An optimized gene knock-out strategy for human CD34+ hematopoietic stem and progenitor cells.

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Abstract
Human CD34$^+$ hematopoietic stem and progenitor cells (HSPCs) are a standard source of cells for clinical HSC transplantations as well as experimental xenotransplantation studies. To further extend the range of applications of these humanized mice, we developed a protocol to efficiently edit the genomes of human CD34$^+$ HSPCs before transplantation. In the past, manipulating HSPCs has been complicated by the fact that they are inherently difficult to transduce with lentivectors, and rapidly lose their stemness and engraftment potential during *in vitro* culture. However, with optimized nucleofection of sgRNA:Cas9 ribonucleoprotein complexes, we are now able to edit a candidate gene in CD34$^+$ HSPCs with almost 100% efficiency, and without affecting their potential for engraftment and multilineage differentiation in mice. The result is a humanized mouse from which we knocked out a gene of interest from their human immune system.

Introduction

CD34 expression serves as a selective marker of immature hematopoietic cells [1]. In clinical practice, CD34 is used to evaluate and ensure rapid engraftment in HSC transplants [2, 3]. CD34+ populations in bone marrow or blood samples are a hematopoietic stem/progenitor mix, of which the majority of cells are progenitors [4]. Human, donor derived CD34+ hematopoietic stem and progenitor cells (HSPCs) populations have become a standard source of cells for allogenic and autologous HSC transplantations [5, 6]. As a result, there has been intense interest in genetic manipulating CD34+ HSPCs for use in the treatment of hematopoietic-related diseases, ranging from sickle cell disease (e.g.,[7, 8]) to severe combined immunodeficiency [9, 10] to HIV/AIDS [11] to cancers [12].

CD34+ HSPCs are also used as *in vitro* and *in vivo* experimental models in conjunction with functional assays (e.g., colony formation, differentiation) and xenotransplantation. For *in vivo* work, novel strains of recipient mice have been developed that better support long term human hematopoiesis and multilineage development of CD34+ cells, including B and T lymphocytes, natural killer cells, monocytes, macrophages and dendritic cells [13].

For gene manipulation experiments in HSPCs, there have been numerous attempts to optimize lentiviral (lv) gene expression platforms, including those incorporating CRISPR-Cas9- or RNAi-based elements, using alternate viral envelop proteins, promoters, viral elements etc. (e.g., [14-17]). However, in our experience such enhanced lv-vectors are still subject one or more biological limitations inherent to HSPCs, either: low infection efficiencies, transgene silencing, and/or transduction associated toxicity.

Others have begun to develop optimized protocols for gene editing using that incorporate delivery of sgRNA:Cas9 nuclease ribonucleoprotein (RNP) complexes to *ex vivo* cultured human CD34+ cells. In some cases, they have reported genomic insertion-deletion (indel) frequencies greater than 90% [18]. However, these protocols still leave room for further improvement. For example, the optimized method reported by Wu. *et al.* requires the use of non-commercially available Cas9, containing an additional NLS [18]. Meanwhile, Modarai *et al.* observed highly variable indel frequencies across patient samples when utilizing the most widely published nucleofection conditions for human CD34+ cells [19].

Here, we report adapting and optimizing a method of sgRNA:Cas9 RNP nucleofection we use for other primary cell systems [20] to human CD34+ cells. With this optimized protocol, we consistently achieve a greater than 95% knockout efficiency using standard commercially available reagents and eliminate donor-specific variability in indel frequency previously reported [19]. Utilizing this protocol, we are also able to simultaneously target at least 3 different genes with minimal loss in knockout frequency. We have also optimized the protocol for transplantation of human CD34+ cells into humanized mice.

Results

Optimization of CD34+ cell nucleofection conditions for delivery of RNA and RNPs.

