Creating CRISPR-Cas9 Knockout Immune Cells using CHIME

Martin LaFleur (lafleur@fas.harvard.edu)
Harvard Medical School
Arlene Sharpe (Arlene_Sharpe@hms.harvard.edu)
Harvard Medical School
Thao Nguyen
Harvard Medical School
Matthew Coxe
Harvard Medical School
W. Nicholas Haining
Dana Farber Cancer Institute

Method Article

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Abstract

Therapies that target the function of immune cells have significant clinical efficacy in diseases such as cancer and autoimmunity. Although functional genomics has accelerated therapeutic target discovery in cancer, its use in primary immune cells is limited because vector delivery is inefficient and can perturb cell states. Here we describe CHIME: CHimeric IMmune Editing, a CRISPR-Cas9 bone marrow delivery system to rapidly evaluate gene function in innate and adaptive immune cells in vivo without ex vivo manipulation of these mature lineages. This approach enables efficient deletion of genes of interest in major immune lineages without altering their development or function. We use this approach to perform an in vivo pooled genetic screen and identify Ptpn2 as a negative regulator of CD8+ T cell-mediated responses to LCMV Clone 13 viral infection. These findings suggest that this genetic platform can enable rapid target discovery through pooled screening in immune cells in vivo.

Introduction

Current approaches to study genes in immune cells using shRNA or CRISPR technology involve stimulating the immune cells in vitro, transducing them with a lentiviral vector, and transferring them back in vivo. These approaches are limited by inefficient vector delivery, the requirement for stimulation which can perturb cell states, and their use on populations that can be easily transferred in vivo. Here we circumvent those issues by transducing hematopoietic stem cells with a lentiviral gene-targeting sgRNA expression vector, which are then used to make bone marrow chimeras. The resulting immune system of these chimeras is deleted for the gene of interest yet is naive and unstimulated. See figure in Figures section.

Reagents

-Cas9-expressing donor mice -WT recipient mice -Kim wipes -ACK lysis buffer \(Thermo Fisher Scientific A1049201) -Retronectin \(Takara Bio T100B) -Falcon® 24 Well Polystyrene Clear Flat Bottom Not Treated Cell Culture Plate, with Lid \(Corning 351147) -RPMI 10% FBS media -Mortar and pestle -CD117 mouse microbeads \(Miltenyi 130-091-224) -SFEM media \(STEMCELL Technologies 09600) -Sort antibodies from Biolegend in PE \(Ter-119 clone TER-119, CD11b clone M1/70, Gr-1 clone RBC-8C5, CD3e clone 145-2C11, CD5 clone 53-7.3, B220 clone RA3-6B2) -TPO \(Peprotech 315-14) -SCF \(Peprotech 250-03) -Flt3-Ligand \(Peprotech 250-31L) -IL-7 \(Peprotech 217-17) -sgRNA-expressing lentivirus \(Addgene plasmid pXPR_053) with defined titer -Filter top FACS tubes \(VWR 352235) -Eppendorf 1.5 mL tubes with caps -96 well flat bottom plate \(VWR 62406-081) -50 mL conicals -70 micron cell strainer \(VWR 21008-952) -70% ethanol -1 mL TB syringe \(VWR BD309623) -Ethanol wipes -Sulfamethoxazole Trimethoprim Antibiotic

Equipment

-BD Aria II \(or equivalent sorter) -Miltenyi magnets -Centrifuge capable of holding 50 mL conicals -Table top centrifuge \(for eppendorf size tubes) -Dissection tools -Caesium 137 irradiator \(or equivalent) -Heat lamp -Mouse restrainer \(Braintree Scientific)

