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Microscopic observation of lymphocyte dynamics in rat Peyer's patches

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TITLE:

Microscopic Observation of Lymphocyte Dynamics in Rat Peyer's Patches

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SUMMARY:

Here, we describe a precise method for collecting thoracic duct lymphocytes and observing the migration of gut-tropic lymphocytes in rat Peyer's patches for 3 hours using time-lapse photography. This technique can clarify how the dynamics of lymphocytes are affected under inflammatory conditions.

ABSTRACT:

Naïve lymphocytes recirculate from the blood to the lymphoid tissues under physiological condition and it is commonly recognized as an important phenomenon in the gut immunity. The stroma of secondary lymphoid organs, such as Peyer's patches (PPs) or mesenteric lymph nodes, are where naïve lymphocytes sense antigens. Naïve lymphocytes circulate through the bloodstream to reach high endothelial venules, the portal of entry into PPs. Some immunomodulators are estimated to influence lymphocyte migration, but the precise evaluation of microcirculation dynamics is very difficult, and establishing a method to observe lymphocyte migration in vivo can contribute to the clarification of the precise mechanisms. We refined the method of collecting lymphocytes from the lymph duct and observing the detailed dynamics of gut-tropic lymphocytes in rat PPs. We chose confocal laser scanning microscopy to observe rat PPs in vivo and recorded it using time-lapse photography. We can now obtain clear images that can contribute to the analysis of lymphocyte dynamics.

INTRODUCTION:

Peyer's patches (PPs) consist of hundreds of lymphoid follicles in the lamina propria of the small

intestine. PPs are divided into follicles, the interfollicular region, and germinal centers located in the lower part of the follicles, where lymphocytes are stimulated by antigen presentation. There are no afferent lymphatic vessels, and the antigens invade the lamina propria from the intestinal lumen via the epithelial cell layer. The epithelial region covering lymphoid follicles is called the follicle-associated epithelium, within which specialized interspersed M cells uptake mucosal antigens. M cells take in antigens from the luminal side and antigens are then captured by dendritic cells and presented toward naïve lymphocytes that flow into PPs through the endothelium of high endothelial venules (HEVs)[1]. PPs play an important role in intestinal immunity and are related with the early stage of inflammation. Many molecular interactions involve the entrance of lymphocytes to secondary lymphoid organs (SLOs), including adhesion molecules, chemokines[2,3], and sphingosine-1-phosphate[4]; thus, there are many expected therapeutic targets. Therefore, observing the lymphocyte dynamics within PPs enables us to catch a glimpse of the very early stage of inflammation and examine the usefulness of several promising drugs.

The method here focuses on the migration of lymphocytes in PPs, which includes several procedures (cannulation into the thoracic duct[5] and collecting lymphocytes and long-term observation after the injection into collected lymphocytes). Since these procedures are complex and it was difficult to see exactly how each procedure was performed in previous reports, we mentioned here some tips to achieve a successful observation. For example, cannulation of the tubes into the thoracic duct was very difficult, and the initial success rate of cannulation was less than 50%. However, we improved the method and achieved a success rate exceeding 80%. We mentioned some other tips in this manuscript that are necessary for the successful observation to enable the quantitative evaluation of the transendothelial migration of lymphocytes under several conditions.

In previous reports, it was difficult to understand the three-dimensional changes over time, such as the intravenous injection of India ink to stain the vascular structure of Peyer's patches[6], or the microscope being monofocal[7]. In recent years, an observational method using some photoconvertible fluorescence protein transgenic animals such as Kaede mice have clarified systematic cellular movements in vivo[8]. The other study clarified CD69 independent shutdown of lymphocyte egress from Peyer's patches[9]. We used confocal laser scanning microscopy (CLSM) because of its high analytical capability. Now we can easily obtain high-resolution images and use them to analyze lymphocyte dynamics.

In this report, we demonstrated a series of methods for evaluating lymphocyte migration in PPs. First, we showed refined methods of thoracic duct cannulation to collect lymphocytes. Second, we improved the observational methods in several ways to maintain objective organs whenever possible under microscopic observation, enabling us to obtain high-quality images for 3 hours. Third, we quantified the cellular movements of lymphocyte migration to evaluate the effects of some medications. These modified protocols will contribute to development of mucosal immunology evaluations.

PROTOCOL:

The experimental protocol was approved by the Animal Research Committee of National Defense Medical College (no. 16058). The animals were maintained on standard laboratory chow (CLEA Japan Inc, Tokyo, Japan). The laboratory animals were treated in accordance with National Institutes of Health guidelines.

1. Collection and separation of lymphocytes

NOTE: Since lymphocytes must be fresh and cannot be stored, they must be collected from rats for each experiment. In addition, gut-tropic lymphocytes must be collected directly from the thoracic duct where they circulate.

1.1. Form a cannulation tube (1 mm diameter) like a hairpin curve in advance a few hours before dipping it in hot water (about 80–90 °C). Then bend and fix it to a plastic board using adhesive tape. Use a mold to draw a curve with a radius of about 5 mm.

1.2. Anesthetize a male Wistar rat (8–12 weeks) with a pentobarbital sodium intraperitoneal injection (30 mg/kg) and cut the body hair of the abdomen using a hair clipper. Remove the hair firmly after shaving so that the hair does not enter the abdominal cavity.

