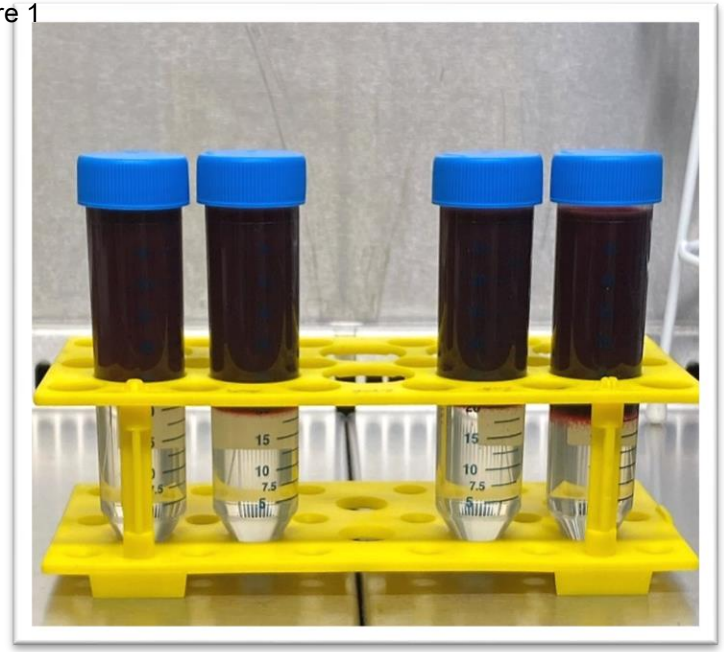
Figure 1



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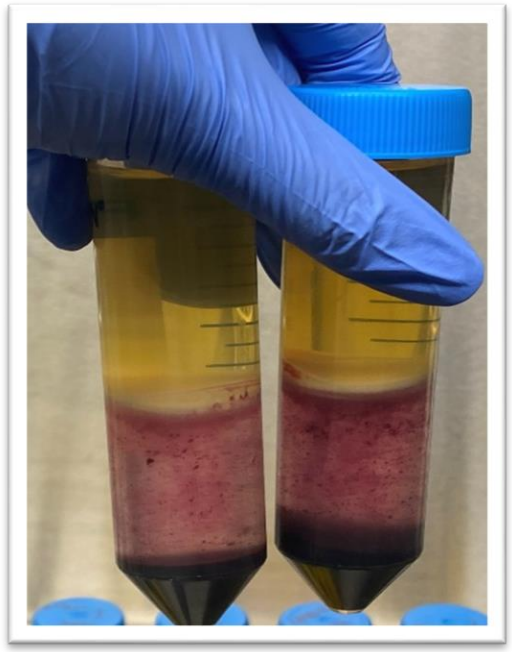

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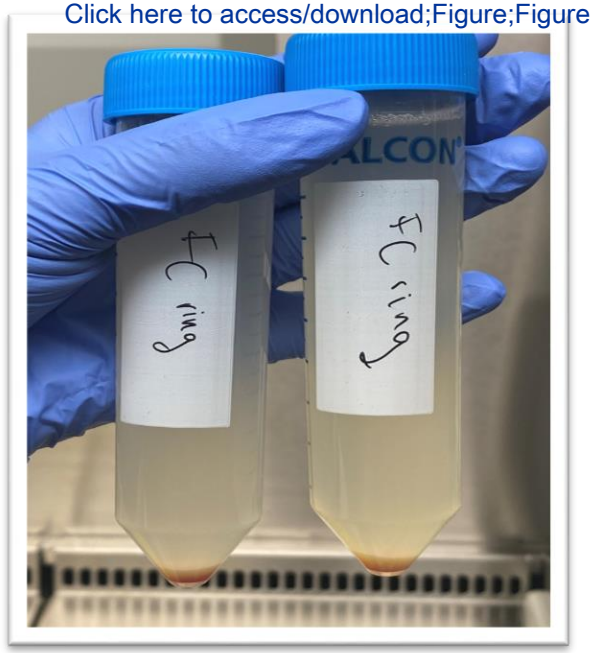

