

**Targeting the untargetable:
Eliminating HIV latency using nanoparticle
delivery systems**

Paula M. Ceva

ORCID iD: 0000-0003-4473-7904

Submitted in total fulfilment of the requirements of the degree of

Doctor of Philosophy

April 2023

Department of Microbiology and Immunology

Faculty of Medicine, Dentistry and Health Sciences

The University of Melbourne

“

Be as persistent as HIV is.

”

Françoise Barré-Sinoussi

(2022)

Abstract

T cells form an important therapeutic target for the development of immunotherapies or the treatment of T cell-implicated pathologies. Specifically, CD4⁺ T cells are subject to human immunodeficiency virus (HIV) infection. Whilst treatment with antiretroviral therapy (ART) successfully represses the viral load to undetectable levels, HIV treatment is lifelong, posing a medical, social and financial burden to those 38.5 million people currently living with HIV globally. The ability of HIV to establish a reservoir of latently infected cells is the foremost barrier to finding a cure for HIV.

One approach towards eliminating HIV latency is the reactivation of viral transcription and subsequent elimination of infected cells through immune-mediated clearance or viral-mediated cytotoxicity. However, this approach so far has suffered from a lack of potency and dose-limiting toxicities due to the use of compounds that affect both host and viral transcription and the inability to specifically target the infected cells. One solution involves the use of nanoparticles for the targeted delivery of existing therapeutics or to advance the development of HIV-specific mRNA-based therapeutics including CRISPR-Cas. However, the generally low rate of endocytosis in CD4⁺ T cells forms a challenge to efficient nanoparticle-based drug delivery to CD4⁺ T cells *in vitro* and *in vivo*.

This thesis describes our efforts towards rationally designing a nanoparticle platform capable of delivering HIV latency-reversing therapeutics to CD4⁺ T cells with high efficiency. We first established a methodology to improve the assessment of nanoparticle performance *in vitro* through the generation of absolutely quantitative, comparable data on nanoparticle-cell interactions. We then used this methodology to screen for nanoparticle designs with enhanced uptake kinetics in CD4⁺ T cells *in vitro*, using a novel, high-throughput assay to quantify nanoparticle internalization over time. We found that targeting sub-100 nm nanoparticles to T cell surface receptors undergoing rapid receptor cycling can be exploited to actively trigger nanoparticle uptake through receptor-mediated endocytosis and identified CD2 and CD7 as potent candidate receptors for future *in vivo* T cell targeting.

We next aimed to use translate these findings to lipid nanoparticles, a well-established platform for the delivery of nucleic acid-based therapeutics. We investigated whether lipid nanoparticles could be used to deliver a next-generation latency-reversing agent based on CRISPR activation,

which specifically targets the HIV proviral genome without affecting host-cell transcription. We identified a novel lipid nanoparticle formulation that can efficiently transfect T cell lines as well as resting CD4⁺ T cells. We showed that this lipid nanoparticle can co-encapsulate the three RNA components of the CRISPR activation system and induce strong latency reversal in a cell line model for HIV latency. We finally presented preliminary evidence that targeting lipid nanoparticles to rapidly cycling surface receptors increases mRNA delivery, further supporting our findings that targeting receptor-mediated endocytosis could be a viable strategy to increase nanoparticle-mediated drug delivery to traditionally hard-to-transfect T cells.

These findings provide a compelling justification for the further assessment of CRISPR activation lipid nanoparticles for the elimination of the latent HIV reservoir, and more broadly contribute to the development of T cell-targeted nanomedicine for HIV and beyond.

I declare that:

- (i) this thesis comprises only my original work towards the degree of Doctor of Philosophy except where indicated in the preface;
- (ii) due acknowledgement has been made in the text to all other material used; and
- (iii) this thesis is fewer than the 100,000 words in length, exclusive of tables, maps, bibliographies and appendices.

Paula M. Cevaal

Preface

The work towards this thesis was carried out under the supervision of Professor Sharon R. Lewin, Professor Frank Caruso, Dr Jori Symons and Dr Michael Roche at The Peter Doherty Institute for Infection and Immunity (Department of Microbiology and Immunology) as well as the Department of Chemical Engineering at The University of Melbourne, Australia.

Science is never done in solitude. Therefore, every chapter of this work was produced as part of a larger team of colleagues and collaborators that provided intellectual input, feedback or practical help with the execution of experiments. For each chapter, these contributions are acknowledged below.

Parts of **Chapter 1. General Introduction** have been published as a narrative review in *ACS Nano* in 2021. The published material is included in this thesis with permission from the below citation (Copyright 2021, American Chemical Society).

Cevaál, P.M., Ali, A., Czuba-Wojnilowicz, E., Symons, J., Lewin, S.R., Cortez-Jugo & C., Caruso, F. In Vivo T Cell-Targeting Nanoparticle Drug Delivery Systems: Considerations for Rational Design. *ACS Nano* **15**, 3736-3753, doi:10.1021/acsnano.0c09514 (2021).

Contributions of each author are as follows: PMC, JS, CCJ outlined the scope of the review article. PMC, AA, ECW, JS and CCJ contributed to literature research and drafting of certain sub-sections of the manuscript. PMC compiled and wrote the first manuscript draft. PMC, SRL, FC, CCJ and JS contributed to subsequent revisions. All authors approved the final manuscript.

