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TITLE:

Isolation of Uterine Innate Lymphoid Cells for Analysis by Flow Cytometry

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pregnancy, uterus, decidua, natural killer cells, innate lymphoid cells, mouse

SUMMARY:

This is a method to isolate uterine lymphoid cells from both pregnant and non-pregnant mice. This method can be used for multiple downstream applications such as FACS phenotyping, cell sorting, functional assays, RNA-seq, and proteomics. The protocol here demonstrates how to phenotype group 1 uterine innate lymphoid cells by flow cytometry.

ABSTRACT:

Described here is a simple method to isolate and phenotype mouse group 1 uterine innate lymphoid cells (g1 uILCs) from individual pregnant uterus by flow cytometry. The protocol describes how to set up time mating to obtain multiple synchronous dams, the mechanical and enzymatic digestion of the pregnant uterus, the staining of single-cell suspensions, and a FACS strategy to phenotype and discriminate g1 uILCs. Although this method inevitably loses the spatial information of cellular distribution within the tissue, the protocol has been successfully applied to determine uILC heterogeneity, their response to maternal and foetal factors affecting pregnancy, their gene expression profile, and their functions.

INTRODUCTION:

Described here is a simple method to obtain a high yield of uterine innate lymphocytes from individual pregnant uterus. This method preserves protein surface expression and

functionality of uterine innate lymphocytes and it is suitable for subsequent applications such as FACS phenotyping, RNAseq, proteomics or functional assays. Here, the focus is on the phenotyping of group 1 uILCs by flow cytometry.

The uterus is composed of three layers: the endometrium, myometrium, and perimetrium (**Figure 1**). The endometrium is the mucosa, lining the lumen of the uterus. Progesterone, produced by the corpus luteum, converts the endometrium into decidua. The myometrium is composed of two layers of smooth muscle that make up the uterine wall. The perimetrium is the serosa that wraps the uterus and connects it to the peritoneum through the broad ligament called mesometrium. In a cross-section of the uterus, the part opposite to the lumen is called the mesometrial side, while the part close to the lumen is called the anti-mesometrial side. A variety of maternal leukocytes populate the endometrium and the decidua, including several types of cells, where the innate immune cells represent the vast majority of cells. Innate lymphoid cells (ILCs), macrophages, dendritic cells (DC), as well as $CD4^+$ and $CD8^+$ T lymphocytes, regulatory T cells (Tregs), and rare B cells, may all play important roles in the regulation of the uterine environment throughout pregnancy[1,2]. ILCs in the uterus are found not only in the mucosa, but also in the myometrium in mice. Including all three groups of ILCs, the uterus is indeed the organ most densely populated by group 1 ILCs. With the structural transformation of uterine tissues throughout gestation, the number and proportion of uterine leukocytes also change (see **Figure 2A** for an example of variations in the percentage of group 1 uILC subsets)[3,4].

When mice are referred to in this paper, the C57BL/6 strain of inbred laboratory mice is meant. Outbred mice (e.g., NMRI mice) are often used in reproductive research because of their high reproductive rate. However, the use of inbred strains is necessary to generate consistent results, and the immunologist's favorite genetic background is C57BL/6, also known as B6.

Approximately 30% of uterine leukocytes in B6 dams at mid-gestation are g1 uILCs, which are defined by flow cytometry as viable $CD45^+CD3^-CD19^-NK1.1^+NKp46^+$ cells (**Figure 2B**): pro-angiogenic tissue-resident NK (trNK), IFN-g producing conventional NK (cNK), and uILC1[4,5]. The percentage of uNK cells is even higher in humans, reaching about 70% in the first trimester[6]. There are more similarities than differences between human and mouse uNK and uILC[7,8]. Although it is important to keep the differences in mind, it is useful to integrate information available on the two species. When one combines information obtained from investigating uILC in humans and laboratory rodents, it is clear that NK cells assist in homeostatic changes essential for the biology of the uterus, including maintenance of arterial integrity[9] and spiral artery remodeling[10], as well as trophoblast invasion[11,12]. They also play specific roles in the defense against pathogens[13,14]. In mice and rats, besides filling the decidua around the implantation site, NK cells accumulate between the two muscle layers of the myometrium of dams in a transient structure known as the Mesometrial Lymphoid Aggregate of pregnancy (MLAp)[15] (**Figure 1B**), also known in the past as the metrial gland, the function of which is yet to be discovered.

Described here is a detailed protocol of the method used in the laboratory to isolate lymphocytes from the uterus of pregnant mice using a combination of mechanical disaggregation and enzymatic digestion. As the whole uterus is used in the method,

lymphocytes isolated from the uterus during gestation are a mixture of decidual and myometrial cells. Further dissection of the decidua from the uterine wall and its MLAp is possible, and it has been described before[16]. The method described here was developed to obtain uterine lymphocytes while preserving protein surface expression, cellular functionality, and viability. The outcome is a single cell suspension with minimal residual cellular debris and a yield typically ranging from 1–5 million cells at mid-gestation (10.5 days) for a pregnant uterus. The applications of this method encompass phenotyping by flow cytometry, cell sorting for subsequent transcriptomic or proteomic studies, functional studies such as intracellular cytokine production, degranulation, ELISPOT or cytotoxic assays. The protocol presented here focuses on identifying group 1 ILCs but can be adapted for other cell types such as other ILCs, T cells, B cells, DC, or macrophages with minor modifications of the antibody panel used for FACS analysis. The protocol can also be used to isolate cells from other tissues and for pooled non-pregnant uteri.

PROTOCOL:

All animal experiments described in this paper were conducted according to the Animals (Scientific Procedures) Act 1986 under PP2363781 issued by the UK Home Office. The protocol below consists of several sections starting from mice husbandry and finishing with staining for FACS analysis. **Figure 3** reflects the main steps of the protocol. The materials used in the protocol are listed in the **Table of Materials**.

1. General mice husbandry, mating, and dissection

1.1. Keep 7–14 week old female mice under specific pathogen-free (SPF) conditions and group-housed (typically 4–6 females according to cage size and animal weight), for 10–14 days to trigger the Lee-Boot effect, which results in estrus synchronization[17].

1.2. Keep the stud males in SPF conditions, single-housed, and rested for at least 48 h between each mating (time for sperm regeneration). It is preferable to use experienced proven 3–4 month old stud males as they are typically more performant than the young ones.

1.3. To increase the likelihood of females becoming pregnant, introduce soiled bedding from a male's cage in the female cage 3 days prior to mating. This triggers the Whitten effect[18] by exposure to male urine pheromones, and results in synchronized estrus as well as enhanced receptivity for mating.

1.4. On Day 0 (D0), set up the mice for mating using one stud male per two females; consider a plug rate of approximately 20%–25%.

NOTE: Mating will likely occur at night since mice are nocturnal animals.

1.5. In the morning following mating (D0.5), check for the presence of a vaginal plug, which is an indicator of copulation (**Figure 4**). The vaginal plug is an aggregate of the male ejaculate and typically persists for up to 8–24 h after mating. Check the plugs early morning.

1.6. Consolidate the plugged females in a new cage and earmark them. Return the males to their cages for resting.

1.7. At D9.5 or 10.5-after mating, prepare 5 mL tubes with 1 mL sterile HBSS 1x (with Mg^{2+} and Ca^{2+}) for tissue collection and place them on ice.

1.8. Proceed to animal euthanasia by cervical dislocation, followed by exsanguination to confirm death.

1.9. Work in a sterile environment if the downstream application requires to do so. Directly after euthanasia, wipe the mouse body with 70% ethanol and proceed to dissection under a laminar flux cabinet with sterile instruments.

1.10. Dissect the pregnant uterus free of mesometrial fat (**Figure 5**) and place the whole uterus in a prepared and appropriately labeled 5 mL tube. Keep the tubes on ice.

2. Mechanical and enzymatic digestion of the uterus

2.1. To prepare the enzymatic digestion solution, mix 3 mL per uterus of sterile HBSS 1x with 30 µg/mL of DNAse and 0.1 Wünsch unit (WU)/mL of Liberase DH or 0.52 WU/mL of Liberase TM. Put the solution in a water bath at 37 °C.

NOTE: Both Liberase TM and Liberase DH can be used. The choice of one over the other must be guided by their potential effect on epitopes recognized by antibodies used for subsequent flow cytometry analysis.

CAUTION: If using lyophilized enzymes, work under the hood.

2.2. Prepare 20 mL of 5 mM EDTA in PBS (no Ca^{2+}/Mg^{2+}). Place half the solution at 37 °C in a water bath and the other half on ice.

2.3. Under a laminar flux cabinet, gently remove the fat surrounding the pregnant uterus with sterile instruments in a sterile Petri dish. Do not allow the tissue to dry.

2.4. Dissect each implantation site with sterile instruments to remove the fetuses (seahorse-shaped translucid structure, around 1 mm in length) (**Figure 6A**). Discard the fetuses.

2.5. Return the uterus to their original collection 5 mL tube and mince the tissue using scissors directly in the 5 mL tube and collection medium. Keep the tubes on ice all the time between the procedures.

2.6. Place the 5 mL tubes containing the minced tissue in a water bath at 37 °C.

2.7. Add 3 mL of warm enzymatic digestion mix to each sample so that the total volume of liquid in the tube is 4 mL (1 mL of collection medium with the minced uterus and 3 mL of

enzymatic digestion solution). Incubate the 5 mL tubes for 30 min at 37 °C with agitation to enhance enzymatic digestion activity.

2.8. Vortex the 5 mL tubes and place them on ice to inhibit the action of enzymes. Then, subsequently, transfer the contents into properly labeled 15 mL centrifuge tubes.

2.9. Flush everything out of the 5 mL tubes into the 15 mL centrifuge tubes using 10 mL of ice-cold 5 mM EDTA PBS solution.

2.10. Centrifuge the 15 mL centrifuge tubes containing the digested tissues for 10 min at 400 x *g*.

2.11. Discard the supernatant, gently flick the pellet, and then resuspend it in 10 mL of warm (37 °C) 5 mM EDTA PBS solution.

