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Methods for the Delivery of Cells, Vaccines, or Infectious Agents into Neonatal Mice in vivo

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Abstract:	Many of the characteristics of the developing immune system are evolutionarily conserved among mammals (1-4). Therefore, studies using animal model systems may have important relevance to the development of the immune system in human neonates and infants. There are reports involving neonatal large animal models, such as neonatal sheep, horses, and pigs (for examples, see (5-7). However, due to the variety of mouse strains and reagents available, the neonatal mouse has most often been the experimental organism of choice for studying the ontogeny of the immune system. To optimally exploit this experimental system, it is ideal to have the technical expertise to manipulate neonates for in vivo studies. Here, we demonstrate several methods of injection of neonatal mice and illustrate how the effects of the treatments can be monitored. The specific reagents administered in this video can be modified to address a variety of research areas, such as vaccine responsiveness, infections with microorganisms, or the maturation status of selected lymphoid lineages. The examples described below are illustrative of the studies that can be performed once the delivery strategies are mastered. First, chimeric mice are created by injecting marked donor cells intravenously, via the facial vein, into 1 day old neonatal mice. Donor cells colonizing the spleen and lymph nodes can be detected by flow cytometry (8-10). Second, systemic vaccination of 7 day old neonatal mice is performed using the intraperitoneal and subcutaneous routes of immunization. Antigen-specific Th1/Th2 responses arising in the draining lymph nodes are visualized by ELISPOT (11, 12). Last, we show a technique for orogastric infection of 7 day old neonates with an invasive enteropathogenic bacterium. The innate immune response is monitored by measuring neutrophilic infiltration of the mesenteric lymph nodes (8, 13).
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METHODS FOR THE DELIVERY OF CELLS, VACCINES, OR INFECTIOUS AGENTS INTO NEONATAL MICE IN VIVO

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SHORT ABSTRACT
This article describes three methods for the introduction of materials into murine neonates. We demonstrate (a) intravenous injection in ≤1 day old neonates, (b) subcutaneous and intraperitoneal injection into 7 day old neonates, and (c) orogastric infusion into 7 day old neonates.

ABSTRACT
Many of the characteristics of the developing immune system are evolutionarily conserved among mammals [1-4]. Therefore, studies using animal model systems may have important relevance to the development of the immune system in human neonates and infants. There are reports involving neonatal large animal models, such as neonatal sheep, horses, and pigs (for examples, see [5-7]). However, due to the variety of mouse strains and reagents available, the neonatal mouse has most often been the experimental organism of choice for studying the ontogeny of the immune system. To optimally exploit this experimental system, it is ideal to have the technical expertise to manipulate neonates for in vivo studies. Here, we demonstrate several methods of injection of neonatal mice and illustrate how the effects of the treatments can be monitored. The specific reagents administered in this video can be modified to address a variety of research areas, such as vaccine responsiveness, infections with microorganisms, or the maturation status of selected lymphoid lineages. The examples described below are illustrative of the studies that can be performed once the delivery strategies are mastered. First, chimeric mice are created by injecting marked donor cells intravenously, via the facial vein, into 1 day old neonatal mice. Donor cells colonizing the spleen and lymph nodes can be detected by flow cytometry [8-10]. Second, systemic vaccination of 7 day old neonatal mice is performed using the intraperitoneal and subcutaneous routes of immunization. Antigen-specific Th1/Th2 responses arising in the draining lymph nodes are visualized by ELISPOT [11, 12]. Last, we show a technique for orogastric infection of 7 day old neonates with an invasive enteropathogenic bacterium. The innate immune response is monitored by measuring neutrophilic infiltration of the mesenteric lymph nodes [8, 13].

INTRODUCTION

During the past several decades, great strides have been made in our understanding of how the immune system develops in both humans and model organisms. One important concept that has emerged is that many of the properties of the neonatal immune system are evolutionarily conserved across many species. The mouse, in particular, provides an outstanding experimental model system because of the many experimental tools and genetically manipulated animals that are available. One additional advantage of the mouse model system is the capacity to conduct experiments *in vivo*. Of course, this requires techniques for manipulating live, small animals. However, it is often difficult to communicate the specifics of animal manipulation through a written protocol alone. Therefore, we have prepared this video to demonstrate several procedures for the introduction of reagents or cells into neonatal mice. First, for colonization of neonates by adoptively transferred cells, we will show how to inject 1 day

old neonates intravenously via the facial vein. Second, for immunization studies, we will demonstrate how to inject 7 day old pups by the intraperitoneal and subcutaneous routes. Lastly, for studies on gastroenteric infections, we will demonstrate how to deliver microorganisms orogastrically. These techniques could be useful for studying a variety of immunological responses, ranging from responses to vaccines or infectious agents to dissecting the cellular and molecular mechanisms underlying immune development.

