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Case Study

Synthetic sgRNAs Enable Researchers to Study Viral Infection in Resting Human CD4+ T Cells

Featuring

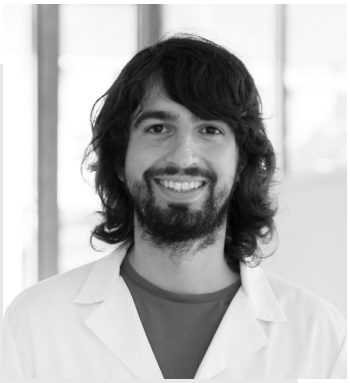
Dr. Manuel Albanese

Postdoctoral Researcher, Max von Pettenkofer Institute and Gene Center, Virology, National Reference Center for Retroviruses, LMU München

APPLICATION
Bioengineering

RESEARCH AREA
Infectious Disease

PRODUCT
Synthetic sgRNA



Dr. Manuel Albanese

Postdoctoral Researcher Max von Pettenkofer Institute and Gene Center, Virology, National Reference Center for Retroviruses, LMU München

> ***We initially started experimenting with guides from some other companies. However, Synthego's modified single guide RNAs always worked best in our hands, so we decided to move ahead entirely with Synthego. We always use a combination of two guide RNAs for best efficiency.***

At A Glance

Executive Summary

Dr. Manuel Albanese and his colleagues used Synthego's synthetic single guide RNA (sgRNA) and Lonza's 4D-Nucleofector® System to optimize Nucleofection® Conditions in resting human CD4+ T cells. Dr. Albanese conducted this study as a postdoctoral researcher together with Adrian Ruhle in Dr. Oliver Keppler's lab at the Max von Pettenkofer Institute and Gene Center in Munich, Germany. The study was published in *Nature Methods* in January 2022, titled, "Rapid, efficient and activation-neutral gene editing of polyclonal primary human resting CD4+ T cells allows complex functional analyses".

Introduction

Resting CD4+ T cells are highly resistant to human immunodeficiency virus-1 (HIV-1) infection, present multiple blocks to active HIV-1 replication, and often act as reservoirs of latent HIV-1 infection. As such, it is important to study the biology of these cells and mimic the viral interaction and replication steps in naïve, resting CD4+ T cells. The authors targeted multiple genes related to viral infectivity (up to six genes were knocked out simultaneously at rates greater than 98% while maintaining high viability), knocked in large reporter gene segments, and performed downstream functional assays to characterize their various gene edits.

Challenge

Previous attempts at gene editing of resting CD4+ T cells have been met with minimal success and low cell viability. Conventionally, it is challenging to maintain the resting states in CD4+ T cells subsequent to gene knockout, thereby limiting the analysis of pathways and factors that trigger CD4+ T cell activation. This is particularly challenging within the context of HIV-1 infection as it restricts the study of pro- and anti-viral infection factors in host immune cells.

Solution

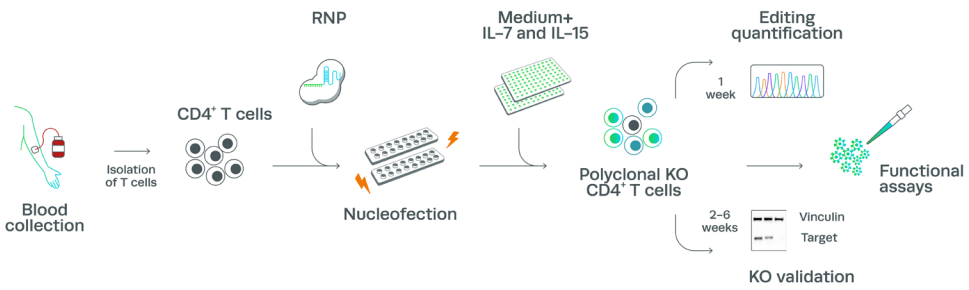
Through a combination of optimized culturing conditions such as interleukin supplementation, Synthego's sgRNA, and Lonza's 4D-Nucleofector® System, the authors were able to optimize the editing conditions for resting human CD4+ T cells and maintain high cell viability for several weeks post Nucleofection®. These edited CD4+ T cells presented a robust system to evaluate the impact of these individual genes on HIV infectivity in resting T cells through a series of downstream functional assays such as T cell migration and fluorescence-activated cell sorting for fluorescence-expressing HIV vectors.

