

TITLE:

A Neonatal Intravenous Transplant Model for Assessment of Early-Life Immune Activities Against Cancer Cells

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SUMMARY:

This protocol demonstrates temporal vein injection for intravenous delivery of pediatric cancer cells to neonatal mice, enabling precise, reproducible modeling of early-life immune responses against cancer. The method enables assessment of the impact of interventions, such as immunomodulation or therapy, when applied during the neonatal period.

ABSTRACT:

More than half of all pediatric cancers are initiated in utero, with progression towards overt disease continuing through the neonatal period. While immunotherapies now offer new treatment options for many children with cancer, how early-life immunity influences cancer progression and therapy response remains incompletely understood. Addressing this gap will require evaluation of therapeutic interventions during the neonatal period, when immune responses differ fundamentally from those in later childhood and adulthood.

Here, we describe a neonatal transplantation model that integrates with bioluminescent in vivo imaging to investigate the impact of early-life immune modulation of pediatric cancers.

Temporal vein injection of luciferase-tagged cells derived from two in utero-initiated cancers achieved reproducible tumour engraftment in 1–2-day-old syngeneic recipients, with no procedure-related mortality and high procedural success as confirmed by bioluminescent imaging. Using infection-driven immune modulation as an experimental variable, we then show that intraperitoneal infection with murine cytomegalovirus five days after transplantation results in a marked reduction in the burden of acute lymphoblastic leukemia (ALL), but not neuroblastoma (NB). This platform enables studying cancer-specific differences in response to early-life immune modulation and underscores the importance of tumor context when modeling immune–cancer interactions in early life.

Collectively, this methodology provides a versatile and highly reproducible platform for interrogating neonatal immune mechanisms relevant to cancer immunosurveillance, infection-driven immune modulation, and therapeutic intervention. Beyond leukemia and NB, this approach can be adapted to additional pediatric malignancies and immunomodulatory strategies, supporting mechanistic studies and preclinical evaluation of early-life cancer interventions.

INTRODUCTION:

Recent advances in immunotherapy have markedly enhanced outcomes for certain cancer patients, yet their optimal application in young children necessitates a thorough understanding of how the developing immune system identifies and eradicates cancer cells. While approximately 50% of childhood cancers originate in utero, including B-cell acute lymphoblastic leukemia (B-ALL) and NB, growing evidence suggests that postnatal events critically influence disease evolution and clinical outcome^{1,2}.

Neonatal immune responses differ quantitatively and qualitatively from those of adults³. In the absence of fully developed adaptive immunity, protection of neonates rely heavily on maternal antibodies and innate immune cells. These innate effectors, however, are functionally less efficient than their adult counterparts due to cell-intrinsic hypo-responsiveness and enhanced activation of immunosuppressive pathways, leaving neonates particularly susceptible to infection^{4–6}. However, the impact of these characteristics on the ability of the immature immune system to mount effective anti-cancer responses remains poorly understood.

Modeling anti-cancer immunity in early life requires the precise and reproducible delivery of cancer cells and immune modulators or pathogens during the short postnatal window, when immune function is limited. Temporal vein injection is a neonatal intravenous (IV) delivery method that best meets these requirements and offers high procedural success, low mortality, minimal handling stress, and compatibility with sequential interventions, such as infection challenge or therapeutic administration^{7–10}.

In this study, we built upon and modified previously validated neonatal intravenous delivery techniques and the versatility of bioluminescent in vivo imaging to describe a transplant model to study the impact of early-life infection on the progression of pediatric cancer cells. This approach enables the intravenous delivery of luciferase-tagged B-ALL or NB cells to 1–2-day-old

pups, followed by immune modulation through intraperitoneal (i.p.) administration of murine cytomegalovirus (mCMV) on postnatal day 7 (P7). Cancer progression was subsequently evaluated 10 days post-infection (P17).

Although access to the temporal vein is restricted to a limited postnatal window (P0–P3), this methodology enhances reproducibility and experimental control, offering a versatile platform to investigate the influence of early-life immune mechanisms on cancer immunosurveillance, infection–cancer interactions, and responses to immunotherapy. Additionally, this approach supports the efficient homing of transplanted cells, thereby enabling the reproducible establishment of disease models for mechanistic investigations.

PROTOCOL:

All procedures (**Figure 1**) were conducted in accordance with the Canadian Council on Animal Care guidelines and were approved by the University of British Columbia Animal Care Committee (protocol A23-0169).

