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Dear Dr. Wu

We are pleased to submit our revised manuscript titled “Unraveling key players of humoral immunity: Advanced and optimized lymphocyte isolation protocol from murine Peyer’s Patches” for consideration in JoVE (JoVE58490).

We would like to thank you for your time on the review of our manuscript. All the points of the reviewers and the editor have been taken into consideration and the manuscript has been revised accordingly.

All the changes are highlighted in the revised manuscript. Below is a point-by-point response to the reviewers’ and the editor’s comments.

Thank you for your kind consideration.

Sincerely

Vicki A. Boussiotis, M.D., Ph.D.

TITLE:

Unraveling Key Players of Humoral Immunity: Advanced and Optimized Lymphocyte Isolation Protocol from Murine Peyer's Patches

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KEYWORDS:

Peyer's patches, follicular T helper cells, germinal center B cell, lymphocyte isolation, tissue preparation, lymphocyte subsets, lymphoid organs, collagenase

SUMMARY:

In this study, we present a novel and effective protocol for the isolation of lymphocytes from Peyer's Patches (PPs), which can be subsequently used for *in vivo* and *in vitro* functional assays as well as flow cytometric studies of follicular T helper and germinal center B cells.

ABSTRACT:

In the gut mucosa, immune cells constitute a unique immunological entity, which promotes immune tolerance while concurrently conferring immune defense against pathogens. It is well established that Peyer's patches (PPs) have an essential role in the mucosal immune network by hosting several effector T and B cell subsets. A certain fraction of these effector cells, follicular T helper (TFH) and germinal center (GC) B cells are professionalized in the regulation of humoral immunity. Hence, the characterization of these cell subsets within PPs in terms of their differentiation program and functional properties can provide important information about mucosal immunity. To this end, an easily applicable, efficient and reproducible method of lymphocyte isolation from PPs would be valuable to researchers. In this study, we aimed to generate an effective method to isolate lymphocytes from mouse PPs with high cell yield. Our approach revealed that initial tissue processing such as the use of digestive reagents and tissue

agitation, as well as cell staining conditions including the selection of antibody panels, have great influence on the quality and identity of the isolated lymphocytes and on experimental outcomes. Here, we describe a protocol enabling researchers to efficiently isolate lymphocyte populations from PPs allowing reproducible flow cytometry-based assessment of T and B cell subsets primarily focusing on follicular TFH and GC B cell subsets.

INTRODUCTION

The entire gastrointestinal tract from the beginning to the end is decked with an extensive lymphoid network that contains immune cells more than any other organ in human and mouse¹. Peyer's patches (PPs) constitute a major component of the intestinal branch of this cellular immune organization, so-called gut-associated lymphoid tissue (GALT)^{2,3}. Within PPs, thousands of millions of antigens derived from dietary materials, commensal microbiota and pathogens are being sampled continuously, and when necessary appropriate immune responses toward them are mounted thus maintaining intestinal immune homeostasis. In that sense, PPs could be named as "tonsils of the small intestine". PPs consist of major sub-compartments: subepithelial dome (SED), large B-cell follicle zones; the overlying follicle-associated epithelium (FAE) and interfollicular region (IFR) where T cells are located⁴. This unique compartmentalization of PPs enables different effector cell subsets to cooperate, thus, confers immunocompetence in the gut. PPs lack afferent lymphatics, and due to this reason, antigens transported to PPs from small intestine are not being conducted through lymphatic vessels in contrast to most of the other lymphoid organs. Instead, specialized epithelial cells located in FAE, so-called M cells, are responsible for the transfer of luminal antigens into the PPs⁵. Subsequently, the transported antigens are picked up by the dendritic cells (DCs) and phagocytes that are located in the subepithelial dome (SED) region beneath the FAE^{6,7}. This antigen sorting process by DCs in the PP is crucial to initiate adaptive immune response⁸ and subsequent generation of IgA secreting cells⁹.

Due to the heavy antigenic burden from commensal flora and dietary material, PPs host endogenously activated effector T and B cell subsets in great abundances such as TFH and IgA⁺ GC B cells¹⁰, suggesting that PPs represent a site of active immune response¹¹. Detection of up to 20-25% TFH cells within total CD4⁺ T cell compartment and up to 10-15% GC B cells within total B cells is possible in PPs collected from unimmunized young C57BL/6 mice¹². In contrast to other T helper cell types (*i.e.*, Th1, Th2, Th17 cells), TFH cells show unique tropism into B cell follicles primarily owing to CXCR5 expression, which promotes TFH cell homing along CXCL13 gradient¹³. In the B cell follicle zones of PPs, TFH cells induce IgA class switch recombination and somatic hypermutation in activated B cells from which high-affinity IgA producing cells differentiate¹⁴. Subsequently, these antibody-secreting plasma cells migrate to the lamina propria (LP) and regulate immune homeostasis in the gut¹⁰.

Identification and characterization of TFH and GC B cell populations within PPs might enable researchers to investigate the dynamics of humoral immune responses under steady-state conditions without the need of time-consuming immunization models traditionally used in TFH-GC B cell studies¹⁵⁻¹⁸. Analyzing TFH cells within PPs is not as straightforward as other cell subsets. Technical challenges include identifying ideal tissue preparation conditions, surface antibody-

marker combination, as well as selecting appropriate positive and negative controls. Both TFH and PP research fields exhibit great variability in terms of experimental procedures and are far from conferring a consensus to establish standardized protocols due to several reasons. First, each cell subset within PPs tends to be affected differentially by tissue preparation conditions requiring further modifications in a cell subset-specific manner. Second, there is a significant discrepancy among the reported methods regarding the details of cell preparation from PPs. Third, the number of protocol-based comparative studies investigating ideal tissue preparation techniques and experimental conditions for PP and TFH research is rather limited.

Current protocol-based studies suggested for PP cell preparation^{19–22} were not TFH- or GC B cell-oriented. Moreover, some tissue preparation conditions recommended for PPs^{19,20} such as collagenase-based digestion were found to affect the outcome of TFH identification by flow cytometry negatively¹⁸. On this basis, we reasoned that an optimized, standardized and reproducible protocol that can be used to study TFH and GC B cell dynamics within PPs would be valuable to investigators working on this topic. This need gave us the impetus to generate an improved and up-to-date protocol for the isolation and characterization of PP lymphocytes that is finely optimized for cellular recovery, viability, and efficiency for flow cytometric characterization of several T and B cell subsets. We also aimed to exclude several laborious preparation steps suggested in previous protocols, thereby, reducing the required manipulations and time for tissue and cell preparation from PPs.

PROTOCOL:

All studies and experiments described in this protocol were conducted under guidelines according to Institutional Animal Care and Use Committee (IACUC) of Beth Israel Deaconess Medical Center.

1. Designing Experimental Set-up and Mouse Groups

1.1. (Optional) Co-house the experimental mice to facilitate horizontal transmission of gut microbiota between experimental mice and to reduce non-specific variability within PP lymphocytes. Additionally, use littermate controls of the same gender to minimize variability.

2. Surgical Excision and Tissue Preparation Steps:

2.1 Surgical removal of the Small Intestine (SI)

2.1.1. Euthanize mice using CO₂ asphyxiation or any equivalent method approved by the institutional animal ethics committee.

2.1.2. Transfer the mouse to a dedicated area for surgical excision. Place backside down and sanitize the abdomen with 70% ethanol. Perform a laparotomy by cutting the abdominal skin and peritoneum along the midline from pubis to the rib cage thus opening the peritoneal cavity.

Note: Perform the first incision on a relatively small area of the skin to avoid penetrating the

peritoneal cavity and damaging intestinal tissue. Continue the excision until desired anatomical border.

2.1.3. Identify the caecum, which is an ideal landmark for the detection of the terminal ileum, which constitutes the distal segment of the small intestine.

Note: Caecum is located on the lower left side of the mouse abdomen.

2.1.4. Pinpoint ileocaecal junction and make a cut at this level as distal as possible to separate the small intestine from the caecum. Throughout the next steps, avoid excessive physical contact with the intestinal wall because fragile PPs collapse easily upon touch.

2.1.5. Gently remove the entire small intestine until the pyloric sphincter by cutting the mesentery using scissors. Identify the junction between pylorus and duodenum, and snip the duodenum at this level, which will lead to complete detachment of small intestine from the abdominal cavity.

Note: (i) Avoid hyperextension as this might cause the rupture of the intestinal wall. (ii) If LP lymphocyte isolation is desired in addition to PP lymphocytes, complete removal of the mesenteric fat is necessary. However, for PP isolation only, remaining mesenteric fat could provide some benefits during the further isolation steps; therefore, it should be preserved.

2.1.6. Place detached small intestines in a 6-well plate filled with cold RPMI + 10% fetal bovine serum (FBS) and gently agitate the tissues manually until all intestinal segments are submerged in the cold media. Maintain the tissues on ice throughout the next steps.

2.1.7. After dissecting the desired number of mouse small intestines, proceed to PP excision from collected small intestines.

2.2. Surgical Excision of PPs and Preparation of Single Cell Suspension:

2.2.1. Gently transfer the small intestine on a paper towel by gripping the mesenteric fat using forceps and place the mesenteric side facing the paper towel. Moisten the entire intestinal segment with cold RPMI + 10% FBS to avoid tissue dehydration and stickiness.

Note: Remaining mesenteric fat can be helpful at this stage because fat tissue segments on the mesenteric site of SI will stick to the paper towel keeping the anti-mesenteric site facing up.

2.2.2. Identify the PPs, which appear as white multi-lobulated aggregates in a “cauliflower-like” shape on the anti-mesenteric side of the intestinal wall.

Note: Flushing out the luminal content is not recommended until all PPs are excised. Emptying the luminal content might cause the collapse of the PPs and will prevent the color contrast between PPs and the intestinal wall, which is very helpful for visual identification of PPs.

2.2.3. After identifying PPs on the anti-mesenteric side, place the surgical curved-end scissor on PPs (curve should face up) restraining the PP from its distal and proximal border. **(Optional)** Push the PP gently toward the blades of scissor using a fingertip. This maneuver will lead to the better exclusion of surrounding non-PP tissue. Excise the PPs gently, excluding the surrounding intestinal tissue.

Note: (i) This step is crucial to obtain maximal PP cell yield while minimizing the cell contamination from neighboring intestinal compartments such as LP and intestinal epithelium, which are also rich in T cells. (ii) From one SI excised from C57BL/6 mouse, 5-10 PPs (average size, multi-lobulated) can be collected. By aiming even smaller PPs (not multi-lobulated), collection of up to 12-13 PPs per mouse (C57BL/6) is possible using this protocol.

2.2.4. Transfer the excised PPs to a 12-well tissue culture plate filled with ice-cold RPMI + 10% FBS and maintained on ice using forceps or curved surgical scissors.

Note: (i) Immediately after excision of PPs, mucus and intestinal content on the PP surface can be cleaned by rubbing the tissue gently on a paper towel. This step will help improve the viability of PP lymphocytes. (ii) Instead of transferring the PPs to a plate, transferring to separated tubes can also be considered depending on total sample number.

2.2.5. **(Optional)** When PP excision and subsequent placement into the well plate are completed, the number and size of PPs collected from different experimental/mouse groups can be documented by taking a picture of the tissue culture plate containing PPs.

2.2.6. Prepare a set of 50 mL conical tubes filled with 25 mL of RPMI + 10% FBS (pre-warmed at 37 °C). Using a pair of scissors, cut the edge of a 1000 μ L tip from the distance that will allow the aspiration of the PPs with 1 mL pipette. Aspirate the PPs with 1 mL pipette and transfer them from the 12-well tissue culture plate to the prepared 50 mL conical tubes.

Note: Use a new tip for each mouse to avoid cross-contamination among the samples.

2.2.7. Secure the lid and place the tubes vertically in an orbital shaker at 37 °C, with continuous agitation at 125-150 rpm for 10 min. Meanwhile, prepare a new set of 50 mL tubes and place a 40 μ m cell strainer on the top of each tube, through which single cell suspension will be prepared.

Note: (i) The agitation step will remove the remaining intestinal content, mucus and cell debris, which decrease the cell viability and recovery of PP lymphocytes if not removed. (ii) Do not apply any kind of digestive enzymes on PP tissue because the digestion process causes a dramatic loss of CXCR5 expression from the cell surface.

2.2.8. After the agitation, transfer the PPs to the 40 μ m cell strainer placed on the top of the newly prepared conical 50 mL tubes. Using the rounded side of a 10 mL syringe plunger, gently disrupt the PPs through the cell strainer to generate single cell suspension. Rinse the strainer

with 15-20 mL of cold RPMI + 10 % FBS.

Note: (i) Before filtering, shake the tubes containing the PPs horizontally. This short shake will facilitate the transfer of PPs into cell strainers. (ii) Using a 70 μ m cell strainer for the isolation of non-lymphoid immune cells (*e.g.*, monocytes, macrophages, DCs) is also desired.

2.2.9. Centrifuge the single cell suspensions at 350-400 x g for 10 min at 4 °C.

2.2.10. Carefully discard the supernatant and resuspend the cells at a concentration of 10×10^6 cells/mL. Count the cells using a hemocytometer.

Note: (i) Prior to cell counting, the total cell number for each mouse PP group can be approximately estimated using the following formula: " $0.5-1 \times 10^6$ cells x (number of PPs) = total cell count". Further dilutions with trypan blue for cell counting might be necessary. (ii) As an alternative to manual counting, automated cell counters can be used.

2.2.11. Transfer $2-2.5 \times 10^6$ cells in appropriate volume (*e.g.*, 200 μ L) into a 96-well round-bottom plate.

Note: For single color and negative control samples, $0.5-1 \times 10^6$ cells might be sufficient.

2.2.12. Centrifuge the plate at 350 x g for 5 min at 4 °C. Flick the plate.

2.2.13. Wash the cells in 200 μ L of Staining Buffer.

3. Surface Antibody Staining

3.1 Viability Staining:

3.1.1. After the final wash, resuspend the cells in 100 μ L of fixable viability dye diluted in PBS (1:1000). Incubate for 30 min on ice or at 4 °C in the dark.

Note: (i) Intracellular staining for the detection of key transcription factors such as Foxp3 or Bcl-6 requires fixation of the cells. In that case, non-fixable viability dyes (*e.g.*, 7-AAD, DAPI) cannot be used. (ii) To dilute fixable viability dye, do not use any staining buffer that contains protein. The media used in this step must be protein-free. (iii) Exclusion of dead cells by using viability staining is crucial because dead cells can cause serious technical difficulties in flow cytometry analysis by emitting autofluorescence and by binding surface antibodies nonspecifically, which might lead to false positive results.

