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TITLE:

Retroviral Overexpression of CXCR4 on Murine B-1a Cells and Adoptive Transfer for Targeted B-1a Cell Migration to the Bone Marrow and IgM Production

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SUMMARY:

Here we describe a method for retroviral overexpression and adoptive transfer of murine B-1a cells to examine in vivo B-1a cell migration and localization. This protocol can be extended for diverse downstream functional assays including quantification of donor B-1a cell localization or analysis of donor cell-derived secreted factors post-adoptive transfer.

ABSTRACT:

As cell function is influenced by niche-specific factors in the cellular microenvironment, methods to dissect cell localization and migration can provide further insight on cell function. B-1a cells are a unique B cell subset in mice that produce protective natural IgM antibodies against oxidation-specific epitopes that arise during health and disease. B-1a cell IgM production differs depending on B-1a cell location, and therefore it becomes useful from a therapeutic standpoint to target B-1a localization to niches supportive of high antibody production. Here we describe a method to target B-1a cell migration to the bone marrow by retroviral-mediated overexpression of the C-X-C motif chemokine receptor 4 (CXCR4). Gene induction in primary murine B cells can be challenging and typically yields low transfection efficiencies of 10–20% depending on technique. Here we demonstrate that retroviral transduction of primary murine B-1a cells results in 30–40% transduction efficiency. This method utilizes adoptive cell transfer of transduced B-1a cells into B cell-deficient recipient mice so that donor B-1a cell migration and localization can be

visualized. This protocol can be modified for other retroviral constructs and can be used in diverse functional assays post-adoptive transfer, including analysis of donor cell or host cell phenotype and function, or analysis of soluble factors secreted post B-1a cell transfer. The use of distinct donor and recipient mice differentiated by CD45.1 and CD45.2 allotype and the presence of a GFP reporter within the retroviral plasmid could also enable detection of donor cells in other, immune-sufficient mouse models containing endogenous B cell populations.

INTRODUCTION:

Recent studies have demonstrated considerable immune cell, and specifically B cell, phenotypic and functional heterogeneity depending on cell localization[1-5]. B-1a cells are one such population with heterogeneous capacity to produce protective IgM antibodies; bone marrow B-1a cells secrete IgM constitutively and contribute significantly to plasma IgM titers[6], while peritoneal B-1a cells have low-level IgM secretion at homeostasis and instead can be activated through innate toll-like receptor (TLR) or cytokine-mediated signaling to rapidly proliferate, migrate, and secrete IgM[7-10]. B-1a cell IgM antibodies recognize oxidation-specific epitopes (OSE) that are present on pathogens, apoptotic cells, and oxidized LDL, and IgM binding to OSE can prevent inflammatory downstream signaling in diseases like atherosclerosis[11]. Therefore, strategies to increase IgM production via increasing peritoneal B-1a cell migration to sites like the bone marrow may be therapeutically useful. However, it is important for such strategies to be targeted and cell-type specific, as off-target effects may negatively impact immune function or health.

Here we describe a method for targeted and long-term overexpression of CXCR4 in primary murine B-1a cells and subsequent adoptive transfer to visualize cell migration and functional IgM antibody production (**Figure 1**). Genetic manipulation of primary B cells is limited by low transfection efficiencies compared to transfection of transformed cell lines. However, as transformed cell lines can significantly deviate from primary cells[12,13], the use of primary cells is likely to provide results that more closely align to normal physiology. Several techniques have been described for gene transfer in primary murine B cells, including retroviral transduction, adenoviral transduction, lipofection, or electroporation-based transfection, which have varying levels of efficiency, transience, and impact on cell health[13-15]. The following method utilized retroviral transduction as it yielded adequate gene transfer efficiency of >30% while minimally impacting cell viability. The CXCR4-expressing retrovirus was generated using the previously described retroviral construct murine stem cell virus-internal ribosomal entry site-green fluorescent protein (MSCV-IRES-GFP; MigR1)[16], into which the mouse CXCR4 gene was sub-cloned[4]. MigR1 (control(Ctl)-GFP) and CXCR4-GFP retroviral particles were generated using calcium phosphate transfection as described in previously published protocols[4,14].

Successfully transduced B-1a cells were then intravenously transferred into lymphocyte-deficient $Rag1^{-/-}$ mice. Both donor and recipient mice additionally contained knockout of the apolipoprotein E (ApoE) gene, which results in increased OSE accumulation and atherosclerosis, thereby providing a model for in vivo B-1 cell activation and IgM production. Moreover, donor and recipient mice differed in CD45 allotype; donor B-1 cells came from CD45.1+ $ApoE^{-/-}$ mice and were transferred into $Rag1^{-/-}$ CD45.2+ $ApoE^{-/-}$ recipients. This allowed differentiation of donor CD45.1 from recipient CD45.2 B cells post-transfer without the need to additionally stain for B

cell markers during flow cytometry analysis. The results provided here demonstrate that targeted CXCR4 overexpression on B-1a cells associates with increased ability of B-1a cells to migrate to the bone marrow, which associates with increased plasma anti-OSE IgM. We additionally provide a method for the enrichment of peritoneal B-1 cells through negative selection and demonstrate the requirement of B-1 cell activation for efficient transduction. This method can be adapted for other retroviral constructs to study the effect of protein overexpression on B-1a cell migration, phenotype, or function. Moreover, the use of CD45.1 versus CD45.2 allotype distinction could theoretically allow transfer into other immune-sufficient murine models containing endogenous B cells.

PROTOCOL:

All animal protocols were approved by the Animal Care and Use Committee at the University of Virginia.

1. Magnetic separation and enrichment of peritoneal B-1 cells

1.1. Euthanize a 12−14-week-old, male, CD45.1^{+}ApoE$^{-/-}$ mouse using CO_2.

1.2. Make a superficial cut in the abdomen using straight surgical scissors and peel back skin using curved scissors to expose the peritoneal wall. Flush peritoneal cavity with 10 mL of 37 °C RPMI-1640 medium using a 10 mL syringe and 25 G needle. Shake mouse to disengage cells by grasping the tail and moving the mouse side to side thoroughly for 15−20 s.

NOTE: Massaging the lavaged peritoneum can also maximize cell recovery.

1.3. Collect peritoneal fluid using a 10 mL syringe and 25 G needle by drawing up fluid at the lower right side of the peritoneum just above the level of the hip, near the intestines. Avoid disrupting epididymal fat depots and underlying organs. Avoid drawing fluid from the mouse's left side as the omental fat can easily be drawn into the syringe.

1.4. Once ~6−7 mL of fluid is collected, dispense into a 50 mL conical tube placed on ice. Next, elevate the mouse vertically by holding the peritoneal wall above the diaphragm using forceps so that any remaining fluid remains at the bottom of the peritoneal cavity. Make a small cut in the peritoneal wall using surgical scissors above the liver (make sure not to cut the liver) and collect any remaining peritoneal fluid using a glass pipet and bulb.

1.5. Pool peritoneal washout cells from all CD45.1^{+}ApoE$^{-/-}$ mice (n = 15−20) into 50 mL conical tubes, and store on ice.

NOTE: For calculating the number of mice needed: B-1a cells comprise 5−10% of the total peritoneal population and yield roughly 2.5−5 x 10^5 B-1a cells per mouse in authors' hands. Transduction efficiency is ~30−40%, as shown below.

1.6. Count live cells using a viability dye such as trypan blue and a hemocytometer (dilute samples 1:5 in trypan blue and load 10 µL into the hemocytometer chamber). Pool cells at up to 1 x 10^8 cells per tube. Centrifuge cells at 400 x *g* for 5 min at 4 °C, then aspirate supernatant.

1.7. Resuspend up to 1 x 10^8 cells in 1 mL of anti-CD16/CD32 antibody (**Table of Materials**) diluted 1:50 in assay buffer (1x phosphate buffered saline [PBS], 0.5% bovine serum albumin [BSA], 2 mM EDTA) in order to block Fc receptors. Scale as necessary based on cell count. Incubate on ice for 10 min at 4 °C.

1.8. Prepare a 2x master mix of the biotinylated antibodies (**Table 1**) in assay buffer for depletion. The 2x master mix accounts for the volume of the liquid the cells are already in when incubating with anti-CD16/CD32. For example, if cells are incubating in 500 µL of diluted anti-CD16/CD32, then add 500 µL of 2x master mix containing 10 µL of biotinylated Ter119, Gr-1, CD23, and NK1.1 antibodies, and 25 µL of biotinylated F4/80 antibody to achieve the final concentrations given in **Table 1**. Add 2x master mix to cells and stain for 20 min at 4 °C.