Procedure

Day 1: Part 1: Retronectin Coating Step 1: Take non-TC treated 24-well polystyrene plate and add 300 microliters per well of Retronectin diluted to 100 microgram/mL in PBS. Note # of wells = # of donor mice x 4. Step 2: Place in fridge overnight wrapped in saran wrap. Note can keep for several days. Part 2: LSK Isolation Step 1: Sacrifice mouse and pin down at the neck. Step 2: Cut skin down legs fully. Pull leg until you hear a pop. Cut away muscle and pull off tibia/femur and place in RPMI 10% FBS on ice. Step 3: Remove pelvic bones and place in RPMI 10% FBS on ice. Step 4: Remove spine and cut into half inch sizes pieces and place in RPMI 10% FBS on ice. Step 5: Clean excess tissue from the bones using a Kim wipe and scissors. Place clean bones in 50 mL conical with RPMI 10% FBS on ice. Step 6: Clean mortar and pestle with 70% ethanol and air dry. Note: treat mortar and pestle and all subsequent steps with sterile technique. Step 7: Dump bones in mortar. Wash 2x with 70% ethanol. Step 8: Wash 2x with RPMI 10% FBS. Step 9: Add 10 mL of RPMI 10% FBS to mortar. Grind up bones with pestle until white. Step 10: Move the supernatant \(leaving bones in mortar) to a 50 mL conical with 70 micron filter top. Step 11: Repeat steps 9 and 10. Step 12: Bring up volume to 50 mL with RPMI 10% FBS. Spin conical at 400g for 8 minutes at 4°C. Step 13: Remove supernatant and resuspend the pellet in 1 mL of ACK lysis buffer per mouse \(4 donors = 4 mL). Incubate for 2 minutes at room temperature. Step 14: Neutralize the ACK lysis buffer with 50 mL RPMI 10% FBS. Pipette through a 70 micron filter into a new 50 mL conical. Step 15: Spin conical at 400g for 8 minutes at 4°C. Step 16: Remove the supernatant. Resuspend the pellet in 2 mL of MACS buffer \(500 mL PBS + 5 mL FBS + 2 mM EDTA). Step 17: Add 120 microliters of CD117 microbes per mouse \(4 mice = 480 microliter) and mix. Incubate on ice for 20 minutes. Step 18: Bring up volume to 50 mL with MACS buffer. Spin conical at 400g for 8 minutes at 4°C. Step 19: During the spin setup Miltenyi MACS magnets with 3 mL LS columns \(2 columns per mouse). Equilibrate with 3 mL of MACS buffer. Step 20: Remove supernatant and resuspend cells in 4 mL of MACS buffer and pipette through a 70 micron filter into a new 50 mL conical. Add MACS buffer such that the final volume in mL = 4 x # of mice \(16 mL for 4 mice). Step 21: Pipette 2 mL into each column and allow the unbound cells to drip into a waste reservoir. Step 22: Add 3 mL of MACS buffer to each column and allow the wash to drip into a waste reservoir. Repeat for a total of 2 washes. Step 23: Add 5 mL of MACS buffer to each column and remove from the magnet. Place the column over a 50 mL collection tube and using the plunger provided with LS columns forcefully plunge the 5 mL into the collection tube. Note: do 4 columns at a time \(maximum) to ensure the 5 mL elution does not drip through into the waste reservoir while plunging other columns. Step 24: Bring up volume to 50 mL with MACS buffer. Spin conical at 400g for 8 minutes at 4°C. Step 25: Remove supernatant and resuspend cell pellet in 1 mL of antibody stain per 4 mice. Antibody stain is composed of 1 mL of MACS and 10 microliter each of the antibodies: Ter119-PE, CD3e-PE, CD5-PE, B220-PE, CD11b-PE, Gr1-PE, cKit-APC, and Sca1-BV421. Cover with tinfoil and stain for 30 minutes on ice. Step 26: Bring up volume to 50 mL with MACS buffer. Spin conical at 400g for 8 minutes

