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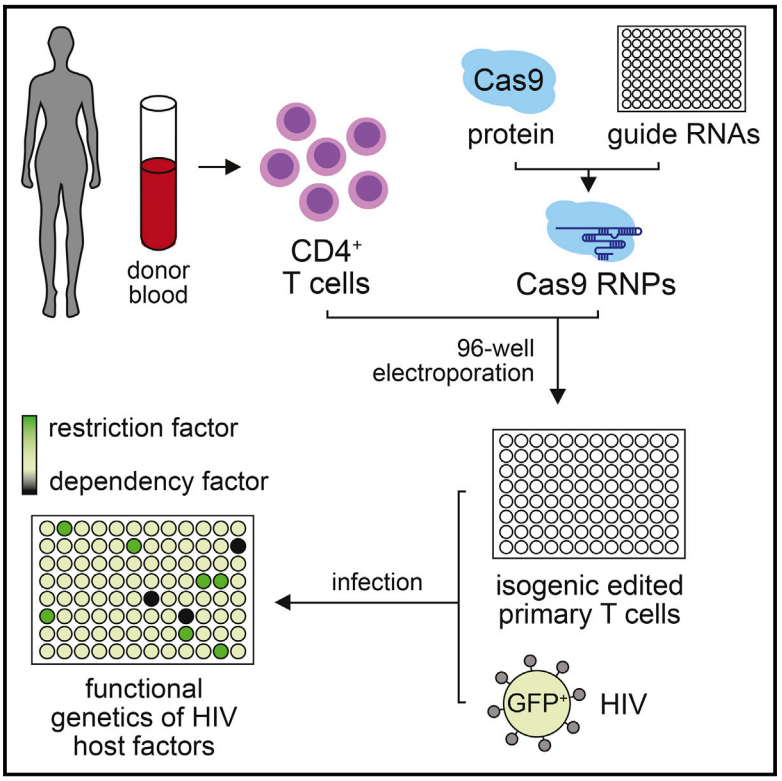
A Cas9 Ribonucleoprotein Platform for Functional Genetic Studies of HIV-Host Interactions in Primary Human T Cells

Graphical Abstract



Highlights

- Cas9 RNP editing of *CXCR4* and *CCR5* protects primary human T cells from HIV
- Arrayed delivery of RNPs confirms *LEDGF* and *TNPO3* as HIV dependency factors
- Method allows for efficient, one-step generation of double-knockout primary cells
- Screen of 45 predicted integrase interactors validates new HIV host factors

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In Brief

Hultquist et al. report a high-throughput platform for the efficient, multiplex editing of host factors that control HIV infection in primary CD4+ T cells. Arrayed electroporation of CRISPR/Cas9 ribonucleoproteins (RNPs) permits the rapid generation of isogenic human cells with ablated candidate factors and identifies gene modifications that provide viral resistance.

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A Cas9 Ribonucleoprotein Platform for Functional Genetic Studies of HIV-Host Interactions in Primary Human T Cells

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SUMMARY

New genetic tools are needed to understand the functional interactions between HIV and human host factors in primary cells. We recently developed a method to edit the genome of primary CD4$^+$ T cells by electroporation of CRISPR/Cas9 ribonucleoproteins (RNPs). Here, we adapted this methodology to a high-throughput platform for the efficient, arrayed editing of candidate host factors. CXCR4 or CCR5 knockout cells generated with this method are resistant to HIV infection in a tropism-dependent manner, whereas knockout of LEDGF or TNPO3 results in a tropism-independent reduction in infection. CRISPR/Cas9 RNPs can furthermore edit multiple genes simultaneously, enabling studies of interactions among multiple host and viral factors. Finally, in an arrayed screen of 45 genes associated with HIV integrase, we identified several candidate dependency/restriction factors, demonstrating the power of this approach as a discovery platform. This technology should accelerate target validation for pharmaceutical and cell-based therapies to cure HIV infection.

INTRODUCTION

Despite extraordinary progress in the development and distribution of antiretroviral drugs, HIV remains a worldwide health threat, infecting millions of new people each year. Even with strict adherence to a therapeutic regimen, patients remain chronically infected with the virus and thus require lifelong treatment (Finzi et al., 1997; Siliciano et al., 2003; Wong et al., 1997). To date, a cure has been achieved in only a single person, the "Berlin patient." In this case, the virus was eradicated by allogeneic, hematopoietic stem cell transplantation from a donor with a natural genetic variant in the *CCR5* gene that prevented HIV entry into these cells (Allers et al., 2011; Hütter et al., 2009). This success has motivated ongoing efforts to engineer human immune cells that lack host factors required for HIV pathogenesis as a means to achieve a permanent cure (Baltimore, 1988; Deeks and McCune, 2010; Leibman and Riley, 2015).

Several clinical trials are currently underway using zinc-finger nucleases (ZFNs) to delete the HIV co-receptors CXCR4 and CCR5 to generate immune cells that are resistant to HIV infection in a manner similar to the Berlin patient (Didigu et al., 2014; Hütter et al., 2009; Tebas et al., 2014). These approaches generally rely on viral-based delivery of a ZFN-expression cassette to generate HIV-resistant T cells or hematopoietic stem cells ex vivo (Maier et al., 2013; Perez et al., 2008; Wilen et al., 2011; Yi et al., 2014; Yuan et al., 2012). Autologous transplantation can then

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be used to repopulate a resistant T cell population while antiretroviral therapies and natural immune responses clear the remaining infection (Baltimore, 1988; Deeks and McCune, 2010; Didigu et al., 2014; DiGiusto et al., 2010; Holt et al., 2010; Tebas et al., 2014). While these represent potentially viable approaches, the use of viral delivery and the degree of off-target editing that may occur over the course of long-term ZFN expression raises concerns in bringing such a treatment to the clinic (Gabriel et al., 2011; Pattanayak et al., 2011; Thomas et al., 2003).

The advent of clustered regularly interspaced short palindromic repeats/Cas9 (CRISPR/Cas9) genome editing has revolutionized our ability to surgically modify the genomes of human cells, but efficient delivery of Cas9 to primary T cells has been a major challenge (Doudna and Charpentier, 2014; Hsu et al., 2014; Mandal et al., 2014; Ran et al., 2013). Recently, we reported that we can overcome this challenge through electroporation of Cas9 ribonucleoproteins (RNPs) directly into primary human $CD4^+$ T cells isolated from the peripheral blood (Schumann et al., 2015). This transient delivery of editing Cas9 RNPs enables high efficiency "knockout" and "knockin" genome editing and could provide a high-throughput method for therapeutic engineering of HIV-resistant human T cells. This approach would have several benefits over the traditional methodologies currently in trial, as it does not rely on viral delivery, does not involve long-term expression off a nucleic acid cassette, and has low rates of off-target editing (Kim et al., 2014; Schumann et al., 2015). As Cas9 technology is further developed, the efficiency and off-target rate should improve, making these advantages even more stark (Doench et al., 2016; Fu et al., 2014; Kleinstiver et al., 2016; Slaymaker et al., 2016).

Beyond CXCR4 and CCR5, other human host factors can affect HIV pathogenesis at different stages of viral life cycle (Brass et al., 2008; Goff, 2007; König et al., 2008; Zhou et al., 2008). However, functional studies of these factors have been limited by significant technical challenges in primary cell types and a subsequent reliance on RNAi and immortalized cell line models (Pache et al., 2011). The limitations of these systems underscore the need for improved technology to knock out specific gene sequences in primary human cells in a manner that is simple, scalable, reproducible, and efficient. Systematic validation of host genes that act as HIV dependency factors could unveil new targets for therapeutic intervention, when targeted either alone or in combination (Didigu et al., 2014; Voit et al., 2013).

Here, we report a high-throughput platform for the efficient editing of host factors that control HIV infection in primary human T cells. Arrayed delivery of Cas9 RNPs permits the rapid generation of isogenic T cells with ablated candidate factors for ex vivo interrogation and investigation. Using this platform, we disrupted the HIV co-receptors *CXCR4* or *CCR5* in multiple donors and reproducibly generated cells resistant to infection in a tropism-dependent manner. Targeting other dependency factors that act after viral entry, including *LEDGF* or *TNPO3*, resulted in reduced infection independent of tropism. Targeting *CXCR4* and *CD4* simultaneously, we demonstrated that this platform also supports multiplex gene editing and can be used to generate double-knockout cells at no cost to the efficiency of the individual Cas9 RNPs. Finally, we used our platform to screen 146 unique Cas9 RNPs targeting 45 genes previously shown to interact with HIV integrase, identifying novel host dependency factors. These studies collectively demonstrate the utility of Cas9 RNP T cell editing as an efficient means to validate host-dependency factors in the context of viral infection and generate HIV-resistant primary human T cells for scientific and potentially therapeutic use.