1.9. Wash cells with 5 mL of assay buffer and centrifuge at 400 x *g* for 5 min at 4 °C and aspirate supernatant.

1.10. Resuspend and incubate cells with anti-biotin microbeads (**Table of Materials**) diluted in assay buffer as per the concentration and protocol recommended by the manufacturer.

1.11. Wash with 5 mL of assay buffer and centrifuge at 400 x *g* for 5 min at 4 °C. Aspirate supernatant and resuspend up to 1 x 10^8 cells in 500 µL of assay buffer.

1.12. Prime magnetic selection columns (**Table of Materials**) with 3 mL of assay buffer. Transfer cells onto primed magnetic selection columns and collect the eluent containing enriched B-1 cells in a 15 mL conical tube on ice. Wash the magnetic selection column with additional assay buffer till the overall volume collected is 10 mL.

NOTE: An aliquot of the pre-purified and post-purified cell fractions should be separately analyzed by flow cytometry for purification efficiency by staining for CD19+ B cells, F480+ macrophages, and CD5+ T cells as shown in **Figure 2**.

1.13. Count the post-purified cell fraction (the eluent containing enriched B-1 cells) by counting live cells as in step 1.6, and resuspend at 1 x 10^6 cells/mL in B cell culture medium (RPMI-1640, 10% heat-inactivated fetal bovine serum [FBS], 10 mM HEPES, 1x non-essential amino acids, 1 mM sodium pyruvate, 50 µg/mL gentamicin, 55 µM β-mercaptoethanol).

2. Peritoneal B-1 cell stimulation

2.1. Split pooled cells for the two transduced conditions (Ctl-GFP and CXCR4-GFP), while setting aside and plating at least 10 x 10^6 cells for a non-transduced control.

2.2. Plate up to 150 µL (150,000 cells) per well into 96-well round-bottom plates.

2.3. Add 100 nM TLR9 agonist ODN1668 to all wells to stimulate cell proliferation.

2.4. Incubate for 16−18 h at 37 °C, 5% CO_2.

3. Retroviral transduction of peritoneal B cells

3.1. Generate Ctl-GFP (MigR1) and CXCR4-GFP retroviral particles using calcium phosphate transfection as described in previously published protocols[14].

NOTE: This will take several days, so prepare and titer retroviral stocks and store aliquots at -80 °C prior to starting this protocol.

3.2. Thaw retrovirus stocks on ice. Use immediately and do not re-freeze as virus titer significantly diminishes during freeze-thaw cycles. Add Ctl-GFP or CXCR4-GFP retroviral supernatants at 20:1 multiplicity of infection (MOI) to cells, in the presence of 8 µg/mL polybrene and fresh β-mercaptoethanol at 55 µM final concentration. Do not add viral stocks to the cells set aside for the non-transduced control.

3.3. Perform spinfection by centrifuging plates at 800 x *g* for 90 min at room temperature.

3.4. Incubate plates with retrovirus at 37 °C, 5% CO_2 for an additional 3 h.

3.5. Harvest and replate cells in fresh B cell medium and incubate at 37 °C, 5% CO_2 overnight.

4. Cell sorting of transduced peritoneal B-1a cells

4.1. Harvest cultured cells and pool into three separate 50 mL conical tubes for each condition: non-transduced, Ctl-GFP transduced, and CXCR4-GFP transduced.

4.2. Count live cells as in step 1.6, then centrifuge at 400 x *g* for 5 min at 4 °C and aspirate supernatant.

4.3. Resuspend cells at 100,000 cells/µL in sort buffer (PBS + 1% BSA) containing 1:50 anti-CD16/CD32 antibody (**Table of Materials**) in order to block Fc receptors. Incubate on ice for 10 min at 4 °C.

4.4. Aliquot cells for compensation controls (~30,000 cells per compensation control): for unstained and single stain controls aliquot from non-transduced sample and for GFP single stain control aliquot from transduced samples.

NOTE: Commercially available compensation beads can alternatively be used for single stain controls if non-transduced cell number is low.

4.5. Prepare a 2x antibody master mix containing the fluorophore-conjugated antibodies in sort buffer (**Table 1**). Add 2x master mix to non-transduced, CTL-GFP transduced, and CXCR4-GFP transduced samples. Add individual antibodies to single stain controls. Incubate for 20 min at 4 °C in the dark.

NOTE: The 2x master mix accounts for the volume of liquid the cells are already in when incubating with anti-CD16/CD32, as in step 1.8. An aliquot of cells from each condition can separately be stained for CXCR4 to confirm CXCR4 overexpression.

4.6. Wash samples with 1 mL of sort buffer and strain through 70 µm filters into polypropylene tubes.

4.7. Centrifuge at 400 x *g* for 5 min at 4 °C and aspirate supernatant. Resuspend samples at 50,000 cells/µL in sort buffer.

4.8. Prior to cell sorting prepare labeled fluorescence-activated cell sorting (FACS) collection tubes containing 1 mL of collection medium (RPMI-1640, 20% heat-inactivated FBS, 10 mM HEPES, 1x non-essential amino acids, 1 mM sodium pyruvate, 50 µg/mL gentamicin, 55 µM β-mercaptoethanol) for each population to be sorted.

4.9. Prior to running samples on the cell sorter add 2x DAPI (prepared as 1:5000 dilution in sort buffer) for dead cell discrimination.

4.10. Sort GFP+ B-1a cells into FACS tubes containing collection medium as $DAPI^{-}$ $CD19^{+}$ GFP^{+} $B220^{mid\text{-}lo}$ $CD23^{-}$ IgM^{+} $CD5^{+}$ cells from the CTL-GFP and CXCR4-GFP samples. Use the non-transduced sample to set the GFP+ gate and to sort $DAPI^{-}$ $CD19^{+}$ GFP^{-} $B220^{mid\text{-}lo}$ $CD23^{-}$ IgM^{+} $CD5^{+}$ non-transduced B-1a cells.

NOTE: Alternatively, non-transduced cells can also be sorted from the CTL-GFP and CXCR4-GFP samples by separately gating B-1a cells within the GFP- fraction. Transduced or non-transduced B-1b cells can also be sorted using this sort strategy as $DAPI^{-}$ $CD19^{+}$ $GFP^{+/-}$ $B220^{mid\text{-}lo}$ $CD23^{-}$ IgM^{+} $CD5^{-}$ cells.

5. Adoptive transfer

5.1. After cell sorting, centrifuge cells at 400 x *g* for 5 min at 4 °C and aspirate supernatant carefully.

5.2. Resuspend cells in cold sterile 1x PBS at 1,000 cells/µL.

5.3. Anesthetize male Rag1$^{-/-}$ ApoE$^{-/-}$ mice using isoflurane and inject 100 µL (100,000 cells) per mouse via intravenous retro-orbital or tail vein injection using an ultra-fine insulin syringe. Inject a few mice with 1x PBS as a control.

6. Quantification of donor cells and plasma IgM

6.1. At the desired time post-adoptive transfer, analyze transferred cells in recipient mice by bone marrow and spleen tissue harvest[4] and flow cytometry. Quantify donor cell localization and CXCR4 overexpression by staining for CD45.1, CD45.2, and CXCR4 and analyze flow cytometry results using a software such as Flowjo.

NOTE: See **Table 1** for antibodies used in this step. Ensure not to use an antibody conjugate that fluoresces in the FITC channel as GFP will be present on transduced donor cells.

6.2. Isolate plasma by adding 10 µL of 0.5 M EDTA to whole blood collected via cardiac puncture at the time of animal sacrifice. Centrifuge whole blood at 7,000 x *g* and aliquot plasma into separate 1.5 mL centrifuge tubes. Store at -80 °C until performing analysis of secreted factors such as IgM by ELISA[4].

REPRESENTATIVE RESULTS:

An overview of the protocol is given in **Figure 1**. **Figure 2** displays enrichment of peritoneal B-1a cells after magnetic depletion of other peritoneal cell types. Live singlet cells in the post-depletion fraction have a greater proportion of CD19+ B cells compared to F4/80^{+} macrophages, lack CD5^{hi} CD19^{-} T cells, and contain an increased frequency of CD19^{+} CD5^{mid} B-1a cells compared to the pre-depletion fraction. **Figure 3** displays the requirement of B cell activation for successful retroviral B cell transduction, and a dose-dependent increase in the frequency of successfully transduced GFP+ B cell subsets with increasing virus MOI using Ctl-GFP retrovirus. **Table 2** displays increased transduction efficiency using 96 well round-bottom plates compared to 24-well or 6-well plates. **Figure 4** displays successful CXCR4 overexpression (>40%) on B-1 cells and increased B-1 cell migration towards CXCL12 in vitro after transduction with CXCR4-GFP retrovirus, without a significant impact on B cell viability. **Figure 5** displays the gating strategy for sorting of live, singlet, CD19+ CD23- IgM+ CD5+ B-1a cells from either a non-transduced condition (GFP-), or the two transduced conditions (GFP+). Note that CD23+ B-2 cells are not present in these samples due to prior magnetic depletion. Transduced live, singlet, CD19+ CD23- IgM+ CD5- B-1b cells can also be sorted using this gating strategy. **Figure 6** displays transferred CD45.1+ donor cells and sustained CXCR4 overexpression on donor cells recovered from bone marrow and spleen of CD45.2 recipient mice 17 weeks post-cell transfer. **Table 3** displays a positive association between CXCR4 expression and donor cell localization to the bone marrow, but not spleen. **Table 4** displays a positive association between donor cell number in the bone marrow and plasma amount of anti-MDA-LDL IgM.