1. Generation of luciferase-tagged leukemic cells

1.1. Isolation of Leukemic Cells from leukemic E μ -Ret mice

1.1.1. Anesthetize mice in a 1 L induction chamber with 5 % isoflurane and 1 L·min⁻¹ oxygen until a deep surgical plane is achieved (loss of righting reflex and slow, regular respiration).

1.1.2. Once deep anesthesia is confirmed, discontinue isoflurane and administer carbon dioxide (CO₂) at a high flow rate (3–4 times the standard gradual displacement rate; e.g., 6–8 L·min for a 10 L chamber) without transferring the animal between chambers. Maintain CO₂ exposure for at least 5 min after respiration ceases to ensure death.

1.1.3. Vent and scavenge the waste gases before opening the chamber.

1.1.4. Confirm death by absence of heartbeat and respiration, then perform cervical dislocation as a secondary physical method in accordance with institutional.

1.1.5. Transfer the carcass to a biosafety cabinet, reconfirm death, record sex and body weight, and spray the exterior with 70 % ethanol.

1.1.6. Make a midline abdominal incision, reflect the skin and peritoneal wall, and excise the spleen into 5 mL sterile PBS.

1.1.7. Dissect tibiae and femora from both legs, removing excess tissue, and excise the spleen under sterile conditions. Immediately transfer the spleen into 5 mL sterile, ice-cold Phosphate Buffered Saline (PBS) solution to preserve cell viability.

1.1.8. Process the spleen.

1.1.8.1. Homogenize spleen tissue in 1 mL of 1× red blood cell (RBC) lysis buffer (use 2 mL for spleens > 200 mg). Incubate for 2–3 min at room temperature.

1.1.8.2. Quench lysis by adding 10 mL PBS + 2 % fetal bovine serum (FBS) (FACS buffer), filter through a 40 µm strainer, and centrifuge at 350 × *g* for 5 min at 4 °C.

1.1.8.3. Resuspend the spleen pellet in 5 mL FACS buffer.

1.1.9. Process bone marrow.

1.1.9.1. Excise both epiphyses and flush each bone with 1 mL FACS buffer using a 1 mL syringe fitted with a 29-gauge needle into a 15 mL tube.

1.1.9.2. Centrifuge the cell suspension, discard the supernatant and resuspend cells in 1 mL of 1× RBC lysis buffer, incubate for 2 min at RT, then quench lysis by adding 10 mL FACS buffer, filter through a 40 µm strainer, and centrifuge at 350 × *g* for 5 min at 4 °C.

1.1.9.3. Discard the supernatant and resuspend the BM pellet in 1 mL FACS buffer by pipetting up and down.

1.1.9.4. Vortex gently and filter through a 40 µm strainer if needed.

1.1.9.5. Resuspend 0.5×10^6 leukemic cells in 200 µL cell culture media (e.g. Dulbecco's Modified Eagle Medium (DMEM)) supplemented with 20 % FBS and interleukin-7 (IL-7; 250 pg·mL⁻¹).

Pause Point: Keep cell suspensions on ice for up to 2 h before staining.

1.2. Lentiviral transduction of leukemic cells:

1.2.1. Seed murine bone marrow–derived stromal cells at 1×10^5 cells per well in a 24-well plate 24 h prior to transduction to allow adherence and achieve 70–80% confluency.

1.2.2. Plate leukemic cells (0.5×10^6 cells in a final volume of 200 µL) in a non-tissue culture treated 24-well plate.

1.2.3. Add a self-inactivating (SIN) lentiviral vector encoding a double-fusion reporter consisting of click beetle red (CBR) luciferase and enhanced green fluorescent protein (eGFP), driven by a constitutive ubiquitin promoter, with the multiplicity of infection (MOI): 10–20, viral titer: $\geq 1 \times 10^8$ TU·mL⁻¹ (transducing units per milliliter) in final transduction volume of 200 µL together with polybrene (8 µg·mL⁻¹; Sigma, H9268-5G).

CAUTION: Polybrene is cytotoxic at high concentrations. Do not exceed 8 µg·mL⁻¹. Handle lentivirus under Biosafety Level 2 (BSL-2) containment.

1.2.4. Seal the plate with parafilm and centrifuge at $1,200 \times g$ for 20 min at room temperature (RT).

1.2.5. Following spinoculation, transfer transduced leukemic cells onto stromal monolayer and culture for 24 h at 37 °C, 5% CO₂ in DMEM + 20 % FBS + IL-7 (250 pg·mL⁻¹) to support cell survival and reporter expression.