RESULTS

A Platform for Editing HIV Host Factors in Primary Human T Cells

Based on our previous success editing primary human T cells via direct electroporation of Cas9 RNPs (Schumann et al., 2015), we established a high-throughput platform for gene editing that is fast, robust, efficient, and readily adaptable to a variety of downstream applications. Unique Cas9 RNPs were synthesized in 96-well format by complexing Cas9 recombinant protein with chemically synthesized CRISPR-targeting RNAs (crRNAs) and *trans*-activating crRNA (tracrRNA) in vitro. These were subsequently delivered to primary human $CD4^+$ T cells by 96-well plate electroporation, allowing for the rapid generation of hundreds of unique, genetically modified pools from a single blood donor (Figure 1). The protocols described here constitute significant improvements to our previous methodology in not only scalability but also enhanced efficiency, as well as improved availability, as all reagents are commercially accessible. This platform therefore represents a valuable tool for the interrogation of genetic factors involved in a myriad of human immune cell processes, including T cell signaling, differentiation, and infection by diverse pathogenic agents.

To test the efficacy of this platform for biological investigation, we targeted a series of HIV host factors for genetic modification. HIV infection has been thoroughly studied in a wide array of biological model systems, but primary cell data in a clean genetic system are difficult to obtain and remain a gold standard in host factor investigation. This is further complicated by variability observed not only between donors but also between investigators, depending on the methodology used for T cell stimulation, infection, and analysis. To account for this variability, we decided to test our platform across multiple loci, in multiple donors, with an array of different stimulation and infection protocols.

Briefly, $CD4^+$ T cells were purified from human blood and activated with anti-CD3/CD28 stimulation. As described above, cells were electroporated with Cas9 RNPs, and genome editing was validated at both the DNA and protein levels. The edited cell populations were then subjected to functional testing with various ex vivo HIV infection assays using both single-cycle reporter viruses and full molecular clones (Figure 1). Provided that this method is efficient and specific, deletion of bona fide host dependency factors is predicted to measurably reduce HIV infectivity compared to control electroporation treatments.

Cas9 RNP Ablation of HIV Co-receptors Blocks Infection

HIV envelope (Env) must bind to CD4 and subsequently one of two co-receptors, CXCR4 or CCR5, on the host cell to mediate membrane fusion and trigger successful entry (Alkhatib et al.,

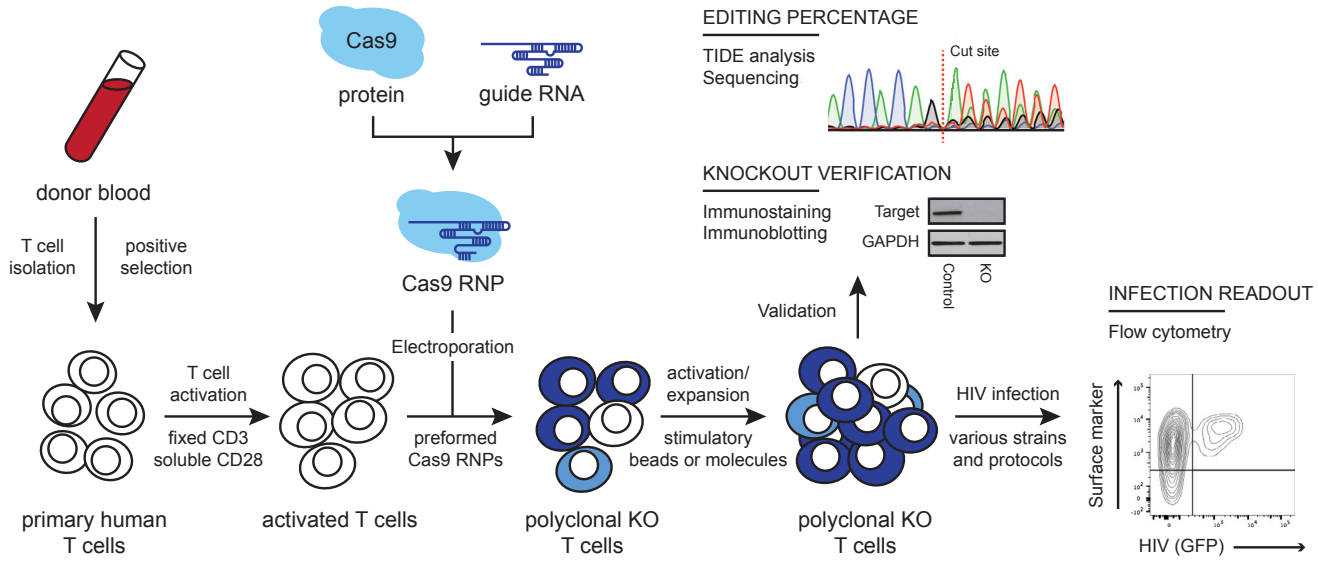


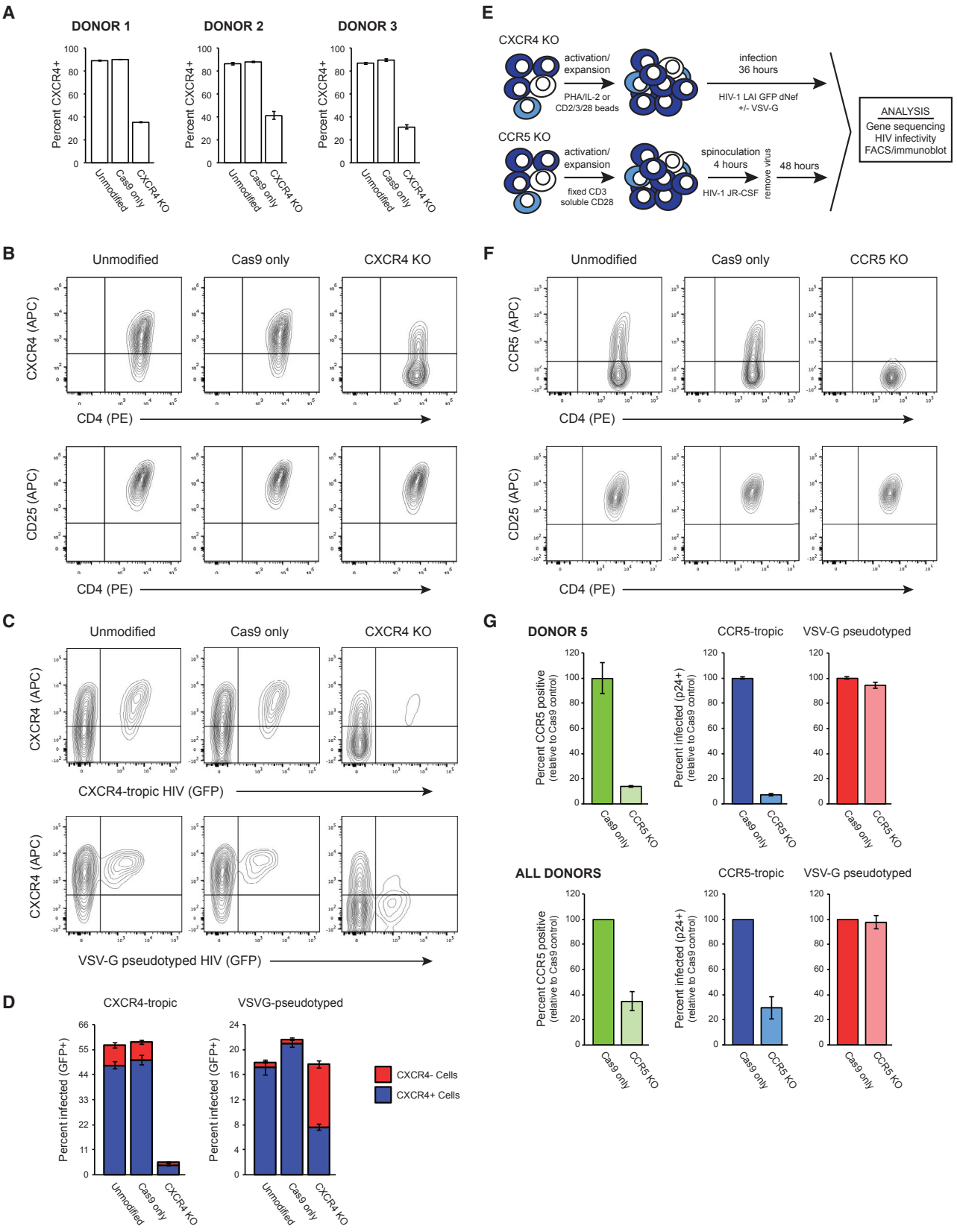
Figure 1. Procedural Overview of the Multiplex Platform for Editing HIV Host Factors in Primary Human CD4$^+$ T Cells