FIGURE AND TABLE LEGENDS:

Figure 1: Schematic of experimental design for retroviral transduction and adoptive transfer. Peritoneal cells isolated from CD45.1 allotype mice are enriched for B-1 cells through magnetic

depletion using biotinylated antibodies and anti-biotin microbeads. Enriched peritoneal B-1 cells are activated to stimulate cell proliferation with TLR9 agonist CpG oligodeoxynucleotide. Activated cells are transduced with Ctl-GFP or CXCR4-GFP retroviral particles. Successfully transduced GFP+ B-1a cells are sorted using FACS and adoptively transferred into CD45.2 allotype host mice.

Figure 2: Enrichment for peritoneal B-1 cells. Representative flow cytometry plots of peritoneal cells pre-magnetic enrichment (top) and post-magnetic enrichment (bottom) for CD19+ B cells. CD19+ F4/80- cells are B cells, CD19- F4/80+ cells are macrophages, CD19+ $CD5^{mid}$ cells are B-1a cells, and CD19- $CD5^{hi}$ cells are T cells.

Figure 3: Retroviral transduction requires B cell activation. Peritoneal B cells were transduced with Ctl-GFP retrovirus at 5:1, 10:1, or 25:1 MOI or left non-transduced in the presence or absence of TLR9 agonist CpG ODN1668. The frequency of successfully transduced GFP+ B2, B1, B-1a, or B-1b cells was quantified by flow cytometry 18 h post-transduction. Error bars represent mean ± SEM.

Figure 4: Confirmation of CXCR4 overexpression and increased B-1a migration in vitro. Peritoneal B cells from $ApoE^{-/-}$ mice with B cell-specific deficiency of CXCR4 were isolated and transduced with Ctl-GFP or CXCR4-GFP retrovirus, or cultured without transduction. (**a**) Representative flow plots of CXCR4 and GFP expression on B-1 cells from non-transduced (upper left), Ctl-GFP transduced (lower left), or CXCR4-GFP transduced (lower right) conditions. FMO-CXCR4 (upper right) used to set CXCR4 positive gate. (**b**) Quantification of the MFI of CXCR4 on GFP+ B-1 cells from non-transduced (n = 1), Ctl-GFP transduced (n = 2), or CXCR4-GFP transduced (n = 2) conditions. (**c**) Frequency of non-transduced (n = 1), Ctl-GFP transduced (n = 2), or CXCR4-GFP transduced (n = 2) B-1 cells that migrated towards CXCL12 as a percentage of the total number of B-1 cells loaded in transwell. (**d**) Representative gating strategy for quantification of viable cells within the successfully transduced B cell population (CD19+GFP+). (**e**) Frequency of live B cells after transduction with Ctl-GFP (n = 2) or CXCR4-GFP retrovirus (n = 2). Error bars represent mean ± SEM. This figure has been modified from our previous publication[4].

Figure 5: Gating strategy for sorting transduced GFP+ B-1a cells. Representative flow cytometry plots for sorting GFP+ or GFP- B-1a cells from a non-transduced sample (top), a Ctl-GFP transduced sample (middle), and a CXCR4-GFP transduced sample (bottom). B-1a cells defined as live, singlet, CD19+ CD23- IgM+ CD5+ cells.

Figure 6: Quantification of transferred donor cells. Representative flow cytometry plots displaying CD45.1+ CD45.2- donor cells from one PBS control (top), one Ctl-GFP+ B-1a cell recipient (middle), and one CXCR4-GFP+ B-1a cell recipient (bottom), and subsequent analysis of CXCR4 expression on donor cells in bone marrow (**a**), or spleen (**b**). Quantification of CXCR4 expression (mean fluorescence intensity, MFI) on donor cells from bone marrow (**c**) or spleen (**d**). Quantification of the number of donor cells recovered in bone marrow (**e**) or spleen (**f**) of recipients. *$P < 0.05$ or **$P < 0.01$ by Mann-Whitney test. Error bars represent mean ± SEM. This figure has been modified from our previous publication[4].

Table 1: Antibodies and their final concentrations used in the protocol.

Table 2: Plate optimization. Frequency of successfully transduced total GFP+ cells or GFP+ CD19+ B cells after transduction of 6 x 10^6 enriched peritoneal B-1 cells in either a 96-well round-bottom plate (40 wells at 150,000 cells per well), a 24-well plate (6 wells at 1 x 10^6 cells per well), or a 6-well plate (3 wells at 2 x 10^6 cells per well) at a 20:1 MOI with Ctl-GFP retrovirus.

Table 3: Association between CXCR4 expression on donor cells and donor cell localization. The mean fluorescence intensity (MFI) of CXCR4 on donor B-1a cells correlated with the number of donor B-1a cells in bone marrow or spleen of Rag1$^{-/-}$ ApoE$^{-/-}$ recipient mice 17 weeks post-adoptive transfer. Data presented as correlation coefficient (r) and statistical significance (p). This table has been modified from our previous publication[4].

Table 4: Association between donor cell localization and plasma amount of anti-OSE IgM
The number of donor B-1a cells in bone marrow of Rag1$^{-/-}$ ApoE$^{-/-}$ recipient mice 17 weeks post-adoptive transfer correlated with circulating amount of anti-MDA-LDL IgM, E06/T15 IgM, or anti-1,3-dextran IgM. Data presented as correlation coefficient (r) and statistical significance (p). This table has been modified from our previous publication[4].

DISCUSSION:

The method provided here enables stable and relatively efficient primary B-1a cell gene delivery, in vivo adoptive transfer, and identification and localization of injected cells. Cells were able to be detected 17 weeks post-cell transfer and retained increased CXCR4 expression. Retrovirus-mediated delivery yielded 30-40% transduction efficiency of primary murine B-1a cells with minimal impact on cell viability in our hands (**Figure 4e**). This is in line with results from a previous study by Moghimi and colleagues which compared techniques for gene transfer into primary murine B cells including retroviral infection, adenoviral infection, nucleofection, or lipofectamine[15]. However, we found that the range of CXCR4 overexpression varied considerably within recipients receiving CXCR4-GFP transduced B-1a cells (**Figure 6c,d**). Therefore, we utilized associative analysis to demonstrate that increased CXCR4 expression correlated with increased B-1a migration and localization to the bone marrow, which associated with increased plasma IgM (**Table 3** and **Table 4**).

Limitations of this method include the large number of mice required to get sufficient numbers of successfully transduced B-1a cells, and the variability in transduction efficiency from one experiment to another. Transduction efficiency is improved by higher titers of viral stocks, which should be at least 2 x 10^7 infectious particles/mL[14]. The use of older mice, aged 12−16 weeks can also improve peritoneal B-1 cell yield, as peritoneal B-1 cell numbers increase with age[17].

It is also important to note that the amount of IgM secreted by transduced B-1a cells post-adoptive transfer was ~5-fold less than the amount secreted by non-transduced B-1a cells after adoptive transfer into the same Rag1$^{-/-}$ ApoE$^{-/-}$ model (data not shown). This may be due to the requirement of B-1 cell activation with TLR9 agonist prior to retroviral transduction (**Figure 3**),

which may limit secondary activation and IgM production in response to OSE in vivo post-adoptive transfer. Therefore, for studies that require robust IgM production by transferred B-1a cells, alternative gene transfer techniques that do not require prior B-1a cell activation, such as lentiviral delivery[18,19], may prove useful. Alternatively, modifications to this protocol that involve activation strategies to induce proliferation but not B-1a differentiation into IgM-secreting cells might also be sufficient for successful retroviral transduction without impacting secondary B cell activation. IL-5 is an important cytokine mediating B-1a cell proliferation and survival, and may be an effective alternative to TLR9 stimulation[20,21].