NOTE: Antibiotics may be added after 24 h if required but should be avoided during viral transduction.

1.2.6. After 24 h, collect non-adherent leukemic cells and wash twice with sterile phosphate-buffered saline (PBS), $350 \times g$ for 20 minutes at 4 °C, to remove residual virus and polybrene.

1.2.7. Resuspend cells in sterile PBS at 1×10^7 cells·mL⁻¹ for transplantation.

Pause Point: After washing, cells can be maintained under the same culture condition up to 48 h before transplantation if needed.

1.3. Transplantation into adult NSG (NOD.Cg-*Prkdc*^{scid} *Il2rg*^{tm1Wjl}/SzJ) mice

1.3.1. Use 4–6-week-old NSG mice as recipient.

1.3.2. Warm mouse for 5 min using a warming pad or heat lamp to dilate the tail veins.

1.3.3. Restrain mouse using an acrylic restrainer.

1.3.4. Inject 1×10^6 cells in 100 µL sterile PBS via lateral tail vein using 29-gauge insulin syringe. No anesthesia required (unless required by institutional guidelines)

1.3.5. Apply gentle pressure to the injection site to prevent bleeding.

CAUTION: Avoid extravasation. Successful intravenous injection results in no visible swelling.

219
220 1.3.6. Monitor mice twice weekly for disease development.

221
222 1.4. Sorting GFP⁺ leukemic cells

223
224 1.4.1. Upon disease onset, harvest BM or spleen from leukemic NSG mice.

225
226 1.4.2. Prepare a single-cell suspension as described above.

227
228 1.4.3. Stain cells with viability dye (7-AAD).

229
230 1.4.4. Use Fluorescence-Activated Cell Sorting (FACS) for sorting GFP⁺ leukemic cells.

231
232 1.4.5. For sorting use high-speed cell sorter and follow the gating strategy of Forward
233 scatter (FSC) vs side scatter (SSC) → exclude debris, FSC-A vs FSC-H → select singlets,
234 Viability dye negative → select live cells, GFP⁺ gate → isolate reporter-positive
235 leukemic cells.

236
237 1.4.6. Collect sorted cells into tubes containing DMEM + 20 % FBS. Target post-sort purity:
238 ≥ 95 % GFP⁺ cells.

239
240 Pause Point: Sorted cells may be kept on ice up to 2 h before cryopreservation.

241
242 1.5. Cryopreservation of sorted cells

243
244 1.5.1. Resuspend sorted cells at 5×10^6 cells/mL in freezing medium containing 90 % FBS
245 and 10 % dimethyl sulfoxide (DMSO).

246
247 1.5.2. Aliquot 1 mL per cryovial and place vials in isopropanol-based freezing container
248 and Store at -80°C overnight (12–18 h).

249
250 1.5.3. Transfer vials to liquid nitrogen (LN₂) for long-term storage.

251
252 CAUTION: DMSO is toxic and readily absorbed through the skin. Handle with gloves and
253 work quickly.

254 Pause Point: Cells may be stored long-term in liquid nitrogen until use in neonatal
255 transplantation experiments.

256
257 2. Generation of luciferase-tagged NB cells

258
259 NOTE: TH-MYCN transgenic mice develop spontaneous NB tumors and were maintained
260 under approved institutional animal care protocols. Lentiviral vector used is the same as
261 described in Section 1.2.
262

2.1. Tumor Harvest and Tissue Dissociation

2.1.1. Euthanize tumor-bearing TH-MYCN mice according to approved animal care guidelines.

2.1.2. Harvest subcutaneous or abdominal NB tumors under sterile conditions.

2.1.3. Weigh tumor tissue and mince into ~1–2 mm fragments using sterile scissors.

2.1.4. Transfer tissue into approximately 5 mL digestion buffer per 0.5 g tumor tissue in a 15 mL conical tube, consisting of DMEM, Deoxyribonuclease I (DNase I; 50–100 U·mL⁻¹), and collagenase-based enzyme blend (10 µg·mL⁻¹ final concentration).

2.1.5. Incubate at 37 °C in a shaking incubator at 150 rpm for 30 min.

CAUTION: Enzymatic digestion reagents may cause skin and eye irritation. Wear gloves and eye protection.