3.1.2. Wash the cells twice with staining buffer. Centrifuge at 350 x g for 5 min at 4 °C. Flick the plate.

3.2 Fc Block and Surface -Layer I:

3.2.1. Prepare the Fc-block solution by diluting anti-CD16/32 antibody (1:200) in FACS buffer.

3.2.2. Resuspend the cells in 20 μ L of prepared Fc-block solution. Incubate for 15 min on ice.

3.2.3. Without washing, add 80 μ L of surface antibody cocktail (see **Table 1** for the antibody summary) prepared at appropriate dilutions. Incubate on ice for at least 30 min.

3.2.4. Wash twice by adding excessive FACS buffer. Centrifuge at 350 x g for 5 min at 4 °C. Flick the plate.

3.3 Surface -Layer II:

3.3.1. Prepare Streptavidin staining solution by diluting fluorochrome-conjugated Streptavidin in staining buffer.

3.3.2. After the final wash, resuspend the cells with 100 μ L of pre-diluted Streptavidin staining solution. Incubate for at least 15 min on ice in the dark.

3.3.3. Wash twice with staining buffer. Centrifuge at 350 x g for 5 min at 4°C. Flick the plate. **(Optional)** If intracellular staining for follicular T regulatory cell detection is not desired, after the last wash, resuspend the cells in 200 μ L of staining buffer and transfer into appropriate tubes to acquire data in flow cytometer. When the samples are not fixed, acquisition of data by flow cytometry should be performed within 3-4 h to obtain accurate results.

4. Cell Fixation

4.1. Prepare fixation/permeabilization (Fix/Perm) working solution using the reagents from Foxp3/Transcription Factor Staining Buffer Set. Mix one-part of fixation/permeabilization concentrate with three parts of fixation/permeabilization diluent to the desired final volume.

4.2. After the final wash, resuspend the cells in 200 μ L of Fix/Perm working solution.

4.3. Incubate the plate on ice or at 4 °C for 20 min. Do not exceed 20 min for this step. Longer incubation time might severely decrease the cell recovery.

4.4. Centrifuge at 350 x g for 5 min at 4 °C. Flick the plate. **(Optional)** After this step, fixed cells can be stored for several days at 4 °C in staining buffer containing bovine serum albumin (BSA) or FBS until subsequent intracellular staining or flow cytometry acquisition.

4.5. Resuspend the cells in 200 μ L of permeabilization Buffer (x1) freshly pre-diluted in water.

4.6. Centrifuge at 350 x g for 5 min at RT.

4.7. Wash once with 200 μ L of permeabilization buffer and centrifuge at 350 x g for 5 min at RT.

5. Intracellular Staining

5.1. Prepare the Fc-block solution by diluting anti-CD16/32 antibody (1:200) in permeabilization buffer.

Note: After the fixation step, the cells must be maintained in permeabilization buffer until the end of the intracellular staining process.

5.2. After the final wash, resuspend the cells in 20 μ L of Fc-block solution. Incubate for 10-15 min at RT in the dark.

5.3. Without washing, add 80 μ L of intracellular antibody cocktail (100 μ L final volume) pre-diluted in permeabilization buffer. Incubate for 30 min at RT.

5.4. Add 100 μ L of Perm Buffer, and centrifuge at 350 x g for 5 min at RT.

5.5. Wash once with 200 μ L of permeabilization buffer, centrifuge at 350 x g for 5 min at RT.

5.6. After the final wash, resuspend the cells in 200 μ L of staining buffer and transfer the cells into appropriate tubes (final volume of 400 μ L in staining buffer) and acquire data by flow cytometry. Samples may also be stained in 96-well plates and run without transferring into tubes if a flow cytometer with plate reader option is available. This step minimizes cell loss during cell transferring.

5.7. Acquire a minimum of 5×10^5 total cells on the flow cytometer to be able to perform reproducible analysis of TFH, TFR as well as GC B cells.

REPRESENTATIVE RESULTS

In contrast to a previous protocol²⁰, we have observed that PPs are not evenly distributed throughout the SI but are localized more densely towards the distal and proximal ends of the SI as shown in **Figure 1A**. Flow cytometric analysis showed that, if followed correctly, our protocol gives a PP lymphocyte population that demonstrates forward-side scatter distribution similar to splenocytes (**Figure 2A, E, and 2D, H**) with > 95% cell viability (**Figure 2B and 2F**). In contrast to other SLOs, in C57BL/6 mice approximately 70-80% of total PP lymphocytes consist of B cells, whereas CD4⁺ T cells constitute only 10-15% of total PP lymphocytes (**Figure 2C and 2G**).

Please note that CD4⁺ CD19⁺ double positive (DP) cells constitute \approx 1% of total PP lymphocytes. With certain precautions during cell preparation such as performing Fc-Block against Fc receptors of B cells and excluding doublets, CD4⁺ CD19⁺ DP cell population could be minimized (**Figure 3A and B**). However, a fraction of DPs that does not demonstrate forward scatter (FSC) characteristics of doublets is unavoidable and should be gated out from the single positive T and B cell gates (**Figure 3B**). Our results revealed that B cells within DP cells highly express CXCR5 on

their surface (**Figure 3C**) that might lead to false positive results in the TFH gate which is adjusted based on CXCR5 expression in CD4⁺ T cells. On the other hand, we found that GL7, a marker of activated B cells, is also expressed in CD4⁺ CD19⁺ DP cells (**Figure 3D**), which might cause interference with GC B cell gating.

Examples of TFH and GC B cell gating algorithms are depicted in **Figure 4**. For GC B cell gating, we use GL7 labeling versus CD38, which identifies GC B cells as GL7⁺ CD38⁻ B cells (**Figure 4A-B**). PNA labeling might be used instead of GL7²³ whereas CD38 can be replaced by one of the following markers: IgD, Bcl-6, and CD95. Alternative gating strategies for GC B cells are demonstrated in **Figure S1**. GC B cell ratio in PPs shows great variability in C57BL/6 mice. However, compared to other SLOs, PPs have significantly higher GC B cell ratio with a range of 2-10% of total B cells under steady-state conditions. Gating strategy for PP TFH cells is described as CD19⁻ CD4⁺ PD-1^{hi} CXCR5⁺ T cells (**Figure 4A, C**). “Zebra plot” was found to be an optimal demonstration method for TFH gating as it depicts PD-1^{hi} population more discretely compared to other plot types (**Figure 4C**). TFR, a subset of TFH cells expressing Foxp3, are gated as CD19⁻ CD4⁺ PD-1^{hi} CXCR5⁺ Foxp3⁺ T cells (**Figure 4D**). Like GC B cells, the TFH cell fraction also varies in unimmunized mouse individuals with a range of 10-20% of total CD19⁻ CD4⁺ PP lymphocytes. Details of alternative gating algorithms for TFH cells are described in **Figure 4E, F and Figure S1C, D**.

To avoid false positivity due to background staining and autofluorescence, isotype controls or alternative equivalent controls such as Fluorescence Minus One (FMO) should be included in the staining panel as a negative control for TFH and GC B cell markers (**Figure 4G-J**).

To optimize the conditions for PP tissue preparation, we developed a novel internally controlled experimental strategy, in which PPs were collected from one individual mouse and pooled separately depending on their anatomical origin (*e.g.*, duodenal, ileal). For performing experiments, pooled PPs were assigned to individual groups so that each experimental and control group included an equal number of PPs from each anatomical region (**Figure 5A**). By this approach, we found that collagenase-based enzymatic digestion (tested at 37.5 mg of collagenase II or IV per 25 mL of digestion mix = 1.5 mg/mL), which is commonly used for lymphocyte isolation from various mucosal tissues, significantly reduced CXCR5 expression in PP lymphocytes (**Figure 5B**), particularly in TFH cells (**Figure 5F-I**), while the expression of other surface molecules (*e.g.*, CD19, CD4) remained intact (**Figure 5C-E**). Reduction of CXCR5 detection was prominent so that the expression of CXCR5 on collagenase-treated TFH cells was almost indistinguishable from isotype control (**Figure 5F-I**). Further experiments showed that the interference level of enzymatic digestion with CXCR5 expression was dependent on the CXCR5 antibody clone used (**Figure 6A-F**). Specifically, upon collagenase II treatment, the detection capacity of CXCR5 antibody clone 2G8 was more significantly impaired, than the detection capacity of the CXCR5 antibody clone L138D7 (**Figure 6A-D**).

Enzymatic digestion not only reduced the expression of CXCR5 but also in the proportion of total lymphocytes within PP cells as determined by the FSC-SSC characteristics, while a considerable fraction of cells isolated from digested PPs exhibited forward and side scatter of a higher intensity than PP lymphocytes (**Figure 7A-C**). The effects of collagenase on cell recovery occurred in a dose-

dependent manner (**Figure 7A, H, I, J**). Enzymatic digestion increased the cell viability (**Figure 7D-E**). However, this benefit was not causatively linked to collagenase digestion because the cells isolated from PPs after the agitation without collagenase were favored similarly (**Figure 7F**). The detection of the nuclear transcription factor Foxp3, which is of particular interest here because it is usually assessed during TFH isolation to identify TFR cells, was improved by the agitation at 37 °C regardless of the collagenase digestion (**Figure 7G**).

FIGURE LEGENDS

Figure 1: Macroscopic structure and anatomical distribution of murine Peyer's Patches. (A). An image obtained from duodenal-end of the small intestine with the stomach. Two closely-located PPs in the duodenum are indicated with black arrows. **(B).** PPs collected from eleven C57BL/6 mice were stored individually in a 12 well-plate. **(C).** Image of freshly excised mouse PPs displayed on a 40 µm cell strainer.

Figure 2: Flow cytometric characterization and basic gating algorithm for PP lymphocytes. (A). Dot plots demonstrate the distribution of freshly isolated unfixed PP lymphocytes along FSC and SSC axes which exhibit great similarity to splenocytes **(D)**. **(B).** Exclusion of dead cells by the means of 7AAD expression. 7AAD⁻ cells represent live cells. **(C).** CD19 and CD4-based immunophenotyping of T and B cells among pre-gated live PP cells. **(E-G).** Representative flow analysis of fixed PP lymphocytes. **(H).** Representative flow characteristics of fixed splenocytes.

Figure 3. CD4⁺ CD19⁺ Double Positive (DP) cells cause false positivity in TFH and GC B cell gating. (A). CD4⁺ CD19⁺ DP lymphocytes within live PP cells are demonstrated. **(B).** Separation of DP cells based on FSC characteristics as doublets and singlets. **(C).** CXCR5 and GL7 expression of DP cells are depicted in histogram plots.

Figure 4. Representative TFH and GC B cell gating strategy. PPs were collected from a 3-month old mouse and lymphocytes were isolated as described in the Protocol section. **(A).** CD19 and CD4 labeling of live PP lymphocytes is depicted. **(B).** CD38^{lo} GL7^{hi} CD19⁺ B cells were gated as GC B cells. **(C).** TFH cells were gated as CXCR5⁺ PD-1^{hi} CD4⁺ cells. **(D).** TFR cells are a fraction of TFH cells expressing regulatory cell-specific transcription factor Foxp3 together with TFH markers and are gated as CXCR5⁺ PD-1^{hi} Foxp3⁺ CD4⁺ T cells. **(E-F).** Gating strategy for TFH surface markers confirmed in splenocytes isolated from NP-OVA-immunized mouse by the means of BCL-6 expression. **(G-J).** Fluorescence Minus One (FMO) controls for key TFH-TFR markers are depicted.

Figure 5: Collagenase-based enzymatic digestion leads to a massive reduction of CXCR5 detection. (A). PPs from a 2-month-old mouse located in the duodenum (≈ 1/3 proximal), jejunum (≈ 1/3 middle) and ileum (≈ 1/3 distal) were collected and pooled separately. Pooled PPs were equally distributed to the experimental groups. Enzymatic digestion with collagenase II or IV at 1.5 mg/mL concentration was performed with agitation for 10 min at 37 °C. **(B-E).** Histogram plots depicting the expression of surface molecules (CXCR5, CD19, PD-1, CD4) of the cells isolated from PPs that were subjected to collagenase-based digestion. **(F-I).** TFH gating within the collagenase-administered and control groups is demonstrated in zebra plots. Data represent three independent experiments.

Figure 6: The effect of collagenase on CXCR5 expression showed great difference depending on the anti-CXCR5 antibody clone. PP lymphocytes collected from a 6-month-old mouse, pooled and separated into different groups regarding antibody clone used and enzymatic digestion application. **(A-F)**. The proportion of TFH cells is depicted in zebra plots within pre-gated live, CD19⁻ CD4⁺ T cells. **(A, C & E)**. PP cells were stained with the Biotin-conjugated anti-mouse CXCR5 antibody (2G8 clone) and Streptavidin conjugated with BV421 fluorochrome. **(B, D & F)**. PP cells were stained with a primary conjugated anti-mouse CXCR5 antibody (L138D7 clone). **(A-B)**. TFH gating plots from undigested PP lymphocytes. **(C-D)**. PPs digested with collagenase II (20 mg/25 mL of digestion mix) and agitated for 10 min at 37 °C. Data represent two independent experiments.

Figure 7. Collagenase-based digestion and agitation at 37 °C influence cell viability, recovery and intracellular staining efficiency. A 3-month-old C57BL/6 mouse was sacrificed; PPs were collected and pooled as described in **Figure 5A**. **(A)**. After the collection of PPs, the tissues were agitated in the presence of collagenase II (37.5 mg/25 mL of digestion mix) for 10 min, FSC and SSC characteristics of fixed PP cells are depicted in **(A)** and viability staining plot is depicted in **(D)**. **(B, E)**. After the collection of PPs, the tissues were kept on ice without agitation or enzymatic digestion; FSC and SSC characteristics of fixed PPs cells are depicted in **(B)** and viability staining is depicted in **(E)**. **(C, F)**. After the collection of PPs, tissues were agitated at 125-150 rpm for 10 min at 37°C in the absence of collagenase; FSC and SSC characteristics and viability staining of fixed PP cells are depicted **(C, F)**, respectively. **(G)**. Comparison of Foxp3 expression in regulatory T cells isolated from agitated and unagitated PPs without collagenase administration is depicted in the histogram plot. **(H-J)**. PPs collected from a 6-month old C57BL/6 mouse and were treated with different concentrations of collagenase II: 25 mg per 25 mL digestion mix, 15 mg per 25 mL digestion mix or no collagenase. FSC and SSC plots which reveal the effects of enzymatic digestion on PP cell recovery and yield are depicted.