Chapter 2 has been published as a methods paper with an accompanying video tutorial by the *Journal of Visualized Experiments* in 2022. The final manuscript has been included in this thesis as such with permission (Copyright 2022, Journal of Visualized Experiments). Due to journal requirements, this section of the thesis is written in American English. The citation for this publication is:

Cevaál, P.M., Roche, M., Lewin, S.R., Caruso, F. & Faria, M. Experimental Quantification of Interactions Between Drug Delivery Systems and Cells In Vitro: A Guide for Preclinical Nanomedicine Evaluation. *J Vis Exp* **187**, e64259, doi:10.3791/64259 (2022).

Contributions of each author are as follows: PMC and MF conceptualized the work and designed

the methodology. MR provided feedback on the method design. PMC and MF wrote the manuscript. PMC, MF, MR, SLR and FC contributed to subsequent revisions. All authors approved the final manuscript. PMC and MF prepared and contributed to the production of the video protocol.

Chapter 3 includes contributions from Dr Christina Cortez-Jugo, Ms Abigail Tan, Dr Matt Faria, Dr Celine Gubser, Professor Frank Caruso, Dr Michael Roche, Dr Jori Symons and Professor Sharon R. Lewin. PMC conceptualized the study and designed the experimental assays. MF and CG provided intellectual input on the various assay designs. PMC, AT and CCJ performed experiments. AT specifically contributed to results presented in Figures 3-7, Figure S4 and Figure S5. CCJ specifically contributed to the results presented in Figure 8 and Figure S3. PMC performed the data analysis. MF provided intellectual input on the data analysis. FC, MR, JS and SRL provided intellectual input at various stages throughout the design, execution and analysis of the work. PMC drafted the manuscript. This work is currently in preparation for publication.

Chapter 4 includes contributions from Dr Stanislav Kan, Ms Abigail Tan, Dr Marvin Holz, Mr Denzil L. Furtado, Professor Damian Purcell, Dr Angus Johnston, Professor Colin Pouton, Mr Thomas Payne, Dr Wei Zhao, Professor Frank Caruso, Dr Jori Symons, Dr Michael Roche and Professor Sharon R. Lewin. PMC, JS and SRL conceptualized the study. DLF was key to the conceptualization of the use of lipid nanoparticles for the study. PMC and AT performed experiments. AT specifically contributed to the results presented in Figure 7-9. MH, DLF and DP provided intellectual input in mRNA synthesis. AJ, CP and TP provided intellectual input in lipid nanoparticle design and synthesis. WZ provided intellectual input on the use of Western Blots, particularly in Figure 7. SK developed the mSM102-LNP formulation. FC, MR, JS and SRL provided intellectual input at various stages throughout the design, execution and analysis of the work.

Chapter 5 includes contributions from Dr Stanislav Kan, Ms Abigail Tan, Dr Marvin Holz, Professor Damian Purcell, Dr Angus Johnston, Professor Colin Pouton, Mr Thomas Payne, Mr Denzil L. Furtado, Professor Frank Caruso, Dr Michael Roche and Professor Sharon R. Lewin. PMC, MR and SRL conceptualized the study. AJ, CP and TP provided guidance on the methodology. PMC and AT performed experiments. AT specifically contributed to the results presented in Figure 2. MH, DLF and DP provided intellectual input in mRNA synthesis. SK developed the mSM102-LNP formulation. FC, MR and SRL provided intellectual input at various stages throughout the design, execution and analysis of the work.

This PhD was supported by a Melbourne Research Scholarship (including fee offset). The research undertaken during this time was supported by the Australian National Health and Medical Research Council (NHMRC; Program Grant No. GNT1149990), the Australian Research Council Centre of Excellence in Convergent Bio-Nano Science and Technology (Project No. CE140100036), two separate project grants from the Australian Centre for HIV and Hepatitis Virology Research (ACH²), the Harold & Cora Brennen Benevolent Trust (Equipment grant No. BREN20122) and The Foundation for AIDS Research (amfAR; TARGET Grant No. 110406-73-RGRL_Lewin).

Acknowledgements

They say that doing a PhD is a journey – and what a journey it has been! Many people have become part of this journey, intentionally or unintentionally, and without their guidance, support, wisdom, love and compassion I would not be where I am today. Nor would this thesis, probably. It is important to me to thank those that have made the past four years the way they were.

First, to my supervisors **Sharon Lewin** and **Frank Caruso**, for the trust you have shown in me. You invited me back for a PhD and you gave me a carte blanche to conceptualize and design a project that I felt most passionate about. You allowed me to persist despite many initial struggles, and you guided me in growing my confidence as an independent researcher. I've come to realize this is not standard, so I am beyond grateful for having been given that opportunity. To **Jori**, for getting me to Australia in the first place, for sharing your love for this beautiful country – but most of all, for teaching me to think big. I've thoroughly enjoyed our many (on- and off-topic) discussions and brainstorming sessions over coffees. To **Michael**, for taking me on as your student in the final stages of my PhD and for teaching me to remain focused amidst those big ideas. It was exactly what I needed in my attempts to finish these projects.