PROTOCOL

All animal usage procedures were in accordance with ethical guidelines laid down by the local IACUC board and university protocol guidelines.

1. Administration of cells intravenously in 1 day old neonatal mice

1.1. Prepare cells of choice in sterile HBSS or PBS. A variety of cell types have been injected using this method, including total spleen cells [10], CD4+ cells [8], regulatory T cells [9], NK cells [14], etc.

1.2. Remove ≤ 1 day old neonatal pups from dams and keep warm under a lamp.

1.3. The neonatal facial vein is located near the outer edge of the eye and extends toward the neck. Firmly hold the back and stomach of the pup between the thumb and index finger to straighten the neck.

1.4. Hold the pup up close to the lamp to optimally visualize the vein.

1.5. Sterilize the area of the facial vein using a sterile cotton tipped applicator soaked with 95% ethanol.

1.6. Angle the needle down slightly (from parallel to the vein) to accommodate the depth of the vein underneath the skin and insert needle.

1.7. Slowly inject the cells (in ≤ 50µl volume) into the facial vein using a 30 gauge hypodermic needle attached to a 1ml syringe. As the cells are introduced, the vein will become clear and then will return to its normal color.

1.8. Slowly remove the needle and immediately apply pressure with a sterile cotton tipped application. It is common for some bleeding to occur. Bruising is normal and will fade within a day.

1.9. Return all pups together back to the dam; monitor to ensure that dam is feeding pups.

2. Systemic immunization of 7 day old neonatal mice

Note: The injection sites will consist of two subcutaneous sites, at the back of the neck and at the base of the tail, and the peritoneal cavity. The maximum volume that can be accommodated per site is 25µl. In these studies, the protein-hapten conjugate dinitrophenyl keyhole limpet hemacyanin was used. However, a variety of other antigens have also been used to immunize murine neonates.

For subcutaneous injections, it is not recommended to disinfect the injection area as there is the risk of introducing residual ethanol.

2.1. Restrain the pup by firmly holding it face down between the thumb and index finger, allowing its stomach to rest on the middle finger. Insert the needle ~ ¼ inch under the skin at the back of the neck and slowly inject 25µl. Remove the needle slowly and repeat this procedure at the base of the tail.

Note: If the injection is successful, there will be no liquid leakage from the injection site. Also note that the skin at the site of the subcutaneous injections will bubble up slightly but this will disappear within a day.

2.2. For intraperitoneal injections, turn the pup over and firmly hold it face up with its head between the index and middle fingers and its lower body between the thumb and ring fingers. This allows the researcher to stretch out its abdomen and keep its legs from interfering with the injection.

2.3. Angling the needle downward just slightly, insert the needle into the peritoneum with the bevel of the needle facing upward. Once the needle is inserted, shift the angle downward slightly and slowly inject 25µl of the antigen. Withdraw the needle slowly.

2.4. Return pups to the dam and ensure that the dam begins to feed the pups again.

3. Orogastric administration of bacteria into 7 day old neonatal mice

3.1. Prepare bacteria of choice in sterile HBSS or PBS and keep on ice. If desired, include 0.1% blue food coloring to visualize delivery of the bacteria to the stomach.

Note: In our studies, we have used *Yersinia enterocolitica* [8, 13]. Neonatal mice are highly resistant to this bacterium, surviving doses exceeding 1 x 10^6 CFU following orogastric infection. However, neonates are generally much more sensitive to other bacteria, including *Citrobacter rodentium* [15], *Shigella flexneri* [16], *Helicobacter pylori* [17], and *Salmonella typhimurium* [18, 19]. Therefore, it may be necessary to decrease the infectious doses with many other types of bacteria.

3.2. Sterilize the tubing to be used to administer the bacteria. Cut the polyethylene tubing into 7 inch-long pieces. Insert a sterile 30 gauge disposable needle into one end,

being careful not to puncture the tubing. Attach the needle to a sterile 1ml syringe containing 500µl of 75% ethanol.