Introduction

Resting human CD4+ T cells are highly resistant to active HIV-1 replication and act as reservoirs of latent infection. As such, it is critically important to study the biology of these cells in naïve, resting CD4+ T cells. The systemic interrogation of the role of canonical genes in viral infection has been challenging as these cells are not compatible with standard transfection protocols and often result in low cell viability following gene editing. Unlike activated CD4+ T cells, resting CD4+ T cells do not proliferate, thus necessitating a highly efficient protocol for generating knockout CD4+ T cells. Previous studies in the field used activated human CD4+ T cells for editing and allowed them to return to resting physiological states for analysis. However, a caveat of using post-activated resting CD4+ T cells is that they do not completely recapitulate the naïve T cell physiology. This has greatly limited scientists from studying the different factors and pathways involved with viral entry and replication in resting CD4+ T cells.

The present case study describes Dr. Albanese's optimized methods to transfect resting human CD4+ T cells using Synthego's single guide RNAs and Lonza's 4D-Nucleofector® System. Using the optimized transfection conditions, Dr. Albanese could knockout up to six genes (*CD46*, *CXCR4*, *PSGL-1*, *CD4*, *TRIM5α*, and *CPSF6*) simultaneously in resting CD4+ T cells with high editing efficiencies and maintained high cellular survivability for several weeks following gene editing. This allowed the authors to interrogate the functionality of these genes in resting CD4+ T cells in a series of downstream assays. Furthermore, the authors utilized this optimized protocol to knock in a green fluorescent protein (GFP) to key genes that regulate viral infection to study alternative pathways for HIV-1 infectivity through simple assays such as fluorescent-activated cell sorting.

Overall, the authors conclude that the impact of this study opens new avenues to investigate virus and human CD4+ T cell interactions and viral latency in physiologically relevant resting CD4+ T cells.



Pipeline Schematic to Establish Polyclonal Knockouts in Human Resting CD4+ T Cells
*The above and following figures were adapted from the original article from Albanese et al, Nat Methods 19, 81–89 (2022) and is licensed under the Creative Commons Attribution License 4.0 which permits the use, sharing, distribution, and reproduction in any format so long as appropriate credit is given to the original authors.

Methods

Dr. Albanese chose Synthego's synthetic sgRNAs and Lonza's 4D Nucleofector® System for his experiments, in combination with optimal cell culture media conditions.
This enabled the team to obtain the following:

- Achieve high and consistent knockout efficiencies in resting primary human CD4+ T cells
- Consistent single gene knockout efficiency enabled experiments studying multi-gene knockouts
- High viability in resting human CD4+ T cells following transfection (unlike prior studies that were limited to 72 hours)
- Ability to perform downstream functional studies due to sustained high viability

Synthego's synthetic sgRNA addressed one of the major challenges for Dr. Albanese's study, by providing consistently high knockout efficiencies (>98%) in resting human CD4+ T cells. This was also critical to the downstream functional characterization studies.

Dr. Albanese used a combination of two synthetic sgRNAs for each target gene knockout in order to achieve the best editing efficiency. For delivery into resting CD4+ T cells Dr. Albanese used the 16-well strip of the Lonza 4D-Nucleofector® X Unit. In a total volume of 20 µl, he transfected $2x10^6$ cells with the pre-complexed RNPs. The nuanced pulsing of the 4D-Nucleofector® allowed him to gain high editing efficiencies while maintaining a high viability.

Please refer to the detailed protocol on how to edit resting CD4+ T cells.

Results

Dr. Albanese's work describes a robust approach to editing primary human resting CD4+ T cells through a combination of Synthego's highly efficient synthetic sgRNAs to ensure successful editing, Lonza's 4D-Nucleofector®, and optimal culturing conditions to ensure high cell survival following Nucleofection®. This optimized approach allows for a greater number of available edited cells for the study of both previously evaluated and novel mechanisms of infection in resting CD4+ T cells.