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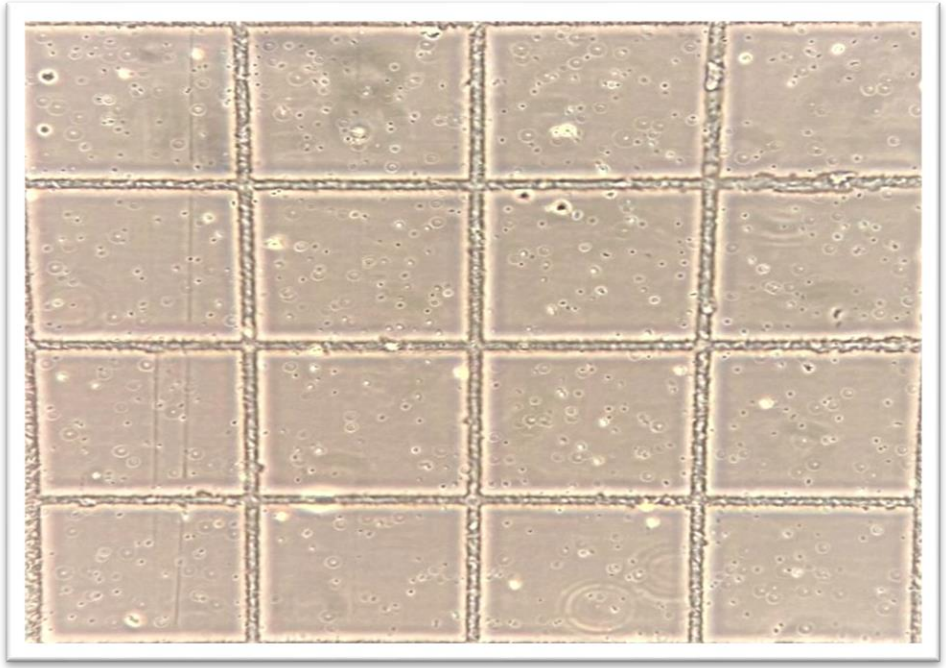