Most publications employing Lonza's 4D nucleofection technology for delivery of sgRNA:Cas9 RNPs or RNA into CD34+ cells utilize the transfection conditions outlined in the publicly available nucleofection database (knowledge.lonza.com) or very similar conditions. This standard procedure uses Kit P3 and nucleofection program EO-100 with some publications using program ER-100 [18, 19]. The reported editing efficiencies under these conditions range from 15-90%, with cell viability between 40 and 80% and significant variability in donor to donor editing efficiency [18, 19]. Based on unpublished publicly presented data, we decided to try and optimize for a more consistent editing efficiency of CD34+ cells across donors and CD34+ cell source (e.g. BM, G-CSF mobilized). Utilizing chemically modified mCherry RNA (Trilink), for quantification of nucleofection efficiency, we compared the standard EO-100 program versus DS-120 and DS-150 (Figure 1a). For both programs we measured a modest increase in nucleofection efficiency versus the standard protocol (EO-100: 83.3%; DS-120: 87.7%; DS-150: 92.7%)(Figure 1A), but a significant increase in levels of mCherry expression within the cells (mCherry high EO-100: 66.3%; DS-120: 79.2%; DS-150: 84%)(Figure 1A). We opted to use the DS-150 program going forward, since it resulted in a larger increase in nucleofection efficiency relative to control conditions (9.4% vs. 4.4%) even though program DS-120 had an overall higher level of mCherry expression. (Figure 1A). We also observe a difference in cell viability between the programs, but our overall cell viability was significantly higher than reported by others (Figure 1A) [18] (knowledge.lonza.com).

Based on previous work in the lab to optimize sgRNA:Cas9 RNP editing in other primary cells and the work of others in CD34+ cells [18], we were able to use a limited range of sgRNAs amounts and a fixed 2:1 sgRNA to Cas9 ratio for further optimization of sgRNA:Cas9 RNP editing in CD34+ cells. We also standardized on using sgRNAs with 2'-O-methyl 3' phosphorothioate modifications (in the first and last three nucleotides) from Synthego and 2xNLS SpCas9 from Aldevron. We tested the efficiency of indel formation and gene KO with sgRNA amounts of 12.5, 25, and 50 pmol per sgRNA and 200 thousand cells in a 20 μl nucleofection reaction (Figure 1b) with dual sgRNA targeting YTHDF2. Indel and KO efficiency was quantified as previous described [20], from Sanger sequencing data and Inference of CRISPR Edits (ICE) analysis provided by Synthego. Maximal gene KO was detected using either 25 or 50 pmol of sgRNA with only a large reduction when using 12.5 pmol (Figure 1B). Interestingly, the reduced editing in the 12.5 pmol sample was due to near complete loss of the fragment deletion between the two sgRNAs. This suggests there is a threshold amount of RNP required to ensure the simultaneous editing necessary for a fragment deletion independent of the activity of the sgRNAs. Given these results, it maybe possible to utilize a smaller amount of RNP than we observed if the sgRNAs are pre-validated to have a high KO efficiency independently.

RNP nucleofection for the simultaneous targeting of 3 or more genes.

The ability to simultaneously KO multiple genes has utility in a variety of situations. Among these is looking for gene dependencies when trying to identify cancer vulnerabilities [21, 22]. and targeting paralogous gene families where loss of one gene maybe functionally compensated for by other genes in the family [23]. The main technical limitation to target multiple genes by nucleofection is that solutions containing

sgRNAs and Cas9 cannot exceed approximately 15-20% of the total volume of the 20 μl reaction. Additionally, very high KO efficiencies must be maintained for all genes, since the more genes that are simultaneously targeted, the greater the effect that small decreases in KO efficiency have on combined targeting efficiency of all the genes. To validate this approach, we simultaneously targeted three YTHDF m6A-binding proteins (DF1, DF2, DF3). Based on our single sgRNA results, we nucleofected 6 sgRNA pools containing 2 sgRNAs per gene and 25 pmol of each sgRNA. The pooled targeting resulted in 100% indel efficiency and 88-100% KO efficiency for all the genes (Figure 1C). When using pre-validated sgRNAs with 95% or greater KO efficiency, this pooled approach should allow the targeting of up to 6 independent genes with approximately 70% of cells being knocked out for all 6 genes and almost all cells having at least a single copy knocked out.

RNP nucleofection recapitulates a lentivirus delivered sgRNA block to erythropoiesis.

To validate the optimized RNP nucleofection conditions in a biological context, we opted to target METTL3 for KO. METTL3 is a core component of the N^6-methyladenosine (m^6A) mRNA methyltransferase (MTase) complex, and we have previously reported that it's knockdown (KD) by lentivirus delivered shRNAs results in a developmental block to erythropoiesis [24]. Human CD34+ HSPCs expanded for 4 days, as previously described, were nucleofected with RNPs generated from a pool of two METTL3 sgRNAs containing 50 pmol of each. Following the nucleofection the cells were placed in erythroid differentiation conditions. Indel and KO frequency was quantified by ICE analysis in cells collected three days post nucleofection and erythroid differentiation by flow cytometry analysis 6-days post nucleofection (Figure 1D). We detected 91.2**%** gene KO and a block to erythroid differentiation of equivalent magnitude to shRNA KD (Figure 1D).