Figure 8: The molecular model and the complete amino acid sequence of CXCR5. **(A)**. Seven-pass transmembrane protein structure of CXCR5 is modeled. **(B)**. The complete amino acid sequence of CXCR5 is demonstrated. The sequence of extracellular regions is indicated in red and extracellular amino-acids with presumable collagenase sensitive chemical bonds are demonstrated in yellow.

Table 1: Antibody summary. The dilutions, clones and fluorochrome conjugations for the relevant antibodies and viability dyes are indicated. The optimal dilution might vary depending on experimental conditions and lot quality of the product. Primary BV421-conjugated L138D7 clone at 1:20 dilution showed comparable CXCR5 detection capacity with the biotin-conjugated 2G8 clone at 1: 50. Therefore, primary-conjugated L138D7 can be used as alternative to biotin-conjugated 2G8 clone.

Figure Supplementary 1 (S1): Alternative gating strategies for TFH and GC B cells. **(A)**. Live PP lymphocytes from a 3-month-old mouse on the C57BL/6 background are depicted. **(B-C)**. GC B cells are gated as CD38⁻ GL7⁺ **(B)** and BCL-6⁺ GL7⁺ B cells **(C)**. **(D-E)**. TFH cells are depicted as PD-

1^{hi} CXCR5⁺ (**D**) and as ICOS⁺CXCR5⁺ (**E**).

DISCUSSION

Here, we describe a protocol optimized for flow cytometric characterization of TFH and GC B cells. One of the major advantages of our protocol is that it enables the isolation of up to 10⁷ (average 4-5 × 10⁶ cells) total PP cells from a single mouse (C57BL/6 strain) without any digestive process. We observed that the total cell yield was positively correlated with the number of PPs and could be estimated from the following simple equation which is helpful for experimental planning: “total PP cell count *per* mouse = 0.5-1 × (number of excised PP *per* mouse) × 10⁶”. This high cell yield was complemented with enhanced cell viability (>95%). Improved cell recovery and viability allowed performing flow cytometric analysis of various lymphocyte subsets in PPs using diverse antibody staining panels in parallel.

Compared to a recent PP isolation protocol published by De Jesus *et al.*²⁰ and previous reports^{19,21}, our protocol gave considerably higher total cell yield and viability, in spite of the absence of the digestion with collagenase. Although the identification of PPs might be “tricky” for the first-time investigators, the learning curve for this protocol is pretty steep, and if mastered, our technique allows the identification and surgical excision of PPs from the small intestine in a short time. If the desired PP number is not reached after the first attempt, we suggest repeating the PP identification step focusing on the distal and proximal end of the SI. In our practice, almost in every mouse, two closely located PPs in the duodenal end (**Figure 1A**) and at least 2-3 PPs within the last 5 cm of the ileum were detectable.

TFH staining can be more demanding than other cell subsets within the PPs, thus requiring extra attention¹⁸. If certain steps are missed during tissue processing and cell isolation, results might be quite misleading. Hence, we suggest researchers to pay attention to the following experimental “tips”. Since key TFH and GC B cell markers such as CXCR5 and GL7 can be expressed in both B and T cells, it is critical to include a B cell marker in antibody panels to avoid false positive results due to the contaminating CD19⁺CD4⁺ DP cells²⁴ (**Figure 2**). Please note that gating strategies based on T cell markers only²⁵ will not provide sufficient exclusion of DP cells since these cells also highly express T cell markers including CD3 (data not shown) and CD4. In addition to the exclusion of DP cells, flow cytometry results should be validated and confirmed by employing appropriate negative controls (*e.g.*, isotype controls, FMOs). Not only false positivity but also misplaced TFH gate can lead to false results and conclusions. Adjustment of TFH gate could sometimes be challenging especially if TFH cell population is not abundant, thereby not appearing as a discrete population in flow cytometry. To circumvent this limitation, the addition of multiple TFH markers (*e.g.*, BCL-6, PD-1, ICOS) into antibody panel might provide alternative gating opportunities and increase accuracy²⁶. For practical reasons, BCL-6 was found to be an ideal co-staining marker for alternative TFH gating as it can be used for gating GC B cells concurrently¹⁶ (**Figure S1C**).

Furthermore, having a positive control sample containing a high TFH proportion could provide researchers good navigation for placing TFH gate correctly. In our experiments, we used PP samples from aged mice (older than 6-months) as a positive control since we have found that

aging causes the enhancement of TFH cells within PPs up to 40% of total CD4⁺ T cells in steady state (data not shown). We also encourage the researchers that have further interest in technical details of TFH staining to look at the methods in the published protocol book for TFH cell staining¹⁸.

Testing experimental hypotheses in PP can be relatively challenging and might require a very high number of mice per group to reach statistical significance²¹. By taking advantage of the high cell yield of our protocol, we circumvented this obstacle with the following experimental strategies. First, we excised PPs and sorted them separately depending on their anatomical origin (*e.g.*, duodenal, jejunal and ileal). Then, we equally distributed these pooled PPs into different experimental and control groups. This internally controlled experimental strategy enabled us to minimize the inter-individual difference in mice while all cells were obtained from a single mouse. Furthermore, variability between different intestinal compartments (*e.g.*, duodenum vs. ileum) - previously reported to be significant² - was prevented as experimental and control groups consisted from an equal number of PPs per anatomical region (**Figure 5A**). By performing replicates of independent experiments, using the same approach, we improved the reliability and significance of our results. Besides eliminating nonspecific variations, this methodology also helps spare mice, reagents and time.

During the development and optimization of our protocol, our results revealed that collagenase II- and IV-based digestion strikingly reduced CXCR5 expression (**Figure 5B**) while other surface molecules for detection TFH and GC B cells remained intact (**Figure 5C-E**). In a recently reported JoVE protocol¹⁹, collagenase II was shown to be very effective for lymphocyte isolation from PPs and LPs. However, collagenase II has previously been reported to have a high tryptic activity, which results in cleavage of several surface molecules. Given its surface-molecule friendly nature²⁷ due to the lower tryptic activity and more common usage in the literature²⁸⁻³⁰, Collagenase IV had also been included in addition to collagenase II in our study and used in the concentrations as recommended in these previous reports. The use of collagenase and equivalent enzymes has been a contradictory issue in mucosal immunology due to the unwanted effects of the digestion on immune cells such as altered surface molecule expression^{31,27}.

It was previously reported that the use of different types of collagenase could interfere with flow cytometric CXCR5 detection within human gut³² and tonsil samples¹⁸. Conversely, other studies demonstrated the isolation of TFH and GC B cells from PPs without the use of collagenases^{24,33}. However, until now no comparative approach had been undertaken to assess whether the inclusion or exclusion of collagenase digestion would represent the optimal condition for isolation of CXCR5-expressing TFH cells from murine PPs. Mouse CXCR5 is a 374 amino acid long seven-pass membrane protein with relatively short extracellular regions compared to other transmembrane proteins such as CD4, CD19 that are abundantly expressed in lymphoid cells in PPs (data obtained from UniProt). In **Figure 8A-B**, a molecular model and total amino acid sequence of mouse CXCR5 are depicted with the presumed target sequences of collagenase-based digestion, which tends to dissociate chemical bonds between glycine (Gly) and neutral amino acids³⁴. The seven-pass transmembrane molecular structure resulting short extracellular domains might be responsible for making CXCR5 molecule vulnerable to enzymatic digestion

(**Figure 8A**). Intriguingly, we found that detection of CXCR5 expression after collagenase treatment is dependent on the antibody clone used. Although 2G8 and L138D7 clones showed similar performance in the undigested PP group, as determined by the detection of comparable TFH fractions, in the digested PP group, L138D7 clone revealed approximately 3x ($\approx 9\%$) higher fraction of TFH cells within CD4⁺ T cells than the 2G8 clone ($\approx 2\%$) (**Figure 6**). This finding suggests that reduced CXCR5 staining might be secondary to modified three-dimensional epitope configuration by the enzymatic activity of collagenase. Our results indicate that collagenase-based digestion must be avoided during PP preparation.

The interference of collagenase dissociation with CXCR5 molecule detection was circumvented by excluding the digestion step. However, it should be noted that for some tissues such as LP, mechanical disruption is not sufficient to isolate the cells and for such tissues, enzymatic digestion would be required¹⁹. In the future, it will be essential to test alternative digestion conditions as well as digestion-compatible antibody clones to bypass this technical limitation for tissues requiring enzymatic digestive processes. Until then, imaging techniques such as immune fluorescence microscopy will stay as the method of choice for TFH detection in these tissues. In addition to isolating LP cells, the isolation of some hematopoietic cell subsets such as DCs and plasma cells from PPs require enzymatic digestion³⁵. When employing our protocol, researchers who desire to isolate these cell subsets from PPs should include the appropriate enzymatic digestion step²⁰. Possible interference of enzymatic digestion with the expression of DC markers should be taken into consideration and digestion conditions should be optimized as needed. When isolation of TFH and GC B cells is desired in addition to a cell subset that requires collagenase digestion³⁶, PPs collected from each mouse could be separated in two groups for processing with and without collagenase treatment in parallel panels.

The effects of collagenase on PP lymphocytes were not restricted to surface molecule expression. Our results revealed that enzymatic digestion caused a relative decrease in the lymphocyte proportion within PP cells in a dose-dependent manner (**Figure 7**). The decrease of the lymphocyte fraction might be a consequence of collagenase-mediated release of other types of hematopoietic cell subsets such as phagocytes and DCs from PPs that are larger and more granular than lymphoid cells. It is also possible that collagenase digestion mediated cell release from neighboring LP and IE compartments, which could contribute to the change of FSC-SSC profile. This cell contamination from other gut compartments might also cause interference in the flow analysis of PP lymphocytes. On that basis, to maximize cell purity, we also avoid the addition of reagents such as EDTA or DTT that are widely used to extract epithelial cells and mucus²⁰, keeping tissue preparation conditions as physiologic and unmanipulated as possible. Despite these adverse effects of enzymatic digestion, beneficial effects on PP lymphocytes were also discovered such as improvement of intracellular staining and cell viability (**Figure 7D-F, G**). However, further analyses revealed that these effects were attributed to the agitation process at 37 °C independently of enzymatic digestion because cell viability and intracellular Foxp3 staining were favored to a comparable extent when cells were agitated in the absence of collagenase. Mechanistically, the beneficial effect of agitation might be due to the improved removal of intestinal content and mucus from PPs.

In summary, we have described a streamlined protocol for the isolation of lymphocytes from PPs. Isolated cells can be stained for flow cytometric characterization of several cell subsets or can be subjected to cell sorting and subsequently employed in various *in vitro* and *in vivo* functional assays.

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DISCLOSURES:

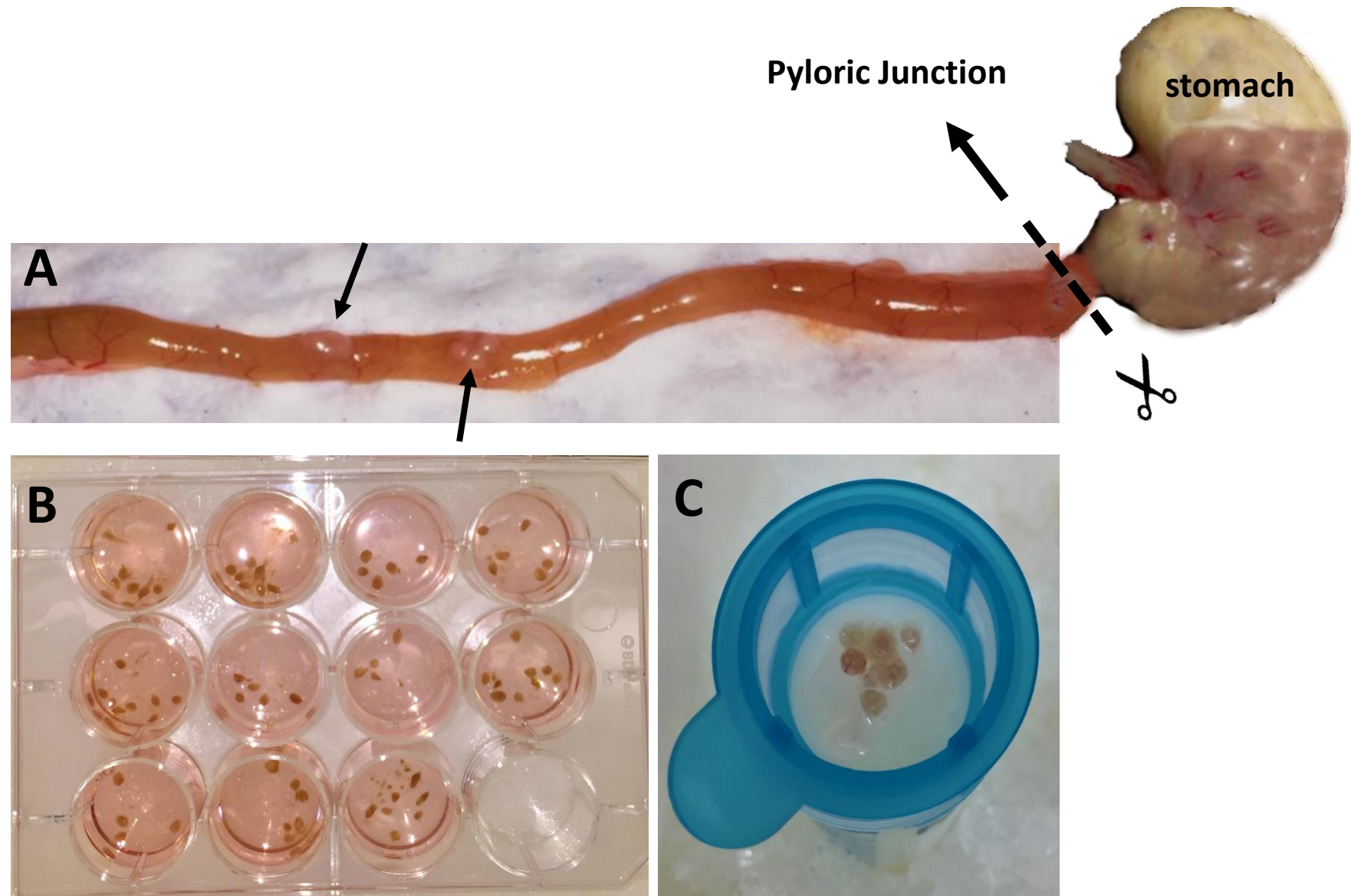
No conflicts of interest declared.