3.3. Pass the ethanol through the tubing and allow to air dry for a few minutes. Remove the needle and insert a new sterile 30 gauge needle attached to a sterile 1ml syringe containing ≥500µl of sterile HBSS. Pass this through the tubing to wash out residual ethanol. Repeat this HBSS wash with a new sterile syringe and needle.

3.4. Sterilize a 50µl gastight Hamilton syringe by passing through 75% ethanol. Wash the syringe multiple times with sterile HBSS to eliminate any remaining ethanol.

3.5. Insert a new 30 gauge needle onto the sterilized tubing. The tubing and void volume of the needle need to be filled with the bacterial solution. Draw up 500µl of the prepared bacteria into a sterile 1 ml syringe (no needle attached). Attach this syringe to the needle on the tubing.

3.6. Place the open end of the tubing in a sterile 1.5 ml microcentrifuge tube. Slowly fill the needle and tubing with the bacterial suspension. Remove the 1ml syringe.

3.7. Draw up 50µl of bacteria into the 50µl Hamilton syringe and attach this to the needle and tubing. Reduce the volume of the solution to 25µl for infusion.

3.8. Place pups in an airtight container with a saturated atmosphere of isoflurane (NDC # 14043-220-050) (be careful to avoid direct contact of mouse skin with the isoflurane as it is a skin irritant). Monitor closely and as soon as the pup becomes limp (≤ 0.5 minutes); the pup is considered to be fully anesthetized if it is non-responsive to pinching the paw with a pair of forceps. Remove and intubate immediately.

3.9. Hold the pup at the scruff of the neck and gently pull back to force the head up, creating a long straight neck.

3.10. Slowly introduce the tubing into the mouth of the pup, angling towards either side of the mouth rather than directly into the center of the throat. Apply gentle pressure until the tubing slides easily down the esophagus. Approximately 1 -1.5cm of tubing will be inserted.

3.11. After infusion of 25µl of the bacterial suspension, place the pup in an upright position and slowly remove the tubing from the esophagus. Keep the pup upright for a few seconds to ensure the solution remains in the stomach.

3.12. Place pup in a new clean cage and proceed to the next pup. Continue until all animals are manipulated and then transfer the dam to the litter.

Representative results

Following i.v. injection of marked adult CD4+ cells into 1 day old neonates, donor cells colonized the lymph nodes (Fig. 1) [8]. Seven days post injection, donor cells were present at similar frequencies in the peripheral lymph nodes (PLN) and mesenteric lymph nodes (MLN). Moreover, their overall representation in the host neonatal animals was proportional to the number of cells injected.

Systemic immunization of 7 day old pups elicited antigen specific primary T helper responses in the draining lymph nodes. The frequencies of antigen-specific IL-4- or IFNγ-secreting lymph node cells were measured by ELISPOT 1 week post immunization (Fig. 2) [11, 12]. Interestingly, the frequencies of IFNγ-secreting cells were similar in neonates and adults while the frequency of IL-4-producing cells was at least 5 fold greater in neonates than in adults. Thus, immunization of neonates under these circumstances led to Th2-skewed responses in neonates.

Oral infection of 7 day old pups resulted in the accumulation of neutrophil-phenotype cells in the MLN. Three days after infection with the enteropathogen *Yersinia enterocolitica*, MLN suspensions were stained with anti-Ly6G and anti-CD11b antibodies and analyzed by flow cytometry (Fig. 3). The neutrophil quality of the detected cells was confirmed by Wright-Giemsa staining of sorted Ly6G+CD11b+ cells [13].

Figure 1. Lymph node chimerism following intravenous injection of CD4+ cells into 1 day old neonatal mice. Total lymph node cells were prepared from GFP transgenic C57BL/6 adult mice and CD4+ cells were isolated [20]. The cells were injected in the indicated numbers into the facial veins of ≤ 1 day old C57BL/6 pups. One week later, cell suspensions were prepared separately from mesenteric lymph nodes (MLN) and peripheral lymph nodes (PLN) and analyzed for GFP+ cells by flow cytometry.