RNP nucleofected CD34+ cell transplantation into humanized mice results in greater than 95% loss of target gene expression *in vivo*.

Previous efforts at gene editing in human CD34+ cells have shown both variability in editing efficiency and persistence of the edits in long-term engrafting HSCs (LT-HSC). Our optimized nucleofection protocol has address the first of these issues. To minimize the loss of LT-HSCs, we further optimized the protocol to minimize the time the cells are cultured *in vitro* before injection into a mouse. In brief (Figure 3a), Human fetal liver HSPCs were thawed out and cultured for 4 hours in our standard HSPC expansion media, to allow them to recover from the thawing process. The cells were then nucleofected utilizing our optimized protocol and after a second recovery period of 4 hours, 10,000 cells were injected into the livers of 2-day old MSTRG mice as previously described [13]. Initial editing was assessed by ICE analysis after *in vitro* culture of a subset of the cells for 3 days. *In vivo*, KO was assessed by flow cytometry of blood 8-weeks post-transplant.

CD33 (SIGLEC3) is a receptor for sialic acid proteins and is primarily expressed on myeloid cells [25]. We targeted CD33 for KO in our initial *in vivo* test since it doesn't have a developmental phenotype when expression is lost and is co-expressed with other common myeloid markers [26]. ICE analysis detected approximately 87% KO of

CD33 in the fetal liver HSPCs 3-days post nucleofection. Interestingly, we detected a significantly more penetrant phenotype *in vivo*. We detected an average of 0.126% of hCD45+ cells expressing hCD33 in KO mice (n=9) versus 12.9% in control mice (n=6) (Figure 2B,C).

N^6-methyladenosine (m^6A) is an abundant RNA modification that affects the methylated transcripts in a variety of ways including altered stability and translation [27]. This regulation occurs through a host of methyltransferases, demethylases and m^6A reader proteins, with METTL3 as one of the core components of the methyltransferase complex [28, 29]. Regulation by m^6A has been shown to play key roles throughout hematopoiesis, with loss of METTL3 leading to enhanced myelopoiesis and reduced erythropoiesis and T cell development [30, 31]. Hematopoietic specific KO of METTL3 in mice results in globally dysfunctional hematopoiesis and a marked reduction in HSPC engraftment [24]. To investigate whether this observation is also relevant during human hematopoiesis we utilized the optimized *in vivo* editing and transplantation protocol from above to target METTL3. Our results recapitulate the observation in mice with a near total loss of human hematopoietic cells *in vivo* in the METTL3 KO mice compared to control (Figure 2D)(CD45+: 0.13% n=11 vs. 27.81% n=8). Similar to the CD33 KO experiment (Figure 2B,C) the *in vivo* phenotype was larger than the KO efficiency predicted by ICE analysis of 90%.

Discussion

Here, we describe an improved method for highly penetrant biallelic indel formation in human CD34+ HSPCs via the use programable sgRNA:Cas9 RNP nuclease complexes. This optimized protocol is higher in efficiency than previously published methods [18, 19], including those currently in clinical trials [7], and works similarly well in adult and fetal HSPCs. A key advantage of this method is that single or multiple genes can targeted. For example, we show complete or complete loss of multiple genes and gene products, including CD33, METTL3, and 3 paralogs (YTHDF1/2/3), singly and together.

Another advantage is that due to highly penetrant targeting, this method can be used to create precise deletions using two nearby sgRNA targeting sequences, either in protein coding genes (e.g., removal of exon) or in non-code DNA elements such as non-coding RNAs and transcriptional enhancers/repressors [32]. Further, given the ability to scale down nucleofections, it is also amenable for small to medium-scale function genetic screens.

There are some limitations and pitfalls to this method, however. First, the nucleation apparatus used is more expensive than traditional electroporators and has proprietary software and settings (e.g., waveforms, capacitance, etc.) that cannot be readily reversed engineered. Second, chemically synthesized sgRNAs are significantly more expensive than the DNA oligos used for standard sgRNA cloning. Third, human CD34+ cells are not widely available and do require special handling and culture conditions. Fourth, it has been shown that nuclease-based gene editing can result in large chromosomal rearrangements in a portion of cells [33]. Thus, both multiple controls are warranted for long-term biological assays are used *in vitro* or, especially*, in vivo*. None-the-less, this should be a useful method for gauging gene or genetic element requirement in pre-clinical studies of human HSPCs and their progeny.