To my PhD Advisory Committee members, **Jason Mackenzie**, **Rob De Rose**, **Sarah Palmer**, for being the absolute best Advisory Committee a student could wish for. Not only have you provided invaluable input and guidance on my research, you have also shown incredible support and care towards me as a person. I am deeply thankful for your presence throughout this journey and for you all as mentors.

To all **Lewin lab members**, for endless training, help and support in the lab as I fought my way through learning a wide range of techniques and skills.. and for bearing with me while I slowly introduced you all to the wonderfully exciting world of nanomedicine! To my fellow PhD students, for forming such a terrific support network for one another – please keep this up, it is so important. I owe particular thanks to a few people in specific. To **Abi**, for helping me out with so many of the experiments I present in this Thesis. I admire your work ethic and kindness to those around you, and a lot of us can learn from how meticulously you execute your work. To **Abdalla**, for being my fellow on this nano journey, and for welcoming me to work, every day, with the biggest smile – it's made so many of my days. To **Rachel**, for being my coffee wife, for letting me vent and for the many pep-talks you've given me. To **Bridget**, for entrusting me with the honourable role of being

your supervisor. It has been an absolute highlight of my PhD.

To the **Caruso Nanoengineering Group**, for giving me a second home and for answering my many, many, many (hopefully not too ignorant) questions about nanoscience. For teaching me the power of cross-disciplinary communication and collaboration. In particular, to **Christina**, for absolutely always being willing to help. To **Denzil**, thank you for that one coffee – without it (and you) I may never have made a single lipid nanoparticle. I owe you more than you can imagine.

To **Matt**, for adopting me during the COVID-19 lockdowns because of pure shared scientific interest, without any hidden agenda. For role modelling how one can thrive in academia without following the norm, for staying true to your own beliefs and speaking up for them rather than falling into the trap of the academic system. For introducing me to coding and mathematical modelling, for believing we can and should improve the way we do science. And for truly challenging me, something I so greatly craved during this PhD.

To Team **Gerulus**, for inspiring me. I am in awe of the wealth of your knowledge (within science as much as beyond), intellect, creativity and, beyond all, vision. I have learnt so much from you three and have enjoyed every chat, coffee or dinner we have shared.

To the beautiful human outside Flagstaff Station, whose name I never got to learn, for randomly yelling a 'good morning, my friend!' one day, which turned into a mutual ritual every time I had the pleasure of seeing you there. If only more people on this planet would exhibit such kindness to strangers. I thank you for inspiring me, and for putting a smile on my face on my way to yet another busy day at work. I hope you are well.

To **my kitesurfing friends**, I apologize for not having nearly as much time for you as I'd have liked! But I'm immensely grateful for being part of such a wonderful community and for the reminders that life outside the lab is pretty amazing too. I've enjoyed every outing with you all into the gorgeous Australian nature. To more of that, soon. To **my friends back home** – Vali, Lucie, Eelco, BJ, Ed, Anke – for not forgetting about me and for continuing to support me from so far away. Visiting home again after the COVID pandemic and having you all still there filled my heart with so much joy. I wish I were better at staying in regular touch, but my love for you has remained constant. I miss you all dearly. Vali & Lucie, I don't know how I would have made it through this PhD without your support, encouragement and love. You are both gorgeous women that I admire and respect immensely.

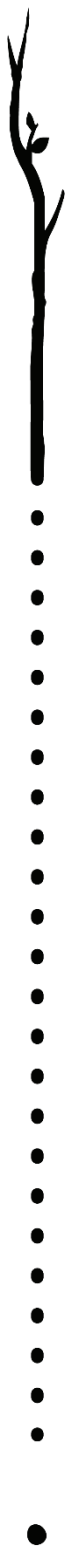
To **my family** – pap, mam, broertjelijf – it's truly incredible that you came all the way here for my Oration, to celebrate this milestone with me. Your support and encouragement to follow my dreams, even if that meant moving so far away and leaving you behind, means the world to me. I hope these weeks down under have allowed you to understand even better why I did move away, and why I'm staying a bit longer in this extraordinarily beautiful country where there is so much opportunity to feel the freedom of nature.

And, of course, to **Rik**, for following me to the other end of the world to be together. No words can describe how grateful I am for the love and support you have given me. You contributed so inconceivably much more to this all than those few references you proudly inserted for me ;). I know how frustrating it has been for you at times, to stand on the sideline, but that knowledge makes me all the more indebted to you for having you by my side.

Lastly, to the sun, the ocean and the winds, thank you for being there when I needed you, for being my meditation, my reminder that there are more important things in life and that really, we are just small human beings on an unimaginably big, beautiful and powerful planet earth.

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Chapter 0

Prologue

Imagine walking into a bicycle shop. You're rather new to cycling but are joining an adventurous, multi-day biking tour through diverse and rough terrain, so you want to make sure you're well prepared. The sign above the bike shop says, "Experts in Bicycle Engineering", so you're confident you've come to the right place.

You're welcomed with a nice coffee and you tell the salesperson about your plans. Passionate as he is, he starts explaining the vast range of bikes that exist, each with specific characteristics, sizes, shapes, technical accessories – and associated price tags. He even shows you their proprietary bikes in the hidden showroom, a futuristic range of self-developed and highly technical prototypes. You are absolutely amazed by their expertise and have never been more excited to get into cycling.