Figure 2. Assessment of the frequencies of antigen-specific Th1/Th2 cells in immunized neonates. 7 day old neonatal or adult BALB/c mice were immunized subcutaneously and intraperitoneally with a total of 25µg or 100µg, respectively, of keyhole limpet hemacyanin (KLH) in PBS. One week later, CD4+ lymph node cells were prepared, re-stimulated in culture with KLH in the presence of splenic antigen-presenting cells, and the frequencies of IL-4 or IFNγ secreting cells were measured in ELISPOT assays, as described [11, 12].

Figure 3. Detection of neutrophilic infiltration into the mesenteric lymph nodes after orogastric infection of neonates. Seven day old C57BL/6 pups were infected orogastrically with 2 x 10^7 CFU of *Yersinia enterocolitica* [8, 13]. Three days later, mesenteric lymph node cells were stained with anti-Ly6G and anti-CD11b antibodies. (A) Representative flow cytometry profiles showing the neutrophil populations in uninfected and infected littermates. (B) Summary of results from a cohort of uninfected and infected pups.

DISCUSSION

As in human development [1], the maturation of many immune processes in the mouse occurs over a prolonged period following birth (for examples, see [21-23]). The challenge for future studies is to identify the mechanisms underlying this developmental regulation. The rate limiting step in ontogeny studies is often the availability of the skills to manipulate the youngest animals in the series. Thus, the goal of this manuscript is to provide visual instruction to help in the establishment of these skills in many laboratories.

The described protocols will allow investigation of both systemic and intestinal immune responses in murine neonates. First, donor CD4+ cells stably populate lymphoid tissues ≥ 3 weeks following intravenous injection into 1 day old neonates [8]. Thus, the effects of CD4+ cells with other properties (e.g., genetically manipulated) or of different developmental ages on the neonatal immune response could be investigated. Second, the capacity to successfully immunize neonatal mice is important for studies that may lead to improved vaccine formulations for human pediatric use. Last, human neonates and infants are particularly vulnerable to pathological bacterial infections at the intestinal mucosa. The technique of orogastric infection in this neonatal mouse model system can be used to develop a better understanding of regional immunity against intestinal pathogens in early life. Clearly, the cell types, antigens, and infectious agents introduced can be varied from those described here. Therefore, this system can be used to investigate neonatal immunity elicited under a variety of conditions in vivo.

There are several technical issues which may affect in vivo experimentation with mouse pups. First, the total volume which can be delivered by any route is small. Depending on the setting, there may be upper limits to the amount of material that can be delivered. For example, when injecting donor cells i.v. into 1 day old neonates, it is advisable to use no more than 50µl. In order to maintain a homogeneous suspension of donor cells, this volume should contain ≤ 1×10^7 normal mouse lymphocytes. This will minimize the possibility of cell clumping which can cause an embolism and the death of the pup. For injection into the peritoneal cavities of 7 day old pups, injection volumes exceeding 25µl may result in leakages of unknown amounts of material. In addition, for vaccines or infectious agents, the total amount infused may be constrained by the maximum concentrations that can be achieved in the working stock solutions. Second, when manipulating suckling mice, the dam must be considered. For example, if the dams are stressed, they may not adequately nurse or shelter the pups. Worse, they may cannibalize the pups, a situation which occurs more frequently in C57BL/6 than in BALB/c mice. We have found that there are fewer losses of pups in experiments when (a) the handlers wear gloves at all times, (b) all the pups are removed from and returned to the dam as one group, at the same time, and (c) the time away from the dam is minimized. To further limit cannibalism, it may be helpful to use foster dams from a relatively docile strain of mouse, such as FvB mice.

DISCLOSURES

All experimental procedures were approved by the University of Miami Animal Care and Use Committee.