Prior studies have utilized splenic B cells isolated through positive or negative selection strategies using antibodies against B220 (B cell marker) or Thy1.2 (T cell marker)[13,14]. However, B220+ splenic B cells are a heterogeneous population containing B-1 and B-2 cell subsets. Moreover, B-1 cell frequency within the total splenic CD19+ B cell population is low (1−2%). In contrast, this method utilizes the peritoneal cavity as a B-1 cell source for transduction, as B-1 cells comprise 60−70% of total CD19+ B cells in this compartment[22], and uses CD23 as a marker for depleting peritoneal B-2 cells. Subsequent sorting of successfully transduced B-1a cells based on GFP, CD19, B220, CD23, IgM, and CD5 expression further allows transfer of a more specifically defined cell type. The magnetic depletion strategy to enrich peritoneal B-1 cells effectively depleted T cells, and reduced F4/80 peritoneal macrophage frequency by ~50% in our hands (**Figure 2**), though further optimization and troubleshooting of this critical step could increase transduction efficiency. For example, using a higher concentration of biotinylated F4/80 antibody for better macrophage depletion might further increase B-1a cell transduction efficiency, as there would be less “off-target” retroviral transduction of other cell types. The use of 96-well round-bottom plates for transduction, instead of flat-bottom 24-well or 6-well plates additionally considerably improved transduction efficiency (**Table 2**), though increases handling and pipetting time.

Overall, this method provides a useful proof-of-concept approach for determining whether targeted gene delivery to B-1a cells can alter B-1a cell localization and functional IgM production. Future applications of this technique could include *ex vivo* delivery of retroviral constructs targeting other proteins, and adoptive transfer to determine its effect on donor or host cell processes in vivo, including cell survival, migration, proliferation, or function. Adoptive transfer into immunocompetent hosts, rather than lymphocyte-deficient hosts, would also be possible with this technique since donor cells (CD45.1+ GFP+) could be differentiated from host cells (CD45.2+ GFP-). Targeting other chemokine receptors using this method could further support the hypothesis that targeting B-1a cell migration towards niches permissive of high IgM production can effectively boost levels of protective IgM.

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DISCLOSURES:

The authors have nothing to disclose.

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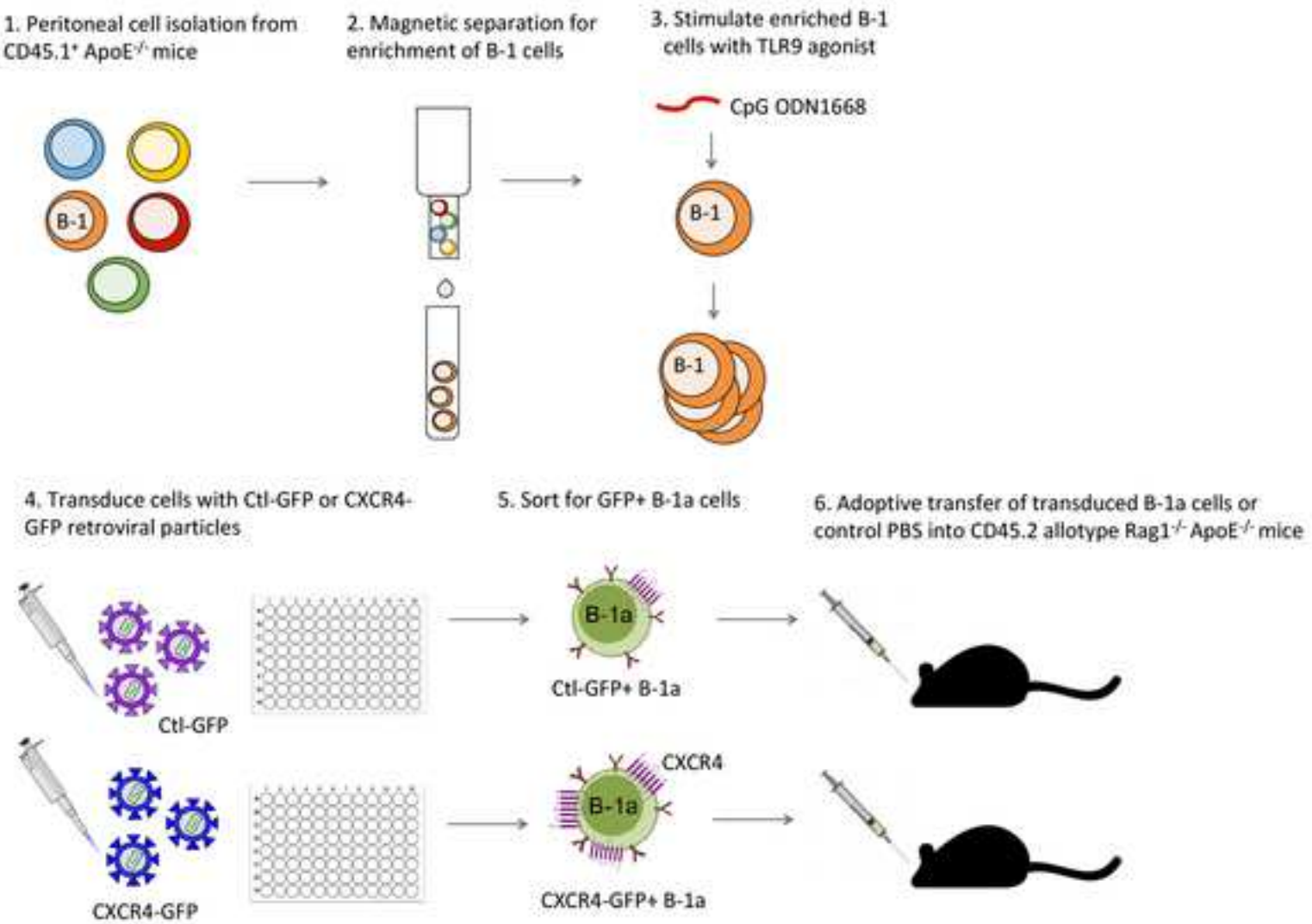