Much as you are enjoying the chat, you're keen to select your new toy. You remind your new friend of the adventure you're planning to undertake and ask him which model will be best suited to your needs. "Good question," he says, "but I'm afraid I'm not quite sure."

The field of nanomedicine has steadily been gaining attention following its emergence roughly two decades ago, with a noticeable spike in popularity since the COVID-19 pandemic. The record-speed development and approval of two lipid nanoparticle-based vaccine candidates was an immense achievement and has made an incredible contribution to bringing life back to normal. If we didn't before, the world has had another glimpse of what nanomedicine has got to offer for the next generation of therapeutics. Yet, these examples of successful translation of nanomedicine are concerningly rare. We have a great understanding of the key gaps in today's medicine, for which nanomaterials could provide solutions – and we have a seemingly infinite stream of new nanoparticle materials in all shapes and sizes. So why do we struggle so much to translate these into the clinic?

One reason could be the disconnect between the research fields involved; the language gap between chemists and medical scientists, between biologists and materials scientists. While together, they should have all the knowledge and more to design the next medical breakthrough, it appears that the challenge of communicating their ideas hinders technological advancements. Should materials scientists be better trained to know which biological assay best puts their

nanomaterial to the test? And how do we get medical scientists to be better at interpreting the information that is available on various nanomaterials?

Furthermore, novel materials are typically explored from an engineering perspective, where the focus is given to novel chemistries, novel characteristics, or novel ways to carry and release a therapeutic drug. Rarely are these materials tested using standardized assays in which the results are reported in a fashion that allows reproducibility. Such standards have only recently been proposed and are only now starting to be adopted by the field. Moreover, novel materials are not usually tested against a benchmark, a golden standard in the field, or even better, previously developed materials. How can we ever rationally select the most promising nanoparticle candidates without such comparability?

And even if we were to fully, reproducibly and comparatively characterize novel nanomaterials *in vitro*, how do we know whether this all translates to effective drug delivery *in vivo*? Or rather, how do we improve the predictability of our *in vitro* assays for *in vivo* efficacy? Attempts at answering such questions naturally bring us to big data, machine learning and mathematical modelling, to identify and start to understand which parameters hold the most predictive power.

Perhaps it's all part of a natural progression, a coming of age of the field of nanomedicine. An evolution from materials science to nanoscience, to translational nanomedicine, to applied clinical science. Trying to bridge this gap, this language barrier, forms an overarching theme of the work that is summarized in this thesis. Most of this thesis is set in the context of HIV/AIDS research – more specifically, research towards a nanoparticle-based therapeutic that can induce long-term HIV remission. However, this work can and should be seen more broadly, as first steps towards a better integration of the knowledge of materials scientists, biochemists, medical professionals, data scientists and biomedical scientists alike.



Chapter 1

General Introduction

1. T cells as important therapeutic targets

The human immune system consists of specialized cells, tissues, and organs that together provide a defence mechanism against a wide variety of pathogens or injury¹. T cells play an essential role in the adaptive arm of the immune system and can be found throughout the body. T cell precursors originate from the bone marrow and migrate to the thymus where they mature before entering the periphery. During maturation, all T cells develop signatory T cell markers including a T cell receptor, coreceptor signalling domain CD3, and the lesser-known costimulatory receptor CD2². In addition, T cells will differentiate into either of the two main subtypes: CD4⁺ helper T cells or CD8⁺ cytotoxic T cells^{1,2}. Naive CD4⁺ and CD8⁺ T cells patrol the bloodstream and lymphoid organs, such as the lymph nodes, spleen, or mucosa-associated lymphoid tissues (Figure 1a), until they encounter their specific antigen. CD4⁺ T cells recognize antigens presented on major histocompatibility complex class II (MHC-II) by antigen-presenting cells such as dendritic cells³ (Figure 1b). Upon activation, CD4⁺ T cells can further differentiate into pro- or anti-inflammatory helper T cell subtypes (T_H1/T_H17 and T_H2/regulatory T cells, respectively), which are characterized by the secretion of different cytokine signatures¹. These cytokines modulate the behaviour of other immune cells to thereby amplify or dampen the immune response. Furthermore, these cytokines can attract other immune cells, including more T cells, to home to the site of inflammation⁴. In contrast, CD8⁺ T cells recognize antigens presented by infected or malignant cells via MHC-I and upon activation excrete cytotoxic granules (granzymes, perforin, interferon- γ (IFN- γ), and other cytokines) to the target cell⁵ (Figure 1b). Once activated, both proliferating CD4⁺ and CD8⁺ effector T cells infiltrate various nonlymphoid tissues during an inflammatory response and they can continue to reside as tissue-resident memory cells (Figure 1a). Together, over the course of an inflammatory response, many T cell subtypes arise, with each subtype exhibiting a specific function and receptor expression pattern and individually implicated in disease¹.

