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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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, 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.

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DISCLOSURES:

The authors have nothing to disclose.

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.

Keywords

Adoptive transfer; cell migration; cell localization; flow cytometry; retroviral transduction; B-1a cells

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 titers⁶, 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 atherosclerosis¹¹. 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. 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-cloned⁴. 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:

Ethics statement: 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 CO₂. 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.
- 1.2. Pool peritoneal washout cells from all CD45.1⁺ApoE^{-/-} mice (n=15–20) into 50 mL conical tubes, and store on ice. For calculating the number of mice needed: B-1a cells comprise 5–10% of the total peritoneal population, and yield roughly

2.5–5×10⁵ B-1a cells per mouse in our hands. Transduction efficiency is ~30–40%, as shown below.

- 1.3.** 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). Pool cells at up to 1×10⁸ cells per tube. Centrifuge cells at 400×g for 5 minutes at 4 °C, then aspirate supernatant.
- 1.4.** Resuspend up to 1×10⁸ cells in 1 mL of anti-CD16/CD32 antibody (see Table of Materials) diluted 1:50 in assay buffer (1X PBS, 0.5% BSA, 2 mM EDTA) in order to block Fc receptors. Scale as necessary based on cell count. Incubate on ice for 10 minutes at 4 °C.
- 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

- 1.6.** Wash cells with 5 mL assay buffer and centrifuge at 400×g for 5 minutes at 4 °C and aspirate supernatant.
- 1.7.** Resuspend and incubate cells with anti-biotin microbeads (see Table of Materials) diluted in assay buffer as per the concentration and protocol recommended by the manufacturer.
- 1.8.** Wash with 5 mL assay buffer and centrifuge at 400×g for 5 minutes at 4 °C. Aspirate supernatant and resuspend up to 1×10⁸ cells in 500 uL assay buffer.
- 1.9.** Prime magnetic selection columns (see 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.

- 1.10. 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 1×10^6 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).

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 10×10^6 cells for a non-transduced control.
- 2.2) Plate up to 150 uL (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 hours at 37 °C, 5% CO₂.

3. Retroviral transduction of peritoneal B cells

- 3.1) Generate control(Ctl)-GFP (MigR1) and CXCR4-GFP retroviral particles using calcium phosphate transfection as described in previously published protocols¹⁴. 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 to cells, in the presence of 8 ug/mL polybrene and fresh β -mercaptoethanol at 55 uM 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 800xg for 90 minutes at room temperature.
- 3.4) Incubate plates with retrovirus at 37 °C, 5% CO₂ for an additional 3 hours.
- 3.5) Harvest and replate cells in fresh B cell medium and incubate at 37 °C, 5% CO₂ 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 1.3, then centrifuge at 400xg for 5 minutes at 4 °C and aspirate supernatant.
- 4.3) Resuspend cells at 100,000 cells/uL in sort buffer (PBS + 1% BSA) containing 1:50 anti-CD16/CD32 antibody (see Table of Materials) in order to block Fc receptors. Incubate on ice for 10 minutes at 4 °C.

- 4.4)** Aliquot cells for compensation controls (~30,000 cells per compensation control):
- 4.4.1)** For unstained and single stain controls aliquot from non-transduced sample
- 4.4.2)** 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 following fluorophore-conjugated antibodies in sort buffer. The 2X master mix accounts for the volume of liquid the cells are already in when incubating with anti-CD16/CD32, as in step 1.5. 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.

Antibody	Final concentration
CD5 PE	1 uL per 100 uL final volume
IgM PECF594	1 uL per 100 uL final volume
CD23 PECy7	1 uL per 100 uL final volume
B220 APC	1 uL per 100 uL final volume
CD19 APCef780	1 uL per 100 uL final volume

NOTE: an aliquot of cells from each condition can separately be stained for CXCR4 to confirm CXCR4 overexpression.

- 4.6)** Wash samples with 1 mL sort buffer and strain through 70 micron filters into polypropylene tubes.
- 4.7)** Centrifuge at 400xg for 5 minutes at 4 °C and aspirate supernatant. Resuspend samples at 50,000 cells/uL in sort buffer.
- 4.8)** Prior to cell sorting prepare labeled FACS collection tubes containing 1 mL of collection medium (RPMI-1640, 20% 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) 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-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-lo} CD23⁻ IgM⁺ CD5⁺ non-transduced B-1a cells. 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.

NOTE: Transduced or non-transduced B-1b cells can also be sorted using this sort strategy as DAPI⁻ CD19⁺ GFP^{+/-} B220^{mid-lo} CD23⁻ IgM⁺ CD5⁻ cells.

5. Adoptive transfer

- 5.1) After cell sorting, centrifuge cells at 400xg for 5 minutes at 4 °C and aspirate supernatant carefully.
- 5.2) Resuspend cells in cold sterile 1X PBS at 1000 cells/uL.
- 5.3) Anesthetize male Rag1^{-/-} ApoE^{-/-} mice using isoflurane and inject 100 uL (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 harvest⁴ 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.