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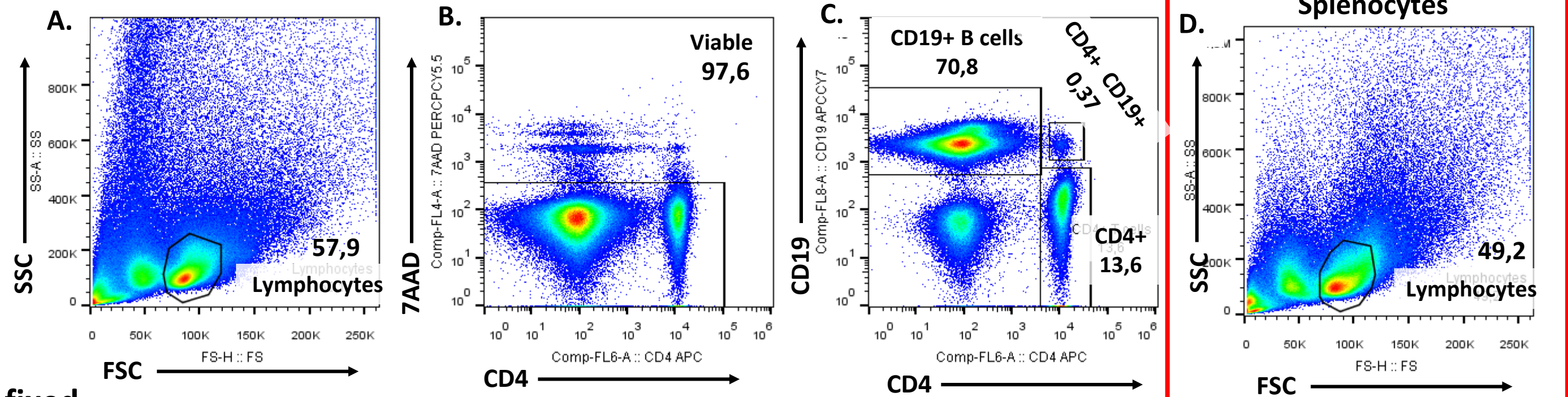
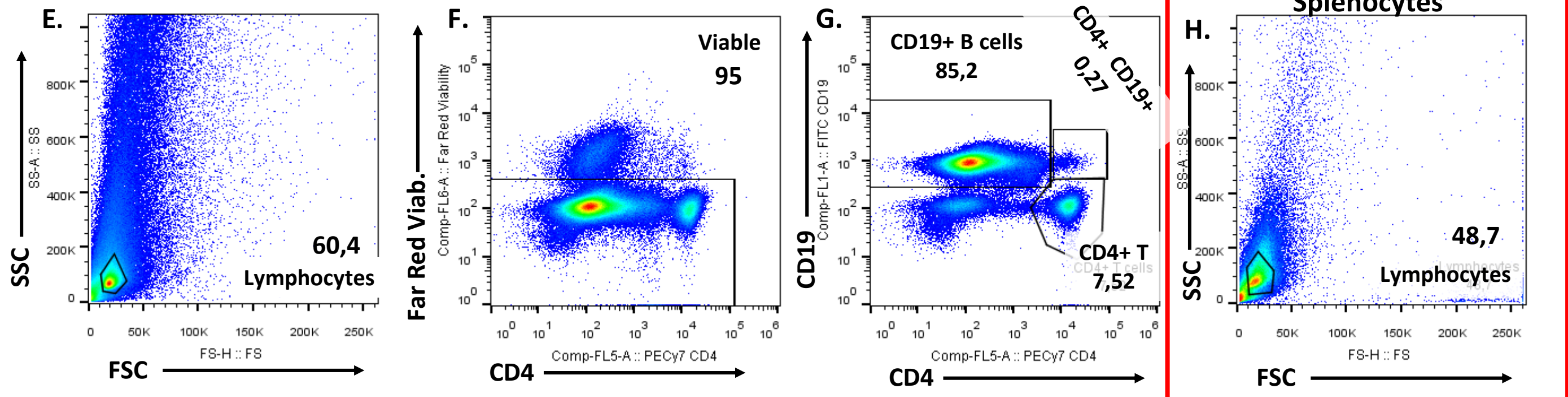
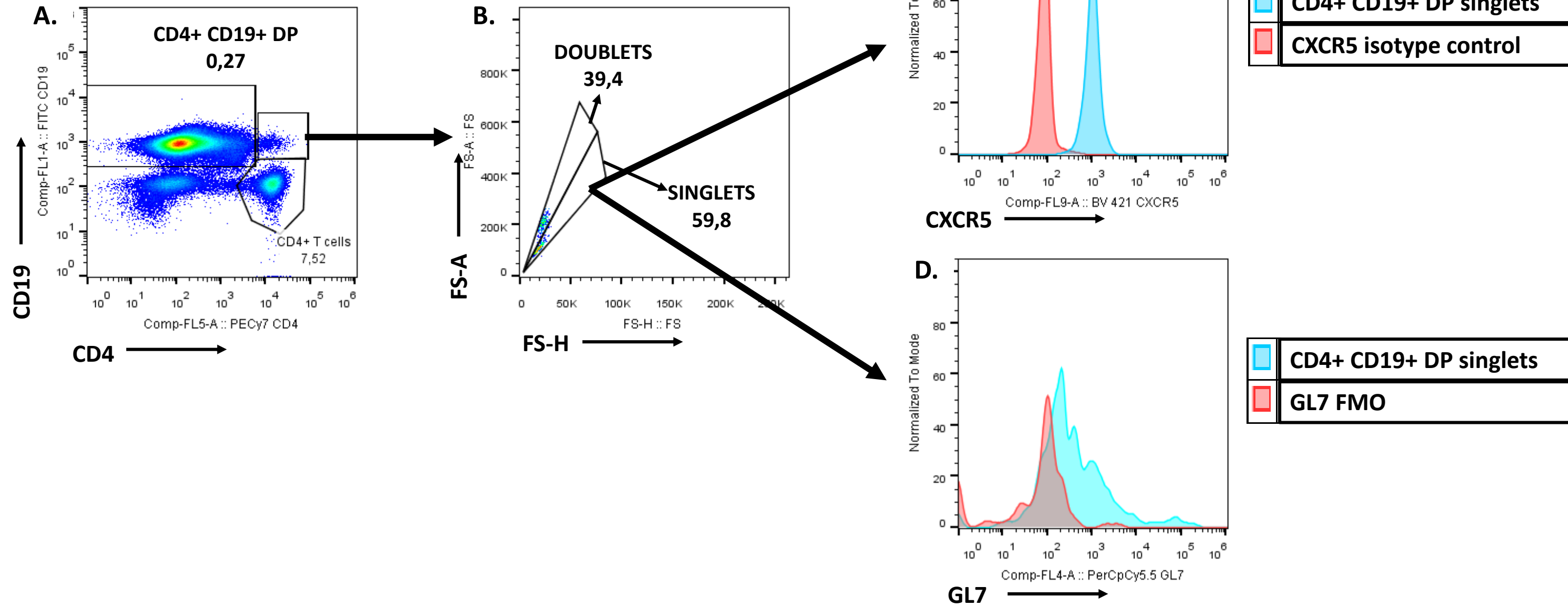
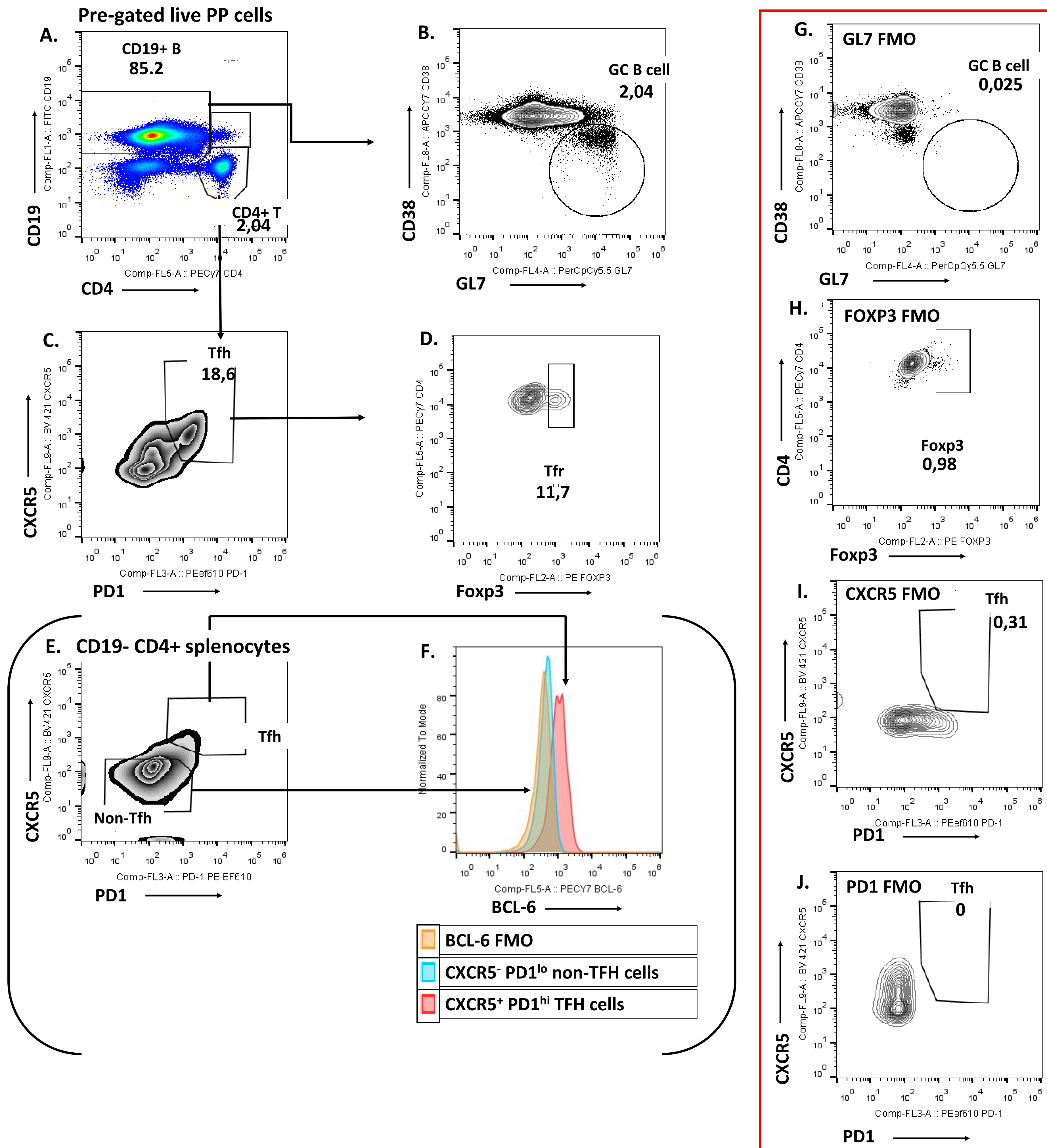
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Figure 3