Due to their important role in adaptive immunity, T cells are of significant interest for the development of a range of immunotherapies⁶. These therapies can aim to deplete specific pro- or anti-inflammatory subsets of T cells⁷⁻⁹, skew the differentiation of naive T cells into one of these subsets¹⁰⁻¹², or use T cells as drug carriers to the site of disease¹³⁻¹⁵. Furthermore, immune checkpoint blockade has gained significant interest as a strategy to boost T cell responses to diseased or infected cells¹⁶. Advances in gene transfer and genome-editing technologies have led to the development of chimeric antigen receptor (CAR)-T cell therapy, in which T cells are

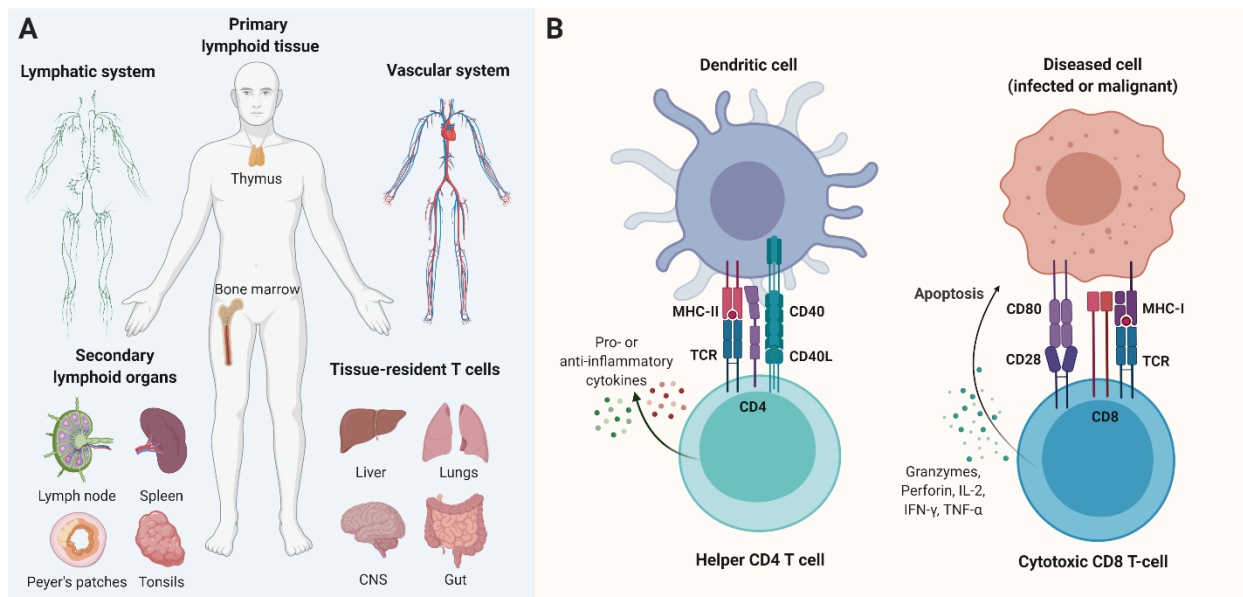


Figure 1. T cell origin, distribution, and function. (A) T cells are derived from primary lymphoid tissues and patrol the lymphatic system, vascular system, and secondary lymphoid organs until they encounter their cognate antigen. Additionally, T cells can enter nonlymphoid tissues to facilitate an inflammatory response or reside in these tissues as tissue-resident memory cells. (B) T cells are divided into two major categories. CD4 T cells, or helper T cells, recognize antigens presented by MHC-II on antigen-presenting cells, such as dendritic cells, and regulate other immune cells. CD8 T cells, or cytotoxic T cells, recognize antigens presented by MHC-I on diseased or infected cells and release toxins to induce apoptosis of target cells. CD, cluster of differentiation; CNS, central nervous system; IFN, interferon; IL, interleukin; MHC, major histocompatibility complex; TCR, T cell receptor; TNF, tumour necrosis factor.

genetically engineered *ex vivo*. Following reinfusion (adoptive transfer), these cells can seek and infiltrate the tumour microenvironment or target infected or autoantibody-producing cells^{6,17-19}. Moreover, T cells are a direct subject of pathology in the form of T cell lymphocytic leukemia, T cell lymphoma, and human immunodeficiency virus (HIV) infection²⁰⁻²³.

In each of these medical indications, significant therapeutic challenges exist in which targeted delivery of therapeutic agents to T cells could have clinical relevance and potential (Figure 2). In brief, delivery vehicles can provide two main advantages. First, targeted drug formulations can enhance the therapeutic index, *i.e.*, by increasing drug delivery to target cells or tissues while reducing off-target toxicity. This strategy could benefit chemotherapeutic interventions in cancers, including T cell leukemia and lymphoma, which suffer from the common side effects of chemotherapy as healthy rapidly dividing cells are eradicated in addition to the cancer cells^{14,24}. Reducing off-target toxicity can also progress gene silencing and immunotherapeutic strategies, *e.g.*, efforts to silence cytotoxic T-lymphocyte-associated protein 4 (CTLA-4), an immune checkpoint inhibitor, *via* targeted delivery of small-interfering ribonucleic acid (siRNA) against CTLA-4 to restore antitumor functions^{25,26}. Furthermore, the reduced off-target effect can potentially warrant the use of more potent therapeutics such as diphtheria toxin or ricin A. Targeted delivery of these pro-apoptotic drugs has been suggested in the context of depleting