2.1.6. After digestion, gently pipette up and down to further dissociate tissue. Filter suspension through a 70 µm cell strainer into a fresh tube.

2.1.7. Centrifuge at 300 × *g* for 5 min at room temperature (20–22 °C).

2.1.8. Discard supernatant and resuspend pellet in DMEM supplemented with 10% FBS.

2.1.9. Perform RBC lysis using RBS lysis buffer as previously described in Section 1.1.

2.1.10. Centrifuge at 300 × *g* for 5 min and resuspend pellet in 10 mL high-glucose DMEM supplemented with 10% FBS and 50 µM 2-mercaptoethanol.

CAUTION: 2-mercaptoethanol is toxic and volatile. Perform handling in a chemical fume hood.

Pause Point: Cells may be kept on ice for up to 1 h before transduction.

2.2. Lentiviral Transduction

2.2.1. Pellet 2 × 10⁶ NB cells at 300 × *g* for 5 min and remove supernatant completely.

2.2.2. Resuspend cell pellet directly in 14 µL concentrated lentiviral vector (same vector described in Section 1.2; viral titer ≥ 1 × 10⁸ TU·mL⁻¹, approximate MOI: 10–20).

2.2.3. Incubate cell–virus mixture for 5 min at 37 °C in a humidified incubator (% CO₂).

2.2.4. Add 200 μ L high-glucose DMEM + 10% FBS and continue incubation for 2 h at 37 °C, 5% CO₂.

CAUTION: Lentiviral vectors must be handled under BSL 2 conditions.

2.2.5. Wash transduced cells four times with high-glucose DMEM to remove residual virus.

2.2.6. Count viable cells using trypan blue exclusion.

IMPORTANT: Keep matrix preparation on ice at all times to prevent premature gelation.

2.3. Subcutaneous Implantation into NSG Mice

2.3.1. Resuspend cells at 5×10^6 cells per 100 μ L in a cold basement membrane matrix preparation (growth factor-reduced extracellular matrix).

2.3.2. Anesthetize 6–8-week-old NSG mice (see Section 1.1) using inhaled isoflurane (2–3% in oxygen).

2.3.3. Load cell–matrix mixture into a pre-chilled 1 mL syringe fitted with a 27-gauge needle.

2.3.4. Inject 5×10^6 cells in 100 μ L total volume subcutaneously into the flank region.

2.3.5. Monitor mice until full recovery from anesthesia.

NOTE: Inject immediately after mixing with the matrix to prevent solidification inside the syringe.

2.3.6. Allow tumors to grow to ~1 cm in diameter.

2.4. Cell sorting

2.4.1. Harvest tumors and generate single-cell suspensions as described in Sections 2.1–2.2.

2.4.2. Enrich luciferase-tagged cells by FACS based on GFP expression, following the gating strategy described in Section 1.4.

2.4.3. Perform two sequential rounds of sorting to achieve $\geq 95\%$ GFP-positive purity.

2.5. Cryopreservation

2.5.1. Cryopreserve sorted cells as described in Section 1.5.

Pause Point: Cells can be stored long-term in liquid nitrogen for future transplantation experiments.

3. Prepare the cell suspension for temporal vein injection

3.1. Thaw cryopreserved luciferase-tagged leukemic cells or NB cells rapidly in a 37 °C water bath until a small ice crystal remains.

3.2. Immediately disinfect the vial exterior with 70 % ethanol, then transfer contents into 9 mL of pre-warmed DMEM + 10 % FBS medium in a 15 mL conical tube.

3.3. Centrifuge at $300 \times g$ for 5 min at 4 °C, aspirate supernatant, and resuspend the pellet in sterile, ice-cold PBS.

3.4. Determine viability using trypan blue exclusion and a hemocytometer (or automated cell counter). Use only suspensions with ≥ 80 % viability.

3.5. Pass the cell suspension through a 40 μ m cell strainer to remove clumps.

CAUTION: Cell clumps increase failed injections and tissue injury.

3.6. Adjust concentration to prepare 20 μ L per neonate containing 1×10^4 leukemic cells (for leukemic transplantation), or 5×10^4 NB cells (for NB transplantation).

3.7. Keep the cell suspension on ice and gently mix immediately before loading the syringe to maintain homogeneity.

Pause Point: Keep cells on ice for up to 2 h before injection if viability remains ≥ 80 %.