2.12. Incubate the samples in the 15 mL centrifuge tubes at 37 °C with agitation for 15 min to remove the left-over digestion medium and reduce the cell clumping.

2.13. Vortex the samples on high for 10 s to further facilitate tissue dissociation.

3. Processing of the uterus into a single cell suspension

3.1. Using the plunger of a sterile 1 mL syringe, force the digested tissue through a 70 µm strainer onto a properly labeled and sterile 50 mL centrifuge tube to remove the cell clumps and the undissociated tissue.

3.2. Wash the strainer several times with a total of 10 mL of cold PBS to collect all the cells.

3.3. Spin the 50 mL centrifuge tube for 10 min at 400 x *g*.

NOTE: Perform the further steps using Option A or Option B. Option A allows better enrichment of the lymphocytes with less debris and stromal cell contamination than Option B. However, Option B gives a higher immune cell yield due to less cell loss and less variability of cell yield between samples. Option B is also easier to perform technically. Therefore, depending on preference, proceed with Option A or Option B.

3.3.1. The steps for Option A are as follows.

3.3.1.1. Label one sterile 15 mL centrifuge tube per sample containing 5 mL of 80% (v/v) isotonic Percoll diluted in PBS.

3.3.1.2. After the spin, discard the supernatant from the 50 mL centrifuge tube. Use a pipet boy to resuspend each pellet in 8 mL of 40% (v/v) isotonic Percoll in PBS.

3.3.1.3. Use a pipet boy on slow speed to carefully overlay the pellet resuspended in the 40% Percoll solution onto 80% Percoll solution. Pipette slowly and continuously; hold the 15 mL tube at an angle of 45° (**Figure 6B**).

3.3.1.4. Without disturbing the overlay, centrifuge the 15 mL centrifuge tubes for 20 min at 850 x *g*, at room temperature (medium acceleration and minimum break).

3.3.1.5. Carefully remove the tubes from the centrifuge without disturbing the Percoll layers (**Figure 6C**).

3.3.1.6. Without disturbing the ring of leukocytes at the interface of the two Percoll solutions, use a sterile Pasteur Pipette to discard all except for approximately 0.5–1 mL of the top Percoll layer.

3.3.1.7. While trying to suck a minimum amount of Percoll solution (up to 4–5 mL total), carefully collect the ring of leukocytes and transfer the cells into a new labeled 15 mL centrifuge tube.

3.3.1.8. Top up each sample with 10 mL of sterile RMPI-1640 medium supplemented with 10% of heat-inactivated FBS.

3.3.1.9. Centrifuge for 5 min at 500 x *g* at 4 °C.

3.3.1.10. Discard the supernatant and proceed to RBC lysis.

3.3.2. The steps for Option B are as follows.

3.3.2.1. Label one sterile 15 mL centrifuge tube per sample.

3.3.2.2. After the spin, discard the supernatant from the 50 mL centrifuge tube. Use a pipet boy to resuspend each pellet with 8 mL of 35% (v/v) isotonic Percoll in RPMI-1640 medium.

3.3.2.3. Transfer the samples into 15 mL centrifuge tubes.

3.3.2.4. Centrifuge the samples at 940 x *g* for 10 min at room temperature with medium acceleration and minimum break.

3.3.2.5. Aspirate the supernatant carefully using an aspirator or pipette boy (not by inverting the tube).

3.3.2.6. Resuspend the pellet in 14 mL of RPMI-1640 medium supplemented with 10% heat-inactivated FBS and then centrifuge the sample at 500 x *g* for 5 min at 4 °C.

3.3.2.7. Discard the supernatant by aspiration and proceed to RBC lysis.

4. RBC lysis

4.1. To lyse the RBCs, resuspend the samples in 3 mL of 1x RBC lysing solution and incubate for 3 min at room temperature.

4.2. Add 10 mL of PBS into the samples to stop the reaction.

4.3. Centrifuge the tubes at 400 x *g* for 5 min and discard the supernatant.

4.4. Add 10 mL of PBS and repeat step 4.3.

4.5. Resuspend each pellet in 1 mL of RPMI-1640 medium supplemented with 5% of heat-inactivated FBS.

4.6. Pass the samples through sterile 70 µm cell strainers.

4.7. Perform the cell count using trypan blue and a Neubauer Chamber as per the manufacturer's instructions.

4.8. Adjust the concentration of the cell suspension to 1–2 million cells in 100 µL of PBS or medium.

5. Panel design strategy and controls

NOTE: The panel described in this paper is suitable for the discrimination of uILC1, trNK, and cNK cells and was designed to be used on a 5-laser BD LSRFortessa. Minor modifications can be made to study different cell populations and use alternative fluorochromes. It is recommended to check the configuration of the instrument, using titrated antibodies for optimal separation, consulting the manufacturer's brightness index and using the brightest dyes for low expressing antigens such as NKp46, and following general guidelines[19]. It is recommended to include a Fluorescence Minus One (FMO) control for NKp46.

6. Innate lymphoid cell staining for FACS phenotyping

6.1. Transfer 1–2 million cells per well into a round-bottom 96-well plate.

6.2. Spin the plate at 400 x *g* for 3 min at 4 °C and discard the supernatant by flicking it into a sink.

6.3. Resuspend the cell pellets into 100 µL of PBS (protein and azide-free) using a multi-channel pipette.

NOTE: Ensure that the PBS contains no sodium azide, no Tris, or any proteins for the subsequent step.

6.4. Repeat step 6.2.

6.5. Resuspend cells in 50 µL of fixable viability dye diluted in PBS (protein and azide-free) (1:1,000). Incubate the cells at room temperature for 30 min in the dark.

NOTE: Ensure that the PBS contains no sodium azide, no Tris, or any proteins such as FBS or BSA as this may result in decreased staining intensity of dead cells and/or increased background staining for the live cells.

CAUTION: If viability dye is powdered, use under the hood.

6.6. Add 150 µL of PBS, resuspend the cells with a multi-channel pipette, and then repeat step 6.2.

6.7. Resuspend the cells in 25 µL of FACS Buffer (PBS supplemented with 1% BSA or 2% FBS) containing 1 µg of locking agent. Incubate the cells for 5 min at 4 °C.

6.8. Add 25 µL of a surface antibody cocktail.

NOTE: Always titrate antibodies and optimize the antibody panel prior to the experiment.

6.9. Incubate the samples at room temperature for 20 min in the dark.

6.10. Add 150 µL of FACS Buffer to each well, mix thoroughly, and then repeat step 6.2.

6.11. Repeat step 6.10.

NOTE: Perform further steps using Option A or Option B. Use option A to stain the cells with surface markers. Use option B to study the intracellular markers by flow cytometry.

6.11.1. The steps for Option A are as follows.

6.11.1.1. Resuspend the samples in 100 µL of 4% paraformaldehyde per well and incubate for 20 min at room temperature.

CAUTION: Use PFA under the hood. Please refer to the safety sheet for discarding PFA waste/objects that have come into contact with PFA (e.g., pipettes) safely.

6.11.1.2. Repeat step 6.2, twice.

CAUTION: Do not discard by flicking into the sink here as it contains PFA. Aspirate with a pipette and discard the waste as per the safety sheet.

6.11.1.3. Resuspend the samples in 200 µL of PBS.

6.11.1.4. Transfer the samples into labeled FACS tubes and top up with 100 µL of PBS. Keep the tubes on ice or in a fridge until processing with FACS analysis. Acquire the samples on a flow cytometer within 24 h.

6.11.2. The steps for Option B are as follows.

6.11.2.1. Resuspend the samples in 100 µL of fixation / permeabilization solution per well (containing paraformaldehyde) and incubate for 20 min at 4 °C.

CAUTION: Use PFA under the hood. Please refer to the safety sheet for discarding PFA waste/objects that have come into contact with PFA (e.g., pipettes) safely.

6.11.2.2. Repeat step 6.2.

CAUTION: Do not discard by flicking into the sink here as it contains PFA. Aspirate with a pipette and discard the waste as per the safety sheet.

6.11.2.3. Add 200 µL of 1x permeabilization / washing buffer, mix well, and then repeat step 6.2.

6.11.2.4. Repeat step 6.11.2.3.

6.11.2.5. Resuspend the fixed and permeabilized cells in 50 µL of 1x permeabilization/washing buffer containing antibodies mix for intracellular staining.

6.11.2.6. Incubate the samples at 4 °C for 30 min in the dark.

6.11.2.7. Add 200 µL of 1x permeabilization / washing solution, mix well, and then repeat step 6.2.

6.11.2.8. Repeat step 6.11.2.7.

6.11.2.9. Resuspend the samples in 200 µL of PBS.

6.11.2.10. Transfer the samples into labeled FACS tubes and top up with 100 µL of PBS. Keep the tubes on ice or in a fridge until processing with FACS analysis. Acquire the samples on a flow cytometer within 24 h.

NOTE: After performing this protocol, the decidual cell suspension is ready for FACS analysis. It is recommended to record as many events as possible per sample; at least 1,000–3,000 events of a parental population have to be obtained to achieve reliable results.

REPRESENTATIVE RESULTS:

The main steps of the method that are described to obtain a single cell suspension of uterine leukocytes are summarized in **Figure 2B**. Demonstrated here are the basic FACS gating strategy used for the identification of three subsets of g1 ILCs in B6 mice: uILC1 ($CD49a^{+}Eomes^{-}$), trNK ($CD49a^{+}Eomes^{+}$), and cNK ($CD49a^{-}Eomes^{+}$) cells. Further analysis of these populations can be performed to study various surface and intracellular markers of g1 ILCs. As an example, the co-expression of IFN-ɣ and self-MHC receptors can be assessed in uILC1, trNK, and cNK cells after stimulation with anti-NK1.1 antibody (**Figure 7**).