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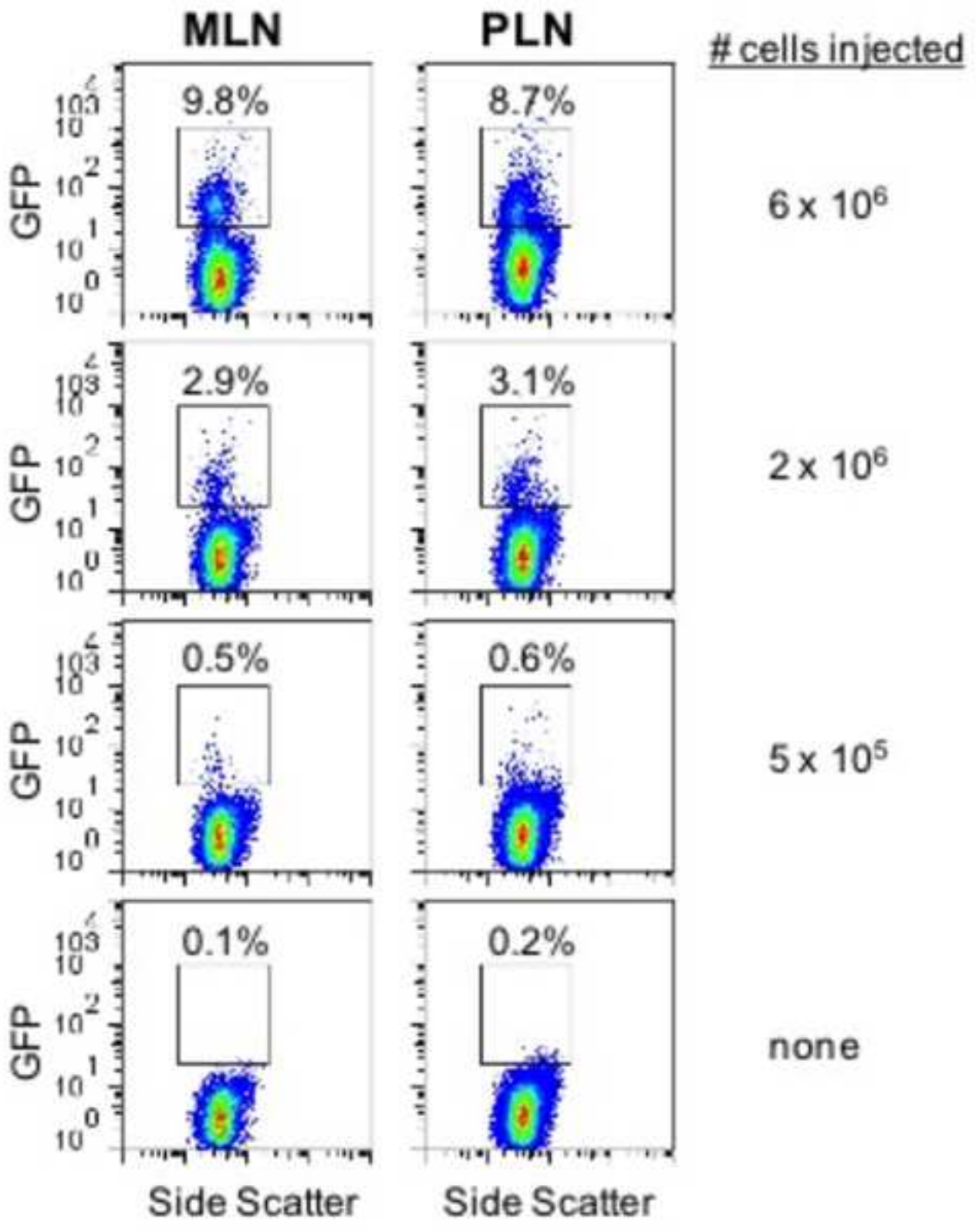