CD4$^+$ T cells are isolated from donor blood by negative selection and activated with plate-bound anti-CD3 and soluble anti-CD28. Cas9 RNPs are formed in vitro by incubating Cas9 protein with the appropriate crRNAs and tracrRNAs. These are electroporated into the activated T cells in a 96-well format to generate a polyclonal pool of homozygous edited (dark blue), heterozygous (light blue), and unedited (white) cells. These cells are activated again and expanded. Editing is verified at the DNA level by TIDE analysis or Sanger sequencing and at the protein level by immunostaining or immunoblot. These cells can then be infected with the appropriate viral strain(s) and infection monitored by flow cytometry either of an integrated GFP reporter or after staining for intracellular p24. See also Table S1.

1996; Berson et al., 1996; Deng et al., 1996; Feng et al., 1996; Wu et al., 1996). The chemokine receptor CCR5 is predominantly used in vivo, but once an infection is established, viral variants that use the co-receptor CXCR4 commonly evolve (Connor et al., 1997; Moore et al., 2004). Several polymorphisms in the *CCR5* gene have been linked with limited disease progression and even resistance to viral infection (Allers et al., 2011; Hütter et al., 2009; Liu et al., 1996; Samson et al., 1996). Targeted inhibition of CCR5 either genetically or chemically has proven to be a viable therapeutic route in vivo, and there is much interest in furthering and expanding such co-receptor-based therapies (Allers et al., 2011; Didigu et al., 2014; Gulick et al., 2008; Holt et al., 2010; Hütter et al., 2009; Tebas et al., 2014). We used our platform to efficiently and specifically knock out *CXCR4* or *CCR5* and measured the impact of this editing on co-receptor expression and HIV infectivity across multiple donors.

We previously demonstrated editing of *CXCR4* in primary T cells (Schumann et al., 2015). Here, we aimed to test the efficacy of this crRNA with our new platform across multiple donors and measure the impact of these changes on HIV infection. We first confirmed that *CXCR4* could be reproducibly and efficiently disrupted in multiple different human blood donors with the modified 96-well plate delivery format. The percentage of CXCR4-positive cells decreased from ~90% in the unmodified- and Cas9-protein-only control populations to ~30%–40% in the populations treated with *CXCR4*-targeting Cas9 RNPs in multiple independent donors (Figure 2A). Editing of the DNA was confirmed by tracking of indels by decomposition (TIDE) analysis (Table S1). Edited cells retained normal cell-surface levels of CD4 and appropriately induced CD25 in response to stimulation with anti-CD2/CD3/CD28 beads (Figure 2B). The same holds true when we stimulate with soluble anti-CD3/CD28 or with PHA/IL-2 (data not shown). After stimulation, we challenged these cells with CXCR4-tropic HIV-1^{LAI}, which includes a GFP marker in the place of *nef*, or an identical virus lacking native Env expression but pseudotyped with pan-tropic vesicular stomatitis virus G protein (VSV-G). Cells were co-stained for CXCR4 surface expression and infection rates quantified by flow cytometry. Unmodified cells and Cas9 control treated (without crRNA or tracrRNA) cells showed no difference in CXCR4 expression or infection with either virus (Figures 2B and 2C). *CXCR4* knockout cells, however, showed a substantial decrease in CXCR4$^+$ cells and a concurrent decrease in infection with the CXCR4-tropic virus. Importantly, most of the cells in this population that did become infected still expressed high levels of CXCR4. On the other hand, *CXCR4* knockout cells showed no difference in infection with the VSV-G pseudotyped virus as compared to the controls (Figures 2B and 2C). This was consistently observed across all donors tested (Figure S1). Therefore, these edited cells retained permissivity to HIV infection but were rendered unsusceptible to specific viral strains in a tropism-dependent manner. These data demonstrate that treatment with Cas9 RNPs successfully ablated CXCR4 expression in human T cells across multiple donors and imposed a specific and targeted block to CXCR4-tropic HIV infection.

While T cells uniformly express high levels of CXCR4, T cells in the peripheral blood generally express low to undetectable levels of CCR5 (Lee et al., 1999). By stimulating these cells with low levels of plate-bound anti-CD3 and soluble anti-CD28 antibodies, however, we were able to induce sufficient expression for infection and analysis (Figure 2D). We designed and tested three *CCR5* targeting crRNAs and found that two of them



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successfully edited the *CCR5* locus (Table S1). While the percentage of CCR5$^+$ cells achieved by our protocol varied slightly between donors, one of these crRNAs was able to routinely decrease this population by ~60%–80% relative to controls (Figures 2E, 2F, and S2). As with *CXCR4* targeting, we saw no significant effects on CD4 expression or CD25 induction when targeting *CCR5* (Figures 2F and 2G). Similarly, these cells showed resistance to HIV infection in a manner dependent on viral tropism. *CCR5*-targeted cells had lower rates of infection with the CCR5-tropic HIV-1 virus JR-CSF, whereas no significant difference was observed upon infection with a VSV-G pseudotyped, pan-tropic virus. This was observed across multiple independent donors (Figure S2).

Altogether, these experiments demonstrate that this platform can be used to efficiently generate HIV-resistant edited primary cells by targeting specific host dependency factors. These cells respond normally to assorted stimuli and retain their permissivity to viral replication if a pathway of infection is present independent of the targeted host factor. More broadly, these data validate this platform as a means to study the effects of genetic perturbations in human T cells on HIV infection.

Validation of Candidate HIV Dependency Factors in Primary Human T Cells

We first demonstrated the utility of this platform to efficiently and reproducibly generate HIV-resistant primary T cells by targeted knockout of *CXCR4* and *CCR5*, host factors essential for virus entry. We next wanted to test the ability of this approach to interrogate host factors that act later in the viral life cycle whose successful editing may also render cells resistant to early infection. Toward this end, we decided to target the chromatin-associated factor LEDGF/p75, a host co-factor shown to be important for HIV integration in several model systems (Ciuffi et al., 2005; Fadel et al., 2014; Llano et al., 2004, 2006a; Shun et al., 2007; Vandegraaff et al., 2006; Vandekerckhove et al., 2006). Knockdown experiments have been difficult to interpret due to residual functional activity of the gene (Llano et al., 2006b), but knockout experiments in the mouse model and in cell lines have shown some effect (Fadel et al., 2014; Shun et al., 2007). While it is likely that there is some functional redundancy with other host factors, the use of LEDGF by the virus is thought to influence the integration site landscape and proviral latency (Gérard et al., 2015; Jordan et al., 2001; Llano et al., 2004, 2006a; Shun et al., 2007; Vandegraaff et al., 2006; Vandekerckhove et al., 2006). The inability to knock out *LEDGF* in primary T cells has greatly limited the study of this factor in ex vivo human systems.

To test the impact of *LEDGF* knockout in primary human T cells, we first designed and tested an array of crRNAs with our multiplex delivery system (Figure 3A). Of the six LEDGF crRNAs we tested, two caused significant decreases in protein level by immunoblot and showed editing at the DNA level by TIDE analysis (Figure 3A; Table S1). These cell populations were infected either with a CXCR4-tropic HIV-1 GFP reporter virus or with a VSV-G pseudotyped virus as described earlier (Figure 2D). While *CXCR4* knockout specifically inhibited infection of the CXCR4-tropic virus, we saw similar inhibition of both viruses in the two populations where LEDGF had been depleted (Figure 3A). The level of inhibition observed is limited by not only the percentage of cells that are effectively edited but also by the length and MOI of the infection. To determine if we see a more pronounced effect using different infection conditions, we repeated these experiments using our most efficient crRNA with a replication competent HIV-1 GFP reporter virus in a spreading infection over 7 days (Figure 3B). Compared to controls, *LEDGF*-targeted cells exhibited a 2- to 7-fold reduction in viral replication across multiple blood donors three days after infection (average 4-fold reduction, Figures 3C and S3). After 7 days of spread, these differences were even greater, with *LEDGF* targeted cells exhibiting a 6- to 12-fold reduction in HIV replication (Figure 3C). Little change was observed in cellular viability over this time course as monitored by live-cell gating (Figure S3). These data collectively confirm a functional role for LEDGF/p75 in HIV infection in primary human CD4$^+$ T cells. Furthermore, they demonstrate the utility of this platform for analyzing HIV host factors at later steps in infection, especially in analyzing those factors resistant to more traditional RNAi approaches.