Depending on the research question, both the protocol (**Figure 3**) and the antibody panel can be adapted. Importantly, it is recommended to use both anti-NK1.1 and anti-NKp46 antibodies in one FACS panel for g1 ILC gating (**Figure 2B** and **Table 1**). It should be noted that g1 ILCs obtained from blood, spleen, or liver have a higher expression of NKp46 on their surface than the uterine counterpart (**Figure 8**). Surface staining for NK1.1 gives a better separation and enables uterine g1 ILCs to be gated easily (**Figure 8**). While NKp46 is expressed by all mouse strains, the NKR-P1C antigen recognized by the anti-NK1.1 antibody PK136 is only expressed by some mouse strains, including C57BL/6 (i.e., B6), FVB/N, and NZB, but not in AKR, BALB/c, CBA/J, C3H, DBA/1, DBA/2, NOD, SJL, or 129. In addition, if the investigator intends to study crucial NK cell receptors such as the MHC receptors Ly49, it is important to be aware of allelic variations in laboratory mouse strains, which recapitulate the high variability of human killer-cell immunoglobulin-like receptors (KIR). Moreover, if the cells are to be stimulated with NK1.1 for a functional assay, as described in Kim, S. et al.[20], it might be desirable to stain the cells with anti-NKp46 rather than anti-NK1.1, as the NKR-P1C antigen may be occupied by the crosslinking anti-NK1.1 or a receptor downregulation may follow the stimulation. Either receptor occupancy or downregulation can impede staining with the same antibody used to stimulate.

A common problem with enzymatic tissue dissociation is the alteration of surface epitopes on cells by enzymes used for a digestion medium. For example, staining for the MHC CD94:NKG2A receptor is poor if Liberase TM is used. However, digestion with Liberase DH preserves NKG2A recognition by 16A11 antibody clone (**Figure 9**). It is recommended to check the influence of enzymes on all epitopes in one's FACS panel. For this purpose, use the suspension of mouse splenocytes obtained by mechanical dissociation (passing the whole spleen through a 70 µm strainer). The sample is then divided into two or more parts followed by incubation with a medium with or without enzyme(s).

As mentioned before, blood-derived cells are present in tissue dissociated samples. If required, blood contaminants can be excluded using an intravascular staining method as developed in Masopust laboratory[21]. **Figure 10** demonstrates that around 6.5% of g1 ILCs present in uterine tissue samples at gestation day 8.5 are blood-derived. Anti-CD45 antibodies used for intravascular staining can be conjugated with a fluorochrome used for a dump-channel; this will exclude blood contaminants without using an extra fluorescence channel. The most common problems and their solutions are presented in **Table 2**.

FIGURE AND TABLE LEGENDS:

Figure 1: Cross-section of a mouse uterus. (**A**) Mouse uterus cross-section (non-pregnant) indicating a variety of maternal leukocytes which populate the uterus**.** (**B**) Mouse uterus cross-section (gestation day 8.5). (**C**) Mouse uterus cross-section (gestation day 13.5). (**D**) Comparison of mouse versus human placenta formation from blastocyst stage onward. Images created with BioRender.com.

Figure 2: Subpopulations of uterine g1 ILC1. (**A**) Percentages of uterine cNK, ILC1, and trNK in mice during early life and pregnancy. W – weeks, gd – gestation day. Modified graph from Filipovic, I. et al.[4]. (**B**) Gating strategy to analyze uterine group 1 ILC subsets by flow cytometry. Lymphocytes were isolated from the uterine tissues at gestation day 10.5. Tissue digestion was performed using a digestion medium containing Liberase TM. Cells were

gated based on their ability to scatter light. Doublets were excluded using a FSC-A versus FSC-H plot, and only $CD45^+CD3^-CD19^-$ viable cells were analyzed further. Within $CD45^+CD3^-CD19^-$ viable cells, the group 1 ILC gate was identified as $NK1.1^+$ $NKp46^+$ cells. Within group 1 ILCs, three subsets can be identified: $CD49a^-Eomes^+$ conventional NK cells (cNK), $CD49a^+Eomes^+$ tissue-resident NK cells (trNK), and $CD49a^+Eomes^-$ uILC1.

Figure 3: A visual guide for the main steps of the protocol. (**1**) Dissect the pregnant uterus free of mesometrial fat. (**2**) Remove the fetuses; return the uterus to the 5 mL tube and mince the tissue. Proceed with the enzyme digestion step: add 3 mL of warm enzymatic digestion mix to each sample. Incubate the 5 mL tubes for 30 min at 37 °C with agitation. (**3**) (i) After digestion, flush everything out of 5 mL tubes into 15 mL tubes using 10 mL of ice-cold 5 mM EDTA PBS solution. (ii) Centrifuge 15 mL tubes containing digested tissues for 10 min at 400 x *g*. (iii) Discard the supernatant; gently flick the pellet and resuspend it in 10 mL of warm (37 °C) 5 mM EDTA PBS solution. (iv) Incubate samples in the 15 mL tubes at 37 °C with agitation, for 15 min. (**4**) Using the plunger of a sterile 1 mL syringe, force the digested tissue through a 70 µm strainer onto a properly labeled and sterile 50 mL tube, and spin for 10 min at 400 x *g*. (**5**) After the spin, proceed with either option A (represented here in diagrams) or B. *Option A:* discard the supernatant from the 50 mL tube and, using a pipet boy, resuspend each pellet in 8 mL of 40% (v/v) isotonic Percoll in PBS. (**6**) (i) *Option A continued:* Using a pipet boy on slow speed, carefully overlay the pellet resuspended in 40% Percoll solution onto the 5 mL of 80% Percoll solution. Pipette slowly and continuously; hold the 15 mL tube at an angle of 45°. (ii) Without disturbing the overlay, centrifuge the 15 mL tubes at 850 x *g* for 20 min at room temperature, acceleration 5, break 1. (**7**) After the spin, while trying to suck a minimum amount of Percoll solution (up to 4–5 mL total), carefully collect the ring of leukocytes. (**8**) Perform red blood cell lysis steps. (**9**) After red blood cell lysis, perform the cell count using trypan blue and a Neubauer Chamber as per the manufacturer's instructions. (**10**) Transfer 1–2 million cells per well into a round-bottom 96-well plate and proceed with spin and viability dye staining. (**11**) After the viability dye step, add 25 µL of a surface antibody cocktail. Proceed with further FACS steps. (**12**) Finally, transfer the samples into labeled FACS tubes and top up using 300 µL of PBS. Keep the tubes on ice or in a fridge until processing with FACS analysis. The samples have to be run on a flow cytometer within 24 h. Images created with BioRender.com.

Figure 4: Vaginal plug (A) and absence of it (B) in C57BL/6 females at 0.5 day post mating.

Figure 5: Dissection to extract the uterus from a pregnant mouse. (**A**) The dam is pinned down with needles on a soft board to wipe the body with 70% ethanol. Two vertical incisions are made, as indicated by the blue dotted lines. (**B**) The skin is lifted to expose internal organs. The intestinal loops are gently moved up to visualize the uterus. (**C**) The uterus is sampled by cutting at three points: next to the ovaries and at the cervix, as indicated by the two blue dotted lines and the blue arrow, respectively.

Figure 6: Preparation of single-cell suspension. (**A**) Mechanical removal of embryos from their implantation site. (**B**) Percoll gradient overlay; the top layer contains the single-cell suspension in 80% of Percoll and the bottom layer 40% of Percoll. (**C**) Lymphocyte ring formation after centrifugation of the percoll gradient.

Figure 7: Representative FACS analysis of functional assay with group 1 ILCs. Intracellular IFN-γ and surface CD107a detection in group 1 ILCs expressing NK receptors for self-MHC (Ly49C, Ly49I, and NKG2A) compared to those that do not, after crosslinking NK1.1 with plate-bound antibodies. The cells were isolated from uterine tissues at gestation day 9.5. Tissue digestion was performed using a digestion medium containing Liberase DH. Shown are the raw values of all four quadrants (corners) as well as the relative percentage of responders among cells expressing receptors for self and responders that do not have self-receptors (bold numbers).

Figure 8: Staining splenic and uterine lymphocytes with anti-NKp46 and anti-NK1.1 antibodies. (**A**) Cell suspensions obtained from mouse spleen and (**B**) uterus at gestation day 10.5 were separated in two; one part was stained with NKp46-APC (red) and the other one with NK1.1-APC (blue). Note that the NKp46 staining of uterine lymphocytes does not separate $NKp46^+$ and $NKp46^-$ cells as neatly as splenic lymphocytes.

Figure 9: Decrease of NKG2A MFI (antibody clone: 16A11) by incubation with digestion medium. Cell suspension of C56BL/6 mouse splenocytes was divided into three parts. One part was incubated in Liberase DH digestion medium (HBSS containing 0.13 WU/mL Liberase DH and 30 µg/mL of DNAse), and another part was incubated with Liberase TM digestion medium (HBSS containing 0.52 WU/mL Liberase TM and 30 µg/mL of DNAse). The third part was treated with neat HBSS for 30 min at 37 °C. Expression of NKG2A marker on g1 ILCs was assessed by flow cytometry. Graph taken from Shreeve N. The role of uterine NK-cell inhibition in pregnancy (Thesis); Supervisor: Colucci F, 2020.

Figure 10: Intravital staining with anti-CD45 antibodies for exclusion of blood-derived g1 ILCs. C57/BL6 mouse at gestation day 8.5 was culled 3 min after intravenous injection with 3 µg of CD45-AF647. The uterus, whole blood, and thymus were harvested and processed for FACS analysis. The X-axis shows the signal from intravenous staining with CD45-AF647, and Y-axis demonstrates signal from *in vitro* stained CD45-BUV395. The percentages of subpopulations are shown in quadrants.

Table 1: An example of FACS panel for conventional 5-lasers cytometer.

Table 2: Troubleshooting guide.