Methods

CD34+ HSPC culture

The G-CSF mobilized CD34+ cells were purchased from the Fred Hutchinson Cancer Center Co-Operative Center for Excellence in Hematology. The cells were quickly thawed in a 37C water bath followed by graduated osmotic equilibration by doubling the total volume with PBS + 0.5% BSA every 2 minutes for a total of 5 doubling. Following centrifugation, the cells were cultured in Stem Span II (Stem Cell Technologies) supplemented with 100 ng/mL each of TPO, IL-6, SCF and FLT-3 ligand. For all the nucleofection optimization experiments the cells were expanded for 3 days prior to nucleofection and cultured in this media for 3 days post nucleofection.

For the erythroid differentiation test the cells were initially expanded as described above. Following nucleofection, the cells were culture as described in Uchida et al. 2018 [34], in erythroid differentiation media consisting of IMDM supplemented with 20% Knockout Serum Replacement (Thermo Fisher Scientific) 2 U/mL EPO, 10 ng/mL SCF, 1ng/mL IL-3, 1 μM dexamethasone and 1 μM estradiol for 5 days. The cells were then transitioned to Erythroid maturation media containing in IMDM supplemented with 20% Knockout Serum Replacement, 2 U/mL EPO, 10 ng/mL insulin and 0.5 mg/mL holo-transferrin for 2 days followed by flow cytometry analysis. EPO was purchased from PeproTech and all other cytokines were purchased from Shenandoah Biotechnology.

Flow cytometry

To monitor erythroid differentiation in the *in vitro* cultures cells were stained with BUV395-CD235a (BD Biosciences 563810) and APC-CD71 (BD Biosciences 341029) and analyzed on a BD Symphony. To evaluate the engraftment levels and multilineage engraftment of human hematopoietic cells in mice, blood was obtained by retro-orbital collection, and RBCs were eliminated by ammonium-chloride-potassium lysis. WBCs were analyzed by flow cytometry, following standard procedures. The following Ab clones were used (all purchased from BioLegend): anti-human Abs CD3-AF700 (HIT3a), CD19-PE-Cy7 (HIB19), CD33-APC (WM53), CD34-PE (581), CD45-Pacific Blue (HI30), and NKp46-PE (9E2) and anti-mouse Abs CD45-BV605 (30-F11) and Ter119-PerCP (TER-119). Human hematopoietic cells were gated on the basis of expression of human CD45 and exclusion of mouse CD45 and Ter119 staining. Dead cells were excluded by staining with 7-aminoactinomycin D.

MISTRG mice engraftment studies

De-identified human fetal liver tissues, obtained with informed consent from the donors, were procured by Advanced Bioscience Resources, and their use was determined as non–human subject research by the Fred Hutch Institutional Review Board (6007-827). Fetal livers were cut in small fragments and then treated for 45 min at 37°C with collagenase D (Roche; 100 ng/ml), and a cell suspension was prepared. Hematopoietic cells were enriched by density gradient centrifugation (Lymphocyte Separation Medium; MP Biomedicals), followed by positive immunomagnetic selection with anti-human CD34 microbeads (Miltenyi Biotec). Purity (>90% CD34+ cells) was confirmed by flow cytometry, and cells were frozen at −80°C in FBS containing 10% DMSO.

MISTRG mice (M-CSFh/h IL-3/GM-CSFh/h SIRPαh/m TPOh/h RAG2−/− IL-2Rγ−/−) were previously reported [13, 35]. Newborn mice (days 1–3) were sublethally irradiated (80 cGy γ-rays in a [137Cs] irradiator), and ~20,000 CD34+ cells in 20 µl of PBS were injected into the liver with a 22-gauge needle (Hamilton Company), as previously described [35]. Engraftment levels were measured as the percentage of human CD45+ cells among total (mouse and human combined) CD45+ cells in the blood. Mice with engraftment levels less than 10% were excluded from further experimentation. All animal experiments were approved by the Fred Hutchinson Cancer Research Center (Fred Hutch) Institutional Animal Care and Use Committee (protocol 50941).