We next tested ablation of three other candidate host dependency factors that act at three other stages of HIV replication to further generalize the applicability of the arrayed targeting method: *TNPO3* (required for efficient cellular trafficking by

Figure 2. Knockout of HIV Co-receptors *CXCR4* and *CCR5* Confers Resistance to Infection in a Tropism-Dependent Manner

(A) Primary T cells were isolated from three different donors and electroporated with buffer alone, Cas9 protein alone, or Cas9 RNPs targeting *CXCR4*. 4 days after electroporation, cells were stained with anti-CXCR4-APC in technical triplicate and analyzed by flow cytometry.

(B) These cells were subsequently stained with anti-CD4-PE and anti-CXCR4-APC or anti-CD4-PE and anti-CD25-APC. Representative plots from one donor are shown above.

(C) These cells were then infected with a CXCR4-tropic (LAI strain) GFP reporter virus or an identical VSV-G pseudotyped, Env-deficient virus in technical triplicate. After 36 hr, cells were co-stained with anti-CXCR4-APC and analyzed by flow cytometry. Representative plots from one donor are shown above.

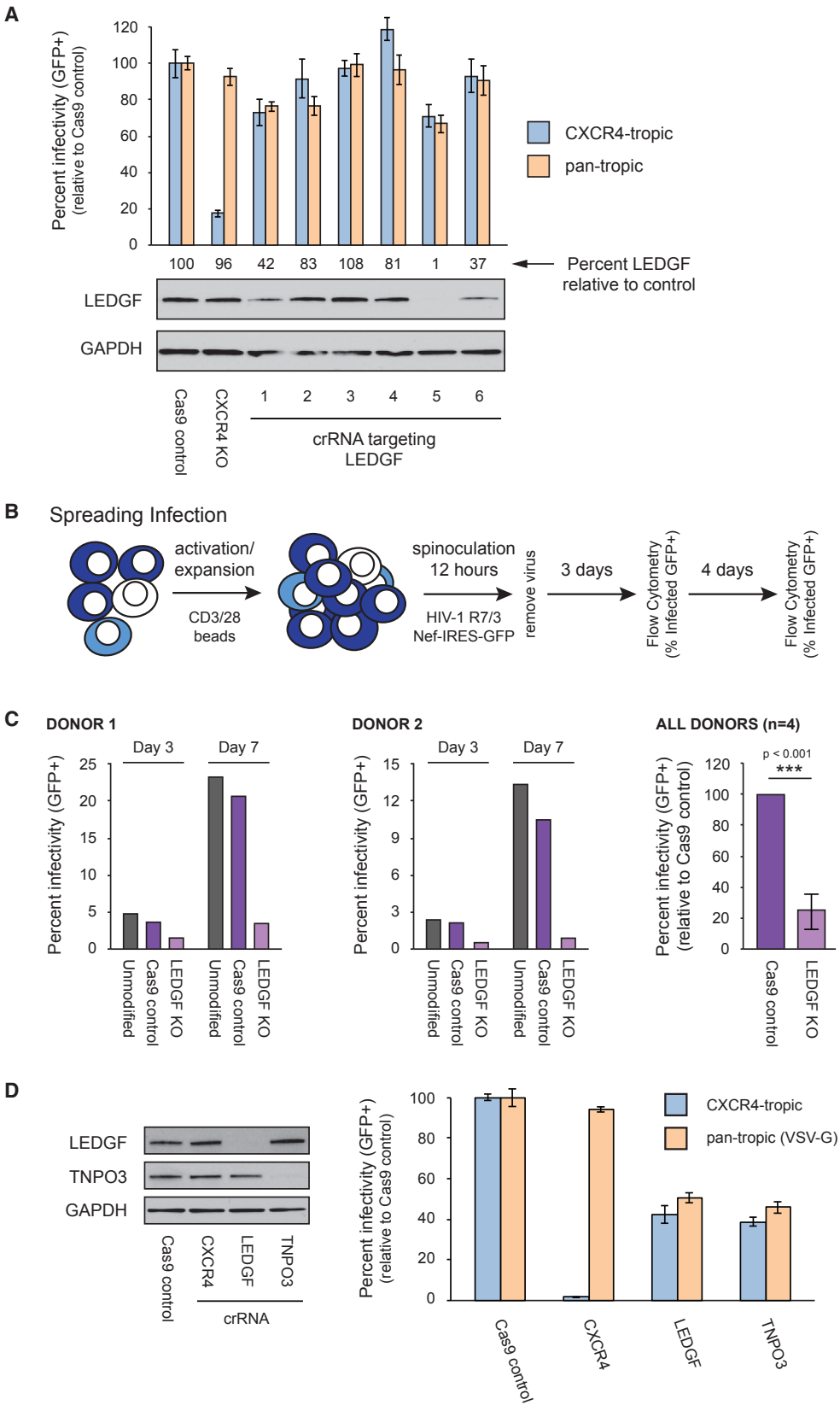
(D) Bar graphs depicting the mean percentage of infected cells across technical triplicates ± SD, including both CXCR4$^-$ cells (red) and CXCR4$^+$ cells (blue). Infections from one representative donor are shown, with CXCR4-tropic virus on the left and VSV-G pseudotyped virus on the right.

(E) Procedural schematic depicting the differences in stimulation/infection protocols for CXCR4 versus CCR5-tropic viruses.

(F) Primary T cells were isolated from six different donors and electroporated with buffer alone, Cas9 protein alone, or Cas9 RNPs targeting *CCR5*. 2 days after electroporation, cells were stained with anti-CCR5-APC or anti-CD4-PE and -CD25-APC and analyzed by flow cytometry. Representative plots from one donor are shown above.

(G) These cells were then infected with a CCR5-tropic (JR-CSF strain) virus or a VSV-G pseudotyped reporter virus in technical triplicate. After 48 hr, cells were co-stained with anti-p24-FITC and -CCR5-APC before analysis by flow cytometry. The top bar graph depicts the mean percentage of CCR5$^+$ cells (green), CCR5-tropic infected cells (blue), and VSV-G pseudotyped infected cells (red) across technical triplicates ± SD relative to the Cas9 control in one representative donor. The mean bar graph depicts the mean across six donors ± SE again relative to the Cas9 control.

See also Figures S1 and S2 and Table S1.



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proper localization of CPSF6) (Brass et al., 2008; Christ et al., 2008; De Iaco et al., 2013), *NUP153* (required for nuclear import of the pre-integration complex) (König et al., 2008; Matreyek and Engelman, 2011), and *CDK9* (required for viral gene expression) (Mancebo et al., 1997; Zhu et al., 1997). For each gene, we designed three crRNAs and tested them for knockout generation in multiple donors by TIDE analysis at the DNA level and immunoblot at the protein level (Table S1). Two of the three crRNAs for *NUP153* and *CDK9* reproducibly led to decreased cell viability and cell death, consistent with essential functions for these genes in cellular health (Albert et al., 2014; Ball and Ullman, 2005; Duheron et al., 2014; Price, 2000). The Cas9 RNPs that did not cause cell death showed low levels of editing at the DNA level but no depletion of the respective protein (data not shown). We interpret these data to mean that these two genes are essential for cellular viability and they were not assayed further. In contrast, cells survived when the nuclear transporter *TNPO3* was disrupted; two crRNAs were identified that generated insertions and deletions at the *TNPO3* locus with ~40%–70% efficiency depending on the donor (Table S1).

To directly compare the efficacy of *CXCR4*, *TNPO3*, and *LEDGF* editing, we used the most efficient crRNA for each gene to generate isogenic knockout populations in multiple, independent donors. Knockout of each factor was specific and did not alter the protein level of the other host factors (Figure 3D). Upon infection with a CXCR4-tropic GFP reporter virus (as in Figure 2D), *CXCR4* knockout nearly ablated infection while *TNPO3* and *LEDGF* knockout inhibited infection with ~60% efficacy. Pseudotyping the virus with VSV-G envelope was sufficient to completely overcome the barrier CXCR4 depletion poses to infection, but knockout of *TNPO3* and *LEDGF* still inhibited infection by the same amount relative to controls (Figure 3D). Thus, while some locus and donor variability in editing efficiency was observed, this Cas9 RNP platform serves as a robust method for disrupting key host factors at multiple stages of infection.