D

Figure 2

Click here to access/download;Figure;Figure 2- Lymphocyte clone.pdf

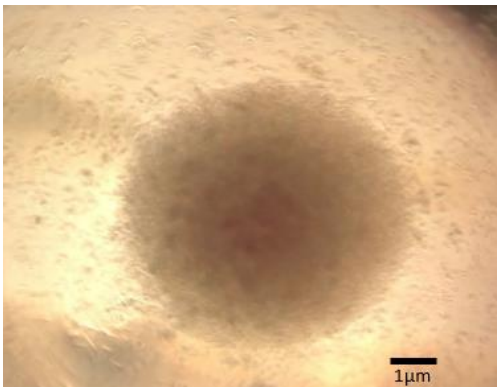


Figure 3

Click here to access/download;Figure;Figure 3- antibodies panel .pdf

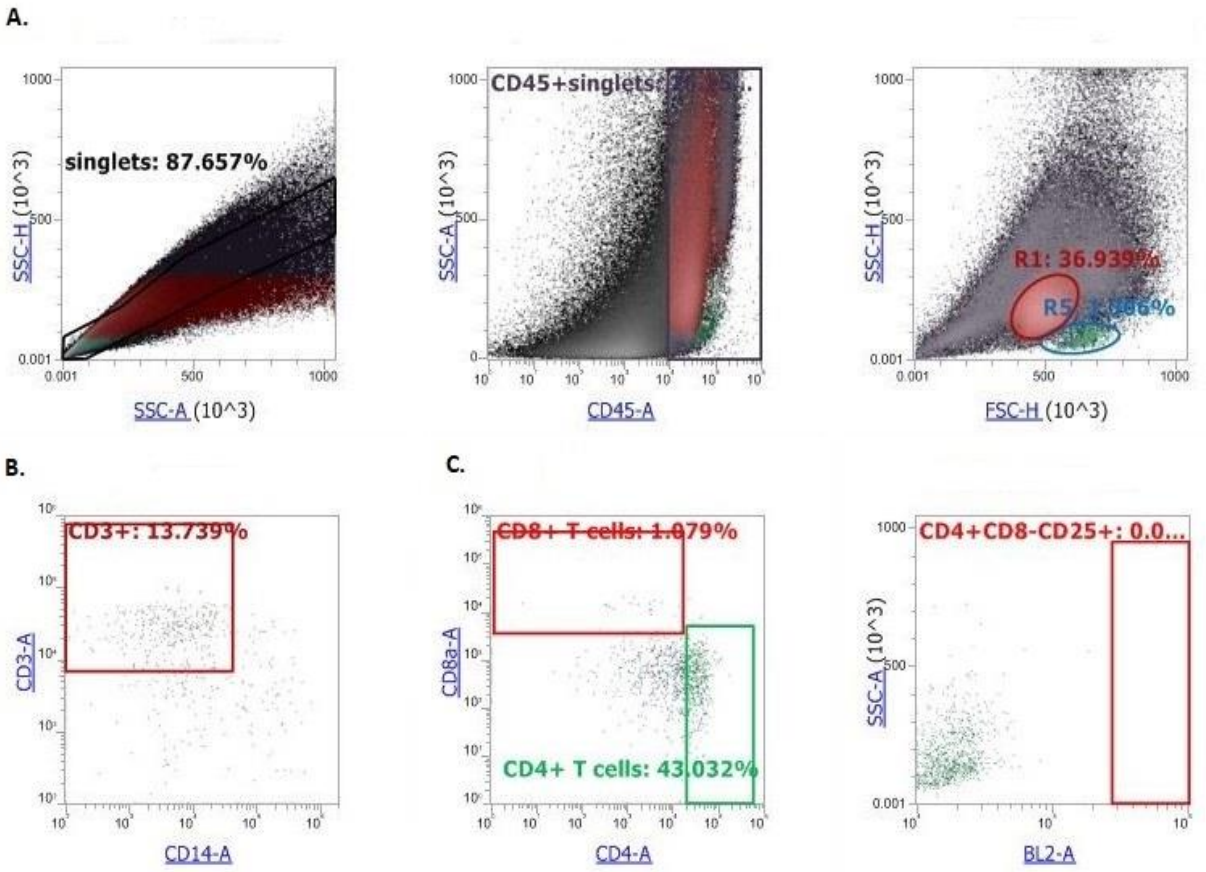
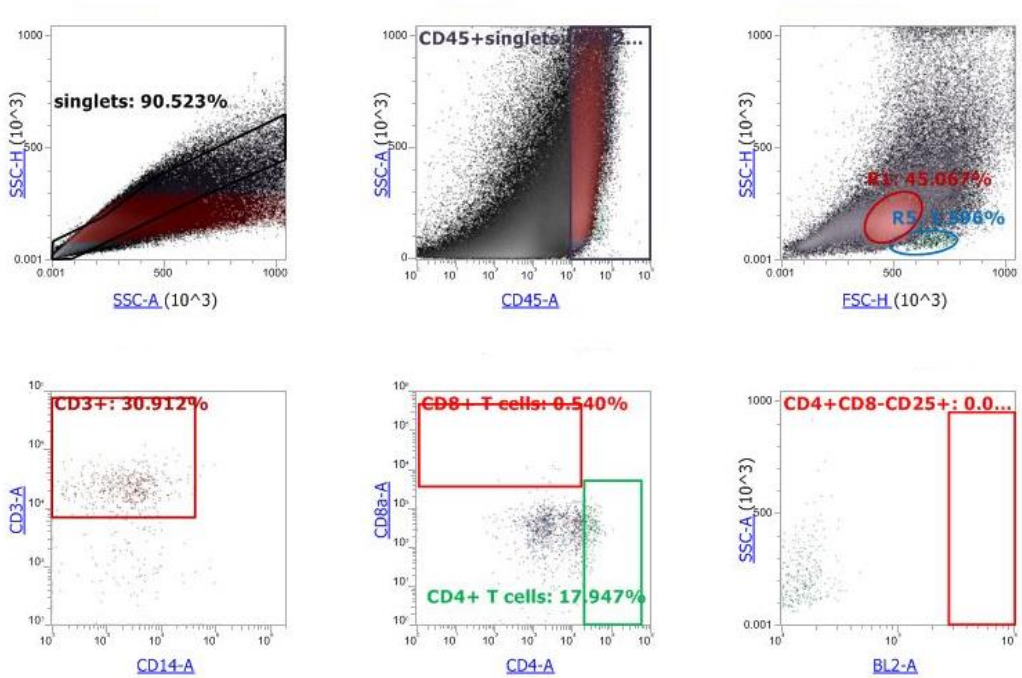


Figure 4

Click here to access/download;Figure;Figure 4- FACS Analysis results of two valve samples.pdf