CD4+ T Cells Retain High Viability Following Single and Multi–gene Gene Knockouts

Dr. Albanese and his team demonstrated robust single and multi-gene knockout of various genes integral for viral infection (*CD46*, *CXCR4*, *PSGL-1*, *CD4*, *TRIM5α*, and *CPSF6*) in resting CD4+ T cells (**Figs 1a-b**). Importantly, cell viability remained relatively high following single and multi-gene knockout (50-80% for 4 weeks following infection, **Fig 1c**). Upon activation, edited T cells continued to express canonical activated T cell markers CD25, CD39 CD69, and HLA-DR (**Fig 1d**).

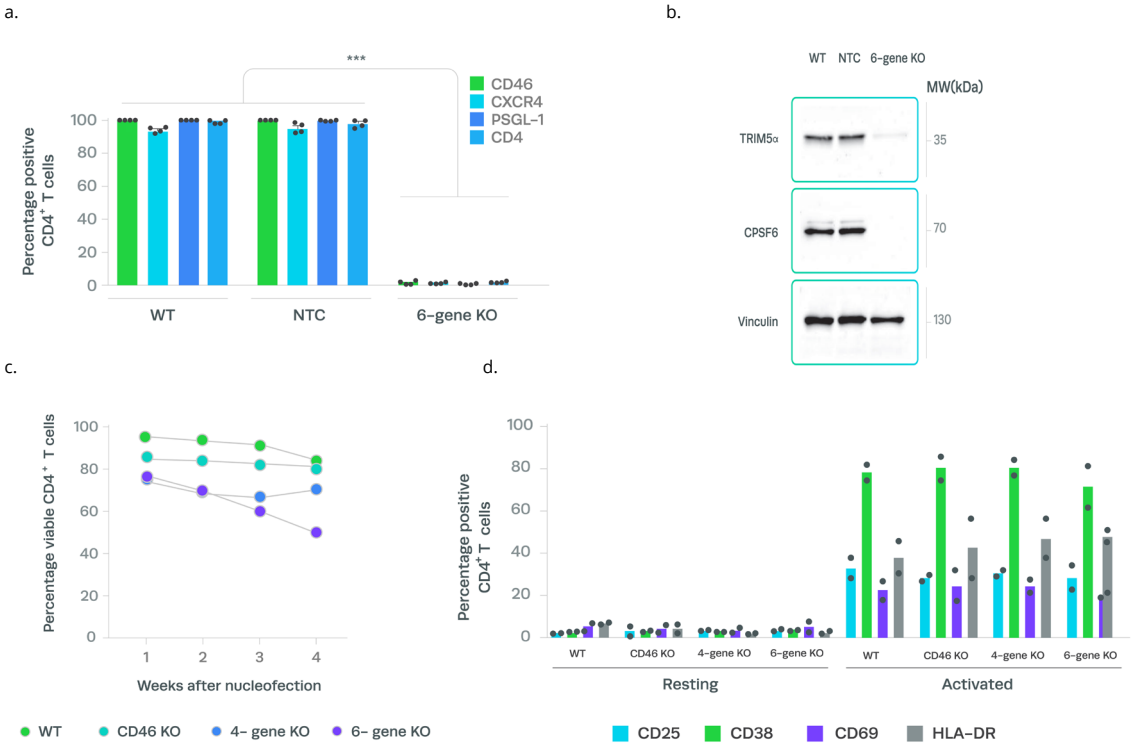


Figure 1. Highly Efficient Multi-gene Knockout in Primary Human Resting CD4+ T Cells Simultaneous, polyclonal six-gene knockout following a single RNP nucleofection with Synthego's sgRNAs resulted in the depletion of 4 cell surface markers (CD46, CXCR4, PSGL-1, and CD4) within 2 weeks via fluorescent-activated cell sorting (**a**) and depletion of cytoplasmic proteins (TRIM5a and CPSF6) within 25 days via immunoblotting (**b**). Viability of both single gene and multi-gene (4- and 6-gene) knockout demonstrated high cell survival 4 weeks following nucleofection as compared to wild-type (WT) controls (**c**). Multi-gene-edited CD4+ T cells demonstrated a typical activated CD4+ T cell profile following stimulation (T-Activator CD3/CD28 beads and IL-2 medium) as assessed by canonical active CD4+ T cell markers CD25, CD69, CD38, and HLA-DR via fluorescent activated cell sorting 2 weeks following nucleofection (**d**). (Figure adapted from Albanese et al, Nat Methods 19, 81–89 (2022).