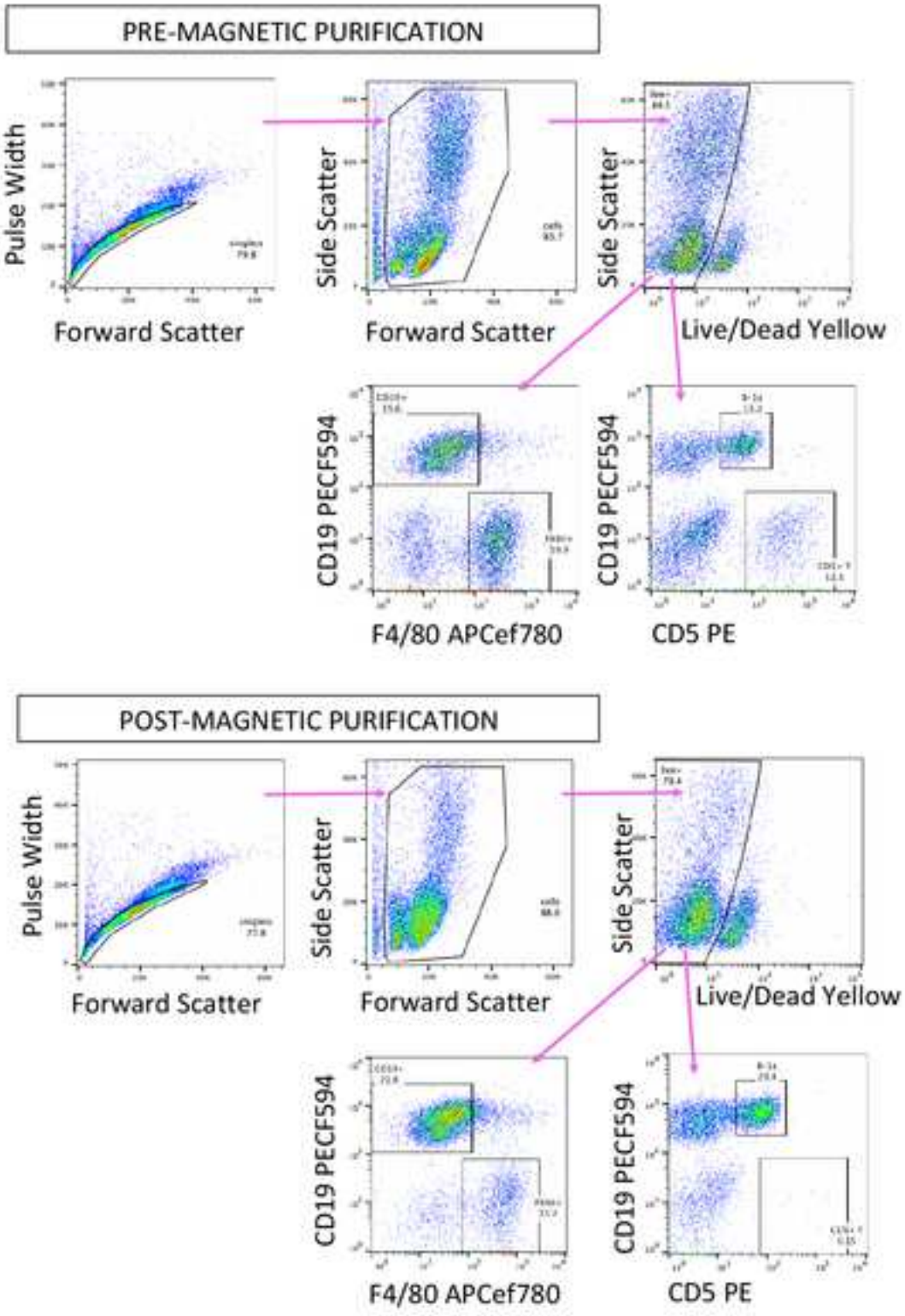

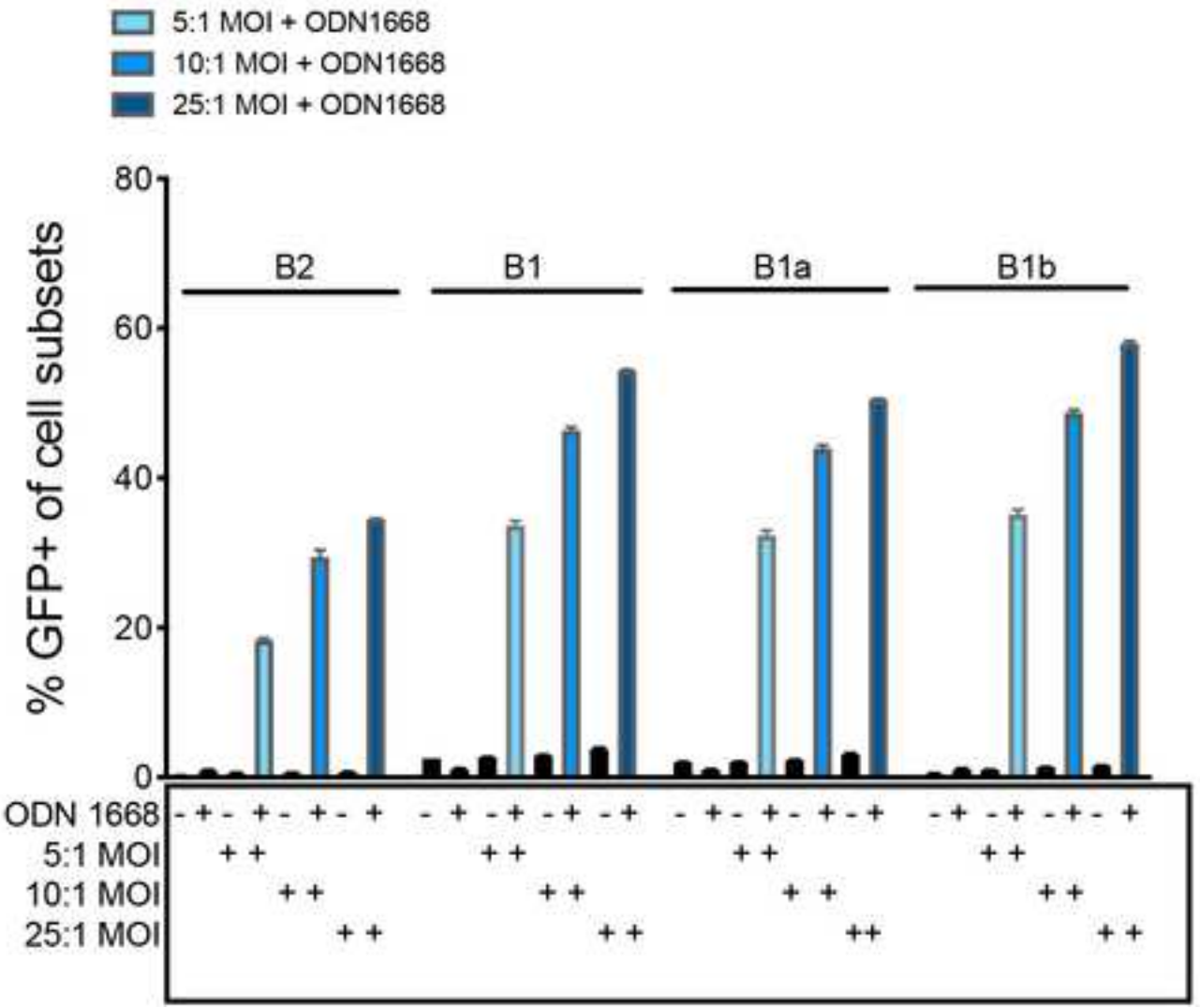

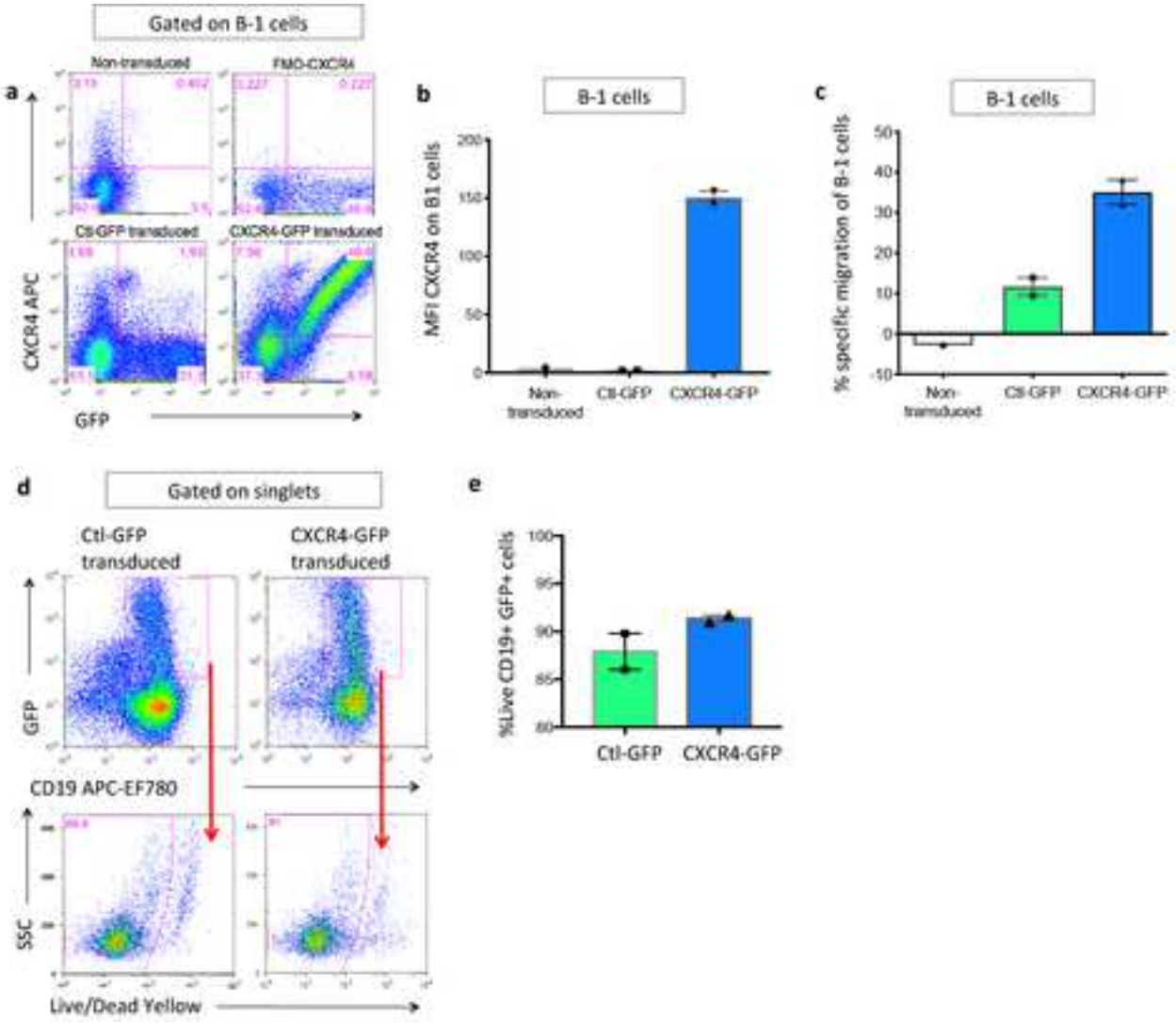

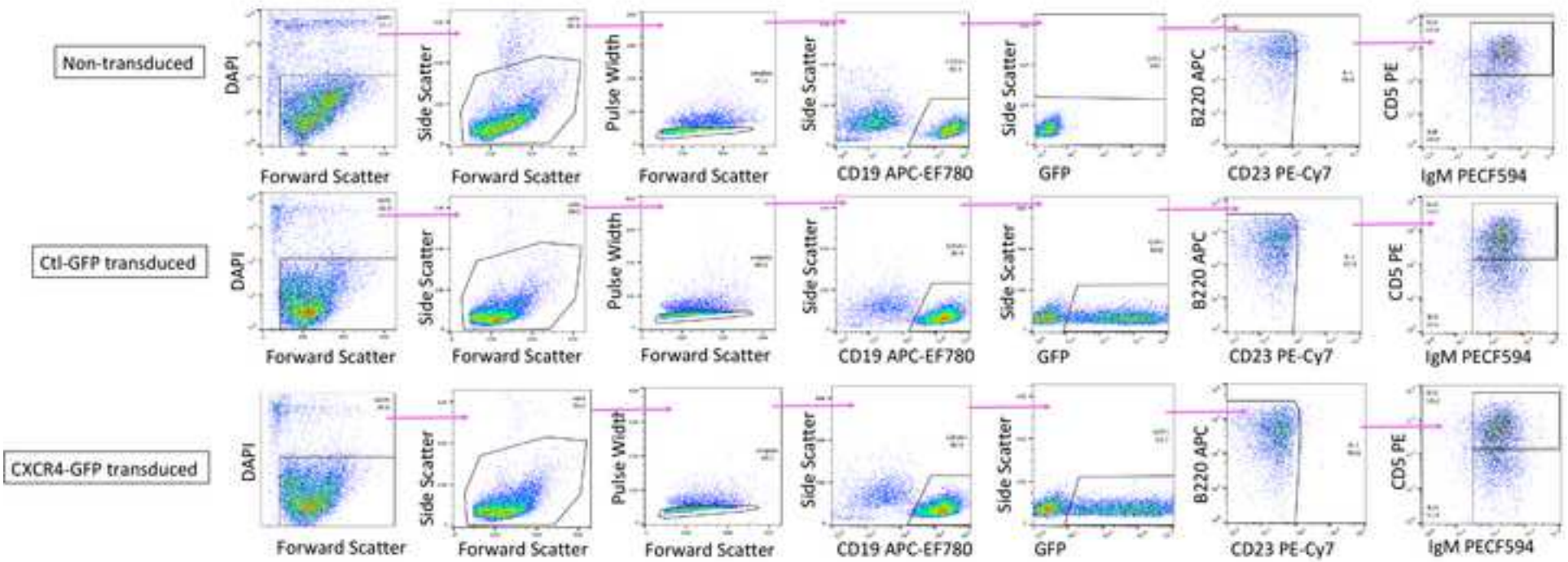

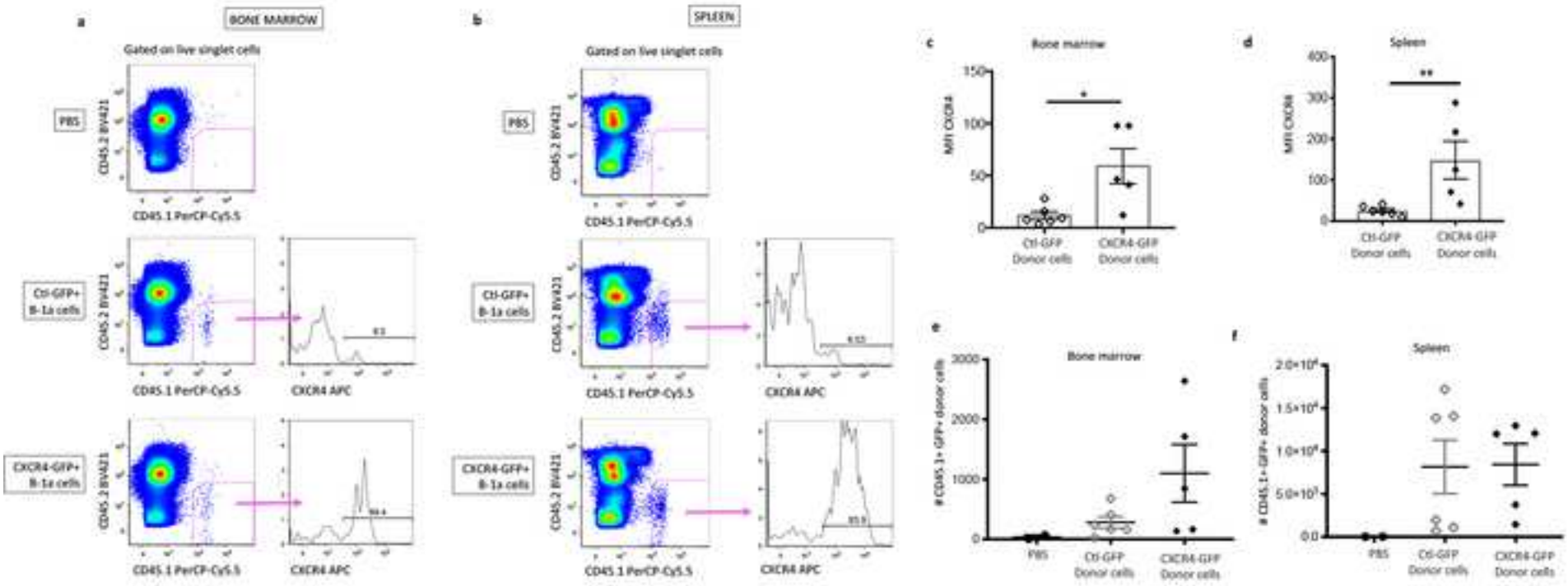

Protocol step	Antibody	Final concentration
Step 1.8	Ter119 biotin	1 µL per 100 µL final volume
	CD3e biotin	1 µL per 100 µL final volume
	Gr-1 biotin	1 µL per 100 µL final volume
	CD23 biotin	1 µL per 100 µL final volume
	NK1.1 biotin	1 µL per 100 µL final volume
	F4/80 biotin	2.5 µL per 100 µL final volume
	Antibody	**Final concentration**
Step 4.5	CD5 PE	1 µL per 100 µL final volume
	IgM PECF594	1 µL per 100 µL final volume
	CD23 PECy7	1 µL per 100 µL final volume
	B220 APC	1 µL per 100 µL final volume
	CD19 APCef780	1 µL per 100 µL final volume
	Antibody	**Final concentration**
Step 6.1	CD45.1 PerCP Cy5.5	1 µL per 100 µL final volume
	CD45.2 BV421	1 µL per 100 µL final volume
	CXCR4 APC	2.5 µL per 100 µL final volume

Condition	%GFP+ of total population
96-well round-bottom plate	30.9%
24-well plate	8.4%
6-well plate	16.2%

%GFP+ of CD19+ B cells
52.7%
21.2%
27.3%

Variable	MFI of CXCR4 on donor B-1a cells	
	r-value	p-value
# of donor B-1a cells in bone marrow	0.71	*0.014
# of donor B-1a cells in spleen	0.43	0.18

Variable	# of donor cells in bone marrow	
	r-value	p-value
Plasma anti-MDA-LDL IgM	0.67	*0.028
Plasma E06/T15 IgM	0.56	0.076