1.3. Make an incision with surgical scissors horizontally from the midline to the left subcostal area. Be careful not to damage the blood vessels in the parietal peritoneum by watching the vessels under light.

1.4. Wrap the stomach with wet gauze and move the stomach gently to the right outside of the body to reveal the retroperitoneal organs. Insert a notch between the erector muscles of the spine and the adipose tissue using surgical scissors and bluntly separate them with fingers.

1.5. Carefully strip off the connective tissue around the thoracic duct. The excess connective tissue increases with age. Expose the thoracic duct from just beneath the left crus of the diaphragm caudally until a 20 mm length of the thoracic duct was visible. Gently separate adhesions between the thoracic duct and aorta using precision tweezers or wet cotton swabs.

1.5.1. Perform this process carefully because some rats have arterioles branching from the abdominal artery crossing over the thoracic duct or a short thoracic duct due to a higher position of the lymph plexus (small thoracic ducts spreading like spider webs). Avoid unnecessary contact to the thoracic duct to avoid inducing edema.

1.6. Apply a ligature by a string (3-0 silk) on the thoracic duct just beneath the left crus of the diaphragm. The caudal thoracic duct becomes distended.

1.7. Make a hole (5 mm diameter) in the abdominal wall by stabbing the cutting edge of surgical scissors, pass the hairpin-curved cannulation tube, and ligate the tube on the iliopsoas muscle at one point. Fill the cannulation tube with normal saline containing 10 U/mL heparin.

1.8. After placing a string (3-0 silk) beneath the distended thoracic duct, stab the thoracic duct with the sharply cut edge a cannulation tube, cannulate it toward the tail about 5 mm, and ligate the thoracic duct with cannulation tube for fixation.

1.9. To supply saline to prevent dehydration, create a hole (3 mm diameter) on the anterior wall of the antrum of the stomach using precision tweezers and pass the silicon tube (2 mm in diameter) to the duodenum through the pyloric ring. After stitching up a wound and maintaining the animals in Bollman's cages, start to infuse sugar-laden saline into each rat's duodenum from the silicon tube at a flow rate of 3 mL/h. Cover the entire cage with a paper towel to keep it warm.

1.10. Fix the cannulation tube to the hole in the center of the lid of a 50 mL conical tube on ice containing 6 U/mL heparin, 10% fetal bovine serum, and RPMI 1640 medium (pH 7.4; see the **Table of Materials**), and collect thoracic duct lymphocytes (TDLs) on ice.

1.10.1. Be careful not to contact the tip of the tube to the wall of the conical tube to prevent cannulated-tube occlusion due to fibrin clot formation inside the tube. The conical tube containing collected lymph fluid is replaced by a new one every 12 hours and stored in a refrigerator. Lymph fluid (30 or 40 mL) can be obtained every 12 hours for about 2 days. Gently flush saline containing heparin every 6 hours to prevent obstruction of the tube by fibrin precipitation.

2. Lymphocyte labeling with carboxyfluorescein diacetate succinimidyl ester (CFDSE)

2.1. Dissolve CFDSE (**Table of Materials**) in dimethyl sulfoxide (DMSO) to 15.6 mM (500 µg of CFDSE dissolved in 60 µL of DMSO).

2.2. Incubate lymphocytes (1×10^9) in 50 mL of RPMI 1640 with 50 µL of CFDSE solution for 30 min at 37 °C as described previously[10].

3. Experimental setup for microvascular studies

3.1. Under continuous anesthesia with 2% isoflurane, open the abdomen of the recipient Wister rat (8–12 weeks) via a midline incision.

3.2. Put a rat on a portable stainless-steel plate (about 120 x 300 mm) with a rectangle hole around the center covered with a glass slide (24 x 50 mm). Choose about 10 cm of the ileal segment including PPs for observation.

3.3. Keep the intestine as warm as possible and moist with phosphate buffered saline (PBS) warmed to 37 °C. Soak gauze that is used to cover the small intestine with PBS.

3.4. Put the slide on the stage of the microscope and choose suitable areas for observation of the microcirculation in PPs where some HEVs are running through the serosa. PPs range in size;

larger ones are suitable for observation of the microcirculation using CLSM. The small intestine is not straight in places; a straight segment at least 2 cm long without any tension is suitable for observation.

3.5. Cover the adjacent intestinal segment and mesentery with absorbent cotton soaked with PBS. Place the intestine segment between two rolled cotton balls and position it as far as possible from the rat's body to prevent it from being vibrated by the rat's heartbeat and breathing.

3.6. Using a 1 mL syringe, slowly inject (over 1 min) CFSE-labeled TDLs (1 x 10^9 cells) into the jugular vein of the recipient rats. A rapid intravenous injection may influence the systemic circulation.

4. Microcirculation of lymphocytes

4.1. Continuously monitor TDLs in the microvasculature of PPs using CLSM and record on a computer for 3 hours using time-lapse photography at 30 s intervals. The depth from the serosa to the HEV of PPs is about 25 µm, enabling the observation of the stroma and capillary lymph vessels to a depth of 30 µm.

4.2. Inject Texas Red–dextran (25 mg/kg) into the jugular vein of each recipient rat to stain the bloodstream and Hoechest 33342 (5 mg/kg) to stain the cell nuclei.