CRISPR editing analysis

Nucleofected cells were harvested at indicated timepoints and genomic DNA was extracted (MicroElute Genomic DNA Kit, Omega Bio-Tek). Genomic regions around CRISPR target sites were PCR amplified using Phusion polymerase (Thermo Fisher Scientific) and primers located at least 250 bp outside sgRNA cut sites. After size verification by agaorse gel electrophoresis, PCR products were column-purified (Monarch PCR & DNA Clean-up Kit, New England BioLabs) and submitted for Sanger sequencing (Genewiz) using unique sequencing primers. The resulting trace files for edited cells versus control cells (nucleofected with non-targeting Cas9:sgRNA RNPs) were analyzed for predicted indel composition using the Inference of CRISPR Edits (ICE) web tool [36].

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Contributions

D.A.K. and P.J.P. conceived of the initial idea. D.A.K., J.L., P.J.P., and A.R. designed follow up. D.A.K. and J.L. performed experiments. D.A.K., A.R. and P.J.P. wrote and revised the manuscript.

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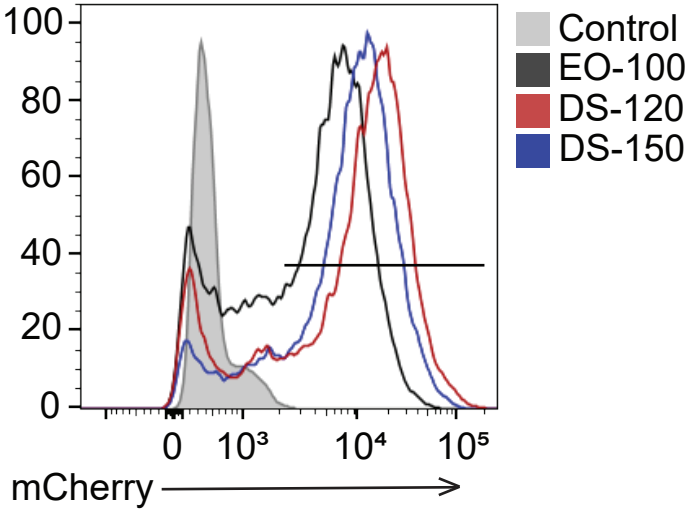
Figure Legends

Figure 1: CRISPR/Cas9 RNP editing of human CD34+ hematopoietic stem and progenitor cells.
A, Optimization of the Lonza nucleofection program selection for CD34+ HSPCs by introducing chemically modified mCherry RNA. Levels of mCherry expression were quantified 2-days post-nucleofection by flow cytometry. **B,** Optimization of input sgRNA amounts when nucleofecting dual sgRNAs for gene knockout. The listed pmole amounts were added for each sgRNA in combination with Cas9 in a 2:1 molar ratio. Three days post-nucleofection gDNA was isolated and CRISPR activity quantified by Sanger sequencing and ICE analysis. **C,** Simultaneous triple gene KO by sgRNA:Cas9 RNP nucleofection. CD34+ cells were nucleofected with a pool of RNPs targeting the three YTHDF RNA binding proteins. Each pool contained 25 pmol each of 6 sgRNAs (2 sgRNAs per gene) with Cas9 in a 2:1 ratio. Three days post-nucleofection gDNA was isolated and CRISPR activity quantified by Sanger sequencing and ICE analysis. **D,** Representative flow cytometry results comparing the *in vitro* erythroid differentiation phenotype when METTL3 is either KO by CRISPR/Cas9 RNPs or lentiviral shRNA knockdown. Cells were assayed by FACS 7-9 days post-nucleofection or post-transduction. All viable cells from the nucleofection are shown and all viable and transduced cells are show for the shRNA KD (n=3).

Figure 2: High efficiency in vivo gene KO following transplantation of CRISPR/Cas9 RNP edited human CD34+ cell into the MSTRG humanized mouse model.
A, The optimized workflow for transplantation of CRISPR/Cas9 RNP edited CD34+ human cells into MSTRG mice. **B,** Representative flow cytometry results from blood of MSTRG mice 8-weeks post-transplantation, comparing the co-expression of CD33 and CD14 on human CD45+ cells in either CD33 RNP KO or control mice. **C,** The frequency of select hCD45+ immune cell populations in blood of MSTRG mice 8-weeks post-transplantation of CD33 KO or control human CD34+ cells. **D,** The frequency of select hCD45+ immune cell populations in blood of MSTRG mice 8-weeks post-transplantation of METTL3 KO or control human CD34+ cells.