Plasma 1,3-dextran IgM	0.29	0.39

Name of Material/Equipment	Company	Catalogue Number
70 micron filter caps	Falcon	352235
anti-biotin microbeads	Miltenyi Biotec	130-090-485
anti-CD16/CD32, or Fc block	Life Technologies	MFCR00
B220 APC	eBioscience	17-0452-83
Beta-mercaptoethanol	Gibco	21985-023
CD19 APCef780	eBioscience	47-0193-82
CD23 biotin	eBioscience	13-0232-81
CD23 PECy7	eBioscience	25-0232-82
CD3e biotin	eBioscience	13-0033-85
CD45.1 ApoE-/- mice	N/A	N/A
CD45.1 PerCP-Cy5.5	BD Biosciences	560580
CD45.2 BV421	BD Biosciences	562895
CD45.2 Rag1-/- ApoE-/- mice	N/A	N/A
CD5 PE	eBioscience	12-0051-83
Ctl-GFP retrovirus	N/A	N/A
CXCR4 APC	eBioscience	17-9991-82
CXCR4-GFP retrovirus	N/A	N/A
F4/80 biotin	Life Technologies	MF48015
Flowjo Software v. 9.9.6	Treestar Inc.	License required
Gentamicin	Gibco	15710-064
Gr-1 biotin	eBioscience	13-5931-82
heat-inactivated fetal bovine serum	Gibco	16000-044
HEPES	Gibco	15630-080
IgM PECF594	BD Biosciences	562565
Insulin syringes	BD Biosciences	329461
Isoflurane	Henry Schein Animal Health	029405
Live/Dead Yellow	Life Technologies	L34968
LS columns	Miltenyi Biotec	130-042-401
NK1.1 biotin	BD Biosciences	553163
Non-essential amino acids	Gibco	11140-050
ODN 1668	InvivoGen	tlrl-1668
PBS	Gibco	14190-144
RPMI-1640	Gibco	11875-093
Sodium pyruvate	Gibco	11360-070
Ter119 biotin	eBioscience	13-5921-82