at 4°C. Step 27: Remove supernatant and resuspend pellet in 0.5 mL per mouse. Pipette through a Polystyrene filter top tube slowly. Wash the top of the filter top tube with an additional 0.5 mL per mouse. Remove the filter top with a solid top and place cells on ice. Step 28: Prepare sort collection tubes. Place 500 microliter of SFEM supplemented with 100 ng/mL of the following cytokines: SCF, TPO, Flt3-Ligand, and IL-7. Keep collection tubes on ice. Step 29: Ensure sorter is setup properly \(Accudrop, test sort, and laser delay). Load sample onto sorter and draw the following sequential gates \(plot attached in images): Live cells \(based on FSC vs SSC) -> Singlet cells \(based on FSA vs FSH) -> Lineage– cKit+ \(based on Lineage-PE vs cKit-APC) -> cKit+ Sca1+ \(based on cKit-APC+ Sca1+). See figure in Figures section. Step 30: Sort your Lineage– cKit+ Sca1+ \(LSK) cells into your collection tube until you run out of sample. Step 31: Once sort is complete spin collection tube at 400g for 10 minutes at 4°C. Step 32: Remove supernatant and resuspend pellet in SFEM supplemented with SCF, TPO, Flt3-Ligand, and IL-7 \(as above). Resuspend with 200 microliter per 100K sorter counted cells. Step 33: Plate the resuspended cells in a 96 well flat bottom plate at 200 microliter per well. Place in a standard 37°C tissue culture incubator overnight. Day 2: LSK Transduction Step 1: Fast temp a centrifuge to 37°C. Step 2: Look at plated LSK and check for contamination \(cloudy media, visible bacteria under microscope). If contaminated will need to repeat the experiment. Step 3: Aspirate liquid from Retronectin coated plate and add 300 microliter PBS to each well pipetting up and down to wash the well. Then aspirate liquid again. Step 4: Pipette the 200 microliter of plated LSK into the Retronectin coated plate wells \(1 96 well goes into 1 24 well). Step 5: Add titered lentivirus on top of LSK into the 24 well plate. Volume of lentivirus is calculated as: Volume in mL = 100,000 LSK ** MOI of 30/ Concentration of lentivirus in viral particles per mL. For example 100,000 LSK ** MOI of 30/10,000,000 particles per mL = .333 mL. Step 6: Add SFEM supplemented with cytokines \(as above) such that the final volume in the well is 400 microliters. SFEM Volume = 400 microliter - 200 microliter \(LSK) - lentivirus volume in microliter. Pipette up and down in the well gently to mix. Step 7: Prepare a balance plate and spin the plate at 650g for 1.5 hours at 37°C with an acceleration of 2 and a brake of 1. Step 8: When spin is complete move the plate to a 37°C tissue culture incubator for 1 hour. Step 9: Pre-warm SFEM supplemented with cytokines \(as above) to 37°C. Prepare 500 microliter * the # of wells in the plate you are using. Step 10: After the hour is complete add 500 microliters prewarmed stem cell media \(with cytokines) on top of the LSK. Place back in the incubator overnight. Day 3: LSK Transfer to Irradiated Recipients Step 1: Look at plated LSK and check for contamination \(cloudy media, visible bacteria under microscope). If contaminated will need to repeat the experiment. Step 2: Irradiate recipient mice with 600 rads in a suitable irradiator. Step 3: Dose the mice with 200 mg sulfamethoxazole and 40 mg trimethoprim per 250 mL of drinking water. Continue this for the next 2 weeks. Step 4: Three and a half hours after the first irradiation pipette stem cells into a 50 mL conical and bring up the volume to 50 mL with PBS. Step 5: Spin conical at 400g for 8 minutes at 4°C. Step 6: Remove supernatant and resuspend in PBS in a volume equal to \(200 microliter * the number of wells that had LSK) + 300 microliter. Place this in a suitable tube on ice. Step 7: Irradiate recipient mice with 600 rads in a suitable irradiator. Step 8: Inject the LSK intravenously \(200 microliter per mouse). For tail vein injections it is recommended to heat the mouse with a heat lamp before injection, place in a restrainer, and wipe the injection site with ethanol. These steps increase your chance of a successful injection. Step 9: After injecting the mice place back into their cage and allow to

reconstitute over the next 8 weeks. Days 4-17: Antibiotic Treatment Step 1: Dose the mice with 200 mg sulfamethoxazole and 40 mg trimethoprim per 250 mL of drinking water. Continue this, changing the drinking water as needed, for a total of 2 weeks post LSK injection.