4.3. (Optional) To quantify lymphocyte dynamics, define lymphocytes adhering to HEVs more than 30 seconds as "adhesive lymphocytes" and lymphocytes emigrating from HEVs to stroma as ''migrating lymphocytes". Then calculate the average percentage of migration (migrating lymphocytes / adhesive lymphocytes + migrating lymphocytes) per field of vision (approximately 0.3 mm^2).

REPRESENTATIVE RESULTS:

Collecting lymphocytes from lymph

To prepare the rat for thoracic duct cannulation, make an incision in the tense thoracic duct as shown in **Figure 1** and then maintain the rat in a Bollman's cage as shown in **Figure 2**.

When the lymphocytes are well collected, we can obtain 30–40 mL/12 h lymph fluid containing about 5 x 10^6/mL lymphocytes. This effluence rate lasts about 2 days. Of the TDLs, 70% express CD4, among which about 90% cells express CD62L, meaning the main composition of TDLs (>60%) consists of naïve gut-tropic lymphocytes. Since the lymphocytes collected here are cells that specifically migrate to the intestinal tract, they are suitable for evaluating the state of migration in Peyer's patches.

Microscopic observation

After injecting an adequate dose of fluorescent lymphocytes, mount the recipient rat's intestine on a glass slide as shown in **Figure 3**. A microscopic image of PPs in the physiological condition is shown in **Figure 4**.

For the micro-observation, rats can be stably anesthetized under laparotomy for about 3 hours. Lymphocytes adhere to HEVs, roll, and migrate to the surrounding stroma, and then migrate to lymph capillaries. We quantified and evaluated the percentage of lymphocytes that migrated in the stroma within the observation period.

After the injection of an adequate dose of fluorescent lymphocytes, gut-tropic lymphocytes begin to adhere to HEVs and migrate across the endothelium into the stroma in about 1 hour. Most move within the stroma, while others migrate to lymph capillaries in about 2–3 hours. The well-developed fluorescence labeling ex vivo in this study resulted high intensity and it enables us to observe lymphocyte dynamics of very deep area. In addition, by using a different kind of fluorescence labelling, we can easily observe dynamics of different kind of lymphocyte simultaneously. This gut-tropic lymphocyte migration can be visually clarified for suppression by some immunomodulators such as FTY720 as shown in our previous report.

Observation of the microcirculatory system itself is extremely difficult, and it is desirable to establish an easy evaluating method. This study has also made it possible to find out where the action site of the drug is in the Peyer's patches. In particular, ex vivo administration of FTY720 to lymphocyte made it possible to limit the site of action only to lymphocytes but not to endothelium. Combined with the sorting of specific lymphocyte, more detailed results would be obtained.

FIGURES AND TABLES:

Figure 1: Procedure to expose the thoracic duct and perform cannulation. After cutting the peritoneum longitudinally, the thoracic duct can be exposed by moving the stomach cranially and dissecting the connective tissue on the dorsal right side of the abdominal aorta. The connective tissue around the diaphragm should be carefully dissected to prevent injury. The thoracic duct should be exposed as wide cranially as possible to create sufficient space for cannulation and then ligated by a 3-0 silk suture just under the diaphragm to stop the flow of lymph and retain the fluid caudally. Milky lymph fluid is visible through the lymph duct, where the catheter can be inserted. The thoracic duct of the rat should be slightly strained to ensure a smooth cannulation.

Figure 2: An image of the entire setting to collect thoracic duct lymphocytes (TDLs). The rats are maintained in Bollman's cages and TDLs are collected through the cannulation tube into each vial. The vials are set on ice and replaced every 12 hours to collect fresh lymph fluid. Sugar-laden saline is administered through a silicone tube using a syringe pump at a rate of around 3 mL/h to prevent dehydration.

Figure 3: The rat is put on the microscope's stage connected to the anesthesia machine, and a fluorescent substance or fluorescently-labeled lymphocytes are injected from the venous tract through the internal jugular vein (**A**). The observation area of the intestinal tract was gently fixed between two rolled cotton balls. The intestinal tract was fixed far from the trunk to avoid vibration by respiratory movements. Rolled cotton balls were soaked in phosphate buffered saline to prevent drying of the intestine during the observation period (**B**).

Figure 4: Microscopic image of Peyer's patches in the physiological condition (**A**). The nuclei of vascular endothelial cells are stained blue. The blood plasma is red, and the blood flow is detected by the flow of unstained cells. Gut-tropic lymphocytes (stained green) adhering to high endothelial venules (**B**).

DISCUSSION:

Here we described a protocol for collecting naïve gut-tropic lymphocytes and observing their migration in rat PPs. These procedures can reveal how lymphocytes move in the microvasculature of PPs and make it possible to visually compare their dynamics under a normal or medicated condition. The direct observation of these dynamics has much merit to obtain a clue for immunological modification by some drugs, although the observational period is limited to only a few hours.