Comments/Description

Clone: RA3-6B2

Clone: eBio1D3
Clone: B3B4
Clone: B3B4
Clone: eBio500A2
Bred in house
Clone: A20
Clone: 104
Bred in house
Clone: 53-7.3
Generated in house using GFP-expressing retroviral plasmid MigR1 provided by Dr. T.P. Bender
Clone: 2B11
Generated in house by cloning mouse CXCR4 into MigR1 retroviral plasmid
Clone: BM8

Clone: RB6-8C5

Clone: R6-60.2

Clone: PK136

Clone: Ter119

Dear Editor and Reviewers,

We thank you for your helpful comments and the opportunity to resubmit our article titled "Retroviral overexpression of CXCR4 on murine B-1a cells and adoptive transfer for targeted B-1a cell migration to the bone marrow and IgM production" to the Journal of Visualized Experiments. We have addressed your concerns as discussed point by point below and have updated our manuscript to reflect this.

Thank you for your consideration,

Aditi Upadhye, Timothy Bender, and Coleen McNamara

Editorial Comments:

• Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammatical errors.

We have double checked and corrected spelling and grammatical errors.

• **Protocol Detail:** Please note that your protocol will be used to generate the script for the video, and must contain everything that you would like shown in the video. **Please add more specific details (e.g. button clicks for software actions, numerical values for settings, etc) to your protocol steps.** There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol.

We have added additional details to our protocol according to the recommendations below. We have not included instrument settings for the flow cytometer as these numbers vary from instrument to instrument and can vary from day to day on the same instrument. We have not included button clicks for software actions for the flow analysis software as this software undergoes routine updates so this information may lose relevance over time.

1) 1.1: Mention animal age, sex, weight. Unclear how flushing is performed. Do you inject using a syringe? Mention needle gauge. What is meant by "shake the mouse"? how vigorous is this action? Please describe it more clearly. Mention puncture site for peritoneal fluid collection. What is the incision made? Mention surgical tools used. How deep do you incise?

We have included the above information to step 1.1 as copied below. We do not typically use or measure weight of donor mice, therefore have not included this parameter.

1.1. Euthanize a 12-14 week-old, male, CD45.1^{+}ApoE$^{-/-}$ mouse using CO_2. Make a superficial cut in the abdomen using straight surgical scissors and peel back skin using curved scissors to

expose the peritoneal wall. Flush peritoneal cavity with 10 mL 37 °C RPMI-1640 medium using a 10 mL syringe and 25-gauge needle. Shake mouse to disengage cells by grasping the tail and moving the mouse side to side thoroughly for 15-20 seconds. Massaging the lavaged peritoneum can also maximize cell recovery. Collect peritoneal fluid using a 10 mL syringe and 25-gauge needle by drawing up fluid at the lower right side of the peritoneum just above the level of the hip, near the intestines. Avoid disrupting epididymal fat depots and underlying organs. Avoid drawing fluid from the mouse's left side as the omental fat can easily be drawn into the syringe. Once ~6-7 mL of fluid is collected, dispense into a 50 mL conical tube placed on ice. Next, elevate the mouse vertically by holding the peritoneal wall above the diaphragm using forceps so that any remaining fluid remains at the bottom of the peritoneal cavity. Make a small cut in the peritoneal wall using surgical scissors above the liver (make sure not to cut the liver) and collect any remaining peritoneal fluid using a glass pipet and bulb.

2) 1.3: Mention counting method.

We have added this information to step 1.3 as below:

Count live cells using a viability dye such as trypan blue and a hemocytometer (dilute samples 1:5 in trypan blue and load 10 uL into hemocytometer chamber).

3) 1.9 NOTE: please supply a reference for the flow cytometry procedure or describe it briefly (e.g., mention gating strategy etc)

We have now described a gating strategy as given below, and the gating strategy is also depicted in Figure 2.

NOTE: an aliquot of the pre-purified and post-purified cell fractions should be separately analyzed by flow cytometry for purification efficiency by staining for CD19+ B cells, F480+ macrophages, and CD5+ T cells as shown in Figure 2.

4) 1.10, 4.2: mention counting method.

We have added this to step 1.10 and 4.2 as given below:

1.10. Count the post-purified cell fraction (the eluent containing enriched B-1 cells) by counting live cells as in step 1.3, and resuspend at $1x10^6$ cells/mL in B cell culture medium (RPMI-1640, 10% heat-inactivated fetal bovine serum, 10 mM HEPES, 1X non-essential amino acids, 1 mM sodium pyruvate, 50 ug/mL gentamicin, 55 uM β-mercaptoethanol).

4.2) Count live cells as in step 1.3, then centrifuge at 400xg for 5 minutes at 4 °C and aspirate supernatant.

• **Protocol Numbering:**All steps should be lined up at the left margin with no indentations.

We have now removed indentations for our protocol steps.

• **Protocol Highlight:** After you have made all of the recommended changes to your protocol (listed above), please re-evaluate the length of your protocol section. There is a 10-page limit for the protocol text, and a 3- page limit for filmable content. If your protocol is longer than 3 pages, please highlight ~2.5 pages or less of text (which includes headings and spaces) in yellow, to identify which steps should be visualized to tell the most cohesive story of your protocol steps.
1) The highlighting must include all relevant details that are required to perform the step. For example, in your protocolstep 4 is highlighted for filming and the details of how to perform the step are given in steps 4.10, then the sub-steps where the details are provided must be included in the highlighting.
2) The highlighted steps should form a cohesive narrative, that is, there must be a logical flow from one highlighted step to the next.
3) Please highlight complete sentences (not parts of sentences). Include sub-headings and spaces when calculating the final highlighted length.
4) Notes cannot be filmed and should be excluded from highlighting.
5) Please ensure that the highlighting best represents the highlighted portion of the protocol.

We have now excluded step 4 from our highlighted portion as it exceeds your given limit.

• **Figures:**
1. Please remove the text "Figure #" from each figure.

We have removed Figure # from our figures and saved as .psd files.

2. Fig 3: define the error bars.
We have now defined the error bars in the Figure 3 legend as mean ± s.e.m.

• **Discussion:** JoVE articles are focused on the methods and the protocol, thus the discussion should be similarly focused. Please ensure that the discussion covers the following in detail and in paragraph form (3-6 paragraphs): 1) modifications and troubleshooting, 2) limitations of the technique, 3) significance with respect to existing methods, 4) future applications and 5) critical steps within the protocol.

We have edited our discussion section to address these topics.

• **References:** Please spell out journal names.

We have spelled out journal names in our references.

• **Commercial Language:** JoVE is unable to publish manuscripts containing commercial sounding language, including trademark or registered trademark symbols (TM/R) and the mention of company brand names before an instrument or reagent. Examples of commercial sounding language in your manuscript are MACS, Fc block, Miltenyi,
1) Please use MS Word's find function (Ctrl+F), to locate and replace all commercial sounding language in your manuscript with generic names that are not company-specific. All commercial products should be sufficiently referenced in the table of materials/reagents. You may use the generic term followed by "(see table of materials)" to draw the readers' attention to specific commercial names.

We have now removed use of the terms "MACS", "Fc block", "Miltenyi", and "LS columns" from our protocol.

2) Please remove the registered trademark symbols TM/R from the table of reagents/materials.
We have removed any TM/R symbols from our Table of materials.

• If your figures and tables are original and not published previously or you have already obtained figure permissions, please ignore this comment. If you are re-using figures from a previous publication, you must obtain explicit permission to re-use the figure from the previous publisher (this can be in the form of a letter from an editor or a link to the editorial policies that allows you to re-publish the figure). Please upload the text of the re-print permission (may be copied and pasted from an email/website) as a Word document to the Editorial Manager site in the "Supplemental files (as requested by JoVE)" section. Please also cite the figure appropriately in the figure legend, i.e. "This figure has been modified from [citation]."

We have received reprint permissions from *Circulation Research* and Wolters Kluwer Health, Inc. and have included this letter as a supplemental file in our resubmission. We have additionally updated figure and table legends to cite our previous publication.