Figure 1

A



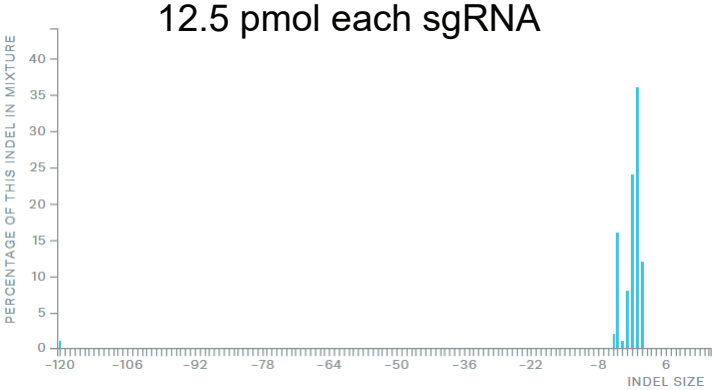
	EO-100	DS-120	DS-150
mCherry%	83.3	87.7	92.7
High mCherry%	66.3	79.2	84
Viability %	62.3	87.8	89.1

B

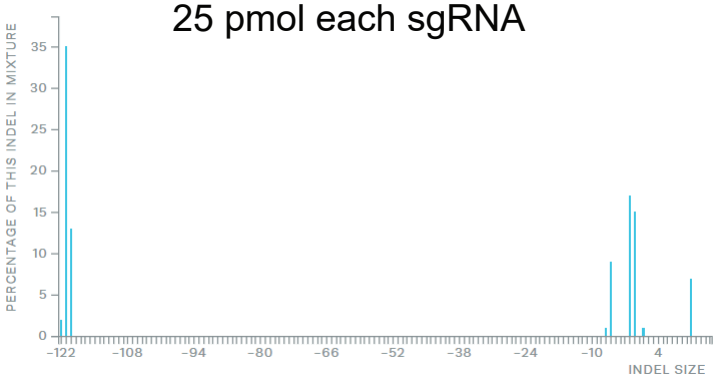
YTHDF2 dual sgRNA

sgRNA	12.5 pmol	25 pmol	50 pmol
Indel %	64	100	100
Knockout %	63	90	92

12.5 pmol each sgRNA



25 pmol each sgRNA



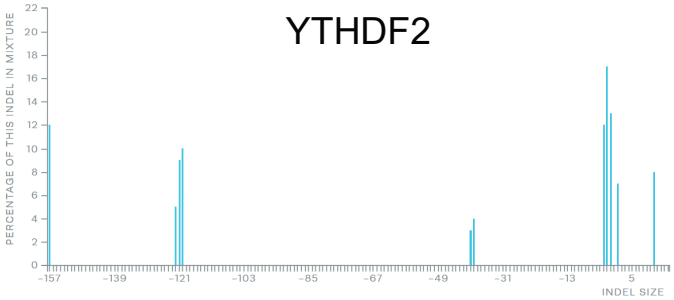
C

YTHDF Triple Gene KO

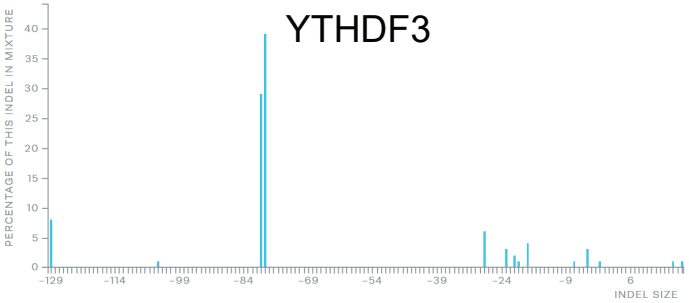
Gene	YTHDF1	YTHDF2	YTHDF3
Indel %	100	100	100
Knockout %	100	88	95