We mentioned some tips in the methods. Among them, one of the most delicate procedures is that the operator must cannulate the thoracic duct quickly and precisely. If an operation takes a long time and involves unnecessary damage, significant bleeding and inflammation occur, resulting in a decreased TDL collection due to edema of the connective tissue and obstruction of the thoracic duct. We used to cannulate the tube after creating a small incision on the thoracic duct using scissors with a small cutting edge. However, this procedure interferes with the cannulation site due to draining lymph. Therefore, now we do not cut the thoracic duct; rather, we stab it with a sharp edge. This method makes the cannulation easier and increases the success rates. In addition, the incision site in the thoracic duct is very important because of the limited abdominal cavity. If the incision site is too close to the diaphragm, the curved part of the cannulation tube would bump against the diaphragm. If it is too far from the diaphragm, it is also difficult to cannulate the tube deeply enough to ensure fixation due to the lymph plexus of the caudal thoracic duct. Even in cases of good cannulation, the cannulation tube can be easily occluded by fibrin formation. Thus, we recommend periodically gently flushing the tube with heparin.

Recent advances in CLSM made it possible to observe the focused area more clearly and precisely in real time compared to a previous study[11]. We now use CLSM in our laboratory, but we can observe the microcirculation more clearly using a multiphoton microscope. Although the protocol requires modification in several points along with technical progress, it enables observation of the localized area of the organ of interest[12, 13].

The merit and key of the method is that it involves separating lymphocytes from recipient animals, incubating them using any kind of compounds in vitro, and observing them after their injection into recipient animals. This makes it possible to elucidate the effects of lymphocytes alone. On the contrary, if we want to elucidate the effects of any kind of compounds on cells other than lymphocytes, such as the vascular endothelium, we can treat recipient animals with any kind of compounds and observe them after the injection of control lymphocytes. To elucidate the same things using mice, we must create conditional genetically manipulated animals one by one. In addition, sorting the isolated lymphocytes by using specific surface marker would make

it possible to study the dynamics of specific types of lymphocytes.

As shown in the video of this study, lymphocytes move indefinitely and laterally through the vascular endothelium of the actual body, so it is difficult to evaluate effects on their behavior using in vivo observations only. Furthermore, if the mechanism of action of an administered drug is to be examined, it is originally unsuitable for examining each site of action. The merit of this study is that by separating lymphocytes and incubating them in vitro, the medicinal effects can be examined separately on the vascular and lymphocyte sides. Fewer variables are easier to analyze.

ACKNOWLEDGMENTS:
This research was supported by grants from National Defense Medical College and by a Health and Labour Sciences research grant for research on intractable diseases from the Ministry of Health, Labour and Welfare, Japan.

DISCLOSURES:
The authors have nothing to disclose.

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patches by sphingosine-1-phosphate lyase inhibitor. *Journal of Gastroenterology and Hepatology*. **33**, 1608-1616 (2018).
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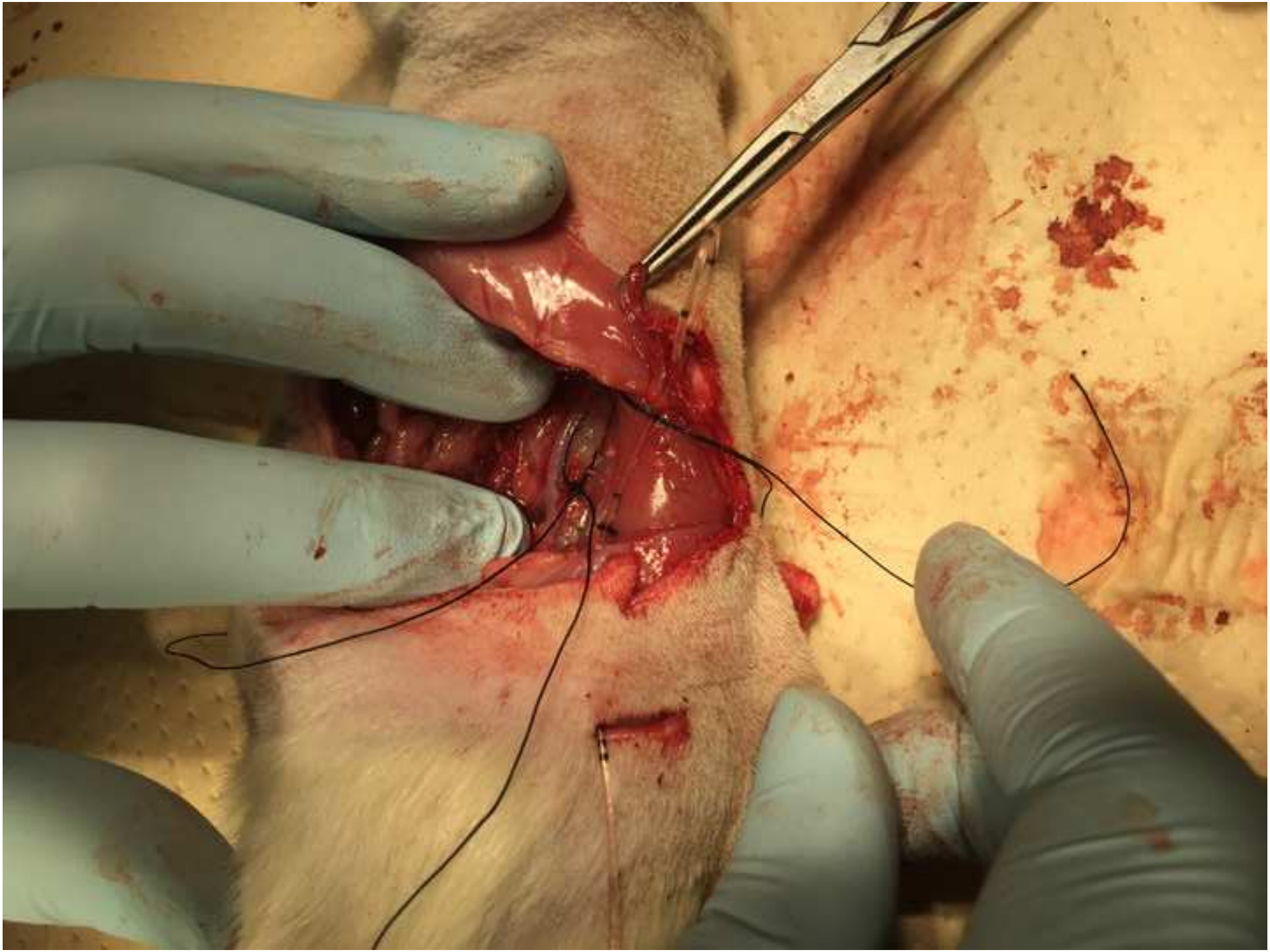
Figure