DISCUSSION:

The method contains several critical steps discussed hereafter. The first critical step is to obtain multiple synchronous pregnancies as the relative frequency of leukocyte populations changes through pregnancy. Having multiple dams at the same gestational day allows for either biological repeats in the same experiments or pooling lymphocytes from individual dams to obtain larger numbers required for downstream applications. Timed mating allows the researcher to pinpoint conception within a 24 h period. Although mice live for around 2.5 years, they will be of reproductive age from 4–7 weeks until 6–8 months old. As younger mice usually produce smaller pups, female mice are generally not mated until they are between 6–8 weeks, and male mice until they are between 8–10 weeks. Given that estrus lasts about 15 h in mice and occurs every 4–5 days, the typical mating rate (revealed by a vaginal plug, see **Figure 4**) is around 25%. It is therefore important to use mice in estrus and

plan sufficient numbers to obtain the required number of dams for a given experiment. The estrus cycle phase can be determined by vaginal smear cytology[22]. The plug rate can be improved by resting males 48 h prior to mating and by taking advantage of the Whitten effect[18]. Alternatively, one can administer pregnant mare serum, which mimics the effect of the endogenous follicle-stimulating hormone, inducing oocyte maturation and, 42–50 h later, human chorionic gonadotropin, which mimics the effect of the endogenous luteinizing hormone, inducing ovulation. This hormonal treatment bypasses the requirement for estrus and makes virtually all treated females receptive.

A second critical step is to ensure the quality of the FACS staining. Antibodies used in flow cytometry must always be titrated and used at the optimal concentration, and it is necessary to check that the enzymatic digestion does not cleave crucial antigenic epitopes. To assess whether an enzyme will cleave an epitope, one could stain two fractions of the same sample in parallel, one undergoing enzymatic and the other mechanical digestion. Similarly, the use of appropriate controls and single stains is crucial to obtain reliable data. For rare events, beads can be used to generate single stain samples. It is recommended not to use beads for setting up voltages but rather a population of cells containing lymphocytes and other leukocytes, such as splenocytes. If beads are used, it is necessary to titrate antibodies for bead staining, so the fluorescence intensity of stained beads will be comparable to the fluorescence intensity of cells. In case of difficulty separating positive from negative cells for a particular marker, an FMO control can also be used to facilitate the gating for a specific marker. In the case of intracellular markers, an isotype control and not FMO control must be used as intracellular staining might result in residual unbound antibodies, which may still be present within cells after the washing steps and therefore increase the background signal. It is recommended to run the samples within 24 h of fixing the cells to obtain the best results in phenotyping by FACS analysis, as autofluorescence increases significantly over time and fluorescence intensity of some antibodies might decline over time.

Another crucial factor to consider is the downstream application of the single-cell suspension obtained with the protocol. For functional assays, it is essential to work in sterile conditions. Similarly, for subsequent omics studies, it is important to work in sterile and RNase-free conditions.

The protocol presented here focuses on phenotyping group 1 ILCs but can be adapted for phenotyping other cell types by modifying the antibody panel or on different tissues. It is recommended that all antibodies are tested against digested and non-digested cell suspension to detect the loss/alteration of surface epitopes by the enzymatic treatment. Similarly, different enzymes can be used to digest the tissue and increase cell yield, but its effect on crucial antigenic epitopes must be carefully studied. While NKp46 is a good marker for splenic NK cells and works in all strains of laboratory mice, the expression of NKp46 on uNK cells in C57BL/6 mice is considerably lower than on spleen NK cells. It is best to stain for both NK1.1 and NKp46 simultaneously. If multiple organs are to be compared directly, it is recommended to treat all samples equally, even if the enzymatic digestion is not required for tissues such as the spleen or the bone marrow. Although the method presented here is applicable to the non-pregnant uterus, the lymphocyte ring isolation by a two-phase Percoll gradient will be challenging, and the yield of isolated cells may be too low for reliable FACS

analysis and therefore will require pooling together cells isolated from the uterus of individual, non-pregnant mice[23].

There are limitations to the protocol to consider for the interpretation of the data. As it is the case for all tissues, circulating lymphocytes coming from the blood will be isolated alongside tissue-resident cells. If the exclusion of circulating lymphocytes is essential for data interpretation, intravital staining can be performed to label circulating cells. Furthermore, a second limitation to the protocol is that some cells will be lost as not all cells can be extracted from the tissue. The most common problems and their troubleshooting are presented in **Table 2**.

Historically, the study of cells in tissues has relied on histological examination of tissue sections. Sandra Peel's excellent review[24] summarizes the work done over more than 100 years until the late '80s. Descriptions of cells later known as uNK cells indeed appear in manuscripts published more than half a century before lymphocytes were even discovered. So, before the discovery of NK cells in 1975, and the uNK cells have been indicated as maternal glycogen cells or granulated metrial gland cells. Anne Croy has made major contributions in the field[25] and kindly taught the team the dissection she had optimized[3], and that is used currently. Although it is instrumental in describing the morphology and tissue location of uNK cells, classical histological examination is limited to the detection of only a few markers on the cells of interest. In 2008, a flow cytometry-based method to simultaneously detect multiple markers on uterine lymphocytes was described[26]. This is essentially the method that has been described in this paper. More recent technologies such as spatial transcriptomics and imaging by mass cytometry combine the power of histology and flow-cytometry, allowing both the simultaneous detection of multiple genes or proteins, respectively, and the preservation of the normal tissue architecture.

The applications of the method described here are multiple and include FACS phenotyping, functional assay (such as ELISPOT, degranulation or cytotoxic assays), cell sorting, and subsequent transcriptomics or proteomics. Further applications that could be developed based on this method include the culture and expansion of decidual NK cells after cell sorting or enrichment by negative depletion. Currently, there is no protocol to culture and expand mouse uNK cells and preserve their viability and functionality for an extended period, in a similar fashion to human NK cells that can be cultivated and expanded for 7–14 days by addition of IL-2 or a combination IL-12 and IL-15. The optimization of such a method for mouse uNK cells would provide more flexibility when performing functional assays and allow for multiple conditions to be tested with a higher cell number. On the other hand, culture conditions are known to modify the unique phenotype of lymphocytes and potentially their function too.

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DISCLOSURE:

The authors have nothing to disclose and no conflicts of interest.

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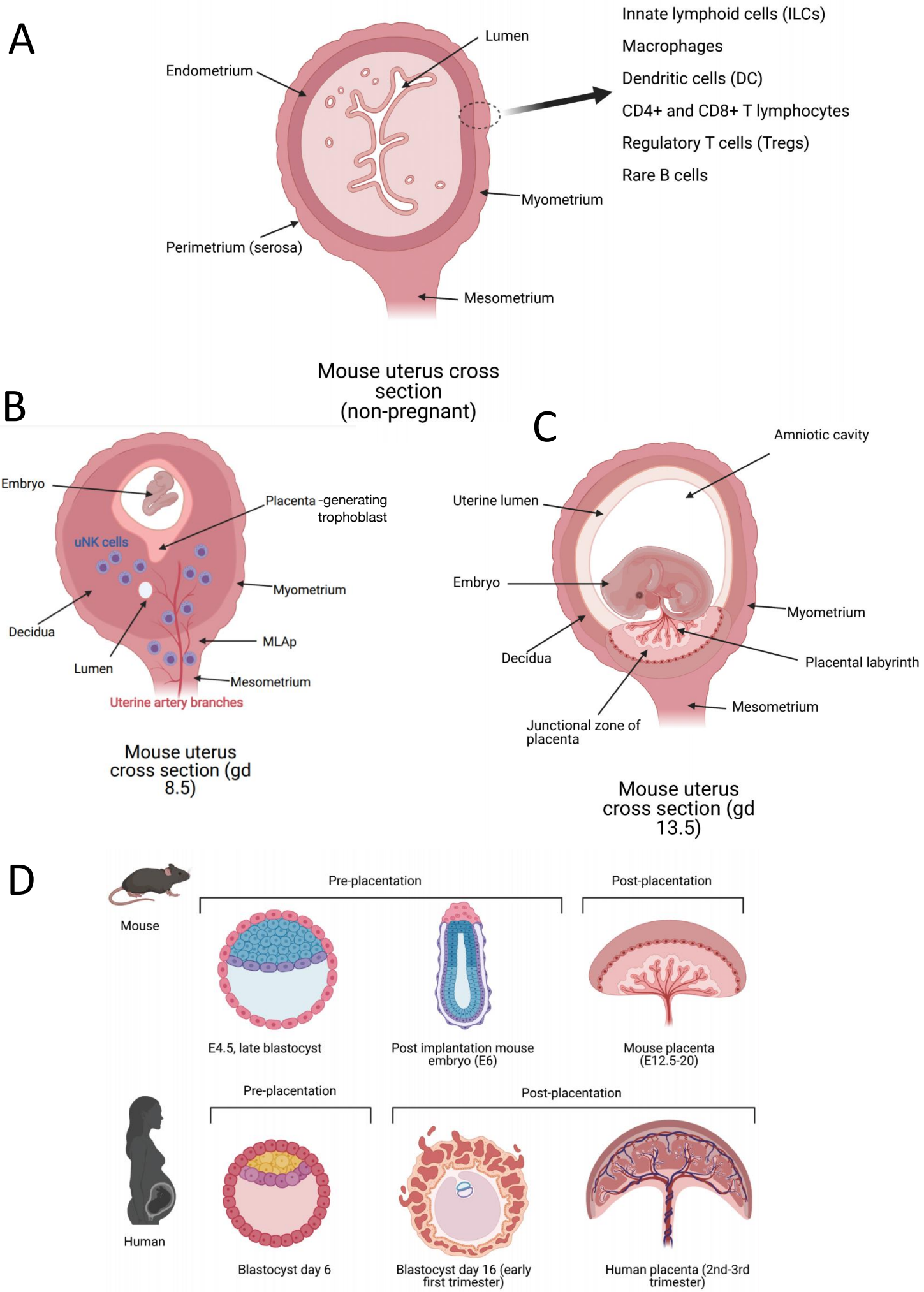
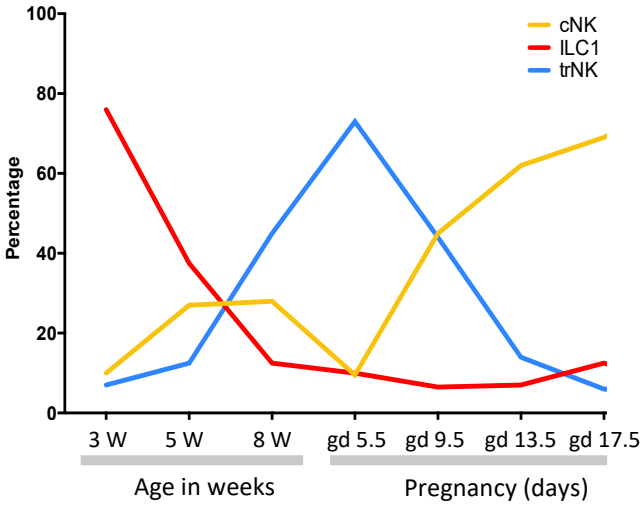


A



Modified graph from Filipovic, 2018, Nat Commun.