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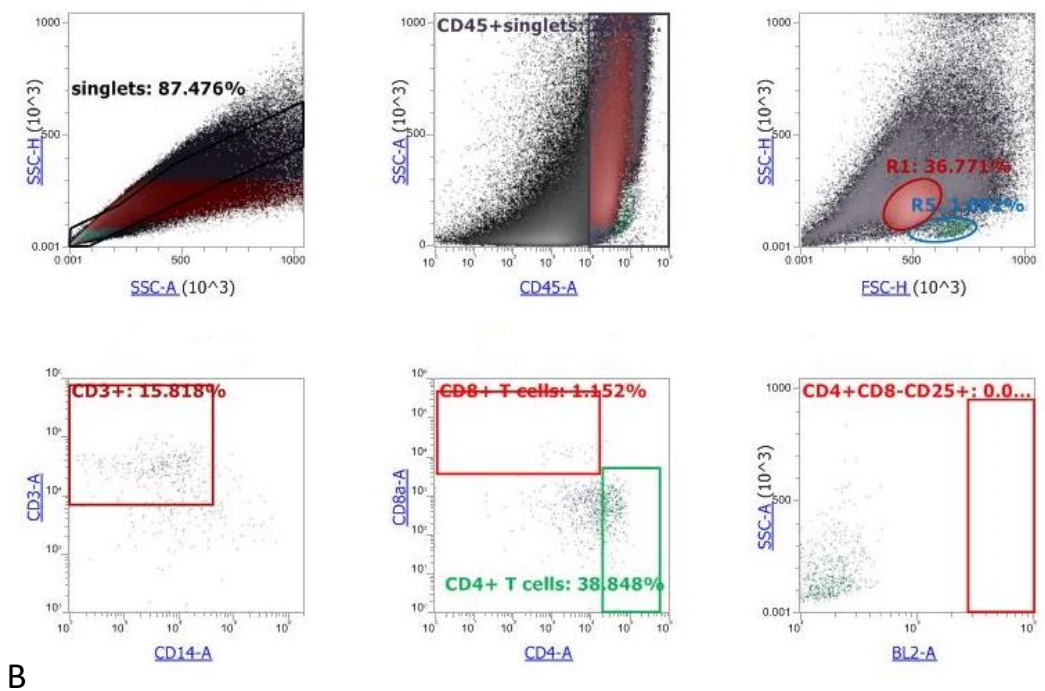


B

Figure 5

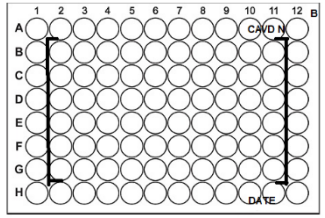
CAVD Experimental Workflow

Phase 1

Lymphocytes isolation from stenotic valve sample

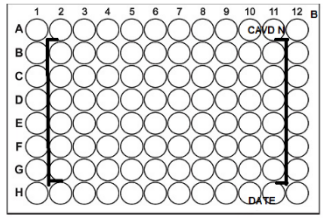
Phase 2

T cells cloning phase and FCs isolation from irradiated buffy coat bag



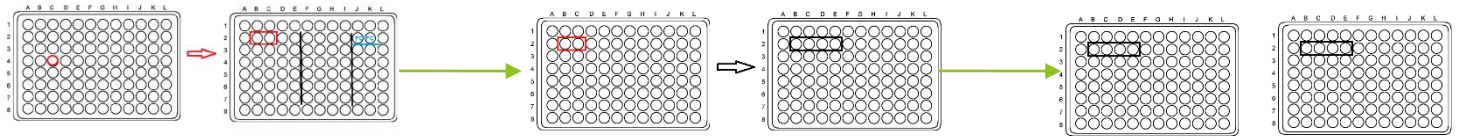
Phases 3-4

Refeeding phase



Phases 4-5-6

Splitting phase



Phase 7

FACS analysis

Table 1

Marker	Fluorophore
CD3	PE/Cy7
CD4	Alexa Fluor 488
CD8	Brilliant violet 510
CD14	Brilliant violet 421
CD25	PE
CD45	Brilliant violet 711