Flourescence Minus One (FMO) Controls



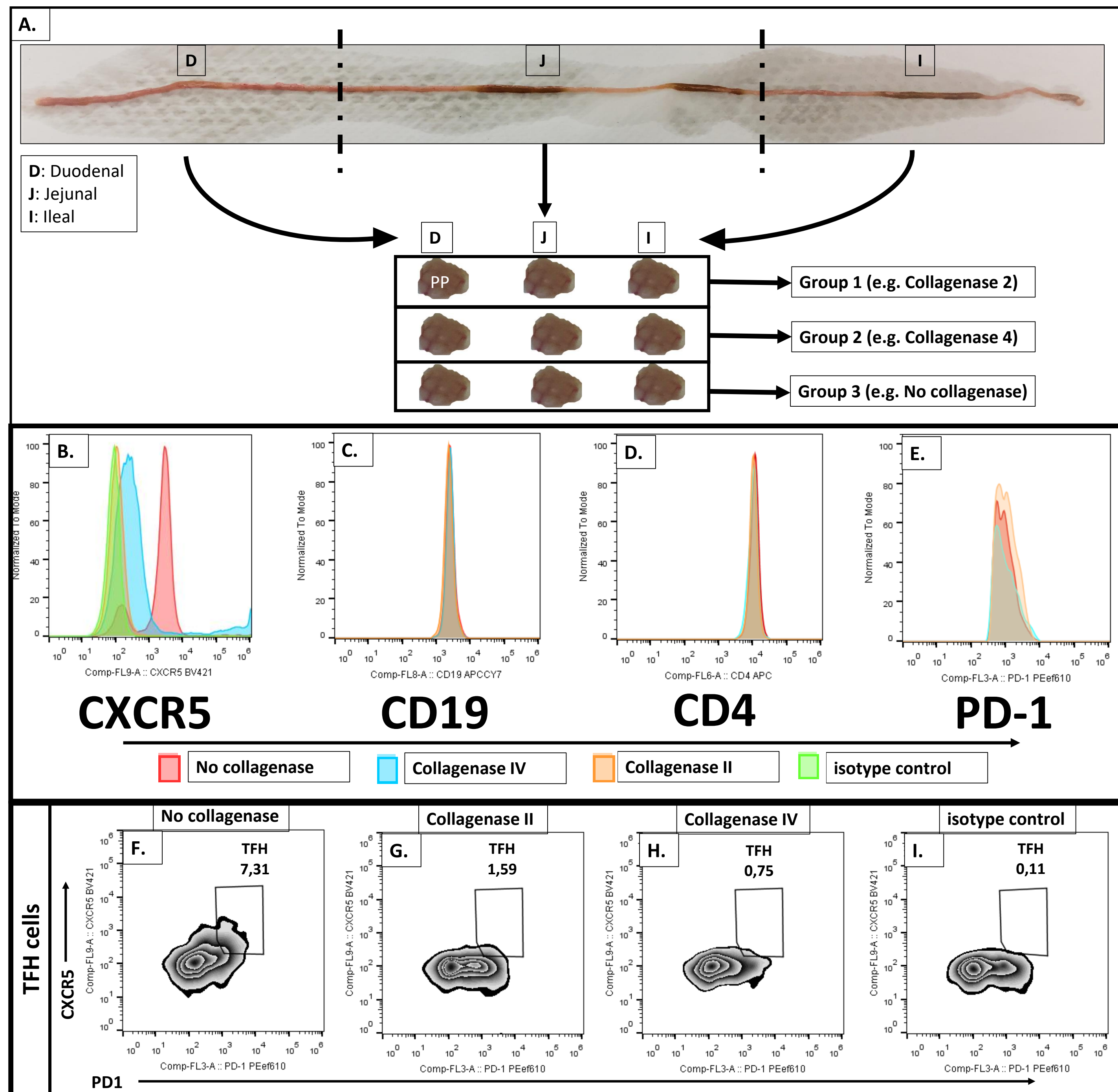
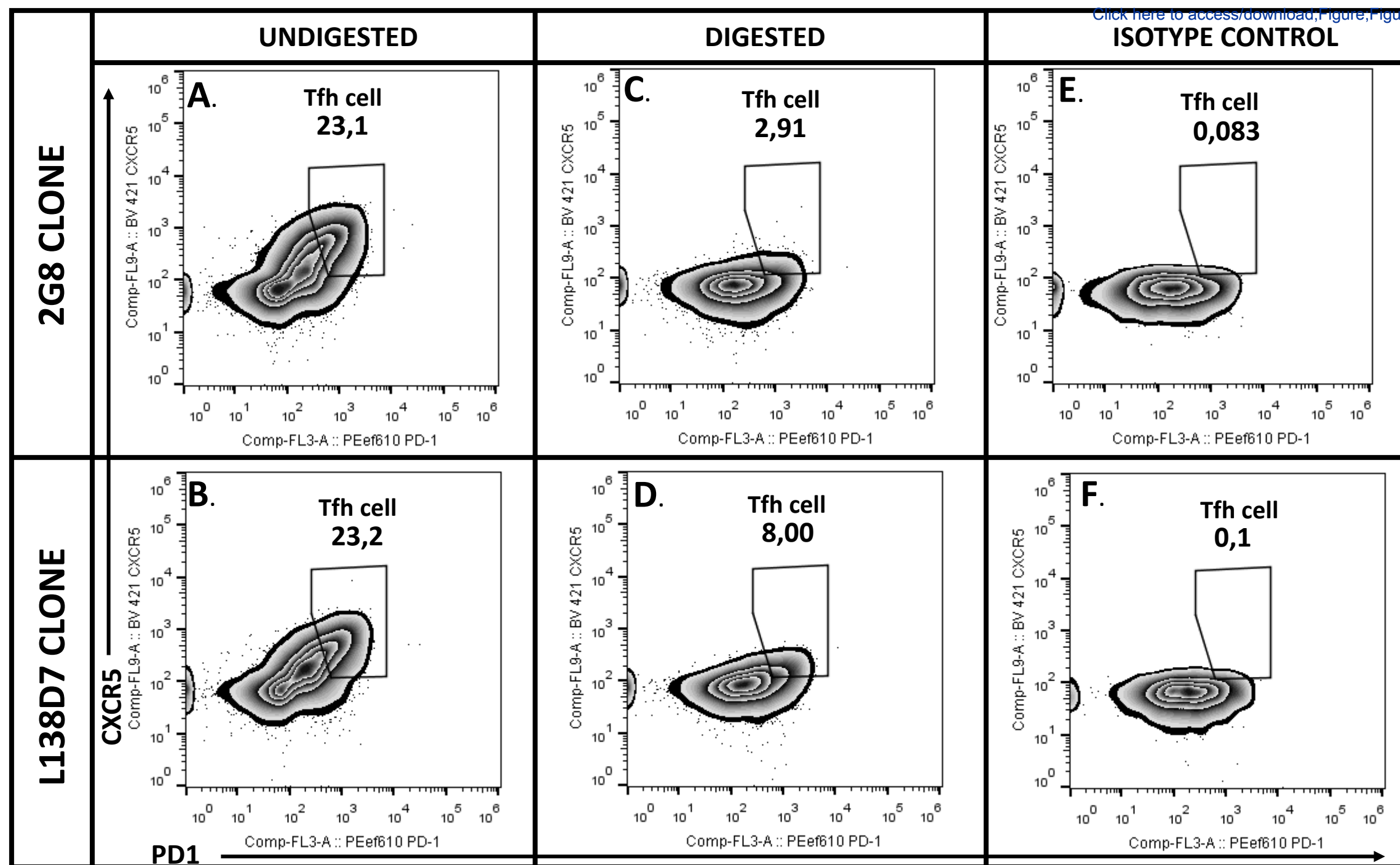
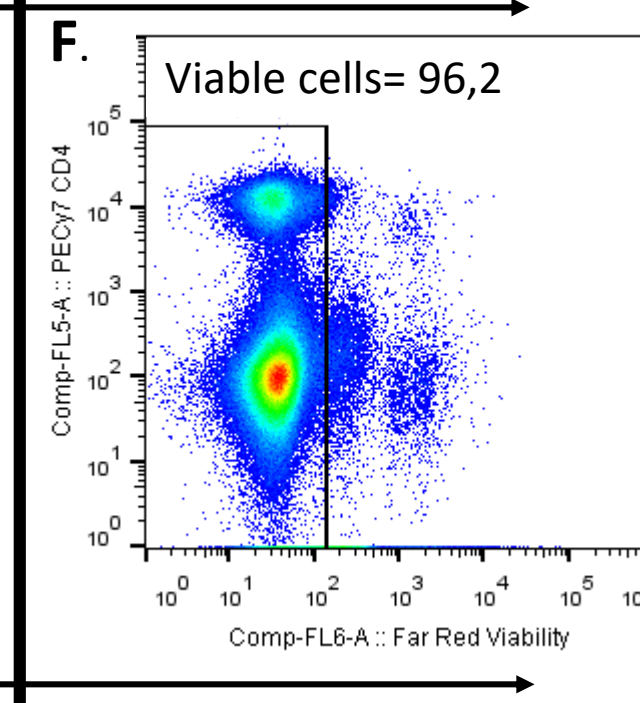
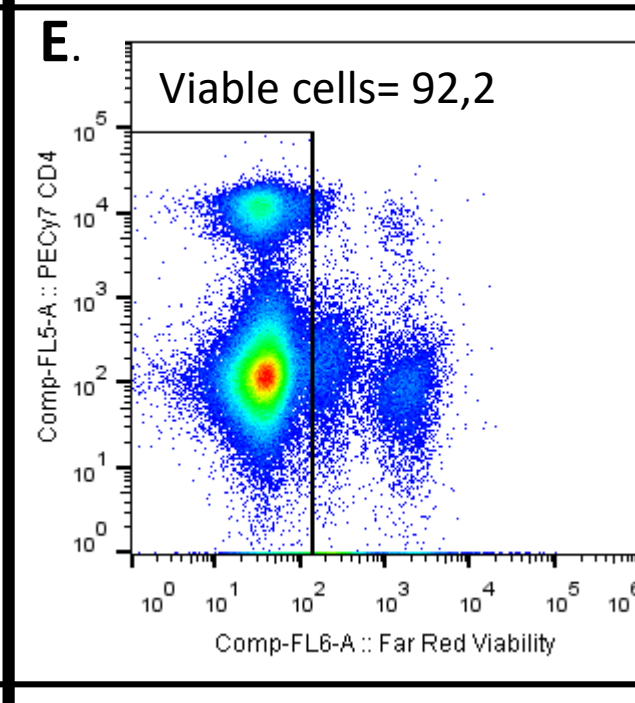
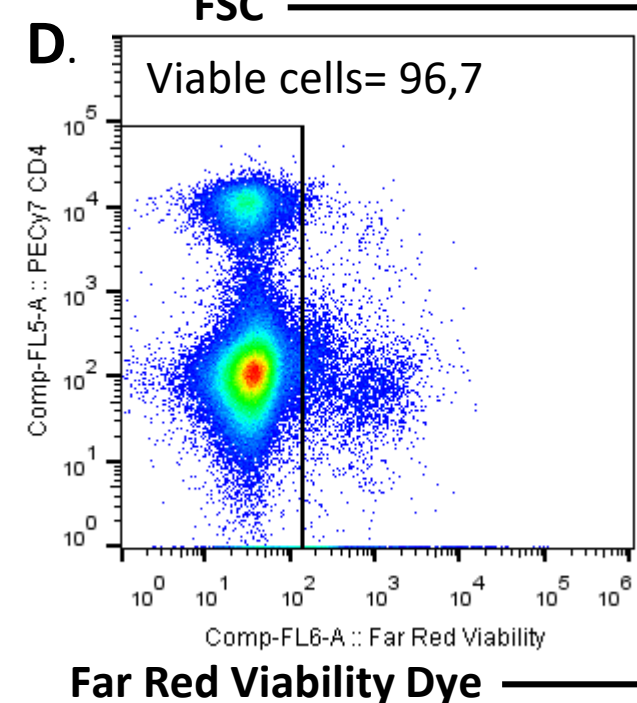
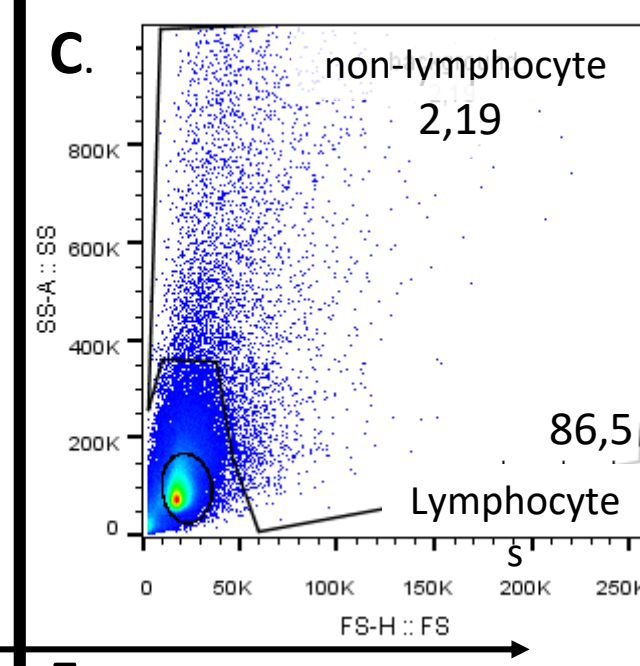
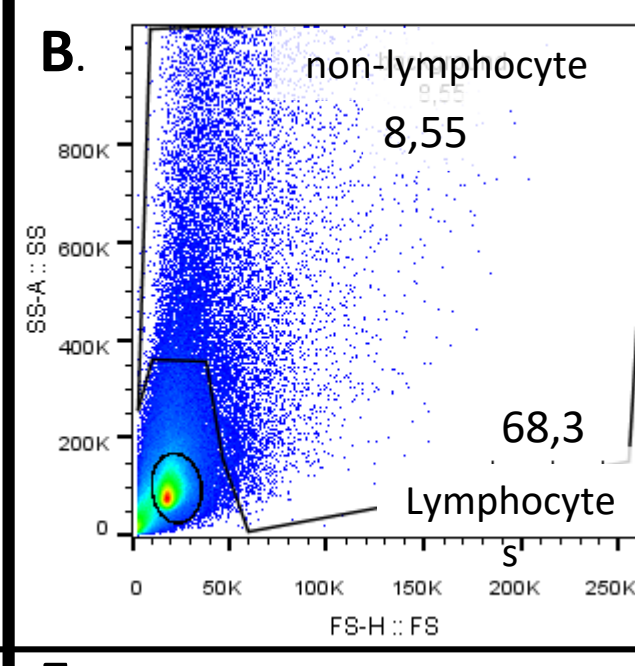
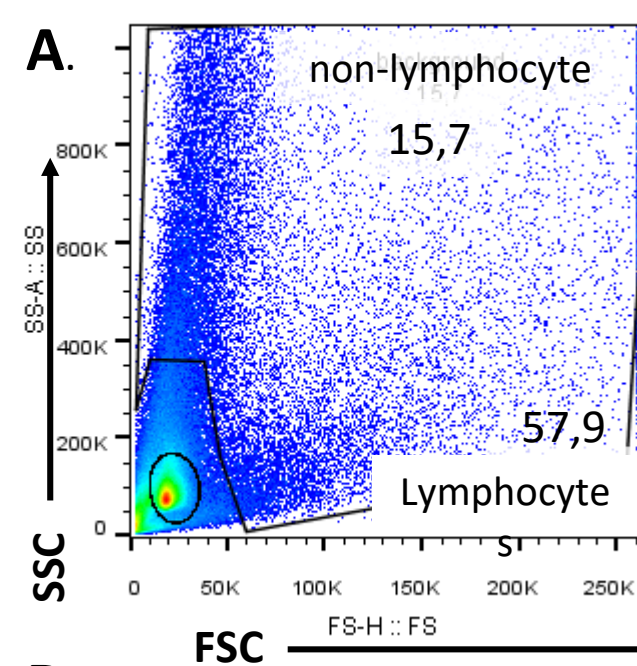
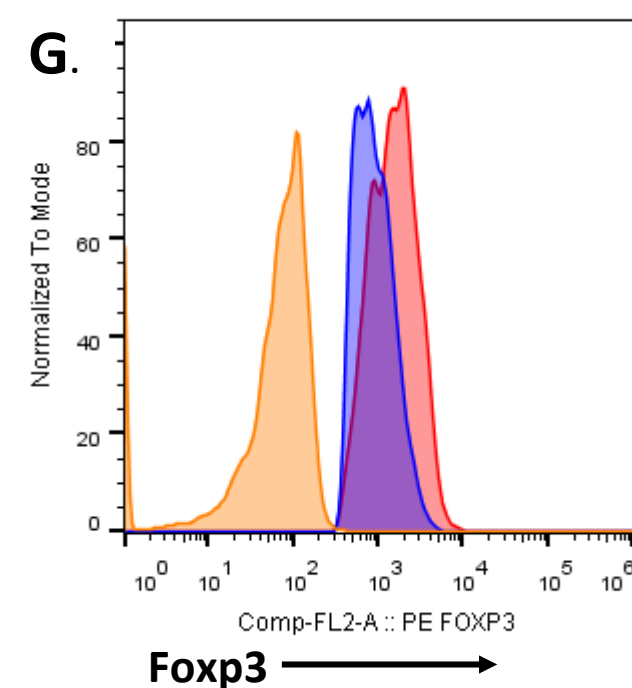
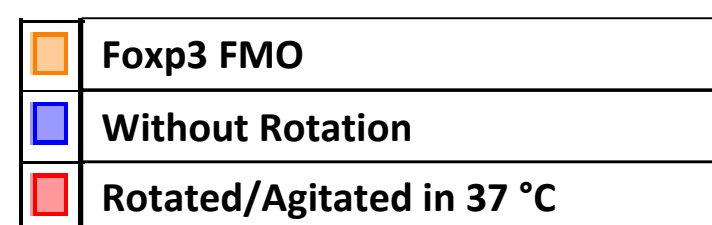