Cas9 RNP Multiplexing Allows for the Generation of Double-Knockout Cells

A potential strength of using in vitro synthesized Cas9 RNPs is the ease with which double knockouts could be generated. Like small interfering RNA (siRNA) methodologies, simply mixing the Cas9 RNPs prior to electroporation would be expected to yield a mixed population of cells, some of which are doubly modified. To test the feasibility of this approach, we attempted to knock out both *CXCR4* and *CD4* by co-electroporation of their respective Cas9 RNPs. For *CD4*, we designed three crRNAs that yielded different efficiencies when electroporated with the respective RNPs singly (Figure 4C). Immunostaining for both CXCR4 and CD4 demonstrated specific depletion of each cell-surface marker only when the Cas9 RNP targeting that gene was included and furthermore demonstrated the clear accumulation of a double-negative population in the multiplexed samples (representative flow plots, Figure 4A). Comparing the efficiency of CXCR4 knockout alone or in multiplex with the CD4 RNPs showed no loss of knockout efficiency in both donors tested (Figure 4B). Similarly, all CD4 RNPs demonstrated equivalent efficiencies with or without inclusion of the CXCR4 RNP (data not shown).

Several methods for delivering nucleic acid or protein to cells result in preferential delivery of multiple reagents to the same cell at once such that a single cell that receives one packaged molecule tends to receive all others in the mixture (Ma et al., 2007). Cas9 RNPs, however, then must direct to the chromosomal DNA, successfully cleave their target site, and introduce a functional mutation to result in a phenotypic readout, a process that may or may not be related to cleavage success at independent loci. Based on the efficiency of each CXCR4 and CD4 RNP when delivered alone, and given that we see no loss in efficiency during multiplexing, we calculated the predicted percentage of double-knockout cells when delivering the Cas9 RNPs at a 1:1 ratio, assuming editing at each locus was independent of the other (Figure 4C). We found that this nearly perfectly reflected the observed percentage of double-knockout cells when staining for CD4 and CXCR4 (Figure 4C). Therefore, the generation of double-knockout cells by Cas9 RNP multiplexing is dependent on the efficiency of each individual RNP, and the probability of editing at each locus appears to be independent.

Given the ability of this platform to generate double-knockout cells, we decided to multiplex two of our most efficient Cas9 RNPs, against *CXCR4* and *LEDGF*, to see if we could generate cells doubly resistant to HIV infection. As observed with CD4 and CXCR4, we saw no loss of efficiency for either the *CXCR4* or *LEDGF* RNPs when delivered simultaneously as opposed to individually (Figure 4D). These cell populations were infected

Figure 3. Knockout of *LEDGF* and *TNPO3* Inhibit Early Events of HIV Infection in Primary T Cells

(A) Primary T cells were isolated from two different donors and electroporated with the indicated Cas9 RNPs, including six different RNPs targeting *LEDGF*. 4 days after electroporation, cells were removed for immunoblotting and genomic DNA isolation. At the same time, cells were infected with a CXCR4-tropic (LAI strain) GFP reporter virus or an identical VSV-G pseudotyped, Env-deficient virus in technical triplicate. After 36 hr, the percentage of infected cells (GFP^+) was determined by flow cytometry. The bar graph depicts the mean percentage of infected cells across technical triplicates ± SD for one representative donor. LEDGF protein levels were quantified and depicted immediately above the immunoblot.

(B) Procedural schematic depicting the modified stimulation/infection protocol for analyzing the *LEDGF* knockout cells over the course of a spreading infection.

(C) Primary T cells were isolated from four different donors and electroporated with buffer alone, Cas9 alone, or the most effective Cas9 RNP targeting *LEDGF* (crRNA #5, see above). Cells were infected as indicated in (B) and percent infection determined by flow cytometry. Results for two donors are depicted at days 3 and 7 in the first two graphs. The final graph depicts the mean percent infection observed in the Cas9 control and LEDGF knockout cells across all four donors at day 3 ± SD.

(D) Primary T cells were isolated from two different donors and electroporated with the indicated Cas9 RNPs. 4 days after electroporation, cells were removed for immunoblotting. At the same time, cells were infected with a CXCR4-tropic (LAI strain) GFP reporter virus or an identical VSV-G pseudotyped, Env-deficient virus in technical triplicate. After 36 hr, the percentage of infected cells (GFP^+) was determined by flow cytometry. The bar graph depicts the mean percentage of infected cells across technical triplicates ± SD for one representative donor.

See also Figure S3 and Table S1.

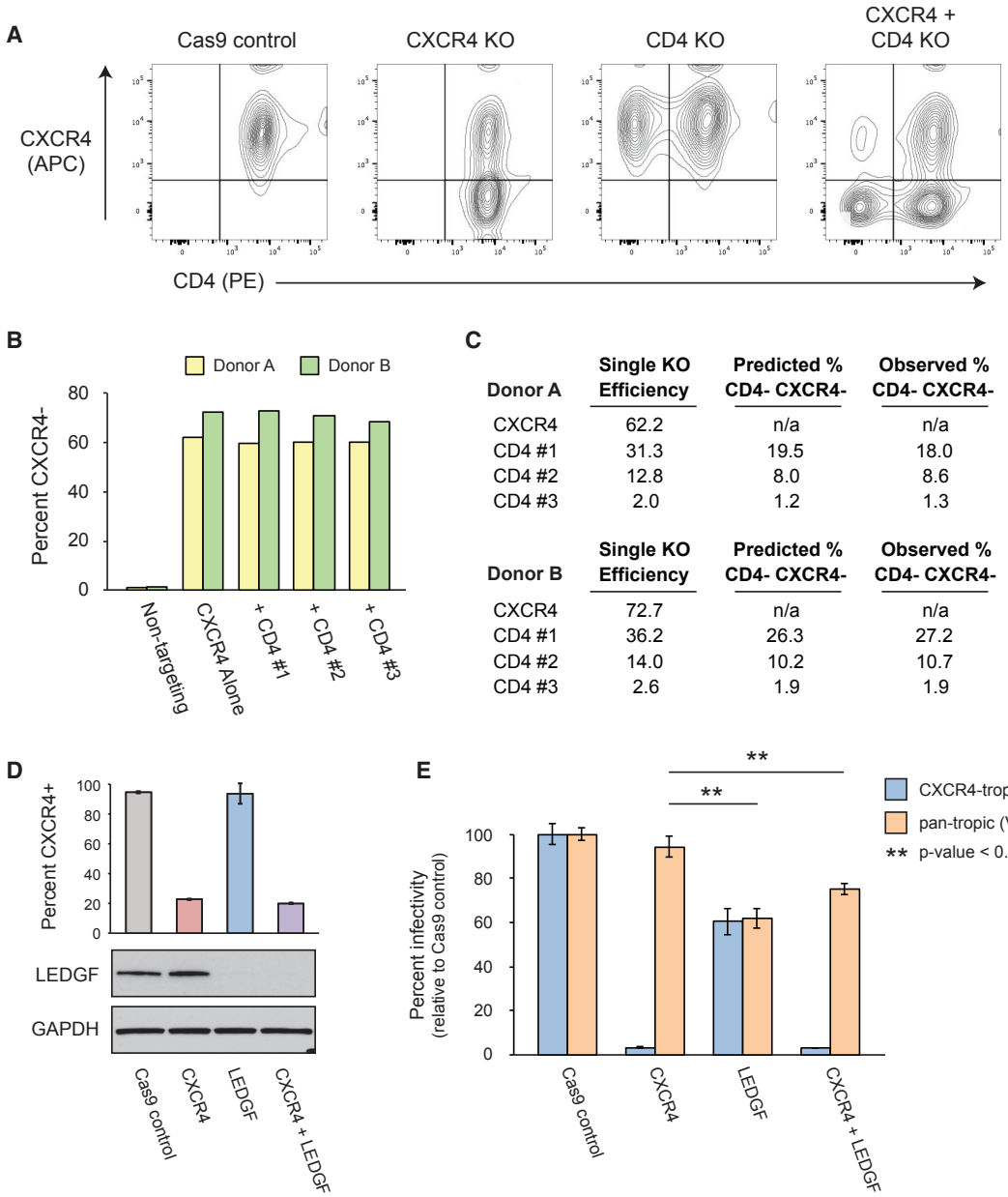

Figure 4. CRISPR RNP Multiplexing Allows for the Generation of Double-Knockout Primary T Cells

(A) Primary T cells were isolated from two different donors and electroporated with the indicated Cas9 RNPs. CXCR4 and one of three different CD4 crRNA were mixed at a 1:1 ratio prior to incubation with the Cas9 protein. Cells were stained with anti-CD4-PE and anti-CXCR4-APC 48 hr after electroporation. Flow plots from one representative donor are depicted.