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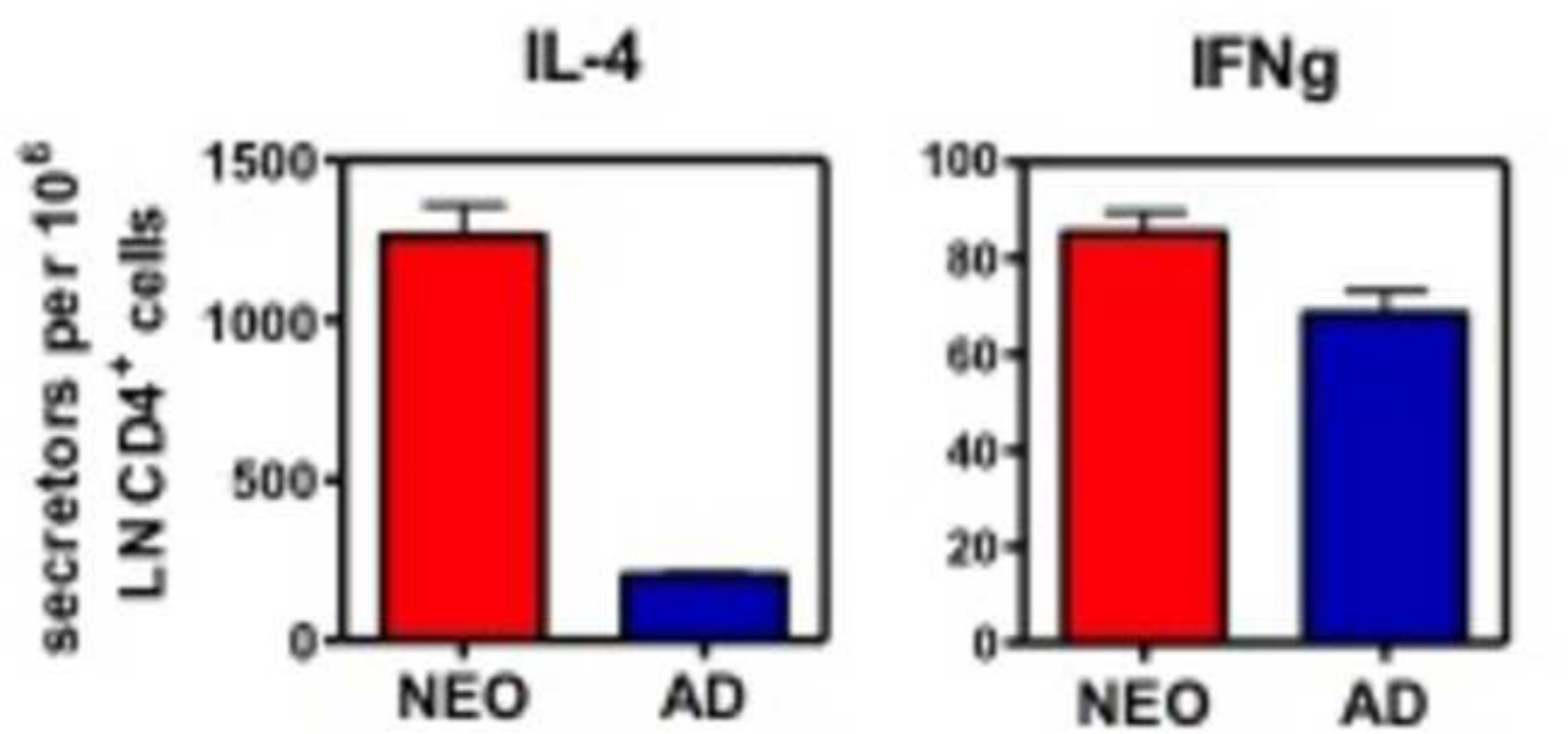

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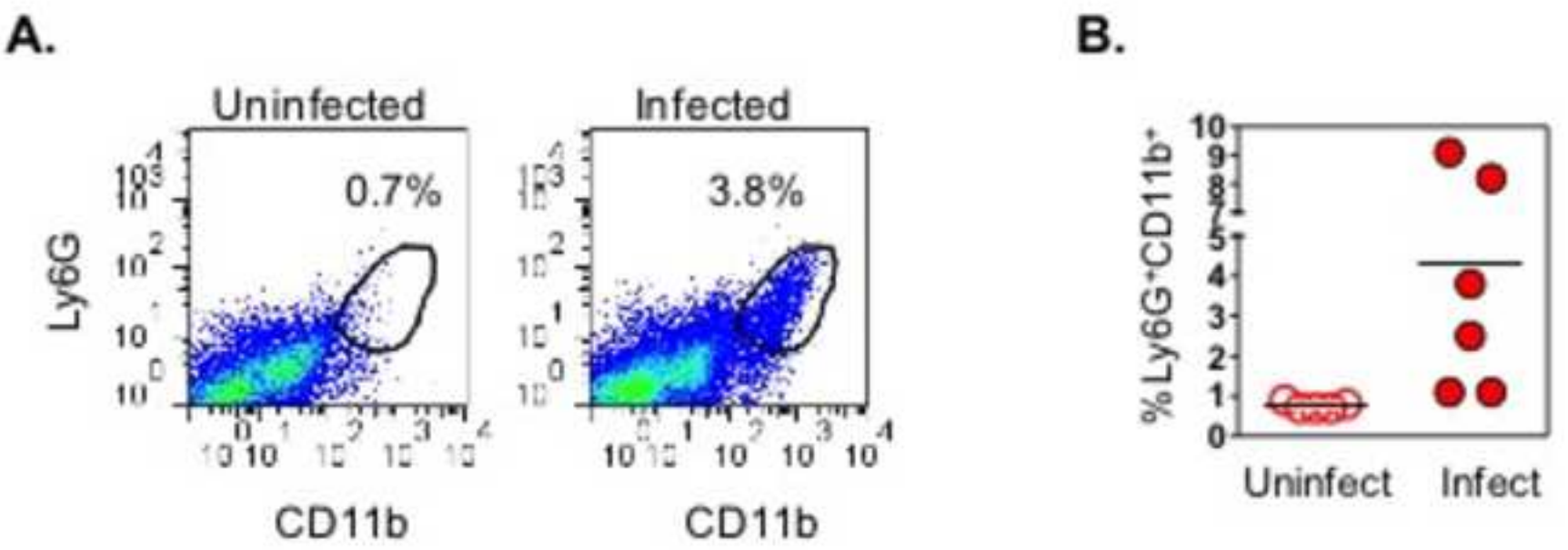