Figure 6

[Click here to access/download;Figure,Figure 6.pdf](#)





Collagenase II (1.5 mg/ml)

✓

✗

✗

Rotation/Agitation in 37 °C

✓

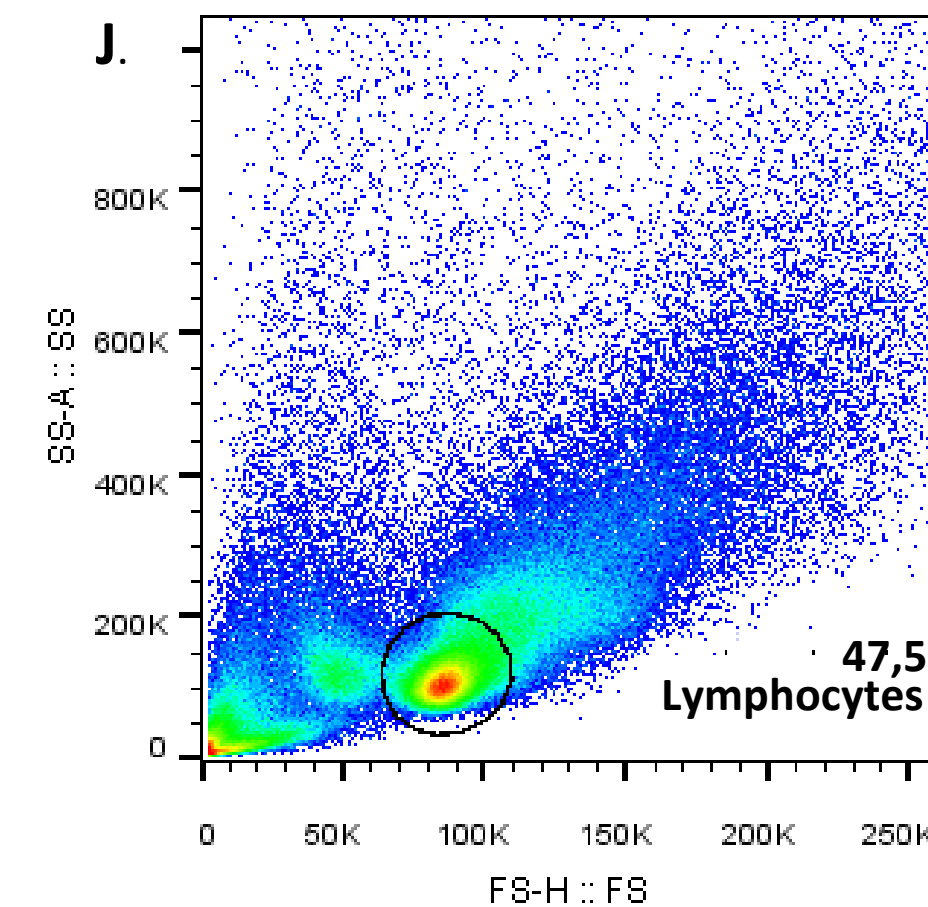
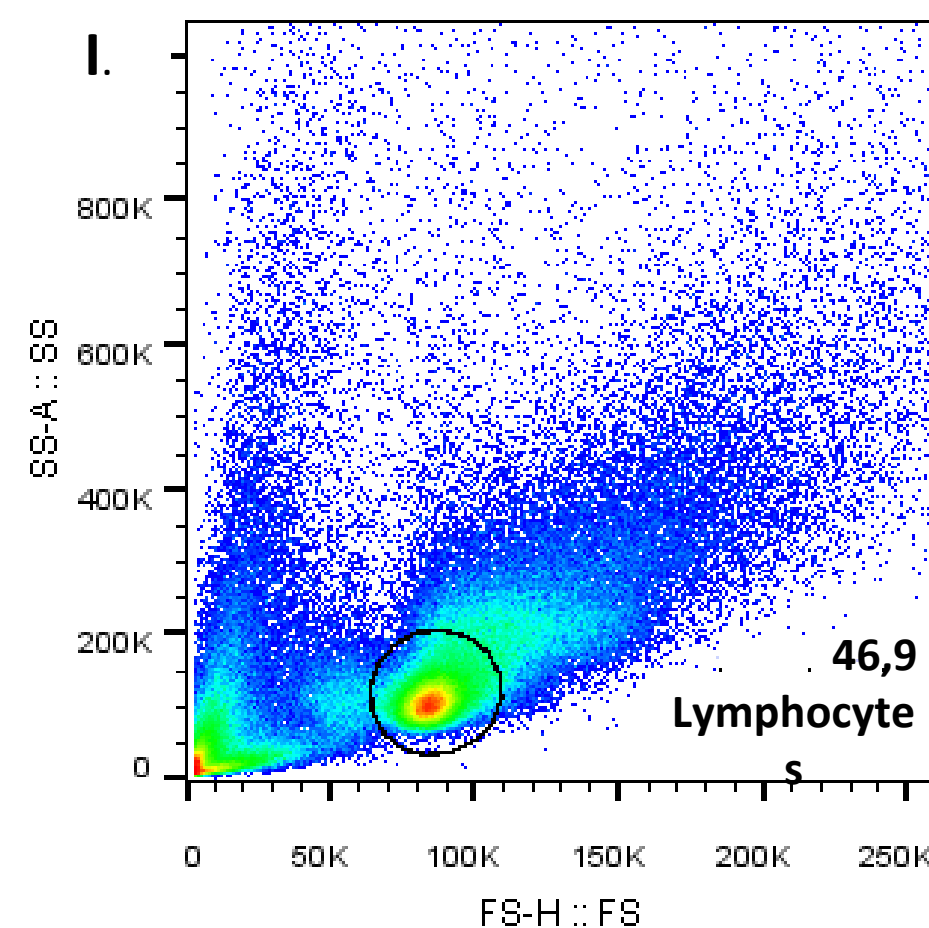
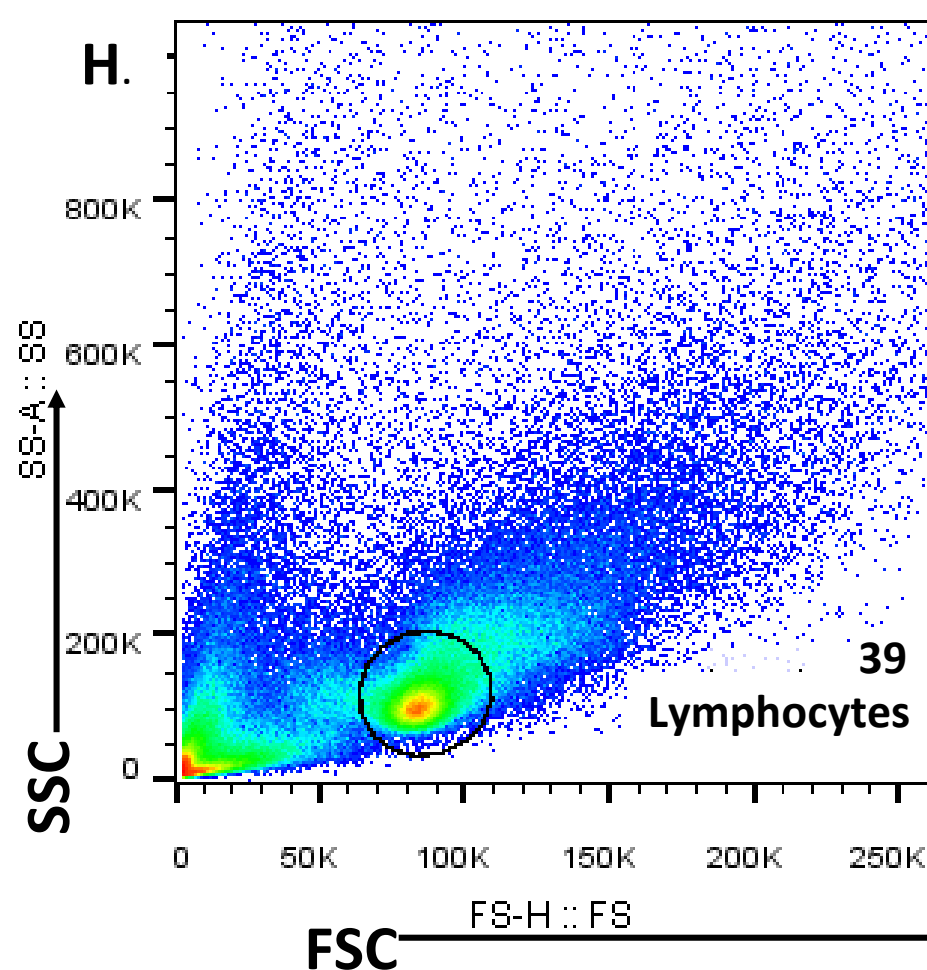
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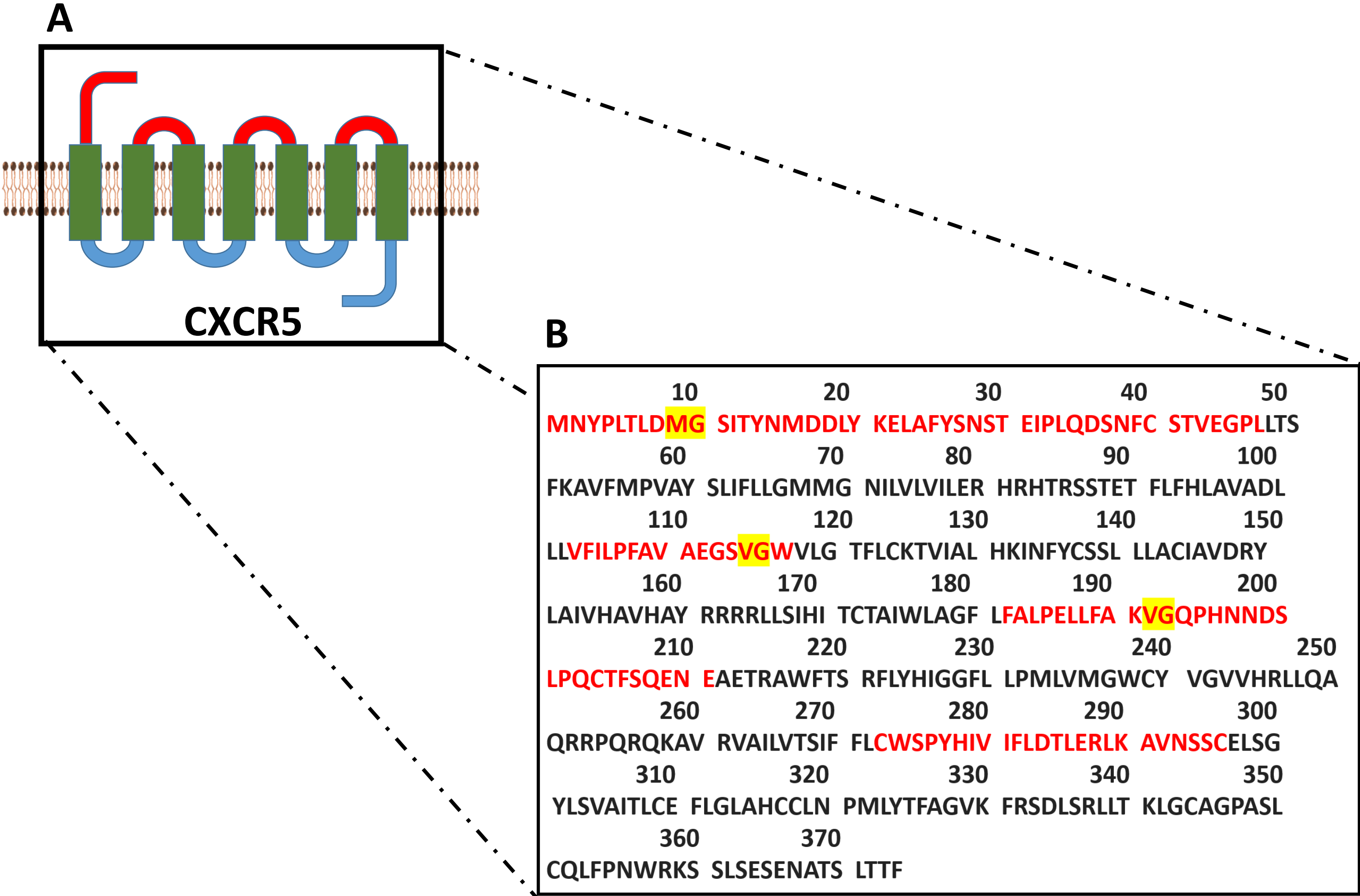
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Collagenase 1 mg/ml

Collagenase 0.6 mg/ml

No Collagenase





Name of Material/ Equipment	Company
anti-mouse CD4 antibody	eBioscience, Biolegend*
anti-mouse CD19 antibody	eBioscience
anti-mouse PD-1 antibody	eBioscience
anti-mouse ICOS antibody	eBioscience
anti-mouse GL7 antibody	Biolegend
anti-mouse CXCR5 antibody	Biolegend*, BD Bioscience
anti-mouse BCL-6 antibody	Biolegend
anti-mouse Foxp3 antibody	eBioscience
Streptavidin-BV421	BD Bioscience
FixableViability Dye	eBioscience
7AAD	Biolegend
FcBlock (CD16/32)	BD Bioscience
Collagenase II	Worthington
Collagenase IV	Worthington
Foxp3/Transcription Factor Staining Buffer Set	eBioscience
6-well,12-well & 96-well plates	Falcon/Corning
50 ml conical tubes	Falcon
40 µm cell strainer	Falcon
10 ml syringe-plunger	Exel INT
RPMI	Corning
PBS	Corning
FBS	Atlanta Biologicals
Orbital shaker	VWR

Curved-end scissor	
Fine Serrated Forceps	
Small curved scissor	

Catalog Number	Comments/Description
17-0041-81 ,10054*	For detailed information see Table 1
MA5-16536	For detailed information see Table 1
61-9985-82	For detailed information see Table 1
12-9942-82	For detailed information see Table 1
144610	For detailed information see Table 1
145512*, 551960	For detailed information see Table 1
358512	For detailed information see Table 1
17-5773-82	For detailed information see Table 1
563259	For detailed information see Table 1
L34957	For detailed information see Table 1
420404	For detailed information see Table 1
553141	For detailed information see Table 1
LS004176	
LS004188	
00-5523-00	
353046,353043/3596	
3520	
352340	
26265	
15-040-CV	
21-040-CM	
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Article Title:

Unraveling key players of humoral immunity: Advanced and optimized lymphocyte isolation protocol from Payer's Patches

Signature:

Vassiliki Boussiotis

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We would like to thank the reviewers for the careful evaluation and their thoughtful suggestions for the improvement of our manuscript. All the points of the reviewers have been taken into consideration and the manuscript has been revised accordingly. All the changes are highlighted in the revised manuscript. Below is a point-by-point response to the reviewers' and the editor's comments.