Functional Characterization of Single Gene Knockouts

Single gene knockouts were systematically evaluated for their role in HIV-1 infectivity. Cell surface marker CXCR4 is well-known for its role in T cell chemotaxis and HIV-1 entry and infection. The knockout of CXCR4 gene resulted in both a reduction in the ability of CD4+ T cells to migrate and also for its ability to be infected by a GFP-expressing HIV-1 (**Figs 2a-b**).

Dr. Albanese and his team further evaluated transcription factors implicated in HIV-1 infection (MX2, CPSF6, PSGL-1) that had been studied in other cell systems but not resting CD4+ T cells. Their work showed that CPSF6 did not mediate pre- and post-entry of HIV-1 into host CD4+ cells. MX2 and CPSF6 are both noted for their role in interacting with the HIV-1 capsid during nuclear entry of the pre-integration complex and proviral integration. Interestingly, the depletion of CPSF6, but not MX2, resulted in resting CD4+ T cells being refractory toward HIV-1 infection (**Fig 2c**). These are novel findings in the field of resting human CD4+ infectivity by HIV-1.

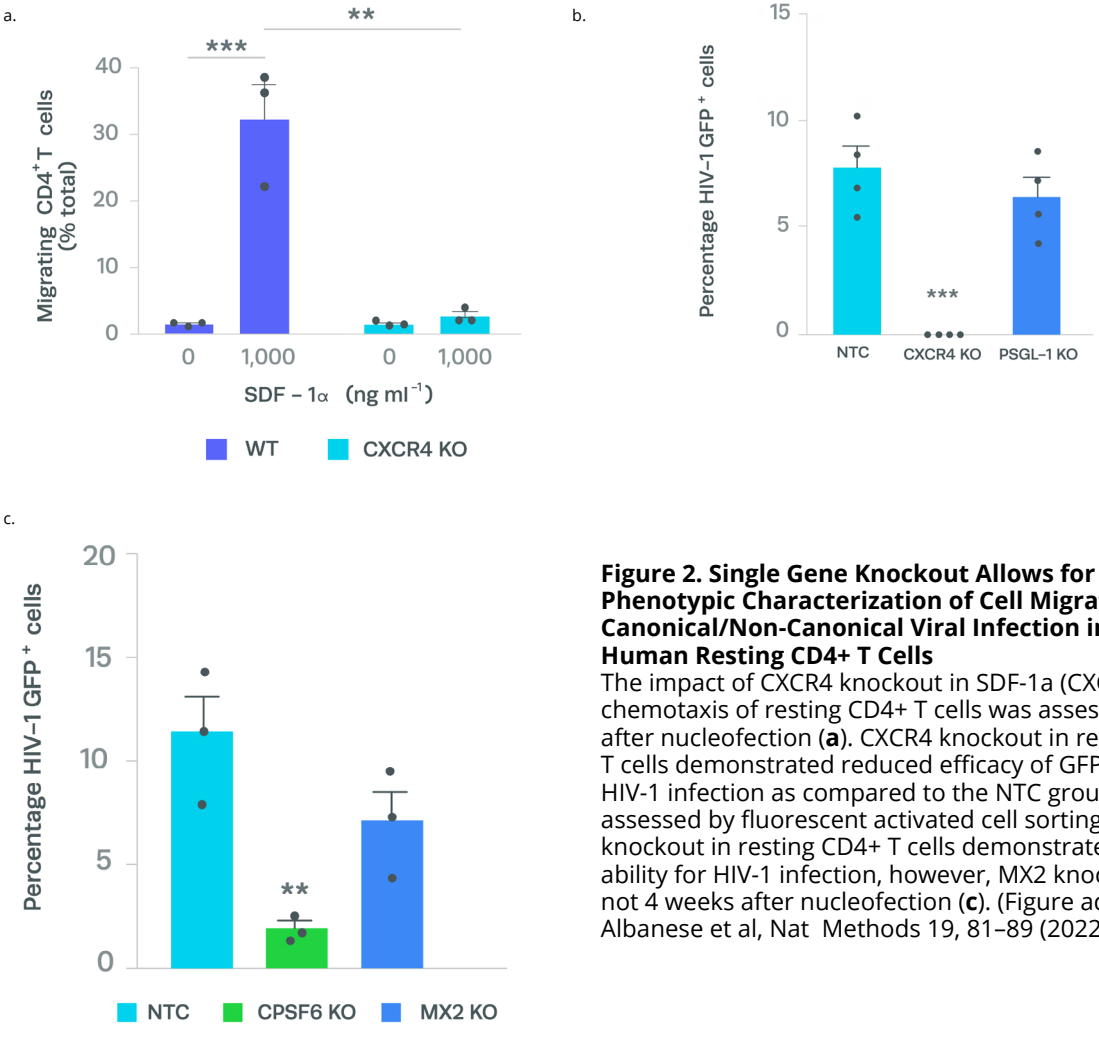


Figure 2. Single Gene Knockout Allows for Phenotypic Characterization of Cell Migration and Canonical/Non-Canonical Viral Infection in Primary Human Resting CD4+ T Cells
The impact of CXCR4 knockout in SDF-1a (CXCL12)-driven chemotaxis of resting CD4+ T cells was assessed 1 week after nucleofection (**a**). CXCR4 knockout in resting CD4+ T cells demonstrated reduced efficacy of GFP expressing HIV-1 infection as compared to the NTC group and assessed by fluorescent activated cell sorting (**b**). CPSF6 knockout in resting CD4+ T cells demonstrate a reduced ability for HIV-1 infection, however, MX2 knockout does not 4 weeks after nucleofection (**c**). (Figure adapted from Albanese et al, Nat Methods 19, 81–89 (2022).