B

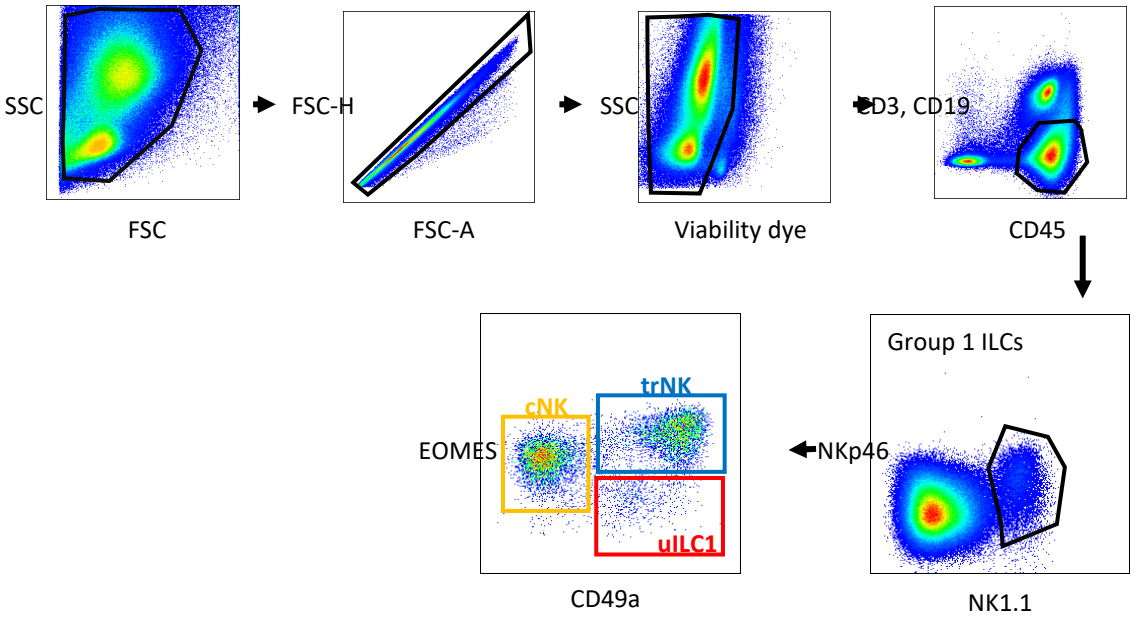


Figure 3

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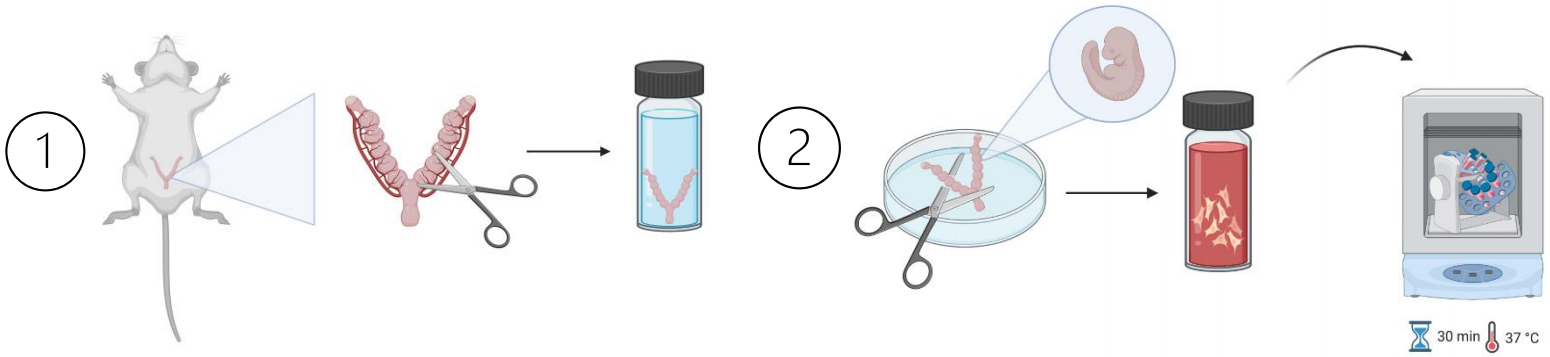
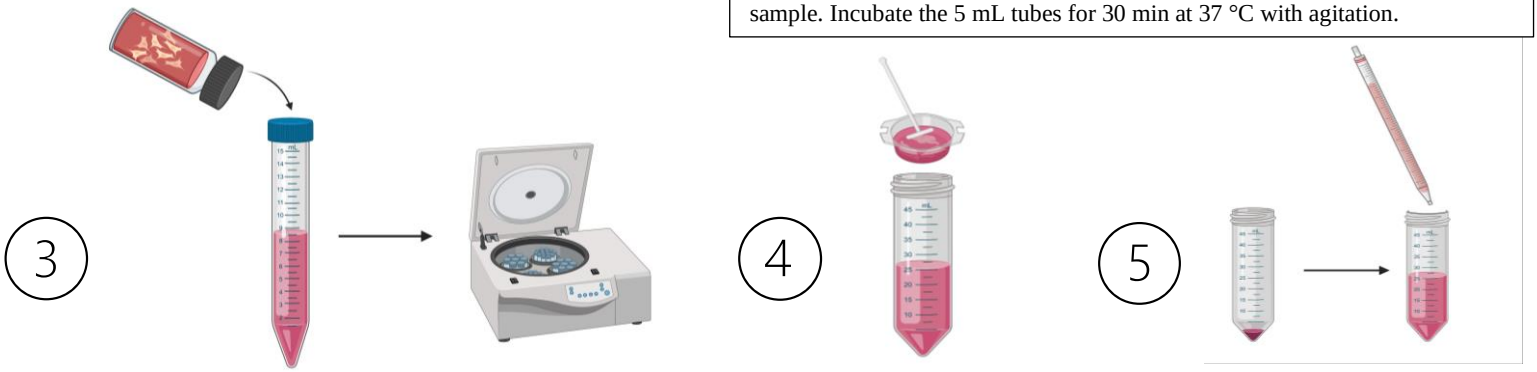
A visual guide to the main steps in the protocol



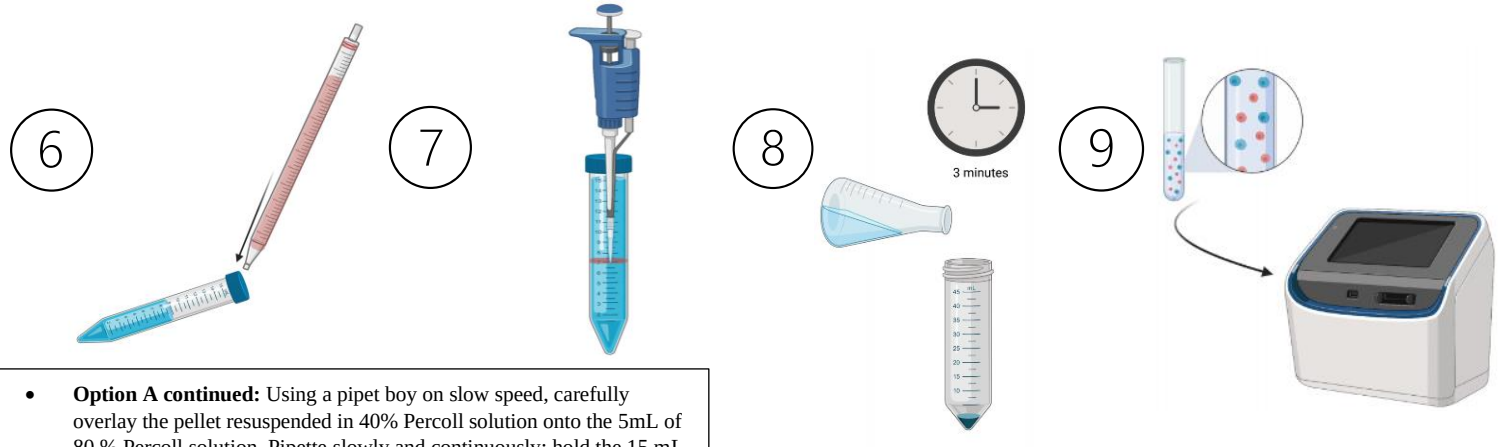
Dissect the pregnant uterus free of mesometrial fat.

Remove the fetuses, return uterus to 5mL tube and mince tissue. Proceed with enzyme digestion step: add 3 mL of warm enzymatic digestion mix to each sample. Incubate the 5 mL tubes for 30 min at 37 °C with agitation.



- After digestion, flush everything out of 5 mL tubes into 15 mL tubes using 10 mL of ice-cold 5mM EDTA PBS solution.
- Centrifuge 15 mL tubes containing digested tissues for 10 min at 400 x *g*.
- Discard the supernatant, gently flick the pellet and resuspend it in 10 mL of warm (37 °C) 5mM EDTA PBS solution.
- Incubate samples in the 15 mL tubes at 37 °C with agitation, for 15 min.

- Using the plunger of a sterile 1 mL syringe, force the digested tissue through a 70 µm strainer onto a properly labelled and sterile 50 mL tube, and spin for 10 min at 400 x *g*.
- After the spin, proceed with either option A (represented here in diagrams) or B. **Option A:** discard the supernatant from the 50 mL tube and using a pipet boy, resuspend each pellet in 8 mL of 40 % (v/v) isotonic Percoll in PBS.