HIV-infected CD4⁺ T cells or depleting suppressive regulatory T cell subsets for immunotherapeutic purposes^{7,27}. Current strategies for *in vivo* drug targeting to T cells include the use of immunotoxins, that is, targeting moieties directly conjugated to the drug or toxin. Denileukin diftitox (Ontak), a bacteria-derived IL-2/diphtheria toxin fusion protein, was one of the earliest T-cell-targeting drugs approved by the US Food and Drug Administration (FDA) in 1999 but has been discontinued since 2014 because of safety issues²⁸. Generally, immunotoxins suffer from poor solubility, limited stability, and rapid clearance, which has prompted efforts in using delivery vehicles to address these challenges²⁹. Second, the use of a delivery vehicle can overcome the need for adoptive transfer of *ex vivo* engineered or drug-loaded T cells. *In vivo* targeting of T cell subsets can greatly reduce the cost of cellular hitchhiking-based drug delivery systems by avoiding the need to individually prepare patients' cells *ex vivo*¹³. The same hurdles hamper widespread applications of CAR-T cell therapy, which currently relies on the costly *ex vivo* genetic engineering of each individual patient's cells prior to reinfusion^{30,31}. Moreover, reinfusion of the expanded

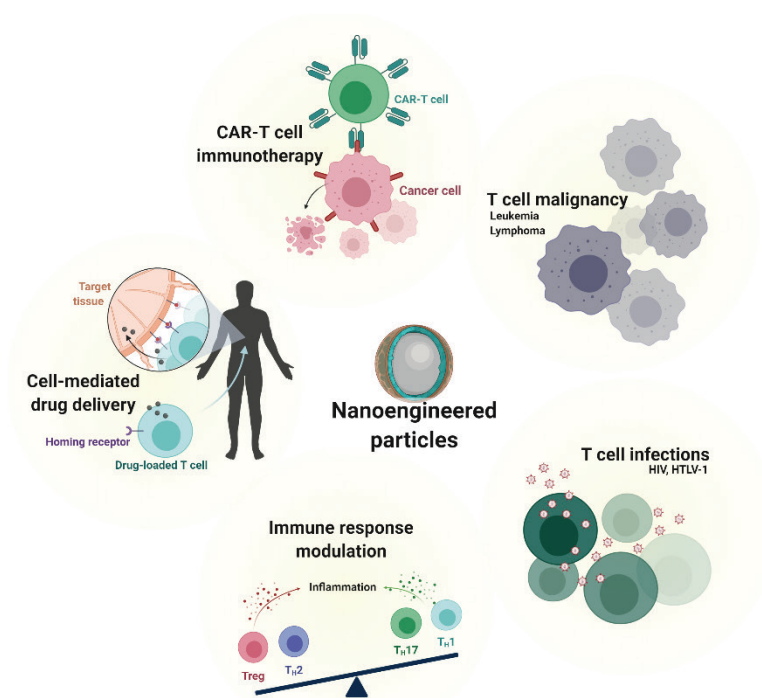


Figure 2. Possible applications of T cell-targeted nanoparticles. Targeting T cells can serve to modulate immune responses by influencing the balance between pro- and anti-inflammatory T cell subsets. The inherent homing capacity of T cells to the site of disease can be exploited for T cell-mediated drug delivery to specific target tissues. CAR-T cell therapy is a promising immunotherapy for cancers and nanoparticles can be used to deliver the CAR-encoding genetic material to T cells *in vivo*. Furthermore, T cells can be the subject of disease themselves and T cell-targeted nanoparticles can be used to treat T cell leukemias, lymphomas, or chronic viral infections. CAR, chimeric antigen receptor; HIV, human immunodeficiency virus; HTLV-1, human T-cell lymphotropic virus type 1; THx, helper T cell subtype x; T_{reg}, regulatory T cell.

engineered CAR-T cell can be accompanied by adverse events such as cytokine release syndrome, resulting in multiple organ failure and neurological toxicity (reviewed therein³²), which potentially can be circumvented by CD8 T cell specific *in vivo* delivery. Furthermore, *in vivo* delivery of gene editing machinery such as the clustered regularly interspaced short palindromic repeats (CRISPR) toolbox to T cells presents potential for therapy and immunomodulation. Although *in vivo* expression of the CRISPR-Cas9 protein has the potential downside of being immunogenic³³, this approach has nevertheless been suggested as a strategy towards an HIV cure or to knock out the immune checkpoint receptor programmed cell death protein 1 (PD-1) in T cells, among others^{22,23,34,35}.

