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:** Trapecar 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 Trapecar, 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 37^0C 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 37^0C, 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 trypic 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 mouse …..PP cell type proportion varies between mouse strains.

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

l. 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).

l. 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: l.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.

l.486-l.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: l.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).