Click here to access/download;Figure;Figure 1.tif



Figure

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Figure

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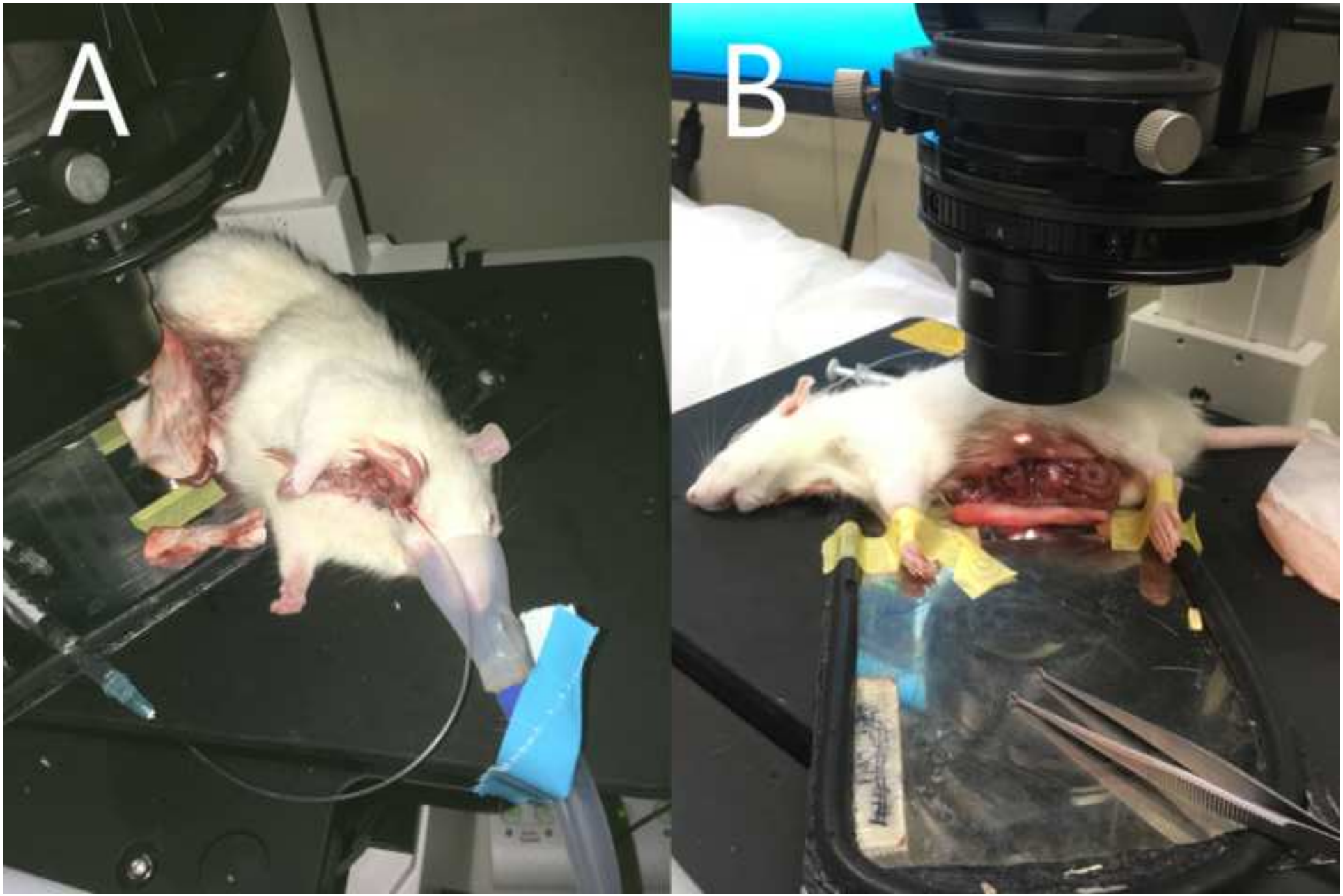