Antibody	Final concentration
CD45.1 PerCP Cy5.5	1 uL per 100 uL final volume
CD45.2 BV421	1 uL per 100 uL final volume
CXCR4 APC	2.5 uL per 100 uL final volume

NOTE: 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 uL 0.5 M EDTA to whole blood collected via cardiac puncture at the time of animal sacrifice. Centrifuge whole blood at 7000xg and aliquot plasma into separate 1.5 mL centrifuge tubes. Store at -80 °C until performing analysis of secreted factors such as IgM by ELISA⁴.

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 multiplicity of infection (MOI) using Ctl-GFP retrovirus. Table 1 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 2 displays a positive association between CXCR4 expression and donor cell localization to the bone marrow, but not spleen. Table 3 displays a positive association between donor cell number in the bone marrow and plasma amount of anti-MDA-LDL IgM. Figure 4, figure 6, Table 2, and Table 3 have been modified from our previous publication⁴.

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 lipofectamine¹⁵. 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 2 and 3).

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 2×10^7 infectious particles/mL¹⁴. 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 age¹⁷.

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 compartment²², 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 1), 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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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, 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 multiplicity of infection (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 hours post-transduction. Error bars represent mean $\pm$ s.e.m.

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 $\pm$ s.e.m. This figure has been modified from our previous publication⁴.

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 ± s.e.m. This figure has been modified from our previous publication⁴.

Table 1.**Plate optimization**

Frequency of successfully transduced total GFP+ cells or GFP+ CD19+ B cells after transduction of 6×10^6 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 1×10^6 cells per well), or a 6-well plate (3 wells at 2×10^6 cells per well) at a 20:1 multiplicity of infection with Ctl-GFP retrovirus.

Condition	%GFP+ of total population	%GFP+ of CD19+ B cells
96 well round-bottom plate	30.9%	52.7%
24 well plate	8.4%	21.2%
6 well plate	16.2%	27.3%

Table 2.
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 publication⁴.

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

Table 3.
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 publication⁴.

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

Table of Materials

Name of Material/Equipment	Company	Catalogue Number	Comments/Description
anti-CD16/CD32, or Fc block	Life Technologies	MFCR00	
Ter119 biotin	eBioscience	13-5921-82	Clone: Ter119
CD3e biotin	eBioscience	13-0033-85	Clone: eBio500A2
Gr-1 biotin	eBioscience	13-5931-82	Clone: RB6-8C5
CD23 biotin	eBioscience	13-0232-81	Clone: B3B4
NK1.1 biotin	BD Biosciences	553163	Clone: PK136
F4/80 biotin	Life Technologies	MF48015	Clone: BM8
anti-biotin microbeads	Miltenyi Biotec	130-090-485	
LS columns	Miltenyi Biotec	130-042-401	
CD5 PE	eBioscience	12-0051-83	Clone: 53-7.3
IgM PECF594	BD Biosciences	562565	Clone: R6-60.2
CD23 PECy7	eBioscience	25-0232-82	Clone: B3B4
B220 APC	eBioscience	17-0452-83	Clone: RA3-6B2
CD19 APCe780	eBioscience	47-0193-82	Clone: eBio1D3
CD45.1 PerCP-Cy5.5	BD Biosciences	560580	Clone: A20
CD45.2 BV421	BD Biosciences	562895	Clone: 104
CXCR4 APC	eBioscience	17-9991-82	Clone: 2B11
Live/Dead Yellow	Life Technologies	L34968	
RPMI-1640	Gibco	11875-093	
heat-inactivated fetal bovine serum	Gibco	16000-044	
HEPES	Gibco	15630-080	
Non-essential amino acids	Gibco	11140-050	
Sodium pyruvate	Gibco	11360-070	
Gentamicin	Gibco	15710-064	
Beta-mercaptoethanol	Gibco	21985-023	
ODN 1668	InvivoGen	tlrl-1668	
70 micron filter caps	Falcon	352235	
Insulin syringes	BD Biosciences	329461	
Ctl-GFP retrovirus	N/A	N/A	Generated in house using GFP-expressing retroviral plasmid MigR1 provided by Dr. T.P. Bender
CXCR4-GFP retrovirus	N/A	N/A	Generated in house by cloning mouse CXCR4 into MigR1 retroviral plasmid
Isoflurane	Henry Schein Animal Health	029405	
PBS	Gibco	14190-144	
Flowjo Software v. 9.9.6	Treestar Inc.	License required	
CD45.1 ApoE ^{-/-} mice	N/A	N/A	Bred in house
CD45.2 Rag1 ^{-/-} ApoE ^{-/-} mice	N/A	N/A	Bred in house