Table of Materials

Name of Material/Equipment	Company	Catalog Number	Comments/Description
50 mL plastic syringes	Fisherbrand	9000701	
96- well U- bottom Multiwell plates	Greiner Bio-One	10638441	
Bag Spike (needle free)	Sigma	P6148	Dilute to 4% with PBS
CD14 Brilliant violet 421	Biolegend	560349	
CD25 PE	Biolegend	302621	
CD3 PE/Cy7	Biolegend	300316	
CD4 Alexa Fluor 488	Biolegend	317419	
CD45 Brilliant violet 711	Biolegend	304137	
CD8 Brilliant violet 510	Biolegend	301047	
Eppendorf tube 1.5 mL	Eppendorf	13094697	
Eppendorf tube 0.5 mL	Thermo Scientific	AB0533	
Falcon 15 mL conical centrifuge tube	Falcon	10136120	
Falcon 50 mL conical centrifuge tubes	Falcon	10788561	
Falcon Round-Bottom Polystyrene Tubes	BD	2300E	
Fast read 102 plastic counting chamber	KOVA INTERNATIONAL	630-1893	
Filters for culture medium 250 mL	NalgeneThermo Fisher Scientific	168-0045	
Filters for culture medium 500 mL	NalgeneThermo Fisher Scientific	166-0045	
HB 101 Lyophilized Supplement	Irvine Scientific	T151	
HB Basal Medium	Irvine Scientific	T000	
Heat-Inactivated FBS (Fetal Bovine Serum)	Euroclone	ECS0180L	
HS (Human serum)	Sigma Aldrich	H3667	
Human IL-2 IS	Miltenyi Biotec	130-097-744	
L-Glutamine	Gibco	11140050	
Lymphoprep	Falcon	352057	
Non-essential amino acids solution	Sigma	11082132001	
Paraformaldehyde	Thermo Fisher Scientific	10538931	
PBS (Phosphate-buffered saline)	Thermo Fisher Scientific	10010023	
Penicillin/Streptomycin	Gibco	15070063	10000 U/mL
PHA (phytohemagglutinin)	Stem Cell Technologies	7811	
Plastic Petri dishes	Thermo Scientific	R80115TS	10 0mm x 15 mm

RPMI 1640 Media	HyClone	15-040-CV
Sodium pyruvate	Gibco by Life technologies	11360070
Syringe Filters 0,45µl	Rotilabo-Spritzenfilter	P667.1
T-25 Cell culture flasks	InvitrogenThermo Fisher Scientific	AM9625
T-75 Cell culture flask	Thermo Fisher Scientific	10232771
β- Mercaptoethanol	Gibco	A2916801

The authors would like to thank the Jove editorial comments and the reviewers for their specific and helpful comments about our manuscript “Investigating Aortic Valve Calcification via Isolation and culture of T lymphocytes using feeder cells from irradiated buffy coat”.

The manuscript has been improved according with the comments suggested.

Editorial comments:

1. Please use numbered superscripts for the references.

Superscripts for all references were used.

Reviewers' comments:

Reviewer #1:

Major Concerns:

There is no more comments.

Reviewer #2:

Manuscript Summary:

The authors have greatly improved the revised manuscript, especially in establishing the premise for evaluating T cells. My only lingering concern is with the figures. As currently presented, the figures are not very informative for someone who is trying to replicate the procedure. It would be helpful to show images of the buffy coat isolation procedure and microscopic images of the cells to help the reader understand what he/she should expect to observe.

A new figure was added, summarizing the key steps of the buffy coat isolation procedure, to make the method more clear and understandable. As suggested, a microscopic image of the cells obtained was included. (Lines 362-364 and 408-410).

Reviewer #3:

Summary:

This is a revised manuscript describing a method to isolate, expand clonal colonies, and then characterize T cells found in a calcified aortic valve. The innate immune response is thought to contribute to CAVD pathogenesis thus the isolation and further study of T cells found in CAVD may help to discern the complex cellular interactions at play. A key step emphasized in this protocol is the use of irradiated blood to produce a feeder cell that allows for the expansion of T cell clones.

Major Comments:

** The schematic in figure is a good improvement, however not addressed were my prior concerns regarding the confirmation of whether the cloning/expansion keeps the T cells in their native state found in the valve? For this protocol to be valid and useful, it needs to show staining or FACs analysis that validates that the T cells found in the native valve share subtype specific markers of the T cells in the final cloned product.*

To show that the same T cell markers are present in both native valve samples and cloned samples, we performed FACS analysis without the cloning phase, as illustrated in figure 4.(Lines 378-380 and 418-422).

We appreciated your suggestions and we hope our modification makes the manuscript suitable for publication.

Yours sincerely.

On behalf of the authors,

Lavinia Curini