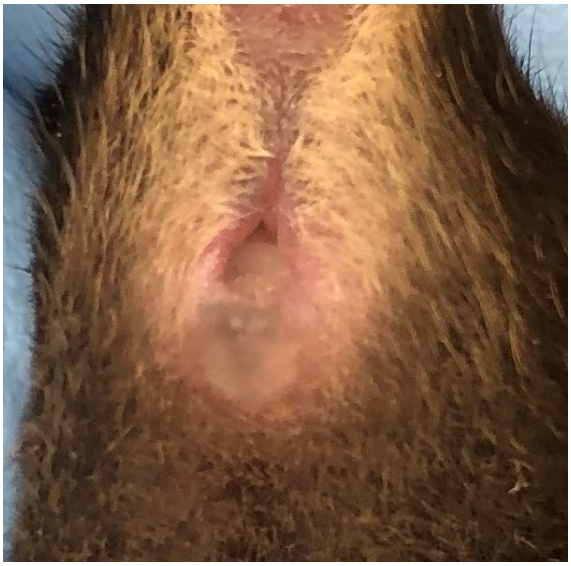
- **Option A continued:** Using a pipet boy on slow speed, carefully overlay the pellet resuspended in 40% Percoll solution onto the 5mL of 80 % Percoll solution. Pipette slowly and continuously; hold the 15 mL tube at an angle of 45°.
- Without disturbing the overlay, centrifuge the 15 mL tubes for 20 min at 850 x *g* at room temperature, acceleration 5, break 1.
- After the spin, while trying to suck a minimum amount of Percoll solution (up to 4-5 mL total), carefully collect the ring of leukocytes.

- Perform red blood cell lysis steps.
- After red blood cell lysis, perform the cell count using trypan blue and a Neubauer Chamber as per manufacturer's instructions.

- Transfer 1-2 million cells per well into a round bottom 96-well plate and proceed with spin and viability dye staining.
- After viability dye step, add 25 µL of a surface antibody cocktail. Proceed with further FACS steps.

Finally, transfer the samples into labelled FACS tubes and top up with 300 µL of PBS. Keep the tubes on ice or in a fridge until processing with FACS analysis. The samples have to be run on flow cytometer within 24 h.

A



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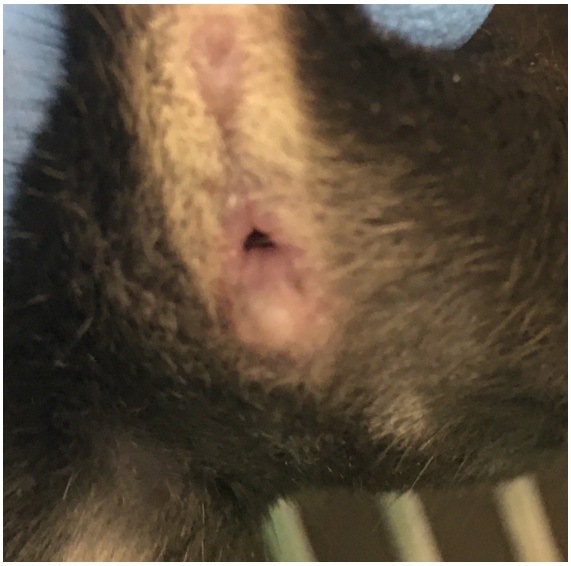
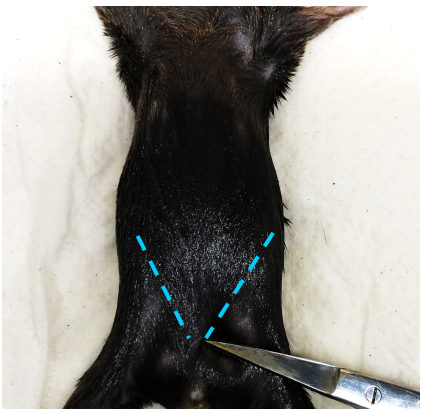


Fig 5

A



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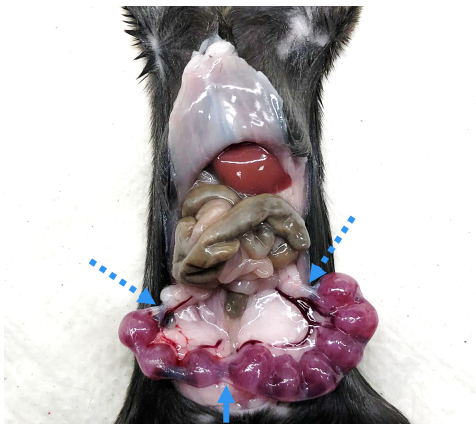

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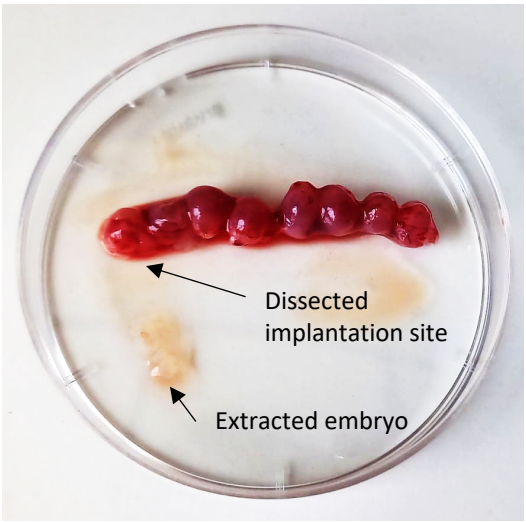

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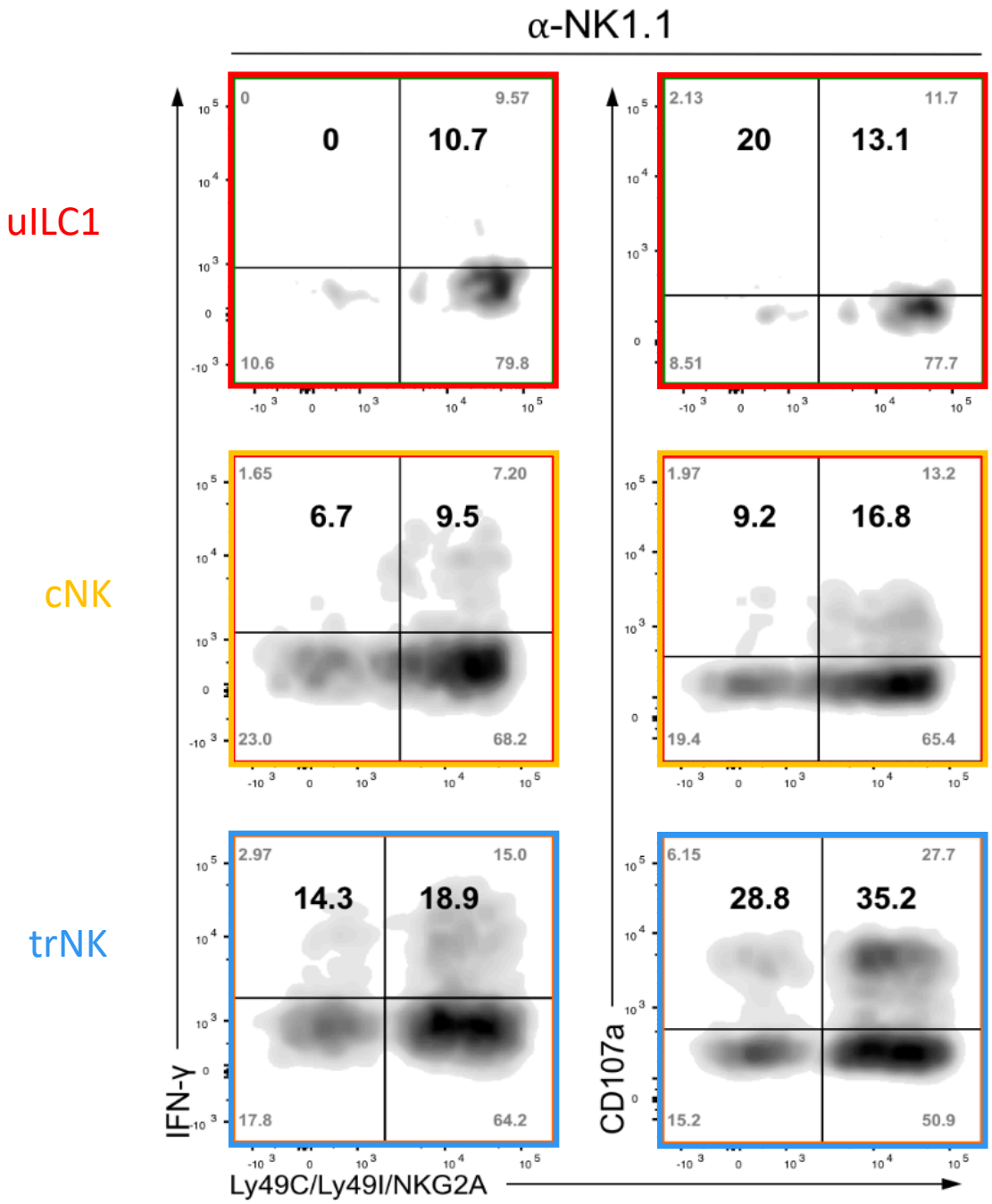
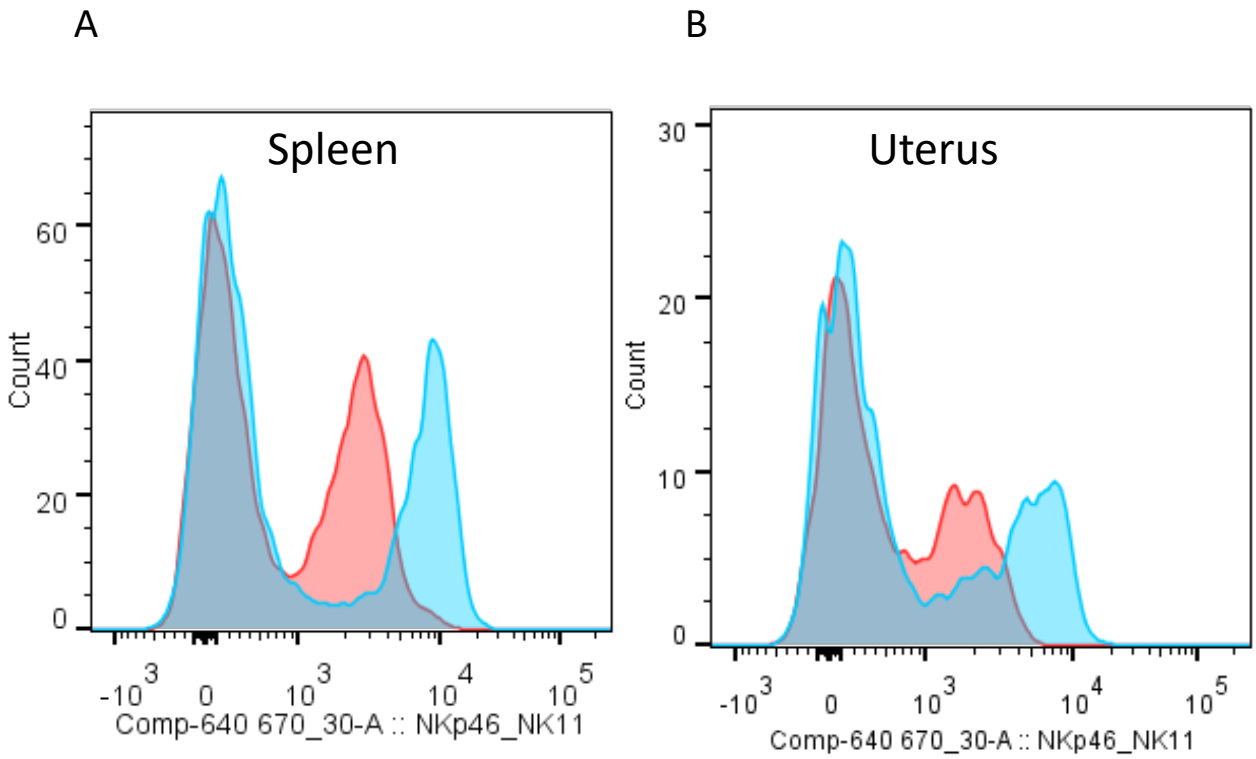
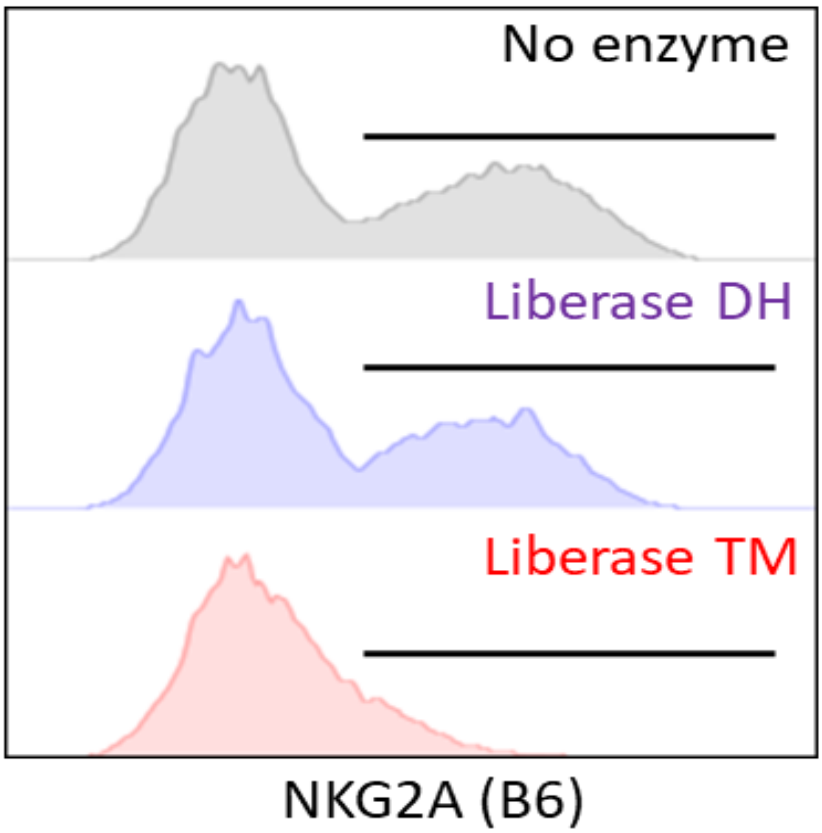