4. Temporal vein injection (P1–P2 pups)

NOTE: Neonatal temporal vein injection can be performed without anesthesia. However, brief hypothermic immobilization is commonly used to reduce movement and improve injection accuracy, particularly for new trainees. The use of hypothermic immobilization is regulated by institutional animal care guidelines and may not be permitted in some institutions. Investigators must consult and follow their local animal care committee policies before performing this procedure. If hypothermic immobilization is approved by your institution, follow the steps below.

4.1. Prepare a small container lined with a sterile napkin and pre-chill it by placing it on ice.

4.2. Place pups (postnatal day 1–2; P1–P2) on the napkin inside the container for hypothermic immobilization for 2–3 min until spontaneous movement is markedly reduced. Use BALB/c and TH-MYCN mice as recipients of leukemic and NB cells, respectively.

393
394 IMPORTANT: Do not allow pups to contact ice directly (use the container and napkin
395 barrier at all times).
396

397 4.3. Load 20 μ L of the cell suspension into a 100 μ L glass syringe fitted with a 30-gauge
398 needle, ensuring no air bubbles.
399

400 4.4. Prepare a transilluminated stage by placing a cold light source beneath a plexiglass
401 platform.
402

403 4.5. Place a sterile napkin with a central hole on the stage and position the pup so the
404 temporal vein is visible.
405

406 4.6. Wear magnifying eyewear (or use a stereomicroscope) to visualize the injection site.
407

408 4.7. Place a small piece of modeling clay (playdough) adjacent to the pup's head to help
409 stabilize the needle during injection.
410

411 4.8. Insert the needle bevel-up into the visible temporal vein and slowly inject the cell
412 suspension, observing vascular filling to confirm successful intravenous delivery.
413

414 4.9. After injection, apply gentle pressure to the injection site using a sterile cotton swab.
415 Place the pup on a warmed recovery surface (approximately 37 °C, e.g., heating pad on
416 low with a towel barrier) until normal movement and pink coloration return.
417

418 4.10. Monitor the pup for 5–10 min after recovery, then return it to the dam.
419

420 CAUTION: Avoid overheating during recovery. Use a barrier layer and monitor continuously.
421

422 4.11. Rinse the syringe immediately with PBS, then with a syringe cleaning solution, and finish
423 with 70% ethanol.
424

425 5. Virus preparation and infection

426

427 5.1. Perform all procedures involving murine cytomegalovirus (mCMV) under BSL-2
428 containment in accordance with institutional biosafety and animal care approvals.
429

430 5.2. Handle virus stocks in a certified Class II biological safety cabinet and perform animal
431 infections in designated BSL-2 procedure areas.
432

433 5.3. Conduct the virus preparation and handling in a certified Class II biological safety cabinet,
434 and perform the animal infections in designated BSL-2 procedure areas.
435

5.4. Wear appropriate PPE, including a laboratory coat or disposable gown, gloves, and eye protection. Treat all virus-containing materials (syringes, bedding, cages, and surfaces) as potentially infectious.

5.5. Minimize sharps use and dispose of needles immediately in approved biohazard sharps containers. Decontaminate work surfaces and equipment after use. Dispose of all solid and liquid waste as biohazardous waste according to institutional procedures.

5.6. Infect mice at P7 (5–6 days after leukemic cell transplantation).

5.7. Thaw a virus aliquot on ice, mix gently by inversion, and keep on ice during preparation. Avoid repeated freeze–thaw cycles (use single-use aliquots).

5.8. Prepare mCMV at 2×10^5 plaque-forming units (PFU)/mL in PBS and inject pups intraperitoneally (i.p.) with 1×10^4 PFU in 50 μ L PBS using a 30-gauge needle. Inject into the lower right quadrant of the abdomen at a shallow angle to avoid abdominal organs.

CAUTION: mCMV is infectious (BSL-2). Use appropriate containment and decontamination.

5.9. Record animal identification, age, experimental group, virus dose, volume, and injection details.

6. Bioluminescence imaging (BLI)

NOTE: Perform BLI at P7 (5–6 days after cell injection and prior to infection) and at P17 (10 days post-infection) as described below.

6.1. Dissolve D-Luciferin in PBS at $10 \mu\text{g} \cdot \mu\text{L}^{-1}$. Filter-sterilize using a 0.22 μm filter, aliquot, and store at -20°C protected from light.

CAUTION: D-luciferin is light-sensitive. Minimize light exposure during preparation and use.