Timing

Day 1: LSK isolation \(6 hours for 4 mice) Day 2: Spin transduction \(3 hours) Day 3: Irradiation and transfer \(5 hours) Time until have reconstituted chimeras: 8 weeks

Troubleshooting

\(1) LSK contamination-This can be due reagents becoming contaminated, improper sterile technique, and excess bubbles during sort collection and subsequent LSK pipetting steps. \(2) Miltenyi column clog-This can be due to overloading column beyond the recommended values above or skipping any of the 3 filtering steps that occur prior to the MACS isolation. \(3) Death of chimeras within 2 weeks post LSK transfer-This can be due to issues with the health or numbers of LSK that were transferred into the mice. \(4) Following reconstitution have low Vex \(transduction marker) %-This can be due to using an inaccurate amount of virus that is much lower than the recommended MOI of 30. \(5) Following analysis of chimeras in an experiment you find that the knockout % of the gene of interest is low. This can be due to a variety of factors including: \(1) poor design of the sgRNA that has low predicted on-target activity or \(2) low expression of Cas9 in the immune lineage of interest or the mouse as a whole. Note we find that there is about half the % knockout when using Cas9 mice that are heterozygous for the Cas9 allele as opposed to homozygous for the Cas9 allele. \(6) Loss of knockout cells following transfer into a WT host. We find that this is because Cas9 is an immunogen in C57BL6 WT mice from Jackson. To circumvent this you can transfer your gene-deleted Cas9-expressing cells to a Cas9-expressing host, which will be tolerized to the antigen.

Anticipated Results

\(1) Sorting about 300K LSK per donor. \(2) Successful survival of the mice 2 weeks post LSK transfer. \(3) A vex transduction marker % in the reconstituted immune system of around 40%. \(4) Approximately 80% of cells that are knockout for the gene of interest in the Vex+ population.