Current research strategies for *in vivo* drug delivery include viral and nonviral methods; however, both methods suffer from low drug delivery efficiencies^{36,37}. Two adeno-associated viral vector-based therapeutics, voretigene neparvovec (Luxturna) and onasemnogene abeparvovec (Zolgensma), have been approved by the FDA. However, the cost associated with large-scale vector production is limiting similar therapeutics being used in the clinic³⁸. Further limitations of viral vector-based delivery methods are associated with the limited choice of cargo, potential oncogenicity³⁹, and immunogenicity⁴⁰. Therefore, the development of safer and more effective drug delivery methods is warranted. The use of engineered nanoparticles as delivery platforms has been suggested as a potential approach to T cell reprogramming and delivery of therapeutics to T cells in general. Nanomedicines have already demonstrated significant innovations in cancer medicine by offering highly versatile drug carriers^{24,41}. In addition, nanoparticle-based vaccines were recently developed and approved by the FDA as a coronavirus disease 2019 (COVID-19) vaccine^{42,43}. However, T cells do not take up nanoparticles as readily as cancer cells because of their small cellular size, high nucleus-to-cytoplasm ratio, non-phagocytic nature, and low rates of endocytosis. In addition, it has been suggested that slow acidification of endosomes in primary T cells reduces transfection efficiency of vectors that rely on pH-dependent release of cargo⁴⁴. Cell exposure to a foreign nanomaterial is likely to induce an immune response or affect immune cell functions depending on the material and cell type⁴⁵. Furthermore, T cell activation has been associated with various cellular changes, including altered expression and spatial reorganization of surface receptors^{46,47}, all of which may have significant implications in nanoparticle-cell interactions. This has created major challenges for materials scientists and biologists aiming to develop T cell-targeted nanomedicines.

Therefore, to rationally design a T-cell targeted nanoparticle system, we require further

understanding of the biological barriers to T cell delivery *in vivo* and the nanoparticle engineering strategies available. The next few sections will first provide detail on the context of HIV infection and the major role nanoscience could play in developing curative strategies. Then, we will explore the nanoparticle engineering strategies available, before diving further into the T cell biology and current knowledge of bio-nano interactions that can influence T cell targeting. Lastly, we will discuss the successes in *in vivo* T cell targeting to date, to finally derive a set of guidelines for our subsequent efforts to rationally design a nanoparticle-based therapeutic towards an HIV cure.

2. Nanomedicine towards an HIV cure

Over the past decades, the HIV pandemic has proven to be one of the major challenges of global health. Despite continuous global efforts, no effective vaccine has been developed as of today. Current treatment regimens of antiretroviral therapy (ART) prove highly effective in preventing HIV replication, reducing the risk of transmission and progression into symptomatic disease (AIDS)^{48,49}. However, ART fails to be curative, treatment is required lifelong and those on treatment can have a reduced average life expectancy as well as an increased risk of co-morbidities⁵⁰. Lifelong treatment also poses a financial burden both to people living with HIV (PLWH) themselves as well as the global health systems⁵¹. In addition, PLWH face ongoing stigma and discrimination⁵².

2.1. The latent HIV reservoir

The HIV genome consists of two single-stranded, positive-sense RNA strands that are coated in nucleocapsid proteins and subsequently encapsulated by capsid (p24) structural proteins. This capsid is surrounded by a lipid bilayer of host-cell origin, lined with viral matrix protein on the inside and studded with the viral envelope protein (Env), a trimer of glycoproteins (gp) 120 and gp41 on the outside⁵³. GP120 mediates HIV entry by binding the CD4 receptor on host cells, which is followed by a conformational change leading to exposure of co-receptor binding sites. Binding of co-receptors C-X-C chemokine receptor type 4 (CXCR4) or C-C chemokine receptor 5 (CCR5) initiates viral entry through the fusion of the viral and host cell membranes. In addition to structural proteins, the HIV genome encodes for the enzymes reverse transcriptase, protease and integrase; regulatory proteins Tat, Rev and Nef; and accessory proteins Vif, Vpr and Vpu. Tat plays an

important role in HIV transcription elongation from the HIV promotor, the long terminal repeat (LTR)⁵⁴. The viral life cycle of HIV consists of several phases; entry, reverse transcription, integration, replication, assembly and release. Antiretroviral drugs can be subdivided into classes according to which of these phases they target: entry inhibitors, integrase inhibitors (II), nucleoside- and non-nucleoside reverse transcriptase inhibitors (NRTIs and NNRTIs, respectively) and protease inhibitors (PI) (reviewed therein^{55,56}). Antiretroviral treatment usually consists of a combination of these drug types to prevent the development of drug resistance.

The major barrier to finding a cure for HIV is the persistence of latently infected CD4⁺ T cells harbouring replication-competent virus^{57,58}. Latency is established when HIV integrates into the host cell genome but remains in (or returns to) a transcriptionally silent state in which no new virions are produced (pre-activation or post-activation latency, respectively). This silent state, however, encompasses a spectrum of transcriptional activity with some cells transcribing no HIV RNA and others producing low levels of cell-associated HIV RNA or proteins⁵⁹⁻⁶¹. These degrees in viral latency likely correlate with blocks in transcription initiation, elongation, completion and splicing that exist in latently infected cells⁶⁰. Furthermore, the degree of latency was found to differ between various T cell subsets^{62,63} as well as between blood and tissue⁶⁴. Latency establishment and maintenance are influenced by a variety of factors, including the site of viral integration, the epigenetic state of the integration site⁶⁵, availability of key transcription factors including Tat and the blocking of HIV RNA export from the nucleus (reviewed therein⁶⁶). This latent state is however reversible, and viral reactivation can occur spontaneously or as a consequence of cellular activation – natural or drug-induced. Consequently, most individuals experience rapid viral rebound within weeks of ceasing or interrupting ART⁶⁷.