YTHDF1

YTHDF2



YTHDF3



D

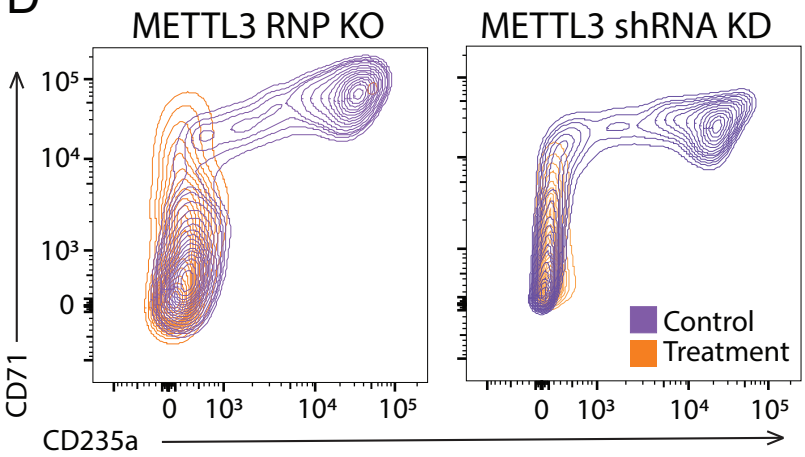
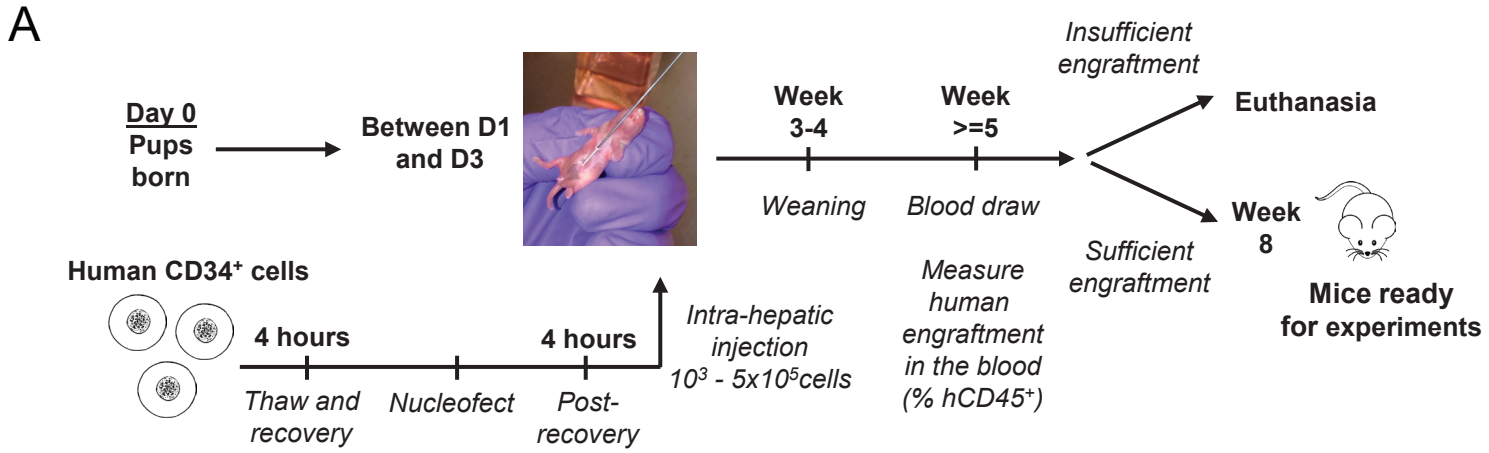