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Reagent	Company	Catalogue #	Comments
50 µl glass Luer tip gastight syringe	Hamilton	1705 LT	
INTRAMEDIC Polyethylene tubing	Clay Adams	427400	0.011 X 0.024” PE10
1cc tuberculin slip tip syringe	Becton Dickinson	309659	
30 gauge hypodermic needle	Becton Dickinson	305106	½ inch length

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Reviewer #1:

Minor Concerns:
1. The order of procedures in the protocol should follow the order discussed in the text and on the video.

All portions of the text have been re-ordered to match the order in the video. In particular, the introduction has been revised so that the order is the same as in the video and throughout the manuscript.

2. The authors may want to suggest filtering of cells through 40-70 µm mesh prior to injecting into the facial vein to make sure particulates are removed. Injecting 108 splenocytes in a 50µl volume seems like quite a high concentration and may lead to clumping which the authors indicate results in blockage of blood vessels and death. Have you actually injected this many cells in the past?

We agree that injecting 10^8 splenocytes in 50µl would be quite high (probably unacceptably high). ~~In the experiments shown in Figure 1, the highest number of cells we injected was 6 x 10^6 in 50µl, or 1.2 x 10^8 per ml.~~ ~~This concentration typically yields a homogeneous suspension with little to no cell clumping.~~ As a general rule, we do not exceed a concentration of 2 x 10^8 cells/ml for injection into neonatal mice. This concentration typically yields a homogeneous suspension with little to no cell clumping. ~~As the reviewer points out, higher concentrations could lead to problematic clumping.~~ Therefore, w~~W~~e do not routinely filter the cells because this results in non-specific losses of unknown numbers of cells. The text in the discussion has been corrected to indicate this important point.

3. In section 2.3 on systemic immunization it reads as if only 25µl can be injected in the peritoneum of 7 day old mice. This seems rather small even for a 7 day old mouse.

Volumes larger than 25µl can be injected and, indeed, there are reports in the literature of i.p. injection of 50µl, even into 1 day old pups. However, there is little "open space" in the peritoneal cavities of murine pups. We have found that injection volumes exceeding 25µl result in leakages of unknown amounts at unacceptably high frequencies (≥20% of injections). Therefore, to ensure 100% delivery, we use the conservative volume of 25µl. This has been explained in the discussion in the revised manuscript.

4. In section 2.1 it indicates that the lack of leakage is an indicator of a successful subcutaneous injection. Is there a trick to preventing leaking?

The only "tricks" are stated in the written procedure – i.e., that the injection and needle removal should be performed slowly.

5. In section 3.5 it is stated that no needle is attached when bacteria are drawn into a 1 ml syringe prior to injecting through tubing. Since this could result in bacteria on the hub and exposed outside the syringe, it might be better to use a large gauge needle to draw up the bacteria and then switch to the needle with the attached tubing. This will reduce the likelihood of contacting the bacteria and potential exposure of personnel. This is only a suggestion for working with infectious agents.

This is a good suggestion but adding it into the protocol at this point would require us to re-film the video footage. Although our current procedure hasn't caused any obvious problems, we will consider incorporating this practice into our protocols in the future.