6.2. Inject D-luciferin i.p. at 50 μ L per pup using a 30-gauge needle.

6.3. Anesthetize pups with isoflurane (induction 3–4 %, maintenance 1.5–2 %) in oxygen ($0.8\text{--}1.0 \text{ L} \cdot \text{min}^{-1}$). Maintain body temperature on a warmed imaging stage (approximately 37°C).

CAUTION: Isoflurane is a volatile anesthetic. Use an approved vaporizer and scavenging system.

6.4. Place anesthetized pups in a small-animal imaging system (instrument details provided in **Table of Materials**).

6.5. Acquire bioluminescent and X-ray images using consistent exposure settings 10 minutes after D-luciferin injection (BLI settings: exposure time 120 s for P7 images and 30 s for P17, binning B4 for P7 and B4 for P17 images, field of view (FOV) 25, f-stop 1.2, object height 1.5 and X-ray settings: resolution high, power high).

Pause Point: Store images files immediately after imaging; analysis can be completed later.

6.6. Analyze images using appropriate BLI imaging software by defining regions of interest (ROIs) encompassing the whole body of each mouse and quantifying emitted light as photons·s⁻¹·cm⁻²·sr⁻¹.

6.7. Export quantified ROI values for downstream statistical analysis.

7. Harvesting leukemic cells from cell transplanted mice

7.1. Harvest leukemic cells and generate single-cell suspensions as described in Section 1.1.

8. Flow cytometry staining and acquisition

8.1. Count cells and centrifuge suspensions at 300 × *g* for 5 min at 4 °C and resuspend at 1 × 10⁷ cells·mL⁻¹ in FACS buffer.

8.2. Transfer 100 µL (1 × 10⁶ cells) into polystyrene flow cytometry tubes.

8.3. Add Fc receptor blocking antibody (anti-CD16/32; 1 µL per tube) and incubate for 5 min at room temperature.

8.4. Prepare antibody master mix in FACS buffer and add to each tube CD19-FITC, BP1-PE, B220-APC, and lineage negative markers (TER119-PerCP, CD3-PerCP, CD11b-PerCP), 1 µL of each antibody per 1 × 10⁶ cells in 100 µL FACS buffer.

8.5. Incubate for 20 min at room temperature in the dark.

8.6. Wash with 5 mL FACS buffer, centrifuge, and resuspend in 200 µL FACS buffer containing 7-AAD (3 µL) and counting beads (6 µL; 6,000 beads).

8.7. Prepare single-stained compensation controls using antibody capture beads or cells and acquire controls alongside experimental samples.

8.8. Acquire samples on a flow cytometer using standard FITC/PE/APC/PerCP detector settings and a medium flow rate. Collect events until 1,000 beads are acquired.

8.9. Export FCS files and analyze using flow cytometry analysis software (listed in **Table of Materials**) using the established gating strategy (**Figure 2D**).

8.10. Calculate absolute cell numbers per spleen or bone marrow using counting beads by multiplying the number of target events acquired at 1,000 beads by a scaling factor of 6 (6,000 beads added), and normalizing to total organ cellularity based on the fraction of cells stained (1×10^6 cells).

9. Plotting graphs and statistical analysis

NOTE: Generate graphs and perform statistical analyses for BLI and flow cytometry data using a statistical software package (listed in Table of Materials).

9.1. Present data as mean $\pm$ standard error of the mean (SEM).

NOTE: No animal was excluded from this study.

9.2. For BLI, compare PBS and mCMV groups separately at P7 and P17 using unpaired Mann–Whitney tests, with Holm–Šídák correction applied to adjust for multiple comparisons across time points.

9.3. Analyze flow cytometry outcomes using a two-tailed unpaired Mann–Whitney test.

9.4. Define statistical significance as $p < 0.05$.

RESULTS:

Temporal vein injection enables efficient neonatal engraftment of leukemia and neuroblastoma cells

To model the impact of early-life infection on pediatric cancer progression, luciferase-tagged B-ALL cells derived from leukemic adult E μ -Ret mice or NB cells derived from moribund Th-Mycn mice were intravenously delivered via the temporal vein into 1–2-day-old syngeneic pups. Successful engraftment was confirmed five days post-transplantation using BLI and X-ray imaging for both leukemia (**Figure 2A**, $n = 7$) and NB (**Figure 3A**, $n = 9$), revealing that temporal vein injection achieved 100% procedural success, with no procedure-related mortality.