Figure 2

A



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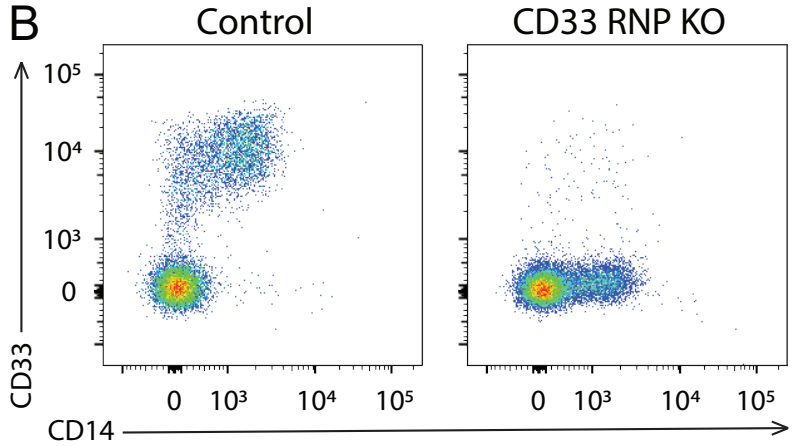
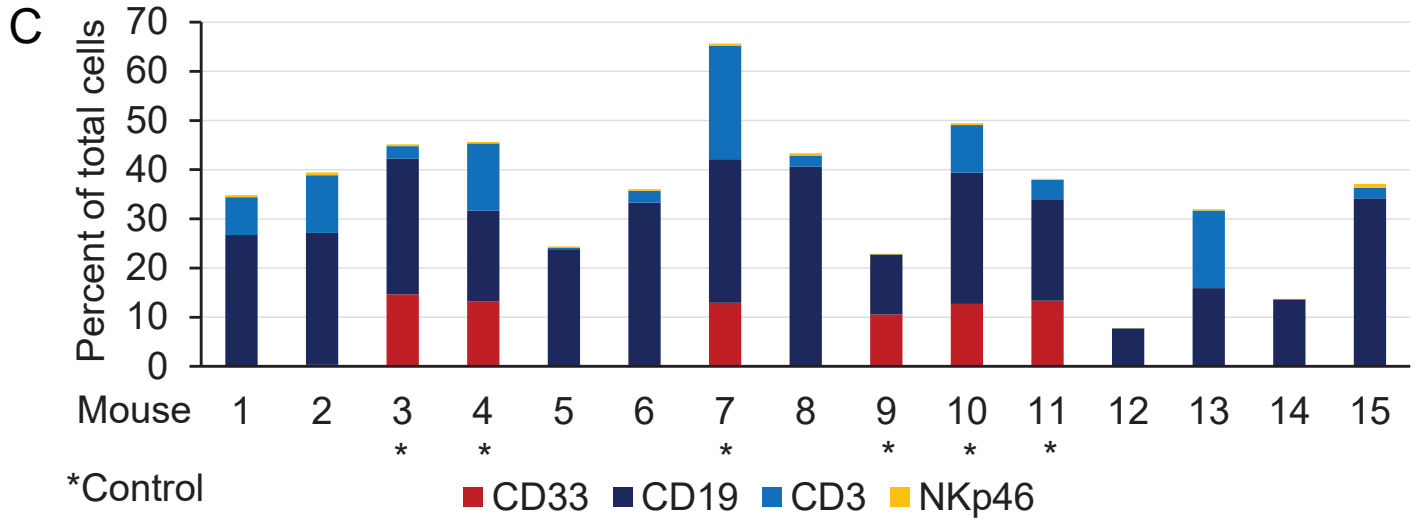
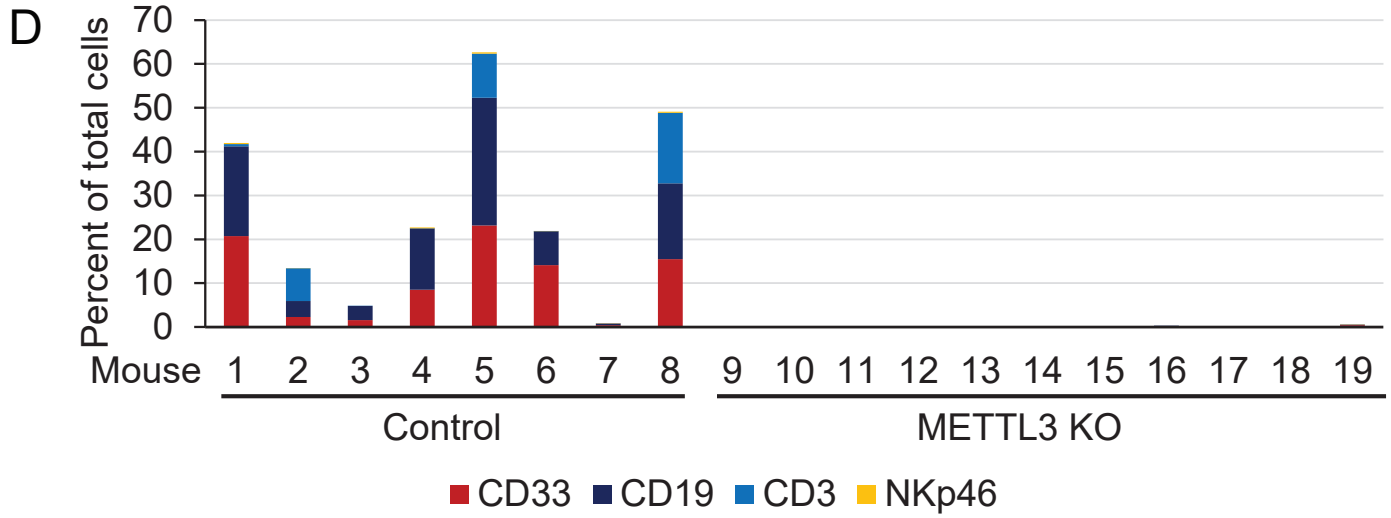


C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