(B) The percentage of $CXCR4^{-}$ cells as measured by flow cytometry in two donors electroporated with Cas9 protein alone, the CXCR4 RNP alone, or the CXCR4 RNP mixed 1:1 with three different CD4 RNPs.

(C) The percentage of $CXCR4^{-}$ or $CD4^{-}$ cells after electroporation with each RNP singly is recorded for each donor under single KO efficiency. The observed percentage of double-knockout cells is then reported alongside the percent editing predicted given an independent probability of editing at each locus.

(D) Primary T cells were isolated from three different donors and electroporated with the indicated Cas9 RNPs. CXCR4 and LEDGF crRNPs were mixed at a 1:1 ratio prior to electroporation in the double targeted population. 4 days after electroporation, cells were removed for immunoblotting as well as immunostaining against CXCR4. The mean percentage of $CXCR4^{+}$ cells in each population across technical triplicate ± SD is depicted in the bar graph above the associated immunoblot for LEDGF.

(E) Cells were infected with a CXCR4-tropic (LAI strain) GFP reporter or an identical VSV-G pseudotyped, Env-deficient virus in technical triplicate. After 36 hr, cells were co-stained for anti-CXCR4-APC and the percentage of infected cells (GFP^{+}) determined by flow cytometry. The bar graph depicts the average percent infected cells across technical triplicates ± SD for one representative donor. p values were calculated by pairwise Student's t test.

See also Figure S4 and Table S1.

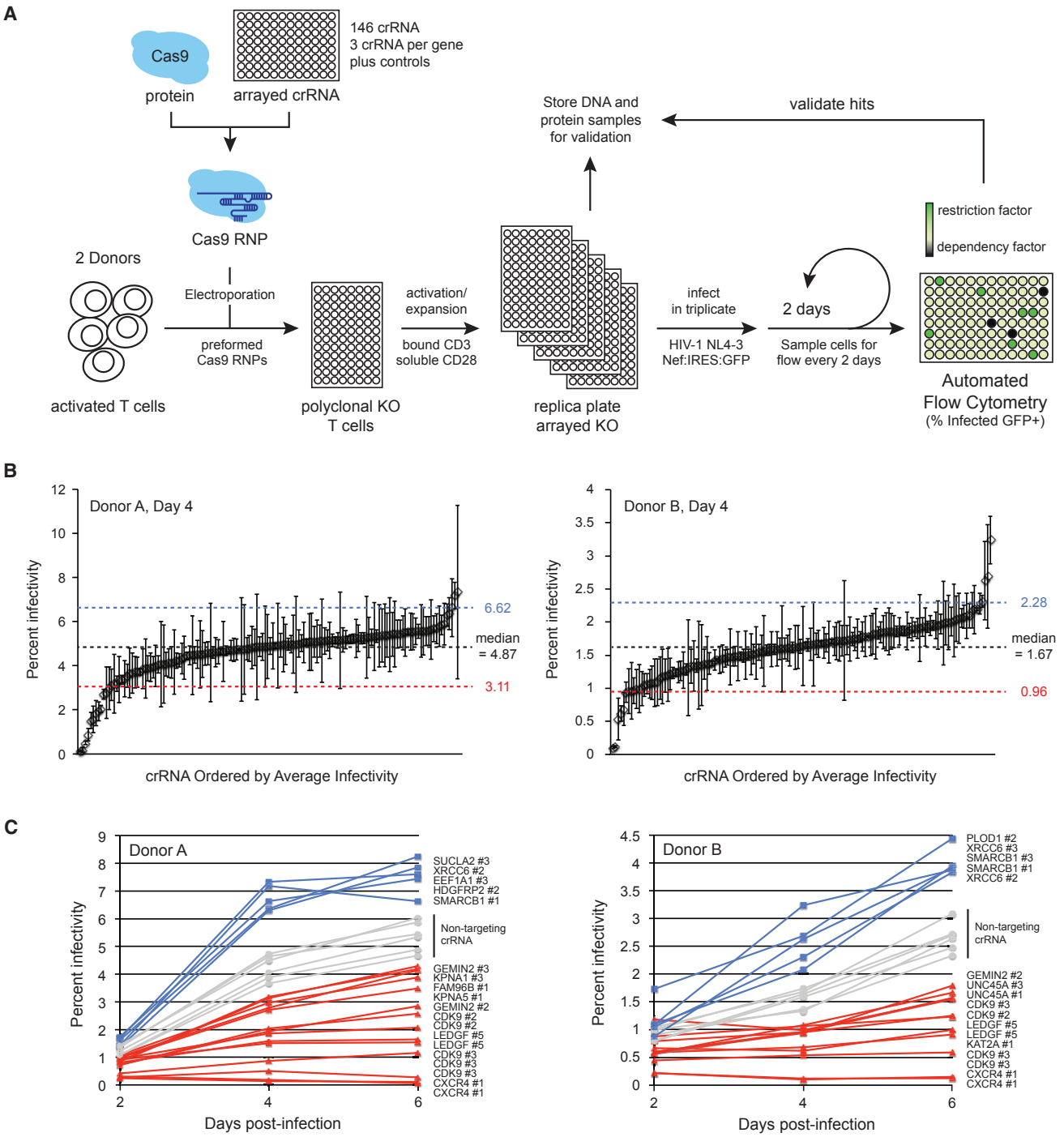

Figure 5. A Primary Cell Knockout Screen of Genes Predicted to Influence HIV Integrase Confirms the Role of Several Proteins in HIV Replication

(A) CD4$^+$ T cells were isolated from the blood of two donors by negative selection and activated with plate-bound anti-CD3 and soluble anti-CD28. 146 independent Cas9 RNPs were formed in vitro by incubating Cas9 protein with the appropriate crRNAs and tracrRNA. These were electroporated into the activated T cells in 96-well format to generate polyclonal pools of edited cells. These cells were activated again and replica plated. Cells were infected in triplicate with replication-competent HIV-1 (NL4-3 strain) with IRES:GFP cloned behind Nef. Every 48 hr for 6 days, half of the culture was removed to monitor infection by flow cytometry while an equal volume of fresh media was replaced.

(B) The percentage of HIV-infected cells (GFP positive) in each polyclonal knockout pool is plotted as the mean across technical triplicates ± SD as measured at day 4 post-infection in each donor. crRNAs are ordered left to right by average infectivity. The median values ± 1.5 SDs across all wells are annotated by the dotted lines.

(legend continued on next page)

with a CXCR4-tropic HIV-1 GFP reporter virus or with a VSV-G pseudotyped virus as previously described and normalized to the Cas9-only control (Figure 2E). As observed before (Figure 3F), *CXCR4* disruption caused cells to become almost completely resistant to CXCR4-tropic HIV infection but had no impact on the infectivity of the VSV-G pseudotyped virus (Figures 4B and 4C). On the other hand, *LEDGF* knockout resulted in a modest decrease in the infectivity of both viruses regardless of their envelope, in agreement with prior results (Figures 3D, 4B, and 4C). The cells treated with both RNPs demonstrated a dual phenotype, restricting the CXCR4-tropic virus as effectively as the *CXCR4* knockout cells and restricting the VSV-G pseudotyped virus as effectively as the *LEDGF* knockout cells (Figures 4B and 4C). These viral replication phenotypes are consistent with our protein level analyses and indicate the efficient generation of double-knockout primary T cells. This was repeated in multiple, independent donors with nearly identical results (Figure S4). Taken together, these data show that our multiplex editing platform can successfully generate double-knockout populations. Not only could this be useful for the analysis of functional redundancy and epistatic relationships among genes, but such combinatorial editing could also be an effective tool for the generation of multiply HIV-resistant cells for curative transplantation therapy (Didigu et al., 2014; Voit et al., 2013).