Comments from Peer-Reviewers:

Reviewers' comments:
Reviewer #1:

Manuscript Summary:
The authors describe a method to transduce B cells in particular B1a cells with the chemokine

receptor CXCR4 using a recombinant retrovirus. To improve transduction efficiency, they isolate peritoneal cells, MACS deplete none-B cells and activate the remainig cells with CpG. After transduction, they sort-purify the CXCR4 expressing B1a cells and adoptively transfer them into lymphopenic mice. After a considerable time, they recover the cells from bone marrow and spleen of the recepient mice. In Addition, they analyse the plasma for antibodies and claim that the bone marrow targeted B1a cells are responsible for the antibodies they find in such mice.

Major Concerns:

Figure 6. Only percentages and FMI are given. This is not sufficient to conclude an enrichment. Abolute number need to be displayed.

We thank the Reviewer for making this important point. We have now included absolute number of donor cells recovered in bone marrow and spleen to Figure 6e and 6f. Importantly, we believe that because CXCR4 overexpression did not occur equivalently on CXCR4-GFP transduced B-1a cells (as seen in Figure 6c), that the correlative analysis showing a positive correlation between CXCR4 expression and # of donor cells in bone marrow in Table 2 is a better representation of our point that increasing CXCR4 expression can result in increased localization specifically to the bone marrow.

Table3: A positive correlation with particular antibody specificites and the number of donor B cells in bone marrow is given. To me it is not clear whether under these condition, i.e. activated B1a cells, the antibodies are produced in bone marrow at all. This could also explain why only 20% of the amount is found under these conditions.

Thank you for bringing up this interesting point. We also performed ELISPOTs on bone marrow and found low but detectable numbers of IgM antibody secreting cells in bone marrow post-transfer (on the order of 10^3 from 1 femur and tibia). This is lower than what was found in spleen (also seen by comparing the number of donor cells in spleen vs bone marrow measured by flow, Figure 6a and 6b), and is also lower compared to the number of IgM ASC seen in wild-type bone marrow from a non-lymphopenic mouse (on the order of 10^5 from 1 femur and tibia after a similar length of Western diet feeding).

However, despite there being fewer overall IgM ASC in bone marrow post B-1a cell transfer, we still see a positive association with the number of donor cells in bone marrow (measured by flow) and the amount of plasma anti-OSE IgM, as well as a positive association with the number of bone marrow IgM ASC (measured by ELISPOT) and the amount of plasma anti-OSE IgM. Our point is not that other tissues do not contribute to overall plasma IgM levels (indeed the spleen likely contributes more in this setting as more donor cells went there), but that donor cells in the bone marrow also contribute to the overall amount of plasma IgM and that increasing their localization to the bone marrow via increasing CXCR4 could boost this.

Minor Concerns:

Methods description:
After 1.4 a washing step seems to be missing

We stain with anti-CD16/CD32 to block Fc receptors for 10 minutes before we directly add our primary staining antibodies, therefore there is no need to wash between steps 1.4 and 1.5.

1.5: the description how the 2x master mix is established and why it is called 2x master mix is unclear. Why was anit-CD11b not included. In addition a cenrifugation step seems to be missing.

We thank the Reviewer for pointing out this confusing language. We add our primary antibody stains directly to the cells that are incubating in Fc block. This saves a wash and centrifugation step and potential cell loss. Therefore, to account for the volume of liquid that the cells are already in (Fc block in assay buffer), we prepare a 2X master mix of our primary antibody stains. We have clarified this in step 1.5 with the following language:

1.5. Prepare a 2X master mix of the following biotinylated antibodies in assay buffer for depletion. The 2X master mix accounts for the volume of liquid the cells are already in when incubating with anti-CD16/CD32, i.e. if cells are incubating in 500 uL of diluted anti-CD16/CD32, then add 500 uL of 2X master mix containing 10 uL biotinylated Ter119, Gr-1, CD23, and NK1.1 antibody, and 25 uL of biotinylated F4/80 antibody to achieve the final concentrations given in the table below. Add 2X master mix to cells and stain for 20 min at 4 °C.

Antibody	Final concentration
Ter119 biotin	1 uL per 100 uL final volume
CD3e biotin	1 uL per 100 uL final volume
Gr-1 biotin	1 uL per 100 uL final volume
CD23 biotin	1 uL per 100 uL final volume
NK1.1 biotin	1 uL per 100 uL final volume
F4/80 biotin	2.5 uL per 100 uL final volume

We do not include CD11b in our depletion because a large proportion of peritoneal B-1 cells express CD11b and would therefore be depleted. The wash and centrifugation step occurs in step 1.6.

3.1: it is not clear whether the tiration of the Virus preparation should be described as it is very important for establishing MOIs.

We thank the Reviewer for this suggestion, and agree that accurate retrovirus titration is a very important step in this process. However, preparation and titration of retrovirus stocks are lengthy processes we believe are outside the scope of this particular protocol. We instead cite previously published protocols that discuss the details of those steps more thoroughly.

3.3: is Polybrene no longer necessary?

We perform spinfection and incubation in the presence of polybrene, and do not replate with fresh B cell media (lacking polybrene) until step 3.5.

4.5 2x antibody mix in not understandable alo centrifugation step seems to be missing.

As in step 1.4/1.5, we add primary antibody straight to cells incubating in Fc block.

5.3: adoptive Transfer is done via retro-orbital injection which is technically very demanding. Why was not a tail vein choosen.

In our hands, retro-orbital injection is easier than tail vein, however either intravenous route would work. We have updated step 5.3 to include tail vein injection as an option.

6.1: many additional antibodies were used besides the three described here.

Actually, for the post-transfer analysis, donor cells in recipients were identified as CD45.1+CD45.2-GFP+, then CXCR4 expression was measured, therefore, only these 3 antibodies were utilized, as shown in Figure 6.

Reviewer #2:

This is an authoritative protocol manuscript from the groups of Coleen McNamara and Timothy Bender, who are well-established experts in the field of B cell biology and Atherosclerosis. I have made a number of comments on the protocol that they may wish to consider at their discretion. I apologize for my delay in reviewing the manuscript

I would emphasize that mice need more than gentle shaking to dislodge the peritoneal cells. Often the lavaged peritoneum needs massaging vigorously to fully realize maximal cell recovery. An option to using a syringe is to cut open the peritoneum and collect the liquid into a beaker or Falcon tube, as recovering all the suspension via syringe can be problematic.

We thank the Reviewer for these comments, and agree that more vigorous shaking is needed to maximize cell recovery. We also agree that collecting all of the peritoneal fluid via syringe is difficult and now include a step to cut open the peritoneum and collect the rest of the fluid via pipet. The updated protocol step 1.1 is given below.

1.1. Euthanize a 12-14 week-old, male, CD45.1^{+}ApoE$^{-/-}$ mouse using CO_2. Make a superficial cut in the abdomen using straight surgical scissors and peel back skin using curved scissors to expose the peritoneal wall. Flush peritoneal cavity with 10 mL 37 °C RPMI-1640 medium using a 10 mL syringe and 25-gauge needle. Shake mouse to disengage cells by grasping the tail and moving the mouse side to side thoroughly for 15-20 seconds. Massaging the lavaged peritoneum can also maximize cell recovery. Collect peritoneal fluid using a 10 mL syringe and 25-gauge needle by drawing up fluid at the lower right side of the peritoneum just above the level of the hip, near the intestines. Avoid disrupting epididymal fat depots and underlying organs. Avoid drawing fluid from the mouse's left side as the omental fat can easily be drawn into the syringe. Once ~6-7 mL of fluid is collected, dispense into a 50 mL conical tube placed on ice. Next, elevate the mouse vertically by holding the peritoneal wall above the diaphragm using forceps so that any remaining fluid remains at the bottom of the peritoneal cavity. Make a small cut in the peritoneal wall using surgical scissors above the liver (make sure not to cut the liver) and collect any remaining peritoneal fluid using a glass pipet and bulb.

The clones of the antibodies used would be a useful addition, perhaps together with the material table at the end of the manuscript.
We have now included antibody clones in our table of materials.

It is my experience that MACS incubations are very sensitive to temperature. Often samples kept on ice perform poorly compared to incubations done in the fridge.

We also find this to be true. The Miltenyi MACS protocol also recommends incubations to be done in the fridge, therefore we have specified in the MACS incubation step (1.7), to incubate as per the concentration and protocol recommended by the manufacturer.

Have other B cell mitogens been tested, or is the use of ODN1668 or IL-5 critical?

We have only used ODN1668 for our B cell transductions, but we hypothesize that IL-5 or a TLR4 agonist like LPS may have similar efficacy for transduction. We discuss alternative activation strategies in the discussion section of the manuscript.

It might be useful to mention that mouse tonic PBS can be beneficial for i.v. injections.

We used commercial PBS (now in our table of materials) for our i.v. injections, which our lab commonly uses for adoptive transfer.

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Nov 14, 2019

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Figures/tables/illustrations used	Figure 4, Supplementary Figure 6
Author of this Wolters Kluwer article	Yes
Will you be translating?	No

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