Fig 8





Graph taken from Norman Shreeve's PhD thesis.

Fig 10

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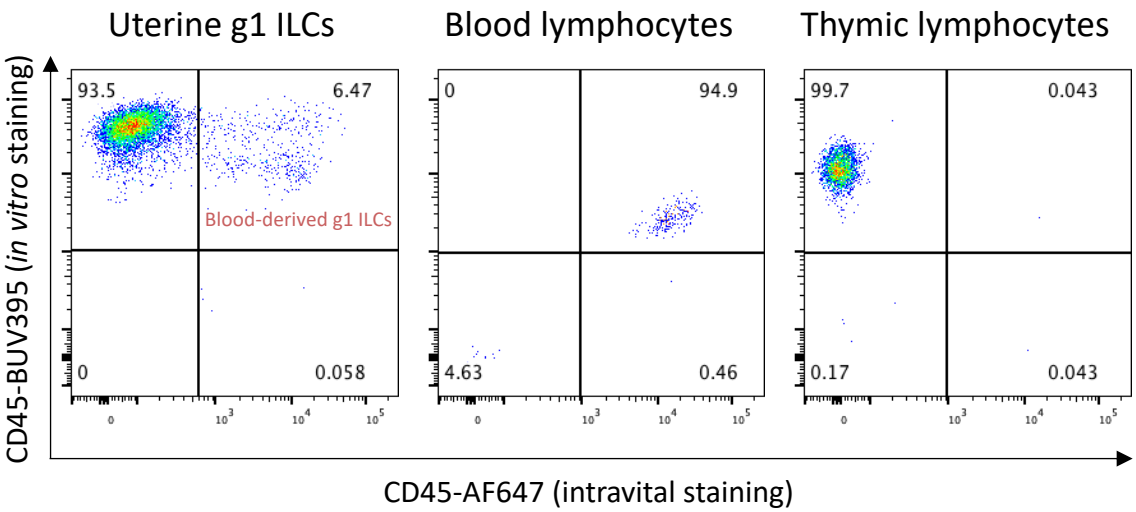

Table 1. An example of FACS panel for conventional 5-laser cytometer

	Antibody / Dye	**Clone**	**Fluorochrome**	**Laser**
1 (Dump channel)	Zombie Violet Fixable Viability dye			Violet
	CD19	1D3	BV421	
	CD3	145-2C11	BV421	
2	CD45	30-F11	FITC	Blue
3	NK1.1	PK136	BV605	Violet
4	NKp46	29A1.4	APC	Red
5	CD49a	Ha31/8	BUV395	Ultraviolet
6	EOMES	Dan11mag	PE	Green

Table 2. Troubleshooting guide

Problem	Possible cause
The cell ring is not visible at the interface of two percoll solutions	Poor layering of percoll solution or mixing two layers during sample handling
	Low numbers of leukocytes (for instance, when using a non-pregnant uteruse)
Incomplete RBC lysis	Cells was not resuspended properly in the lysing buffer
	Lysing solution is cold
Low cell yield	Poor enzymatic digestion
	Cell loss during washing steps
	Tissue sample contains low numbers of lymphocytes (for instance, when using a non-pregnant uteruse)
High variability of absolute leukocyte numbers obtained from mice of the same group	Inconsistent collection of cells at the interface of 40% and 80% percoll solutions
Not able to detect expected lymphocyte subpopulations/markers or unusually low MFI for some cell surface markers	Enzymatic digestion affected surface expression of some epitopes or their degradation
High background noise in the flow cytometer	High proportion of cell debris or RBC contamination

Suggestion
Take extra care to not break the 80% percoll cushion during overlaying. Pay attention during sample handling: do not disturb the percoll interface
The interface can be seen even when the cell number at the interface is very low. Even if the ring is not visible, collect liquid at between 40% and 80% percoll solutions, as there might still be enough cells for further processing
Pipette cells up and down to break the clumps and fully resuspend cells in lysing buffer
Equilibrate the lysing solution to room temperature before use
Prolong time of incubation with the lysing solution up to 15min
RBC lysis step can be repeated
Check if enzymes are not out of date and have been stored according to their manuals
Inspect the cell pellet after each washing step: the opaque pellet at the bottom of the well after spin. Using V-bottom instead U-bottom plates, swing rotor centrifuge, longer centrifugation time may reduce cell loss
Pool several uteruses to obtain enough events for analysis. Consider using Option B of the protocol for cell isolation
Make sure to collect whole cell fraction on the percoll interface. Consider using Option B of the protocol for cell isolation
Optimize enzymatic digestion by changing: enzyme (e.g. to different type of liberase or collagenase) and/or length of incubation and/or enzyme concentration
Adjust FSC thresholding parameter. Consider using Option A of the protocol for cell isolation

Name	Manufacturer	Catalog Number
70 µm cell strainers	Falcon	352350
BSA	Sigma	A9647-100G
CD19 antibody	BD	562701
CD3 antibody	BD	562600
CD45 antibody	BioLegend	103108
CD49a antibody	BD	740262
DNase I	Roche (Sigma)	10104159001
EOMES antibody	eBioscience	12-4875-82
Fc block Trustain fcx	BioLegend	101320
Fetal Bovine Serum	Gibco	10217-106
Fix/Perm buffer (part of BD Cytofix/Cytoperm Fixation/Permeabilization Kit)	BD	554714
HBSS, calcium, magnesium, no phenol red	Gibco	14025092
Liberase DH	Roche (Sigma)	5401089001
Lysis buffer Pharmlyse	BD	555899
NK1.1 antibody	BioLegend	108739
NKp46 antibody	BioLegend	137608
Paraformaldehyde 16% Solution (methanol-free)	Agar Scientific	AGR1026
PBS 10x	Gibco	14030-048
PBS 1x (no Ca2+ or Mg2+)	Thermo Scientific	14190144
Percoll	VWR internationa	17-0891-01
Phosphate buffered saline	Sigma-Aldrich	P5368-10PAK
Pre-Separation filters	Miltenyi	130-095-823
RMPI-1640 medium + GlutaMAX	Gibco	61870-010
UltraPure 0.5M EDTA, pH 8.0	Thermo Scientific	15575020
Zombie Violet Fixable Viability dye	BioLegend	423113

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Response to Editors

Dear Dr Krishnan,

We have revised the manuscript according to your advice and the point-by-point-response is included below (in blue).