Neonatal mCMV infection suppresses leukemia progression

Following confirmation of B-ALL engraftment, neonatal pups were randomized to receive either PBS ($n = 3$) or mCMV (1×10^4 PFU per pup, $n = 4$) via intraperitoneal injection at 7 days of age. Ten days post-infection, mCMV-infected pups exhibited a significant reduction ($p < 0.001$, unpaired Mann–Whitney test) in leukemia burden compared with uninfected controls, as assessed by BLI and X-ray imaging (**Figure 2B, C**). Significant reduction in leukemia burden was

further confirmed by flow cytometric quantification of leukemic cells in the spleen and bone marrow ($p < 0.02$ and $p < 0.05$, respectively, unpaired Mann–Whitney test) (**Figure 2D**). These findings are consistent with our previous observations demonstrating an age-dependent protective effect of neonatal viral infection against B-ALL leukemogenesis¹¹.

Neonatal mCMV infection does not affect neuroblastoma progression

In contrast to the leukemia model, neonatal mCMV infection did not alter NB burden. BLI and X-ray imaging performed ten days post-infection revealed comparable tumor progression in infected and control NB-bearing pups with no statistically significant difference (**Figure 3B, C**, $n = 4$ control, $n = 5$ infected), indicating that early-life viral infection exerts cancer-type-specific effects.

FIGURE AND TABLE LEGENDS:

Figure 1. A schematic diagram of the overall experimental workflow

Figure 2. Neonatal mCMV infection suppresses B-ALL progression following temporal vein transplantation.

Luciferase-tagged leukemic cells were transplanted into postnatal day (P) 1–2 neonatal mice, followed by intraperitoneal injection of murine cytomegalovirus (mCMV) or PBS control at P7.

(A) Representative bioluminescence imaging (BLI) combined with X-ray imaging showing leukemic cell engraftment at P7 (prior to infection). (B) Leukemia burden at P17 (10 days post-infection). (C) Quantification of BLI signal at P7 and P17 demonstrates a significant reduction in leukemic burden in mCMV-infected mice compared with PBS-treated controls. Data are shown as mean $\pm$ SEM. Quantified BLI signal at P7 and P17 was compared between PBS and mCMV groups at each time point using unpaired Mann–Whitney tests, with Holm–Šidák correction applied across the two between-group comparisons. ($*p < 0.001$). (D) Gating strategy for analyzing flow cytometry data to identify leukemic cells 1.1. (FSC vs SCCs $\rightarrow$ singlets $\rightarrow$ lineage[−] 7-AAD[−] CD19⁺ $\rightarrow$ B220⁺ BP1⁺). (E) Flow cytometric quantification of leukemic cells in the spleen and bone marrow of PBS- or mCMV-treated transplanted mice at P17. Data are shown as mean $\pm$ SEM (unpaired Mann–Whitney test, $n = 3$ –4 mice per group; $**p < 0.02$, $*p < 0.05$).

BLI and X-ray images were acquired using consistent exposure settings 10 minutes after D-luciferin injection (BLI settings: exposure time 120 s for P7 images and 30 s for P17, binning B4 for P7 and B4 for P17 images, field of view (FOV) 25, f-stop 1.2, object height 1.5 and X-ray settings: resolution high, power high)

Figure 3. Neonatal mCMV infection does not affect neuroblastoma progression

Luciferase-tagged NB cells were transplanted into postnatal day (P) 1–2 neonatal mice, followed by intraperitoneal injection of murine cytomegalovirus (mCMV) or PBS control at P7. (A) Representative BLI combined with X-ray imaging showing NB cells engraftment at P7 (prior to infection) and (B) NB burden at P17 (10 days after infection); (C) Quantification of BLI signal at P7 and P17 demonstrates a comparable burden NB cells with no statistically significant difference in mCMV-infected mice ($n = 5$) and PBS-treated controls ($n = 4$). Data are shown as mean $\pm$ SEM.

Quantified BLI signal at P7 and P17 was compared between PBS (n = 4) and mCMV (n = 5) groups at each time point using unpaired Mann–Whitney tests, with Holm–Šídák correction applied across the two between-group comparisons.