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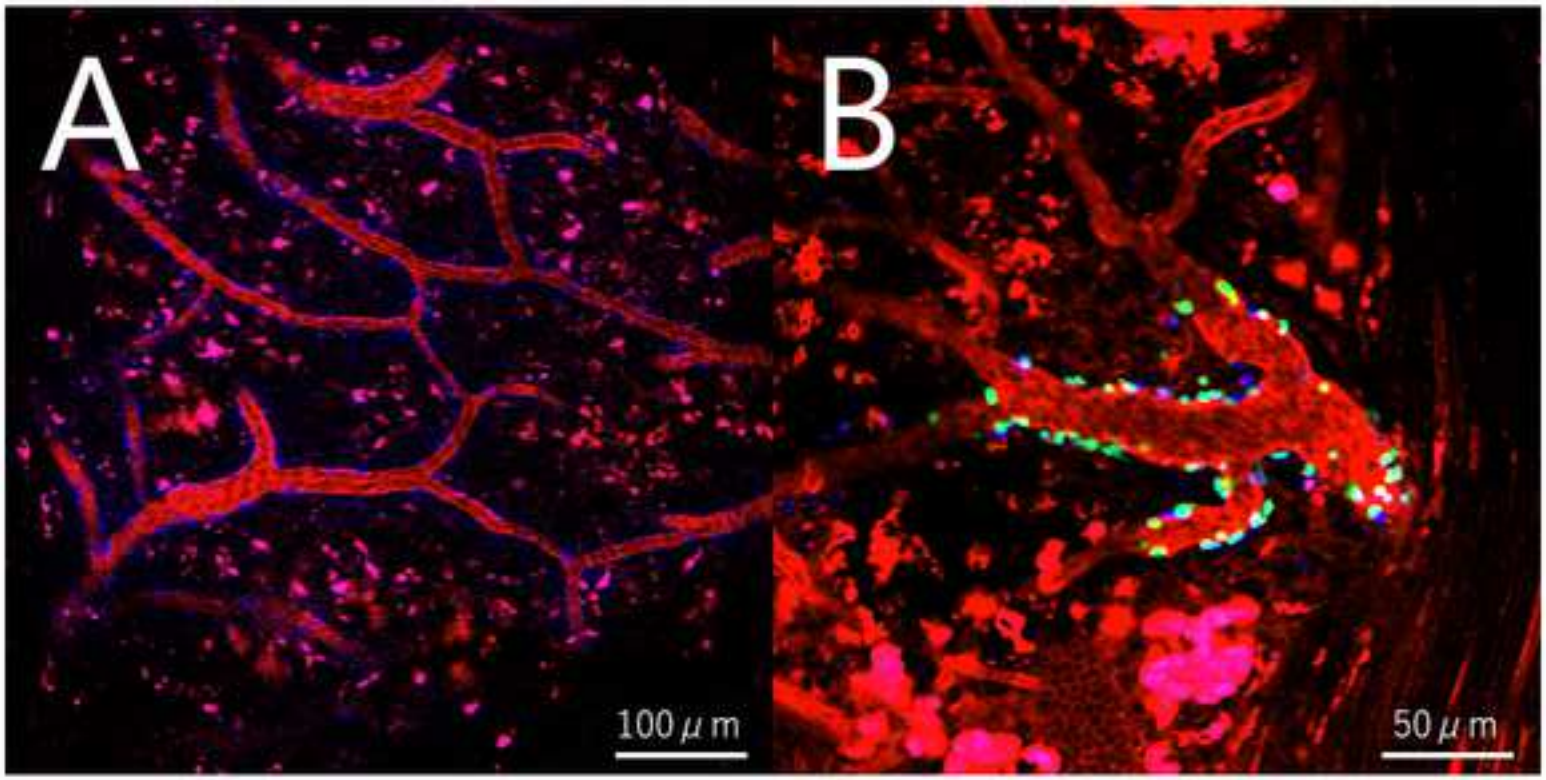

Name of Material/ Equipment	Company	Catalog Number
A1R+	Nikon	
Carboxyfluorescein diacetate succinimidyl ester	Thermo Fisher Scientific	C1157
Hoechest 33342	Thermo Fisher Scientific	H3570
Isoflurane	Wako Pure Chemical Industries	099-06571
RPMI 1640 medium	GIBCO	11875093
Texas Red–dextran	Thermo Fisher Scientific	D1863

Comments/Description

Comfocal Laser Scanning Microscopy

To Editor-in-Chief,
Journal of Visualized Experiments

Thank you for providing such helpful comments for improving our manuscript. We have revised the paper in accordance with your comments. We would like to resubmit the revised manuscript "Microscopic observation of lymphocyte dynamics in rat Peyer's patches" by Shirakabe et al. for publication in the Journal of Visualized Experiments. Our specific responses to the Reviewers' comments are included below.

Editorial comments:

Changes to be made by the Author(s) regarding the written manuscript:
1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. The JoVE editor will not copy-edit your manuscript and any errors in the submitted revision may be present in the published version.

Thank you for your comments. We reviewed spelling and grammar issues for all manuscripts.

2. 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 sort the Materials Table alphabetically by the name of the material.

Thank you for your comments. We revised the table of the Material Table.

Reviewers' comments:

Reviewer #1:

Minor Concerns:
Page 1, line 26: A few hours is imprecise

Thank you for your comment. We corrected 'a few' to '3' hours to be more precisely.

Page 1, line 26, 27: What is time-lapse photography?