An Arrayed Platform for Primary T Cell Genetics Using CRISPR/Cas9 RNPs

One major benefit to this approach is the ability to perform high-efficiency gene editing in primary T cells in an arrayed format for screening and high-throughput phenotypic analysis. Unlike pooled CRISPR libraries that rely on selection and sequencing for the identification of impactful genes, this system allows for the generation of hundreds of physically distinct cellular pools for analysis and a myriad of downstream applications. This is especially ideal for high-throughput screens that may require the interrogation of hundreds of genes with specific and specialized readouts not suitable for pooled analysis.

As proof of principle, we designed a screen of 45 genes described in the published literature that either directly or indirectly affect the function of HIV integrase. These included 21 genes supported by at least two publications in the HIV-1, human interaction database as well as 24 genes identified by HIV-1, human protein-protein interaction mapping by affinity-purification mass spectrometry (AP-MS) (Ako-Adjei et al., 2015; Jäger et al., 2011). Three crRNAs were designed per gene alongside multiple non-targeting controls and several previously analyzed crRNAs, including those targeting *CXCR4* and *LEDGF* as positive controls and *CDK9* as a toxicity control. In total, 146 crRNAs were designed and synthesized in 96-well plate arrayed format (Tables S1 and S2). These crRNAs were incubated with tracrRNA and Cas9 protein to form Cas9 RNPs, which were subsequently electroporated into activated primary T cells from two donors (Figure 5A). These cells were reactivated and replica plated into five 96-well plates. 48 hr later, one plate was lysed for protein, one was lysed for genomic DNA, and three were infected with HIV-1^{NL4-3} with IRES:GFP immediately following the Nef reading frame. Every 2 days for 6 days, half of the cultures were removed and fixed for analysis by automated flow cytometry and the cultures supplemented with an equal volume of fresh media (full dataset provided in Table S2).

At each time point, we observed a roughly normal distribution of infection rates tightly clustered around the median value (Figure 5B). Cells treated with non-targeting controls distributed evenly around the median, while the cells treated with *CXCR4* RNPs consistently resulted in the lowest rate of infection. Any RNP that scored more or less than 1.5 SDs from the median at any given time point was considered significantly impactful, and the full spreading infection profile for these hits was plotted alongside the non-targeting controls (Figure 5C). Of the RNPs with significant effects, including the positive control RNPs targeting validated host dependency factors, more resulted in decreased rates of infection (1.5 SDs less than the median; red line), but some resulted in increased rates of infection (1.5 SDs more than the median; blue line).

For both donors, the strongest blocks to infection were dictated by knockout of the positive controls: *CXCR4*, *CDK9*, and *LEDGF* (Figure 5C). *CDK9* likely exerted its effect based on both toxicity of the editing event and specific blocks to the HIV replication cycle. No other RNP elicited cellular toxicity as severe as those targeting *CDK9* as determined by flow cytometry measuring cellular density (data not shown). *GEMIN2* knockout also consistently resulted in blocks to infection in both donors, as predicted from studies in cell lines (Hamamoto et al., 2006; Nishitsuji et al., 2009). *KPNA1*, *KPNA5*, and *KAT2A* also significantly decreased replication in at least one donor, confirming previously published studies (Armon-Omer et al., 2004; Gallay et al., 1997; Levin et al., 2010; Terreni et al., 2010). *FAM96B* and *UNC45A* resulted in significant blocks to infection as well, but no role for these proteins in HIV infection has been published beyond an interaction with HIV integrase (Jäger et al., 2011).

In contrast to the above factors, *SMARCB1* (*INI1/SNF5*) and *XRCC6* knockout both resulted in strong increases in infection in both donors (Figure 5C). SMARCB1 is known to inhibit HIV replication in cell lines, possibly through the stabilization of pre-intregration complexes in non-reactive conformations (Das et al., 2009; Maroun et al., 2006; Pyeon et al., 2015). XRCC6 (Ku70) and other components of the non-homologous end-joining (NHEJ) pathway, however, have an unclear role in the HIV life cycle, though some results suggest a role in post-integration expression (Baekelandt et al., 2000; Jeanson et al., 2002; Li et al., 2001; Manic et al., 2013). These results suggest a possible role for Ku70 as a restriction factor in primary T cells and highlight the importance of this approach to directly test the impact of genetic alterations in relevant primary models. Additionally,

(C) The percentage of HIV-infected cells plotted over 6 days in primary T cells nucleofected with the indicated CRISPR Cas9 RNPs. Six non-targeting controls included in the plates are annotated in gray. The spreading infection results are displayed for all RNPs that scored above (blue) or below (red) 1.5 SDs from the median infectivity across the plate at any given time point in the series.
See also Figure S5 and Tables S1 and S2.

PLOD1, *SUCLA2*, *HDGFRP2*, and *EEF1A1* each scored significantly as a potential restriction factor in at least one of the two donors.

Importantly, we cannot rule out the role of any gene that failed to score as significant in this assay without validation of the crRNAs used at the DNA and/or protein level. Conversely, correlation between crRNA effectiveness at the protein level and the functional impact on infection can provide secondary validation in screens of untested crRNAs. For example, *XRCC6* crRNA 2 resulted in the most efficient knockout by immunoblot, crRNA 3 was of intermediate efficiency, and crRNA 1 was ineffective (Figures S5 and 5C). This correlates well with the spreading infection data, where we see crRNA 2 as effective in both donors, crRNA 3 as effective in one donor, and crRNA 1 failing to score significantly. Similar parallels are observed with *UNC45A*, where crRNAs 1 and 3 are the only two to effectively result in gene knockout by immunoblot and are the only two to result in significant inhibition of HIV infection (Figures S5 and 5C). TIDE analysis of editing for each gene observed to alter HIV infectivity further validated these findings. In every case, only those crRNAs that caused a phenotypic change in infection were successful at inducing DNA edits at the corresponding genetic loci (Table S1).

Overall, these data demonstrate the strength of this platform for the high-throughput screening of genes directly in primary cells. This approach has the potential to unveil a number of new host factors playing essential roles in HIV infection and pathogenesis. Given the ease of use, effectiveness of editing, and universal availability of the reagents, we hope this platform can be broadly adapted for the study of a broad variety of T cell processes.

DISCUSSION

In this study, we describe an efficient, high-throughput method for systematically editing genes in primary human lymphocytes to facilitate the study of viral host factors, T cell genetic interactions, and therapeutic approaches for the cure of HIV. This arrayed Cas9 RNP platform is notable for its efficiency, adaptability, and scalability. Each reaction consists only of the primary T cells, recombinant Cas9 protein, electroporation buffer, tracrRNA, and crRNA. We found targeting to be robust across different donors and different loci, with one to two of every three crRNAs tested achieving significant editing and detectable decreases in protein expression. The T cells must be activated and generally require 24 to 48 hr for recovery after electroporation, but they are otherwise amenable for use in a wide variety of downstream applications. We found minimal adverse effects on cell replication, surface marker expression, response to stimulation, or infection broadly. These experiments were performed with 300,000 cells per reaction in 96-well plate format, but the cell number could be even further reduced to scale up target discovery efforts. Coupled to the chemical synthesis of crRNAs in 96-well plates, this platform allows for the screening of hundreds of genes in isogenic sets of primary cells from a single donor. The method should be readily adaptable to diverse primary human cell types that are targets of a variety of pathogenic organisms.

In this study, we used this Cas9 RNP platform to knock out genes that are required for HIV infection in T cells. Targeting of the HIV co-receptors CXCR4 and CCR5 elicits strong protective phenotypes to infection that strongly correlate with the effectiveness of the RNP in a tropism-dependent manner. CCR5 has already been developed as a target for treatment with the antiretroviral drug maraviroc, and cell-based therapies with CCR5-deficient cells are under development (Didigu et al., 2014; Gulick et al., 2008; Tebas et al., 2014). However, the natural variant in CCR5 that confers resistance to HIV may increase susceptibility to West Nile virus, suggesting that CCR5 may have pleotropic effects on T cell function and infection with diverse pathogens (Glass et al., 2006). Similarly, CXCR4 is essential in hematopoietic stem cells but appears largely dispensable in T cells, suggesting multiple roles in a cell-type-dependent manner (Berson et al., 1996; Feng et al., 1996; Zou et al., 1998). The ability to readily and reproducibly generate knockout cells ex vivo provides a system for testing pleiotropic effects of these modifications, an essential consideration for the development of next-generation therapeutics.