eGFP Knock–In Upstream of *SAMHD1* Enables Studying Alternative Pathways for HIV–1 Infection

Dr. Albanese created a functional reporter system to study the mechanism of SAMHD1 in regulating viral infection. SAMHD1 is a human anti-viral protein that prevents viral replication of HIV-1. The specific knockout of the *SAMHD1* gene has been studied in other diseases, and loss-of-function mutations in *SAMHD1* have resulted in a greater vulnerability to viral infection.

In order to study the role of *SAMHD1* in resting CD4+ T cells, Dr. Albanese and team knocked in an N-terminal eGFP protein into the *SAMHD1* gene by using one sgRNA from Synthego and a double-stranded plasmid DNA donor template with approximately 550 base pair homology arms (**Fig 3a**). This resulted in a 2.3% knock-in efficiency of GFP into T cells that were then subsequently enriched for downstream assays via FACS. This GFP-SAMHD1 CD4+ T cell line was then used to study alternative infectivity pathways with high resolution by the researchers. The team used a reporter HIV-1-BFP/SAMHD1-GFP CD4+ T cell system where BFP and GFP were used to demonstrate shifts in the status of T cells following infection.

GFP-SAMHD1 resting CD4+T cells were largely refractory to infection by a BFP-expressing HIV-1 vector unless a known degrader for SAMHD1, Vpx, was introduced which resulted in a decrease in the number of SAMHD1-GFP+ cells and a concomitant increase in HIV-1-BFP+ cells (**Fig 3b, left 4 panels**). To further explore additional pathways of HIV-1 infectivity, Dr. Albanese and colleagues introduced EFV, a reverse transcriptase inhibitor, to Vpx-treated GFP-SAMHD1 CD4+ T cells resulting in an abrogation of BFP+ cells while still maintaining a depletion of GFP+ T cells (**Fig 3b, right 2 panels**) thus allowing for high-resolution analysis of alternative pathways for viral infection.