Thank you for comment. Time-lapse photography is a technique whereby the frequency at which film frames are captured (the frame rate) is much more spread out than the frequency used to view the

sequence. We added some sentences explaining “time-lapse photography”. We added the following sentences to the Summary.

Here we describe a precise method for collecting thoracic duct lymphocytes and observing the migration of gut-tropic lymphocyte in rat Peyer’s patches for 3 hours using time-lapse photography. Time-lapse photography, a technique whereby the frequency at which film frames are captured is very low compared with the frequency to view the sequence, can clarify how the dynamics of lymphocytes are affected under inflammatory conditions.

Page 1, line 83: For many hours: also very vague.

Thank you for your comment. We corrected ‘for many’ to ‘3’ hours to be more precisely too.

Figure 4: How is this identified as a Peyer´s patch?

Thank you for your comment. When observing the rat intestine from the serosa side, the Peyer's patches can be visually confirmed as white round particles (about 1~2 mm in diameter). Aiming at that point and adjusting the depth of focus with a microscope, you can find a cluster of spherical blood cells in vessels as shown in Figure 4.

Additional comment: It would be interesting to sort the isolated lymphocytes to be able to study the dynamics of specific types of lymphocytes.

Thank you for your comment. We added description to the Discussion reflecting your suggestions.

The merit and key of our method is that it involves separating lymphocytes from recipient animals, incubating them using any kind of compounds in vitro, and observing them after their injection into recipient animals. This makes it possible to elucidate the effects of lymphocytes alone. On the contrary, if we want to elucidate the effects of any kind of compounds on cells other than lymphocytes, such as the vascular endothelium, we can treat recipient animals with any kind of compounds and observe them after the injection of control lymphocytes. If we want to elucidate the same things using mice, we must create conditional genetically manipulated animals one by one. In addition, sorting the isolated lymphocytes by using specific surface marker would make it possible to study the dynamics of specific types of lymphocytes.

Reviewer #2:

Major Concerns:
Introduction:
1/ The introduction contains a lot of approximations. Almost not a single sentence is correct. Just as a few examples: a/Numbers of PPs given by the authors are for humans. It is much less for rodents; b/ Parafollicular regions are termed interfollicular regions in PP; c/ The germinal center is located in the lower part of the follicle; d/Spleen and lymph have nothing to do with lymph nodes and PP; e/ As it is written, one could understand that M cells are captured by DCs; etc…

Thank you for your comments. We revised necessary parts of the introduction as shown below. As you pointed out, we have shown the number of follicles in the Peyer's patches in the human case. In addition, description about the spleen and lymph was deleted. Description about M cells was changed.

In humans, Peyer's patches (PPs) consist of hundreds of lymphoid follicles in the lamina propria of the small intestine. PPs are divided into follicles, the interfollicular region, and germinal centers located in the lower part of the follicles, where lymphocytes are stimulated by antigen presentation. There are no afferent lymphatic vessels, and the antigens invade the lamina propria from the intestinal lumen via the epithelial cell layer. The epithelial region covering lymphoid follicles is called the follicle-associated epithelium, within which specialized interspersed M cells uptake mucosal antigens. M cells take in antigens from the luminal side and antigens are then captured by dendritic cells and presented toward naïve lymphocytes that flow into PPs through the endothelium of high endothelial venules (HEVs)[1]. PPs play an important role in intestinal immunity and are related with the early stage of inflammation. Many molecular interactions involve the entrance of lymphocytes to secondary lymphoid organs (SLOs), including adhesion molecules, chemokines[2, 3], and sphingosine-1-phosphate[4]; thus, there are many expected therapeutic targets. Therefore, observing the lymphocyte dynamics within PPs enables us to catch a glimpse of the very early stage of inflammation and examine the usefulness of several promising drugs.

2/ Location of HEV, B and T cells inside the tissue should be specified especially since authors study lymphocyte migration;

Thank you for your comment. We are afraid that our change is what the reviewer intended to point out. Because we did not separated lymphocytes into T / B, we could not show difference in distribution of T / B. However, it is very easy to do that by using our system and the reviewer 's comment highlighted it clearly. If we stained T / B by different fluorescent, we can observe dynamics of T / B

simultaneously. We added it in the discussion.

The merit and key of our method is that it involves separating lymphocytes from recipient animals, incubating them using any kind of compounds in vitro, and observing them after their injection into recipient animals. This makes it possible to elucidate the effects of lymphocytes alone. On the contrary, if we want to elucidate the effects of any kind of compounds on cells other than lymphocytes, such as the vascular endothelium, we can treat recipient animals with any kind of compounds and observe them after the injection of control lymphocytes. If we want to elucidate the same things using mice, we must create conditional genetically manipulated animals one by one. In addition, sorting the isolated lymphocytes by using specific surface marker would make it possible to study the dynamics of specific types of lymphocytes.

3/ Previous works using the same type of approaches are still not well referenced. (e.g. "Some methods that were previously developed to observe lymphocyte migration have some advantages and disadvantages" with no references and no explanation of what could be these advantages and disadvantages).
As well, authors should reference works that have used photoconvertible transgenic animals to study lymphocyte egress from PP.

Thank you for your comments. The points you pointed out were rewritten as follows.