Reviewer #1

We would like to thank this reviewer for their favorable comments *"The protocol provides valuable technical advice, is clearly written and I would recommend its publication after a minor revision."*

Minor Concerns: *Trapezar et al. have published a protocol describing isolation of lymphocytes.... An Optimized and Validated Method for Isolation and Characterization of Lymphocytes from HIV+ Human Gut Biopsies M Trapezar, S Khan, NR Roan, TH Chen, S Telwatte, M Deswal, M Pao,...AIDS research and human retroviruses 33 (S1), S-31-S-39.*

This citation is included in our revised manuscript as reference #32.

Reviewer #2

The authors seek to establish an advanced and optimized lymphocyte isolation protocol from Peyer's Patches: they show that collagenase digestion can critically impair the ability to detect germinal center and Tfh cells, as several surface markers are degraded.

Major Concerns:

1. Germinal center and Tfh cells are usually isolated from peripheral LN and spleen without the need of digestion. Moreover, it is widely accepted that germinal center B cells are extremely sensitive to digestion, as they quickly die at 37C. So, to any B cell biology, digesting a tissue to isolate GC cells would be a non-starter: a quick literature search will show that GC analysis in PP does not require digestion (see Bergqvist et al. J Immunol 2006; 177:7772-7783; Kawamoto et al.Science 336 (6080), 485-489). The only reason to digest PP is to isolate Dc and plasma cells that tend to adhere stronger to stromal cells, exactly in the same way this is done in spleen.

We agree with the reviewer that several studies in the literature did not use digestion for isolation of GC and TFH cells. However, in other studies collagenase digestion was used in this context (Couter, C. J. et al. *J. Vis. Exp.* 5411454114, 1–5 (2016)). In the reference mentioned by the reviewer (Kawamoto et al.Science 336 (6080), 485-489), no detailed information is provided regarding collagenase digestion for GC lymphocyte isolation from PP but a previous publication is cited (Fagarasan et al *Nature* 413, 639 (2001)), which employed the method described in Kamata et al. *J Immunol* August 1, 2000, 165 (3) 1387-1394). Notably, in this publication, although isolation was performed without collagenase digestion, incubation with EDTA and agitation at 37°C for 15 minutes was employed. This approach closely resembles the preferred method determined by the comparative studies described in our protocol.

Previous studies that reported isolation of PP lymphocytes with collagenase digestion (e.g. Couter, C. J. et al. *J. Vis. Exp.* 5411454114, 1–5 (2016)) did not perform a comparative analysis between the efficacy

of methods (i.e. with and without collagenase treatment). Thus, it has remained unclear which was the optimal approach. However, studies, which examined the contribution of DCs to the GC reaction in PPs and used collagenase II digestion (Reboldi, A. *et al. Science* (80). **352**, (2016): <http://dx.doi.org/10.1126/science.aaf4822>), showed that the TFH fraction within PPs was only 3.8% of total T cells, which is significantly lower than the physiological fraction (approximately 20%). Our studies and the development of our protocol were triggered by such previous observations. We have added information in the discussion (lines 545-556 and ref # 33) as suggested by the comments of this reviewer.

Regarding the impact of temperature on B cell viability, which is the second concern of this reviewer, we would like to clarify several points: In our experimental conditions, incubation at 37°C with gentle agitation for 10 minutes rather improved instead of compromising viability of GC/PP lymphocytes (Figure 7E and F). In addition, previous studies on *in vitro* GC differentiation cultured at 37°C up to 6 days did not compromise cell viability (Sage et al. *Nat Immunol* 14(2):152–161. doi: 10.1038/ni.2496). Similarly, collagenase-based digestion, which requires incubation for 30-40 min at 37°C, did not have an impact on cell viability in this protocol (Couter, C. J. et al. *J. Vis. Exp.* **5411454114**, 1–5 (2016)). Notably, in the GC B cell publication mentioned by the reviewer (**Kawamoto et al. Science 336 (6080)**), although isolation was performed without collagenase digestion, incubation with EDTA and agitation at 37°C for 15 minutes was employed.

We agree with the comment of the reviewer ***“The only reason to digest PP is to isolate Dc and plasma cells that tend to adhere stronger to stromal cells, exactly in the same way this is done in spleen”***.

Since our studies showed a significant impact of collagenase digestion on CXCR5 expression, in our protocol, we suggest that when DC isolation is desired, half of the PPs can be processed with collagenase digestion, while the other half can be processed without collagenase digestion and used for TFH cell isolation, avoiding the compromise of CXCR5 expression. Because CXCR5 expression is a key marker for TFH cell identification, we have added few points the discussion of our revised manuscript regarding the impact of collagenase digestion on TFH cell characterization (lines 558-578).

2. On the same line: it is known that Collagenase type II has high clostripain and tryptic activity and is rarely used for lymphocyte isolation; Collagenase type 4 or Collagenase D are preferred and should only be used. It many figures it is unclear if the one used is collagenase type II or type IV: in Fig.7H for example, in 15 mg of collagenase (unknown kind) does not affect the lymphocyte population. Many groups use 0.5 mg/ml of Collagenase IV or D to isolate LP as well to isolate splenic DC and PC: the authors should try this concentration before arguing that *any* digestion is bad for PP lymphocytes.

The reason for which we used primarily Collagenase II was that a recent Jove paper (Couter, C. J. et al. *J. Vis. Exp.* **5411454114**, 1–5 (2016)) recommended usage of Collagenase II for LP and PP preparation at a concentration of 37.5 mg/25 ml. Because we were also concerned about the high tryptic activity of collagenase II, in our experiments, we also included Collagenase IV, which is known to have milder effects on surface molecules. We found that collagenase IV reduced TFH fraction (identified by the expression of CXCR5 and PD-1) more severely than Collagenase II (Figure 5G-I), suggesting that cleavage of surface molecules is not only dependent on the enzymatic activity of collagenase but also

on the amino acid sequence and structure of individual surface molecules. Consistent with our findings, previous studies have shown that collagenase IV digestion caused massive decrease in surface expression of CXCR5 in the lymphocytes isolated from human gut biopsies (Trapecar et al AIDS research and human retroviruses 33 (S1), S-31-S-39). We have outlined the differences in the effects induced by the two types of collagenase and the justification for comparing these digestion outcomes in lines 545-556 of our revised manuscript. Also, we added all details regarding collagenase type and concentration in the relevant figure legends.

Regarding collagenase concentration, we tested Collagenase type II at 0,6 mg/ml and noticed that it interfered with TFH identification. Collagenase IV reduced TFH fractions more significantly than Collagenase II when used in similar concentrations (fig. 7G-7H). We would like to clarify that we did not claim that “any digestion is bad”, as mentioned by the reviewer. Instead, in the discussion of our manuscript we stated that further studies could identify alternative conditions of enzymatic digestion that do not interfere with TFH cell identification (lines 581-595).

3. The authors argue that the possible digestion of neighbor lamina propria or intraepithelial lymphocytes makes a strong rationale to avoid digestion: this is not a strong rationale, it is quite a nonsense. Despite digestion not being not required for GC and Tfh, GC and Tfh are easily distinguishable from LP and IEL: GC B cells express CD19 as well as B220, whereas the only resident B cell origin cells in LP, plasma cells, do not express B220 and have an intermediate to low CD19 level. Similarly, no Tfh cells are present in LP or IEL. In addition, the frequency of T cell in the LP, even considering the whole SI is not that high compare to other hematopoietic and non-hematopoietic cells: if the PPs are excised properly, contamination from IEL is not an issue. This can be easily tested by freezing freshly excised PP, cut section and stain for IF/IHC with anti T cell antibody.

We respectfully disagree with the reviewer’s comment that our concern about the effects of digestion on LP and IEL “is quite a nonsense”. The presence of TFH cells in LP has been documented in previous publications in mouse and human systems (Hirota, K. *et al. Nat. Immunol.* 14, 372–379 (2013); Trapecar, M. *et al. AIDS Res. Hum. Retroviruses* 33, S-31-S-39 (2017)), which noted that distinguishing TFH cells isolated from LP vs. PPs after digestion might be challenging.

We agree with the second comment of the reviewer in point 3 that most of the resident B cells in LP are plasma cells and can be distinguished from GC B cells located in the PPs based on B220 or CD138 expression. However, when collagenase is applied, plasma cells from LP will be released, which will result in B cell contaminating the PP plasma cell pool. This will preclude analysis of plasma cell compartment specifically within PPs.

Although the frequency of T cells in LP and the whole SI are not as high as in other tissues (e.g. spleen or lymph nodes), a significant number of T cells is present in the LP and IEL. Couter, C. J. *et al. (J. Vis. Exp.* 5411454114, 1–5 (2016) showed that 10^4 CD8⁺ T cell can be isolated per cm LP of small intestine. Other studies have shown that 50% of total LP cells are CD3⁺ T cells (Boll *et al. Scand. J. Immunol.* 42, 191-201, 1995) and CD4⁺ T cell fractions were found to be comparable to CD8⁺ T cell fractions. In PPs, up to 10% of total lymphocytes are CD4⁺ T cells and in young mouse max. 20% of these CD4⁺ T cells are TFH cells. Previous studies (Reboldi *et al:* <http://dx.doi.org/10.1126/science.aaf4822>) indicated that

the high density of T cells in neighboring gut tissues (LP and IEL) will likely lead to contamination of excised PP cell populations if appropriate technical precautions are not taken. For the above reasons, we think that it will be helpful to the users of this protocol to clarify these issues. We have added a relevant point in the discussion (lines 596-606).

Minor concern:

CXCR5 downregulation prevents the authors to identify Tfh: however, PD1 staining is still quite good, and it could be used in combination with Bcl6.

Although PD-1 is expressed by TFH cells, CXCR5 expression is indispensable for the identification of TFH & TFR cells and cannot be substituted by either PD-1 or BCL-6. These molecules can only be used in combination with CXCR5 to detect TFH cells. (Sage, P. T. & Sharpe, A. H. *Trends Immunol.* **36**, 410–418 (2015); Espéli, M. & Walker, J. M. *Methods and Protocols.* (2015). doi:10.1007/978-1-4939-2498-1).

Reviewer #3

1. Manuscript Summary: In this manuscript, authors describe a method to isolate B and T cells from Peyer's patches (PP) use very strong terms, such as "revolutionize the PP research field", which should be avoided in any rigorous scientific publication.

In the discussion of the revised manuscript (lines 581-594) we added a comment regarding the type of cells that can be isolated from PP by using or not using digestion. Also, we rephrased the overstated comments and toned down the strong terms used in the original manuscript.

Major concerns:

1. Introduction: PP are very poorly introduced without any notion of their structure (i.e. FAE, SED, follicles, IFR) and their specificity (i.e. M cells, specialized SED phagocytes, initiation of the mucosal immune response and IgA-secreting cell generation). Many references are lacking (at least some recent reviews on the topic and seminal works on CXCR5 role in B and Tfh cell recruitment in the follicles and on the role of PP Tfh in IgA-secreting cells generation, etc...). This should be added in the introduction to highlight the importance of working on PP immune cells.

As requested by the reviewer, in the introduction of the revised manuscript we added the background information in the field together with the relevant references (lines 60-82).

2. Introduction: Throughout the introduction (l.95-96; l.98-102; l.106-108), in order to promote their own method authors, tend to criticize previous methods...very strong words throughout the manuscript, such as "enormous", "revolutionize", "dramatic", which should be avoided.

These issues have been properly corrected and rephrased and the contribution of our method to the field has been outlined.

3. Protocol: Mouse strain should be indicated since numbers and positioning of the PP vary greatly between mousePP cell type proportion varies between mouse strains.

Mouse strains were indicated in the relevant figure legends and in the protocol section (lines 185-187).

I. 155: the anatomical location of the caecum is NOT highly variable in mice....only occasionally found in the lower center or on the lower right side.

We corrected this point in the revised manuscript (line 139).

I. 160: the best way to collect Peyer's patches from the gut is to take them out without removing the small intestine, just by following the anti-mesenteric side of the small intestine from the caecum to the stomach. By doing so, there is no risk to miss one PP or to damage it. The scissors are placed flat on the intestinal wall surrounding the PP, so that only PP is excised. It is a much faster process than the one proposed by the authors (avoid steps 5 to 7 of (i) and 1 and 2 of (ii)) and the tissue is better preserved (only one step to the dissociation process).

We respectfully disagree with the with Reviewer on this point because excising the PPs without removing the small intestine will require excessive handling of the small intestine to position PPs properly for the excision. As we indicated in our protocol, excessive handling might cause collapse of PPs, and for this reason, collecting PPs without SI removal would not be a safe approach, especially for non-experienced researchers. When small intestine is completely removed, which requires less than 1 minute, PPs can be defined and eventually excised more efficiently. Also, if the desired number of PPs is not achieved, one can quickly repeat several times the PP identification step in the entire SI.

4. Results: I.370, by adding collagenase other cell types are released, such as phagocytes and stromal cells. These cells are of course larger and more granular than lymphocytes.... a misinterpretation of their isolation procedure comparison.

We clarified this issue in result section (581-594).

5. Discussion: The discussion is too long for very little interest and bibliography... collagenase IV, collagenase II has been used for years to isolate cells from PP).