References

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Figures

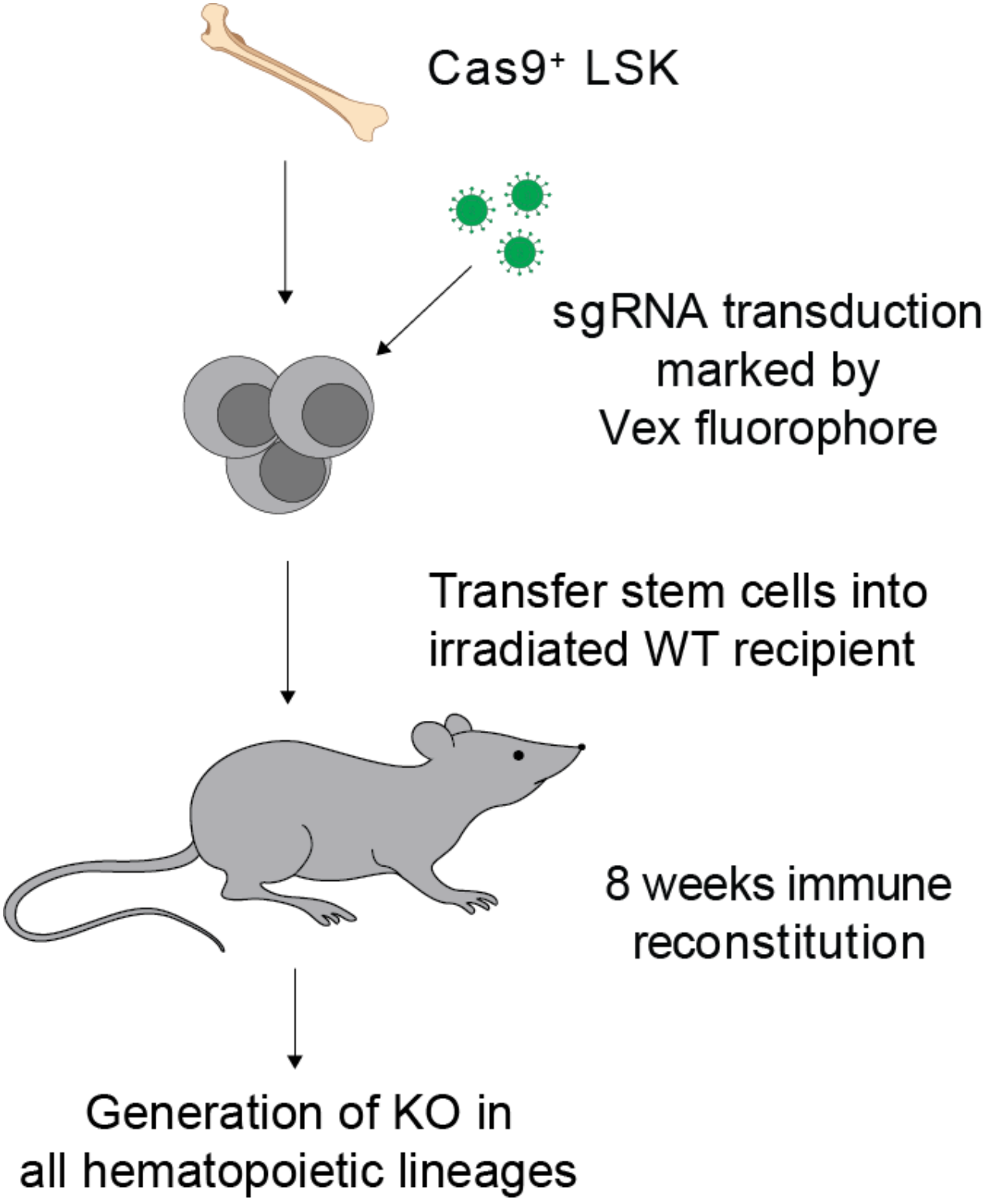

Figure 1

CHIME Schematic Cas9-expressing mice are sacrificed and bones isolated. LSK cells are isolated from the bones. The next day the LSK are spin-transduced in vitro with a sgRNA and Vex-expressing lentiviral vector. The following day the cells are injected intravenously into irradiated recipient animals.

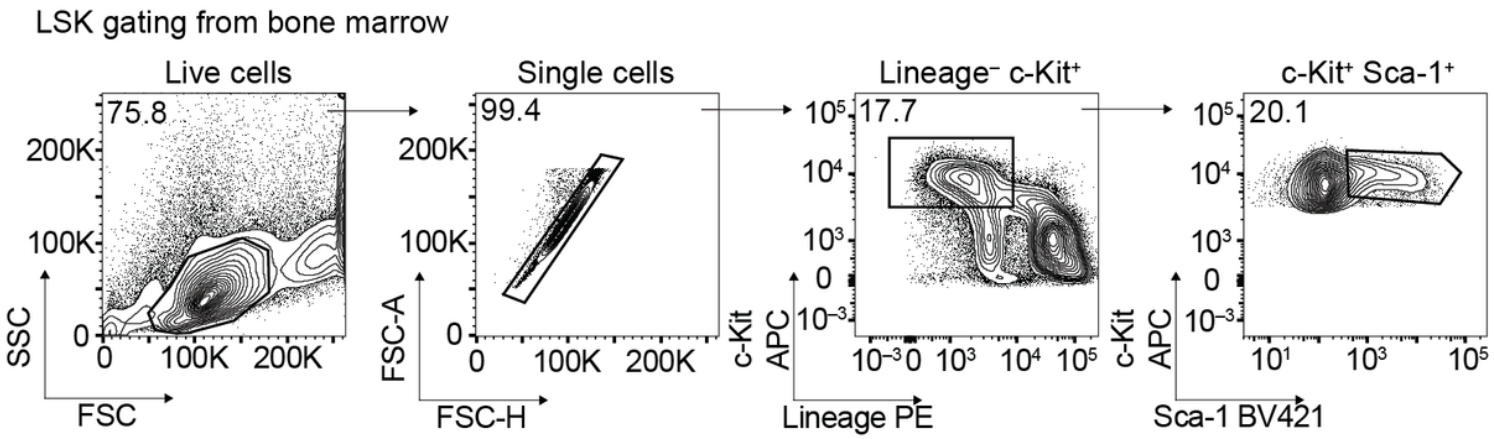

Figure 2

LSK sort gates from bone marrow LSK cells are gated first on live cells, then singlets, then on cKit+ Lineage–, and then on cKit+ Sca1+.