This approach was also successful in targeting genes required at later stages of initial infection. Disruptions of *LEDGF* and *TNPO3* were well tolerated in primary T cells and both resulted in clear defects in HIV replication. The ability to measure the impact of *LEDGF* on infection demonstrates the advantage of this technique over RNAi approaches. Even treatment with ubiquitously penetrating RNAi can leave cells with persistent low levels of target gene expression, which has complicated functional validation of factors required at only low concentrations, such as *LEDGF* (Llano et al., 2006a, 2006b). In contrast, Cas9 RNPs can generate true genetic knockouts in a substantial subset of targeted cells. While still a heterogenous pool, a large fraction of Cas9 RNP-treated cells are completely devoid of the target protein, allowing for functional interrogation in a clean genetic system. Furthermore, unlike siRNA electroporation, transient treatment with Cas9 RNPs generates permanent genetic modifications in the T cells, allowing for the examination of later time points in infection where more robust effects may be observed.

While *LEDGF* and *TNPO3* knockouts resulted in clear replication defects upon single-round infection, neither inhibited infection fully, even after adjusting for observed editing efficiency. This strongly suggests alternate routes to infection may exist independent of these factors or that some level of functional redundancy with other unidentified host factors is occurring in primary T cells (Levin et al., 2010; Vandegraaff et al., 2006; Vandekerckhove et al., 2006). The ease in generating double-knockout populations using this system makes it an attractive platform for analyzing these redundant factors and perhaps for systematically determining epistatic relationships among host dependency genes to better identify gene pathways (Roguev et al., 2013). The fact that editing at different loci appears to occur independently of each other, however, mandates the validation of highly efficient RNPs to ensure high efficiency of double editing. Such multiplex editing could generate cells that are resistant to HIV infection through multiple independent mechanisms, analogous to current combinatorial antiretroviral therapies (Didigu et al., 2014; Voit et al., 2013).

In contrast to *LEDGF* and *TNPO3*, *NUP153* and *CDK9* appeared essential for T cell survival ex vivo. While not unexpected,

these results rule out therapeutic ablation of these factors. While genes that impact cell viability or growth are obviously excluded from this possibility, ablation of any gene would need to be studied more thoroughly in a mouse model prior to consideration for any cell-based therapy. As mentioned above, while *CXCR4* and *CCR5* deletion may not cause an obvious deficiency in vitro, deletions of these genes have been linked to immune defects in vivo. One potential way around this problem is to focus on identifying and engineering specific, naturally occurring polymorphisms that are known to be well tolerated and potentially protective against infection. We have previously demonstrated that Cas9 RNPs can generate knockin edits in primary T cells in addition to the knockout disruptions that were the focus of the current studies (Schumann et al., 2015). While the efficiency of knockin edits is lower than that of knockouts, as the technology improves, we envision this platform could be just as readily employed to study the impact of point mutants and haplotypes on infection.

Beyond using this CRISPR/Cas9 RNP approach to explore the roles of known host factors, genetic relationships between genes, and functional redundancy, the ease of scalability in performing these manipulations in primary human T cells opens up the possibility of discovery-based screening. As a proof of principle, we screened 45 genes with known or suspected roles in HIV integrase function as curated from principally cell-line-based data in the literature. Beyond confirming the expected phenotype of several host factors in primary cells, we also uncovered novel roles for genes with no previously published replication phenotype, including *XRCC6* and *UNC45A*. While much more follow-up work remains to be done with these factors, it demonstrates the power of the platform to achieve high-quality genetic perturbation on the medium- to high-throughput scale.

This platform has multiple applications well beyond the identification of genes involved in HIV replication. The capacity to perform high-content screens in human T cells allows for the genetic dissection of pathways essential for immune cell effector function in a variety of healthy and diseased states. One major concern in applying genome editing to primary human cells has been that CRISPR/Cas9 generates a heterogeneous mixture of cells with various phenotypes as a result of imprecise repair mechanisms at the targeted site (Doudna and Charpentier, 2014; Ran et al., 2013). While this platform can target genes with 70%–90% efficiency, not every cell is edited in the final population. Moving forward, we will need improved ways of purifying edited cells for both research and therapeutic purposes. Nevertheless, this study demonstrates that we can already link specific, edited genotypes to complex phenotypes, including HIV infection. The efficiency of gene disruption is high enough that we are able to observe quantitative changes in HIV infection with multiple in vitro assays. Heterogeneously edited cells may actually prove beneficial for experiments performed under selective conditions, identifying genetic variants that protect against infection while leaving normal gene function unperturbed. Efforts to understand these and other questions are ongoing and promise to shed more light on the potential of CRISPR/Cas9 technology for therapeutic purposes.

Multiplex Cas9 RNP-mediated editing of primary $CD4^+$ T cells is a powerful method for the study of T cell processes and the identification and analysis of next-generation cell- and drug-based therapies. It allows for interrogation of microbial dependencies on human host genes directly in the relevant primary cells, identifying pathways that could potentially be exploited for therapeutic development. Specifically coupling Cas9 RNP editing with cell-based therapies could provide a direct path from target discovery and validation to the engineering of therapeutic cells for the cure of HIV infection and other disease states.

EXPERIMENTAL PROCEDURES

Detailed experimental procedures and associated references are available in the Supplemental Experimental Procedures.

Cas9 RNP-Mediated Editing of Primary Human T cells

In brief, primary $CD4^+$ T cells were isolated from human whole blood by Ficoll gradient centrifugation and negative selection via the Easysep Human $CD4^+$ T cell enrichment kit (STEMCELL Technologies). T cells were stimulated with bound anti-CD3 and soluble anti-CD28 in the presence of interleukin 2 (IL-2) for 48 hr prior to electroporation. Electroporation was performed using the Amaxa P3 Primary Cell 96-well Nucleofector kit and 4D-Nucleofecter (Lonza). Recombinant *S. pyogenes* Cas9 protein used in this study expresses a C-terminal HA tag and two nuclear localization signal (NLS) peptides that facilitate transport across the nuclear membrane. The protein was expressed and purified as described previously (Anders and Jinek, 2014) and obtained from the QB3 Macrolab, University of California, Berkeley. Purified Cas9 protein was stored in 20 mM HEPES (pH 7.5) plus 150 mM potassium chloride, 10% glycerol, and 1 mM tris(2-carboxyethyl)phosphine (TCEP) at −80°C. crRNA for each gene were designed using the online tool developed by the Zhang lab at the Massachusetts Institute of Technology (http://crispr.mit.edu/) (Hsu et al., 2013). Each crRNA and the associated tracrRNA were chemically synthesized (Dharmacon) and suspended in 10 mM Tris-HCl (pH 7.4) to generate 80 μM RNA stocks.

Cas9 RNPs were prepared fresh for each experiment. crRNA and tracrRNA were first mixed 1:1 and incubated 30 min at 37°C to generate 40 μM crRNA:tracrRNA duplexes. An equal volume of 40 μM Cas9-NLS was slowly added to the crRNA:tracrRNA and incubated for 15 min at 37°C to generate 20 μM Cas9 RNPs. For each reaction, ~300,000 stimulated T cells were pelleted and resuspended in 20 μL P3 buffer (Lonza). 3 μL of 20 μM Cas9 RNP mix was added directly to these cells and the entire volume transferred to the 96-well reaction cuvette. For double-editing reactions, 3 μL of each Cas9 RNP was added to 20 μL cells. Cells were electroporated using program EH-115 on the Amaxa 4D-Nucleofector (Lonza). 80 μL pre-warmed, complete RPMI was added to each well, and the cells were allowed to recover for 30 min at 37°C. Cells were then re-stimulated on plates coated overnight with 10 μg/mL anti-CD3 (UCHT1, Tonbo Biosciences) and 10 μg/mL anti-CD28 (CD28.2, Tonbo Biosciences) for 24 hr prior to infection.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, five figures, and two tables and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2016.09.080.

AUTHOR CONTRIBUTIONS

J.F.H., K.S., N.J.K., and A.M. conceived the project. Primary cell isolation, crRNA design, and knockout cell generation were conducted by K.S. Target identification, HIV infection, and subsequent analyses were performed by J.F.H. and M.J.M. TIDE analysis was performed by J.W. LEDGF spreading infections were performed by L.M. The figures were designed and assembled by J.F.H. The text was written by J.F.H. and A.M. with input from K.S., L.M., J.D., N.J.K., and V.S. M.J.M., J.F.H, K.S., and A.M. edited the manuscript for publication.

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