1. The manuscript has been formatted to fit the journal standard. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.
   Done

2. The numbering of the Protocol steps has been adjusted to the journal standard. Please check the step numbers, especially the steps where the reader is directed to perform a step described earlier in the protocol (Lines: 287, 323, 335, 346, 348, 361, 380, 388, 396, 398, etc.)
   Done

3. Figure 2: Please revise the x-axis legend in Figure 2A from "3W" to "3 W" to maintain a single space between the numeral and (abbreviated) unit. ("gd9.5" to "gd 9.5", etc.)
   X-axis revised and new updated figure uploaded

4. Figure 3: Please revise the text in the figure to follow the standard format. Please maintain a single space between the numeral and (abbreviated) unit, except in cases of %, x, and ° (i.e., the degree sign; excluding temperature), and ensure that standard abbreviations are used. (e.g., 5 mL,15 mL, 40%, 3°, 100 °C, 3 x g, 5 mM, 4–5 mL, 25 µL, 24 h). Italicize g in centrifugation speeds.
   Done

Sincerely

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Rebuttal letter

Isolation of uterine innate lymphoid cells for analysis by flow cytometry

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Point-by-point response

We thank the editors and the reviewers for their comments and suggestions. Please find below (in blue), our point-by-point response.

Editorial comments:

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. Spelling and grammar reviewed.

2. Please revise the text to avoid the use of any personal pronouns (e.g., "we", "you", "our" etc.). Addressed in the whole document.

3. Please define all abbreviations before use (gd). Addressed in the whole documents.

4. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials. For example: Falcon, Heraeus Megafuge 40R, Percoll, BD Perm/Wash, BD Pharm Lyse, Liberase, etc. Addressed in the whole document except for Percoll, Liberase DH and Liberase TM. “Percoll”, “Liberase DH” and “Liberase TM” cannot be replaced with generic terms without significant alteration of the protocol to the point that readers will not be able to reproduce it. For instance, replacing “Percoll” with "separation medium" does not make any sense, as all physical properties of this “separation medium” have to be listed, which is not possible to do as it is not disclosed by the company. Replacing Liberase TM / Liberase DH also does not make sense: replacing them with the phrase “digestion medium containing Collagenase I, II, Thermolysin / Dispase” is not good enough as we do not know the proportion of enzymes in these mixes (it is not disclosed by the company). Therefore, the

concentration of “digestion medium containing Collagenase I, II, Thermolysin / Dispase” cannot be specified.

5. Please ensure that all text in the protocol section is written in the imperative tense as if telling someone how to do the technique (e.g., “Do this,” “Ensure that,” etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as “could be,” “should be,” and “would be” throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a “Note.” However, notes should be concise and used sparingly. Please include all safety procedures and use of hoods, etc. Done

6. For SI units, use standard abbreviations when the unit is preceded by a numeral. Abbreviate liters to L to avoid confusion. Examples: 10 mL, 8 µL, 7 cm2. Addressed in the whole document.

7. Line 134/141: Please replace “bijoux” by “microcentrifuge” tubes or any other generic term. Done.

8. Line 136: Please provide more details to the euthanasia step. Please mention how animals are anesthetized and how proper anesthetization is confirmed prior to cervical dislocation. Done, lines 149

9. Line 144: When times is used as a unit (i.e., for concentrations and magnifications), use lowercase x throughout after the numerical value. Examples: 10x magnification, 2x concentration. Done

10. Line 177-182/ 236-242/276-278: Please ensure that the Protocol section consists of numbered steps. We cannot have non-numbered paragraphs/steps/headings/subheadings. Consider adding these lines under “NOTES” Done.

11. Please include a one-line space between each protocol step and then highlight up to 3 pages 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. Remember that non-highlighted Protocol steps will remain in the manuscript, and therefore will still be available to the reader. Done.

12. Please move the Figure and Table legends to the end of Representative Results. Done.

13. Please do not use the &-sign or the word “and” when listing authors in the references. Please title case and italicize journal titles and book titles. Do not use any abbreviations. References reviewed and edited accordingly.

14. Please obtain explicit copyright permission to reuse any figures from a previous

publication. Explicit permission can be expressed in the form of a letter from the editor or a link to the editorial policy that allows re-prints. Please upload this information as a .doc or .docx file to your Editorial Manager account. The Figure must be cited appropriately in the Figure Legend, i.e. "This figure has been modified from [citation]." We have A permission to reproduce figures from the Nature group is included.

15. Figure 2: Please consider revising the x axis legend to "3 weeks, 5 weeks, etc. Provide the define the term "gd" in the figure legend description. Changing "w" for "weeks" on the x axis impairs clarity of the figure, thus "gd " and "w" were clearly defined in the figure legend.

16. Figure 3: Please remove the commercial terms (Falcon, Percoll, BD Pharm Lyse) from the figure and replace them with generic terms. Addressed except for Percoll; please see the explanation above (point 4).

17. Figure 6: Please replace the commercial term Percoll by generic term. Not replaced, please see the explanation above (point 4).

18. Figure 7/10: Please define the numbers represented in the quadrants in the Figure/Figure description as percentage. Done.

19. Figure 9: Please remove the commercial term "Liberase" from the figure and replace it with generic term. Not replaced, please see the explanation above (point 4).

20. Table 1: Please remove trademark (™) and registered (®) symbols from the Table. Done.

21. Please sort the Table of Materials in alphabetical order. Done.

Reviewers' comments:

Reviewer #1:

Manuscript Summary:

This manuscript described the method to isolate and phenotype mouse group 1 uterine innate lymphoid cells (g1 uILCs) by flow cytometry. The description is clear and the Protocols are useful.

Major Concerns:

Most researchers know the protocols to isolate uterine innate lymphoid cells, however, they may be more interested in how to isolate Myo/ MLAp and decidua lymphoid cells respectively. The authors isolate Myo/ MLAp and decidua lymphoid cells respectively in their previously published article (PMID: 26371244), but the description is not very clear.

So it is better to add the protocols of isolating Myo/MLAp and decidua lymphoid cells respectively in this manuscript.
The protocol is applicable for the isolation of both decidual and Myo/MLAp lymphoid cells. The method to dissect the decidua from the Myo/MLAp has been described before in reference 3 and, in more details, in Croy et al, 2010, PMID: 20033660. We include this paper in the list of references.

Minor Concerns:

line 284: cNK (CD49a-s) should be cNK (CD49a-Eomes+) edited, see line 408.

Table 1 should not be separated in two pages. Table 1 was not designed to be separated in two pages. The table is provided as an excel file as requested by JoVE.

Reviewer #2:
Manuscript Summary:
This manuscript clearly outlines in text and figures a protocol to obtain lymphoid cells from pregnant and non-pregnant mouse uterine tissue. Whilst mainly focussed on assessment of cells by flow cytometry, and centred on g1 uILCs, the protocol is clearly indicated to be readily adaptable to obtain other cell types, and for other assessment purposes such as omics or functional assays.

Major Concerns:
None

Minor Concerns:
Is the protocol 'designed' for isolation of cells from individual uterus, or pooled uteri?
The protocol is designed for isolation of cells from individual uterus. When the yield is low, we pool cells from multiple uteri, however each uterus is treated individually. See lines 106-107 and 582-583.

Reasons why step 12 and 13 are included in the enzymatic digestion protocol section should be included. Done, see lines 190, 192, 205.

A reader may wonder why cells move from 37 degrees for digestion, to 4 degrees, then back to 37 degrees for resuspension, then additional 37 degree incubation in buffer and 10s vortex, then pushed through a filter with plunger. We appreciate the concern of the reviewer. The digestion is done at 37C to allow for the enzymatic reaction. The passage at 4C is intended to stop the enzymatic reaction. We agree with the reviewer that the subsequent step at 37C may not be necessary, however, because we have not tested other temperatures, we advise to use this step at 37C. Readers may want to test them.

In Figure 3, minor protocol steps are suggested to be added throughout, eg '...carefully collect the ring of leukocytes' could be changed to '...carefully collect the ring of leukocytes and spin' to aid clarity. This then aids interpretation of the next group of

steps. Another eg, 'Incubate the bijoux tubes for 30 min at 37 °C with agitation' is suggested to be changed to 'Add digestion mix and incubate for 30 min at 37 °C with agitation'. It is recommended to revisit all text in this figure to add additional words and phrases and remove those that are duplicated. Details were added to legend and the text has been clarified.

In part 6, it should be clarified why no azide, Tris, or protein is recommended in the PBS. See line 324-327.

The figure legend for figure 2 indicates that Liberase TM was used - is this a mistake? If not, then it should be indicated somewhere why this is still appropriate.
Not a mistake, both can be used. Liberase TM was used for that experiment as it leads to higher yield than Liberase DH and did not damage any crucial epitopes used in this specific experiment. We have also added a comment in the protocol stating that both can be used but the choice must be guided by their effects on important epitopes involved in antibody binding (see line 165-167).

It is suggested that the software created X-axis title of the overlay histograms in Figure 8 be replaced for clarity. Done.

Table 1 includes paraformaldehyde, however this is not referred to in any section. Added, see line 350.

In the discussion, it is suggested that the authors may like to include reference to cytological evaluation of vaginal smears as a way of increasing plugging rates. Done, lines 535-536.

Page 8, the authors should elaborate on why beads are not recommended. This would be of benefit to the reader, especially those attempting to assess low numbers of cells in this way for the first time. Done, lines 553-555.

Page 8, perhaps the authors could expand on why flow cytometric assessment within 24h is optimal, ie what happens after this, and if intracellular staining is not to be performed, indicate whether still recommend some sort of fixation, and what this might be. Done, lines 561-562.

Sincerely,
Francesco Colucci (for the Authors)

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