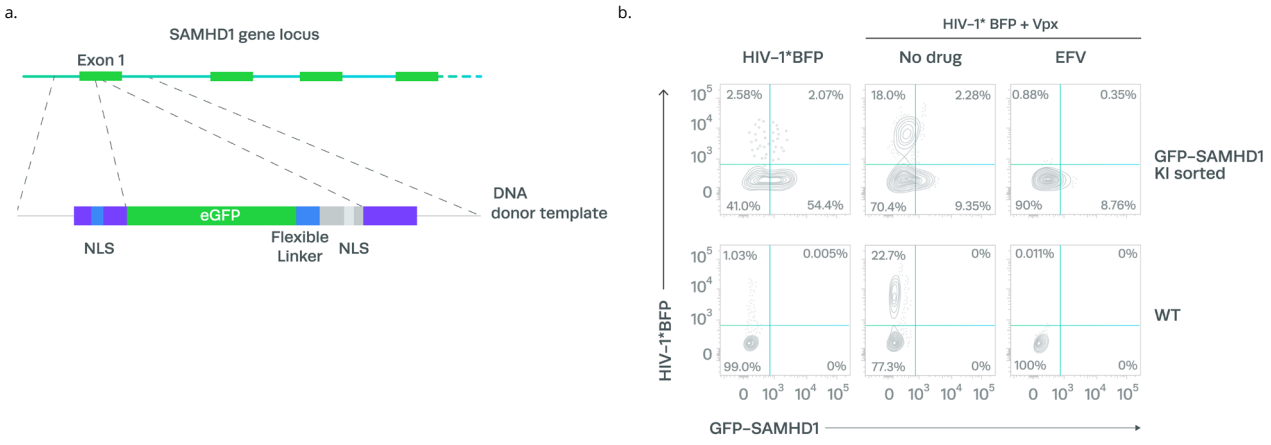


Figure 3. Large Knock-in of eGFP into anti-viral protein SAMHD1 Loci Allows for Study of HIV-1 Infection in Primary Human Resting CD4+ T Cells
SAMHD1, an innate human protein that blocks viral replication of HIV-1 in dendritic cells, macrophages, monocytes, and resting CD4+ lymphocytes, was studied for its role in CD4+ T cell infectivity by HIV-1. eGFP was knocked in upstream of the SAMHD1 loci utilizing a double-stranded plasmid DNA donor template with 550 bp homology arms for exon 1 of SAMHD1 to generate GFP-SAMHD1 expressing resting CD4+ T cells (**a**). Infection of the GFP-SAMHD1 CD4+ T cells with BFP-expressing HIV-1 virions is inhibited except when Vpx protein is introduced which degrades SAMHD1 protein (**b, middle column**). The inclusion of EFV, a reverse transcriptase inhibitor, resulted in an inhibition of BFP-HIV-1 infection despite SAMHD1 depletion (**b, right column**). (Figure adapted from Albanese et al, Nat Methods 19, 81–89 (2022).

Synthego's Synthetic Single Guide RNAs Enable High–Efficiency Knockouts in Resting CD4+ T Cells

When Dr. Albanese and his team compared Synthego's synthetic sgRNAs (freshly prepared or frozen and then thawed) against another major vendor's synthetic sgRNAs, Synthego's sgRNAs resulted in consistently greater (more than 2x) knockout efficiency than the other vendor's (**Fig 4**).

This efficient gene editing in resting CD4+ T cells is important as these assays require the use of valuable donor cells from humans. A gene-editing protocol that results in **high cell viability and high gene editing efficiency** is critical in enabling downstream experiments. Furthermore, high editing efficiency is crucial for future downstream clinical applications. A large amount of successfully edited donor cells will impact how many cells are available for patient use. To ensure enough viable cells for the downstream functional assays, Dr. Albanese and the team started out with 2.5x more cells than typically required for these assays.