BLI and X-ray images were acquired using consistent exposure settings 10 minutes after D-luciferin injection (BLI settings: exposure time 120 s for P7 images and 30 s for P17, binning B4 for P7 and B4 for P17 images, field of view (FOV) 25, f-stop 1.2, object height 1.5 and X-ray settings: resolution high, power high)

Table 1: Table of Materials

DISCUSSION:

Understanding how early-life immune development influences cancer progression is essential for designing age-appropriate immunomodulatory strategies. In this study, we describe a reproducible neonatal transplantation platform that enables precise temporal control of cancer cell engraftment and immune modulation during a critical window of postnatal immune immaturity. By combining temporal vein-mediated intravenous delivery with BLI, this model allows systematic interrogation of how early-life infection shapes pediatric cancer progression across distinct disease contexts.

Using this approach, we demonstrate that neonatal mCMV infection markedly suppresses leukemia progression following neonatal intravenous transplantation, whereas NB progression remains unaffected under identical experimental conditions. These contrasting outcomes highlight the context-dependent nature of infection-driven immune modulation in early life and underscore the importance of disease-specific biology when modeling pediatric cancer-immune interactions.

The observed reduction in leukemic burden following neonatal mCMV infection is consistent with our previous study¹¹. We have shown that neonatal viral infection can induce broad activation of innate immune pathways, including interferon gamma (IFN- γ) response which can induce apoptosis in preleukemic cells. In contrast, NB is a solid tumor derived from neural crest-origin cells and may be less susceptible to systemic immune effector mechanisms induced by neonatal infection or may possess intrinsic resistance mechanisms that counteract interferon-mediated immune pressure. The lack of effect observed in the NB model emphasizes that early-life immune activation does not uniformly impact all pediatric cancers.

Importantly, the strength of this study lies not only in the biological observations but also in the methodological framework it establishes. Temporal vein injection offers clear advantages over intraperitoneal and retro-orbital approaches for neonatal studies^{7–9,12,13}. In contrast to intraperitoneal delivery, which may result in delayed or variable systemic dissemination due to absorption kinetics within the peritoneal cavity, temporal vein injection provides direct intravenous access, enabling rapid and uniform systemic distribution of transplanted cells while minimizing procedure-related variability in neonatal models. Moreover, compared with retro-

orbital injection which can be technically challenging in neonatal pups and carries a risk of ocular injury, temporal vein injection is less invasive and more reproducible in early postnatal animals.

In this work, we introduce several practical refinements that further enhance the robustness and reproducibility of the technique. Specifically, the use of a Hamilton syringe coupled with a 30-gauge needle, together with playdough-based stabilization, enables smooth, controlled injection and improves precision. In addition, we implemented a simple transillumination setup, consisting of a napkin with a central aperture placed over a plexiglass platform with a cold light source positioned beneath, which substantially improves contrast and facilitates visualization of the temporal vein. As demonstrated by bioluminescent imaging, these refinements collectively led to high procedural success and efficient systemic distribution of transplanted cells, with no procedure-related mortality, thereby enabling reliable disease establishment across models.

The compatibility of this approach with sequential interventions such as infectious challenge, immune modulation, or therapy administration, provides flexibility for studying dynamic immune–tumor interactions during early development. Furthermore, the use of BLI permits longitudinal, non-invasive monitoring of disease progression, reducing inter-animal variability and enhancing experimental reproducibility. This approach can be readily adapted to other pediatric malignancies, infectious or inflammatory stimuli, and immunomodulatory strategies.

Several limitations should be considered. The immune responses elicited by mCMV in neonatal mice may not fully recapitulate those induced by common human childhood infections, and species-specific differences in immune development should be acknowledged. In addition, this study focused on short-term cancer progression, and longer-term outcomes, including immune memory or tumor relapse, were not assessed. Future studies integrating immune profiling, tissue-resident immune cell analysis, and extended longitudinal follow-up will be valuable for elucidating the mechanisms underlying the observed disease-specific effects. From a technical standpoint, the temporal vein is reliably accessible only during a limited postnatal window (P0–P3), after which venous access becomes impractical, representing an inherent limitation of the technique. Nonetheless, this model provides a robust and versatile framework for studying early-life immune influences on pediatric cancer progression and for evaluating immune-based preventive or therapeutic strategies.

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The authors have nothing to disclose.

AUTHOR'S CONTRIBUTION:

Maryam Aletaha, Zahra Savoji, and Nakaw Young developed the methodology and prepared the initial draft of the manuscript. Ali Farrokhi and Gregor S. D. Reid conceived and designed the study.

Maryam Aletaha and Ali Farrokhi performed data analysis and interpretation, including statistical and computational analyses. Ali Farrokhi supervised the study and prepared the final version of the manuscript. Gregor S. D. Reid reviewed and edited the manuscript.

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