In previous reports, it was difficult to understand the three-dimensional changes over time, such as the intravenous injection of India ink to stain the vascular structure of Peyer's patches[6], or the microscope being monofocal[7]. In recent years, an observational method using some photoconvertible fluorescence protein transgenic animals such as Kaede mice have clarified systematic cellular movements in vivo[8]. The other study clarified CD69 independent shutdown of lymphocyte egress from Peyer's patches[9]. We used confocal laser scanning microscopy (CLSM) because of its high analytical capability. Now we can easily obtain high-resolution images and use them to analyze lymphocyte dynamics.

6 Gastroenterology. 1981 Sep;81(3):481-91.
7 Gastroenterology. 1995 Oct;109(4):1113-23.
9 Mucosal Immunol. 2014 Jul; 7(4): 892–904.

4/l.80 to l.86 should be completely rewritten. Authors describe a method, they do not demonstrate a series of methods, etc…

Thank you for your comment. We already demonstrated a series of methods in the Protocol section, so we dare avoid mentioning it in the Introduction.

5/ Authors claimed that "they quantified the cellular movements of lymphocyte migration" but there is nothing related to quantification in their manuscript.

Thank you for your comment. As you pointed out, we added some sentences in the Protocol to quantify lymphocyte dynamics as below.

4.3) (Optional) To quantify lymphocyte dynamics, define lymphocytes adhering to HEVs more than 30 seconds as “adhesive lymphocytes” and Lymphocytes emigrating from HEVs to stroma as “migrating lymphocytes”. Then you can calculate the average percentage of migration (migrating lymphocytes / adhesive lymphocytes + migrating lymphocytes) per field of vision (approximately 0.3 mm^2).

Results:
1/ The authors still do not provide sufficient information on what they can get as results from their approach. This strongly limits potential interest of readers for their method.

Thank you for your comments. Regarding the merit of the experimental method obtained by this research, we added some descriptions to the Results as below.

Collecting lymphocytes from lymph

To prepare the rat for thoracic duct cannulation, make an incision in the tense thoracic duct as shown in **Figure 1** and then maintain the rat in a Bollman’s cage as shown in **Figure 2**.

When the lymphocytes are well collected, we can obtain 30–40 mL/12 h lymph fluid containing about 5×10^6/mL lymphocytes. This effluence rate lasts about 2 days. Of the TDLs, 70% express CD4, among which about 90% cells express CD62L, meaning the main composition of TDLs (>60%) consists of naïve gut-tropic lymphocytes. Since the lymphocytes collected here are cells that specifically migrate to the intestinal tract, they are suitable for evaluating the state of migration in Peyer's patches.

Microscopic observation

After injecting an adequate dose of fluorescent lymphocytes, mount the recipient rat’s intestine on a glass slide as shown in **Figure 3**. A microscopic image of PPs in the physiological condition is shown

in **Figure 4**.

For the micro-observation, rats can be stably anesthetized under laparotomy for about 3 hours. Lymphocytes adhere to HEVs, roll, and migrate to the surrounding stroma, and then migrate to lymph capillaries. We quantified and evaluated the percentage of lymphocytes that migrated in the stroma within the observation period.

After the injection of an adequate dose of fluorescent lymphocytes, gut-tropic lymphocytes begin to adhere to HEVs and migrate across the endothelium into the stroma in about 1 hour. Most move within the stroma, while others migrate to lymph capillaries in about 2–3 hours. The well-developed fluorescence labeling method *ex vivo* in this study resulted high intensity and it enables us to observe lymphocyte dynamics of very deep area. In addition, by using different kind of fluorescence labelling, we can easily observe dynamics of different kind of lymphocyte simultaneously. This gut-tropic lymphocyte migration can be visually clarified for suppression by some immunomodulators such as FTY720 as shown in our previous report.

Observation of the microcirculatory system itself is extremely difficult, and it is desirable to establish an easy evaluating method. This study has also made it possible to find out where the action site of the drug is in the Peyer's patches. In particular, the *ex vivo* administration of FTY720 to lymphocyte made it possible to limit the site of action only to lymphocytes but not to endothelium. Combined with the sorting of specific lymphocyte, more detailed results would be obtained.

2/ Langerhans cells have nothing to do with PPs. Why are authors suddenly mentioning these typically skin-related phagocytes?

Thank you for your comment. As you pointed out, I deleted it and replaced it with the following text.

After the injection of an adequate dose of fluorescent lymphocytes, gut-tropic lymphocytes begin to adhere to HEVs and migrate across the endothelium into the stroma in about 1 hour. Most move within the stroma, while others migrate to lymph capillaries in about 2–3 hours. This gut-tropic lymphocyte migration can be visually clarified for suppression by some immunomodulators such as FTY720 as shown in our previous report.

Minor Concerns:
Protocol:
Although much well detailed, the protocol is still poorly written and difficult to follow. What is mcg?

Thank you for your comment. We corrected it because it is a typographical error of 'μm' instead of 'mcg' as below.

4.1) TDLs can be continuously monitored in the microvasculature of PPs using CLSM and recorded on a computer for 3 hours using time-lapse photography at 30-sec intervals. The depth from the serosa to the HEV of PPs is about 25 μm, enabling the observation of the stroma and capillary lymph vessels to a depth of 30 μm.

We would be grateful if this manuscript could be reviewed as an original article for publication in the *Journal of Visualized Experiments.*

Thank you again for considering our manuscript.
With my best regards,

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