We corrected these issues and added the relevant citations (lines 545-556). The original Discussion section was shortened but new points were added, as requested by the other reviewers.

I.486-I.500: Contamination of PP cells by other small intestine compartments when collagenase is used ... in absence of tissue-dissociating enzymes many cell types, among which phagocytes and stromal cells, are not released from the tissue ... observed by the authors.

We agree with this statement of the reviewer and clarified this issue in lines 581-606 in the Discussion section.

Minor Concerns:

1. Protocol: I.276: this step is cell fixation. Thus, there is no cell viability to consider here.

We changed viability to “recovery”.

2. Results: l.319, it's been known for decades that PPs are not evenly distributed in the mouse small intestine...."in contrast to previous protocols" should be avoided.

We agree with this comment of the reviewer regarding the uneven distribution of the PPs in the mouse small intestine. Our goal was to make a distinction from a previously published protocol, which claimed that PPs are evenly distributed in the small intestine (De Jesus, M., Ahlawat, S. & Mantis, N. J. Isolating And Immunostaining Lymphocytes and Dendritic Cells from Murine Peyer's Patches. *J. Vis. Exp.* 1–8 (2013). doi:10.3791/50167). We properly clarified this point.

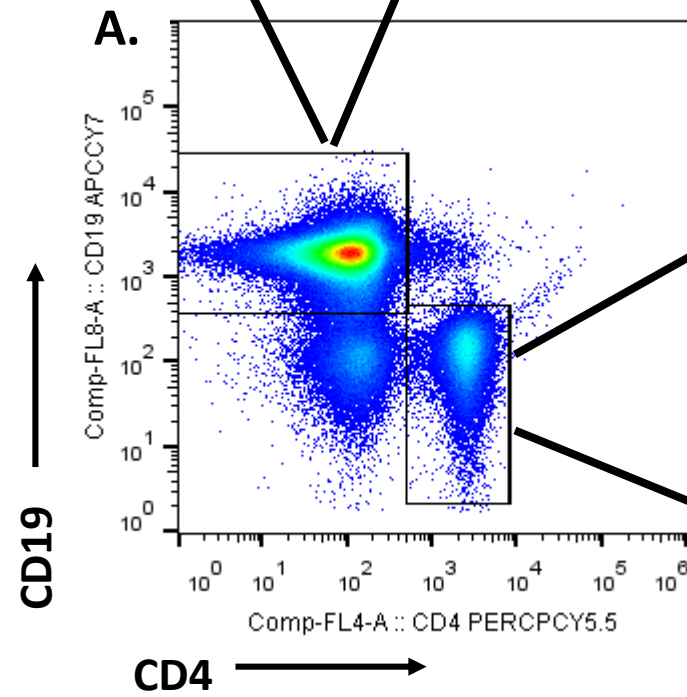
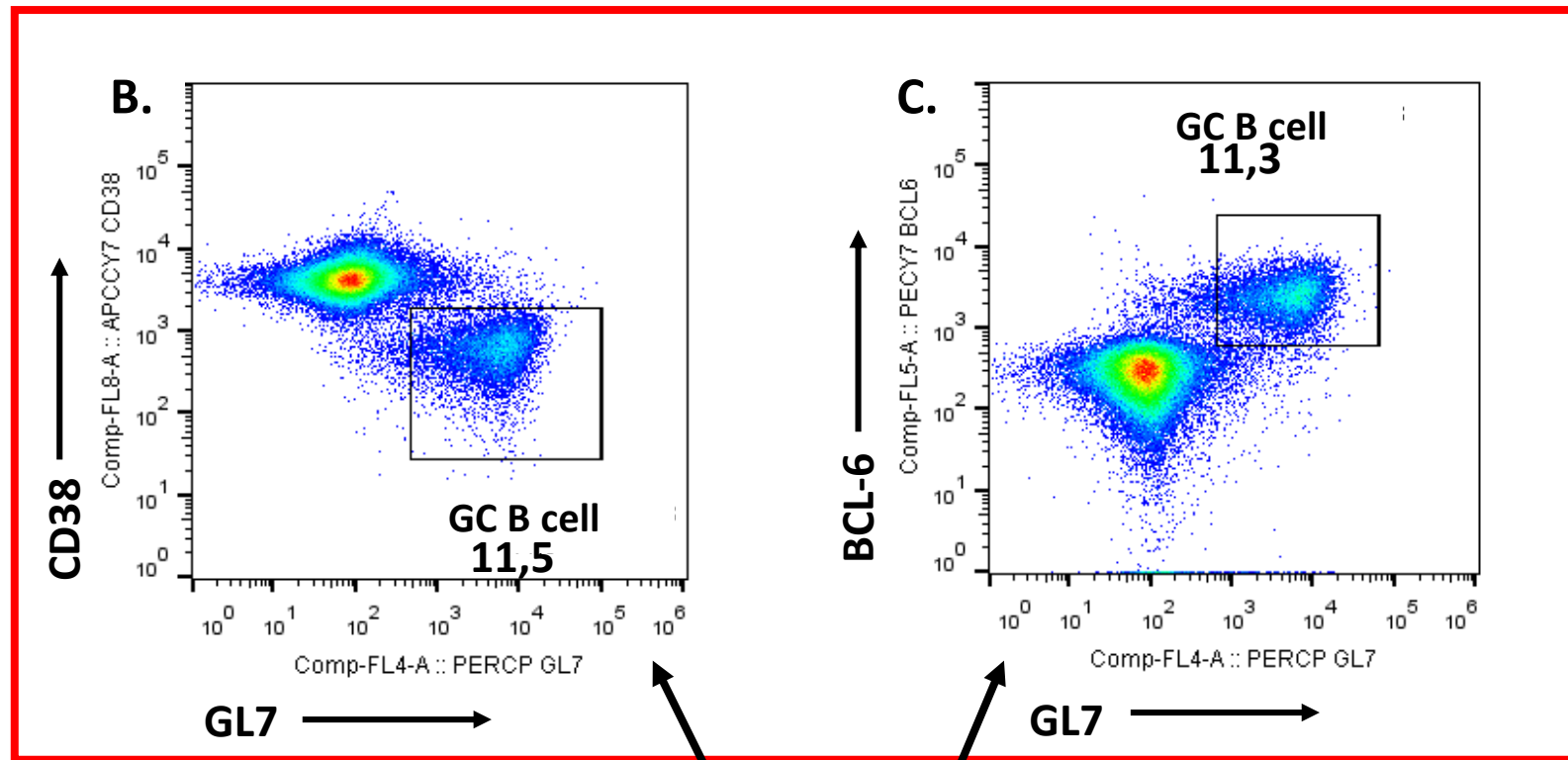
3. Discussion: l.406, the last 5 cm of the terminal ileum contains at least two PP and more often three.

It is our understanding that in sources of mouse anatomy, terminal ileum is described as an anatomical region that is shorter than 5 cm -rather around 1 cm. **(Revised guides for organ sampling and trimming in rats and mice Part 1, Exp Toxic Pathol 2003; 55: 91–106, URBAN & FISCHER <http://www.urbanfischer.de/journals/exptoxpath>).**

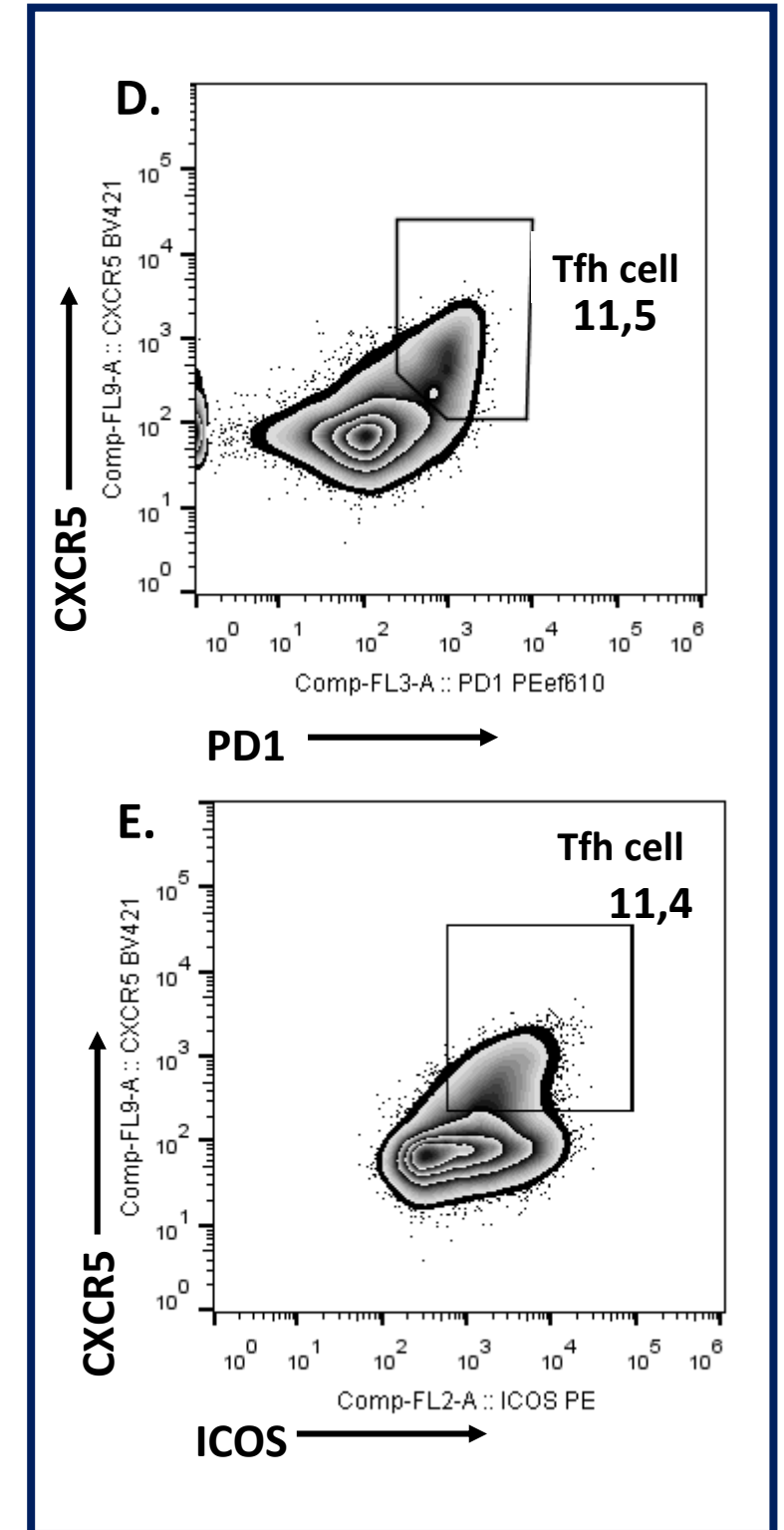
We agree that one can find 2-3 PPs in the last 5 cm of the intestine, as the reviewer indicated, but we think that this region does not represent the terminal ileum. As indicated by the reviewer, in the revised manuscript we changed our description to reflect this point (lines 501-504).

Antigen	Clone	Fluouochrome	Dilution
CD4	GK 1.5, RM4-5	APC, PECy7, PerCpCy5.5, FITC	1:100
CD19	6D5	FITC, APC/Cy7	1:100
PD-1	J43	PE ef 610 (Texas Red)	1:100
ICOS	15 F9, 7E.17G9	PE	1:100
GL7	GL7	PerCp/Cy5.5	1:75
CXCR5	2G8, <u>L138D7*</u>	Biotin	1:50
BCL-6	7D1	PE/Cy7	1:50
FOXP3	FJK-16	APC, PE	1:100
Streptavidin	-	BV421, PE	1:100
FixableViability Dye	-	BV 510 (AQUA)	1:1000
7AAD	-	PerCp/Cy5.5	1:500
FcBlock (CD16/32)	2.4G2	-	1:200

GC B cells



Tfh cells



Editors Comments:

Changes to be made by the Author(s):

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We performed these quality checks as indicated by the editor.

2. Figure 6: There is no panel G as described in its figure legend. Please revise either Figure 6 or its figure legend.

We corrected the figure legend and removed description of panel G.

3. Please provide Table 1 as an .xls or .xlsx file.

We provided the requested table.

4. Please provide an email address for each author.

We provided an email address for each author.

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

We rephrased the abstract as requested.

6. Please rephrase the Introduction to include a clear statement of the overall goal of this method.

We rephrased the introduction to clearly state the goal of the method, as requested.

7. Please use SI abbreviations for all units: L, mL, μ L, h, min, s, etc.

We used the standard international unit abbreviations as requested.

8. Please include a space between all numbers and their corresponding units: 15 mL, 37°C, 60 s; etc.

We corrected this format in the relevant places of the text as requested.

9. Please adjust the numbering of the Protocol to follow the JoVE Instructions for Authors. For example, 1 should be followed by 1.1 and then 1.1.1 and 1.1.2 if necessary. Please refrain from using bullets, dashes, or indentations.

We corrected the protocol accordingly.

10. Lines 134-144, 232-236, 267-270, 288-291: The Protocol should contain only action items that direct the reader to do something. Please either write the text in the imperative tense as if telling

someone how to do the technique (e.g., “Do this,” “Ensure that,” etc.), or move the solutions, materials and equipment information to the Materials Table.

These sections were removed and materials and equipment were included in the appropriate table.

11. Please specify all surgical instruments used.

We included information about the surgical instruments.

Information about the type of surgical instruments used has been provided in the materials list.

12. Lines 293-294: Please specify the amount/volume of antibody and buffer added.

As requested, we included this information in the relevant sections of the protocol.

13. Please include single-line spaces between all paragraphs, headings, steps, etc. After that, please highlight 2.75 pages or less of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol.

We made these changes as indicated.

14. Please ensure that the highlighted steps form a cohesive narrative with a logical flow from one highlighted step to the next. Please highlight complete sentences (not parts of sentences). Please ensure that the highlighted part of the step includes at least one action that is written in imperative tense. Please do not highlight any steps describing euthanasia.

We made these changes as indicated.

15. References: Please do not abbreviate journal titles. Please include volume and issue numbers for all references.

We made these changes in the citation format as indicated.

16. Please revise the table of the essential supplies, reagents, and equipment. The table should include the name, company, and catalog number of all relevant materials in separate columns in an xls/xlsx file. Please provide lot numbers and RRIDs of antibodies, if available.

We revised the table as indicated and provided all the available information. Lot numbers are not available.