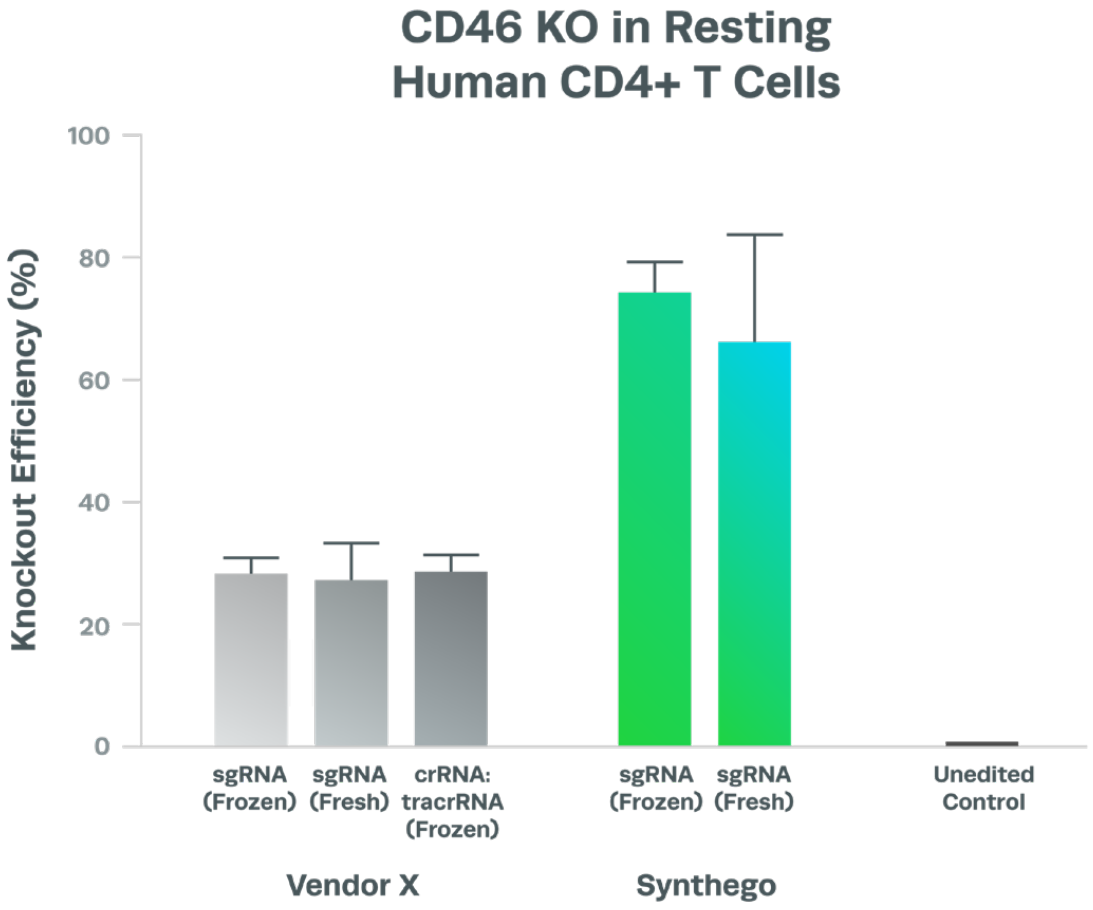


Figure 4. Synthego's Synthetic Guides Outperform Another Vendor's in Editing Resting CD4+ T Cells The editing efficiency of both frozen and freshly prepared Synthego synthetic sgRNAs were directly compared against another vendor's sgRNA and crRNA:tracrRNA in editing the CD46 loci in resting CD4+ T cells. Synthego's synthetic sgRNAs demonstrated consistently high (60%+) knockout efficiency as assessed by TIDE analysis regardless of preparation condition. (Unpublished data, Albanese et al.)

Conclusions

Previous viral interaction studies have utilized CRISPR editing approaches in post-activated CD4+ T cells as they can be readily expanded following gene editing, whereas maintaining viability in resting CD4+ T cells post-gene editing is a persistent problem that has challenged the field. Also, such studies have largely only targeted single cellular factors and are often extremely time-consuming. The few studies that have looked at CRISPR-mediated genetic manipulation in resting T cells are greatly limited due to cytotoxicity. In many cases, the cells do not survive beyond three days after transfection.

In this study, Dr. Albanese and his colleagues utilized optimized media conditions and the Lonza 4D-Nucleofector® System, which resulted in high cell survival of edited resting human CD4+ T cells. This optimized protocol, combined with the efficacy of Synthego's sgRNAs in generating highly efficient knockouts for both single and multiple genes, enabled the successful analysis of several genes that mediate HIV-1 infectivity in resting CD4+ T cells. Learnings from this study could be extended beyond virus-host cell interactions, including areas such as immuno-oncology and T cell biology.

To learn more about Synthego's CRISPRevolution products, go to **Synthego.com/products/crispr-kits**

To learn more about Lonza's 4D-Nucleofector® System, go to **Bioscience.lonza.com/nucleofector-technology**

> ***Our work is vital to the larger T cell community because now scientists can knock out genes in resting T cells and continue to maintain their resting states. This will allow scientists to study T cell activation factors. Our multiple gene knockout approach allows researchers to study T cell signaling pathways with redundant protein functions.***
>
> *- Dr. Manuel Albanese*

About Synthego

Synthego is a genome engineering company that enables the acceleration of life science research and development in the pursuit of improved human health. With its foundations in engineering, the company leverages machine learning, automation, and gene editing to build platforms to advance both basic research and therapeutic development programs.

Lonza Cologne GmbH – 50829
Cologne Germany