The term 'latent reservoir' is used to describe all cells, tissues and organs that harbour latent HIV during suppressive treatment. Finding and identifying these cells is complicated by the lack of a surface biomarker for the latent reservoir⁶⁸. As such, measurements of the latent reservoir depend on polymerase-chain reaction (PCR)-based quantitation of integrated HIV DNA or, established more recently, specific quantitation of 'intact' proviral DNA. This 'intact reservoir' is here defined as those cells harbouring proviruses that lack large deletions and hypermutations, as only these are hypothesized to contribute to viral rebound upon cessation of ART⁶⁹⁻⁷¹. Originally, resting memory CD4⁺ T cells were considered the main cellular latent reservoir for replication-competent HIV^{57,58}. Since then, HIV DNA has been detected in all memory T cell subsets as well as naïve CD4⁺

T cells^{60,61,72-76}. In addition to the T cell compartment, HIV is known to infect CD4-expressing cells of monocyte/macrophage lineage^{77,78}. While most studies on the latent reservoir have been performed on circulating T cells, most T cells reside in secondary lymphoid tissues, including lymph nodes and gut-associated lymphoid tissue (GALT). Indeed, lymphoid tissue is a major HIV reservoir with a higher level of transcriptional activity, potentially due to lower drug levels in lymphoid tissue⁷⁹⁻⁸². Follicular T helper cells, in particular, have been identified to be enriched for HIV in viremic individuals and replication-competent HIV in ART-treated, aviremic individuals^{83,84}. Other tissue reservoir sites include the intestinal tract, female genital tract and the brain^{80,82,85-89}. In this regard, CD69⁺ tissue-resident memory T cells (T_{RM}) inhabiting these tissues have been suggested as preferential targets for HIV infection⁸⁷. A recent study focusing on the cervical mucosa indeed identified T_{RM} cells as key contributors to the HIV reservoir with long-term persistence⁸⁷.

2.2. Strategies towards an HIV cure

Traditionally, HIV cure is defined as a state in which all (replication-competent) HIV provirus is eliminated from an individual. This definition was based on the notion that a single provirus can lead to a viral rebound. The report of Timothy Ray Brown ('the Berlin patient') as the first-ever person to be cured of HIV reignited the search for such an 'eradicating' cure (formerly referred to as 'sterilizing cure')⁹⁰. This eradication was achieved through an allogeneic stem cell transplantation from a donor with a CCR5Δ32/Δ32 homozygous deletion, which rendered the newly reconstituted immune system resistant to *de novo* HIV infection⁹⁰. Since then, four likely further cases of an HIV cure through a similar procedure have been presented – Adam Castillejo (the 'London patient')^{91,92}, two anonymous individuals termed the 'Dusseldorf patient'⁹³ and the 'City of Hope patient'⁹⁴, and a woman of mixed race (the 'New York patient')⁹⁵ – whereas a fifth and sixth individual appear to have cleared all replication-competent HIV without stem cell transplantation^{96,97}. Nonetheless, this curative strategy is not scalable to the global population of PLWH due to the significant morbidity and mortality associated with stem cell transplantation.

Most efforts have since shifted towards achieving a long-term, ART-free remission rather than a complete eradication of the HIV provirus. Sparked by these initial case reports, gene editing approaches have been explored to generate a knock-out of CCR5 or a CCR5Δ32 mutation in host cells, to induce resistance to infection with CCR5-tropic HIV. Since the development of the clustered regularly interspaced short palindromic repeats (CRISPR) Cas9 toolbox^{98,99}, these gene-editing strategies have been gaining further momentum. CRISPR-based approaches in this regard

aim to generate CCR5 Δ 32/ Δ 32 stem cells *ex vivo* prior to transplantation. The first-in-human study (single case) by Xu *et al.* reported successful and sustained donor engraftment and chimerism without any adverse events, however, the achieved percentage of CCR5 disruption was at 5% insufficient to induce HIV remission¹⁰⁰. Alternatively, the CRISPR-Cas system has been studied to directly disrupt the HIV proviral genome by targeting the HIV LTR or protein-encoding sequences¹⁰¹⁻¹⁰⁴. In particular, combinatorial gRNA approaches have been found to completely excise the HIV provirus and exhibit superiority in preventing viral escape¹⁰⁵⁻¹⁰⁷. Further T-cell targeted gene editing approaches include the generation of CAR-T cells recognising specific HIV epitopes, to enhance the immune response against HIV-infected cells. CD8⁺ T cells transduced with such CAR constructs have been shown to exhibit potent antiviral responses *in vitro* and *in vivo*^{108,109}. In a recent open-label clinical trial, adoptive transfer of broadly neutralizing antibody (bNAb)-derived CAR-T cells was found to be well-tolerated and resulted in a reduction in the size of the intact proviral reservoir¹¹⁰. As these gene editing approaches are generally based on nucleic acids encoding the CRISPR machinery or CAR, their advancement and eventual success depend on efficient and non-toxic delivery of these constructs to T cells *in vivo*.

Another strategy towards HIV remission aims to reactivate HIV transcription using latency-reversing agents (LRAs) to increase the expression of viral proteins, potentially resulting in virion production. This process, termed 'shock and kill', should subsequently induce death through viral cytopathic effects or immune-mediated clearance of the infected cell^{21,56}. Latency reversal is

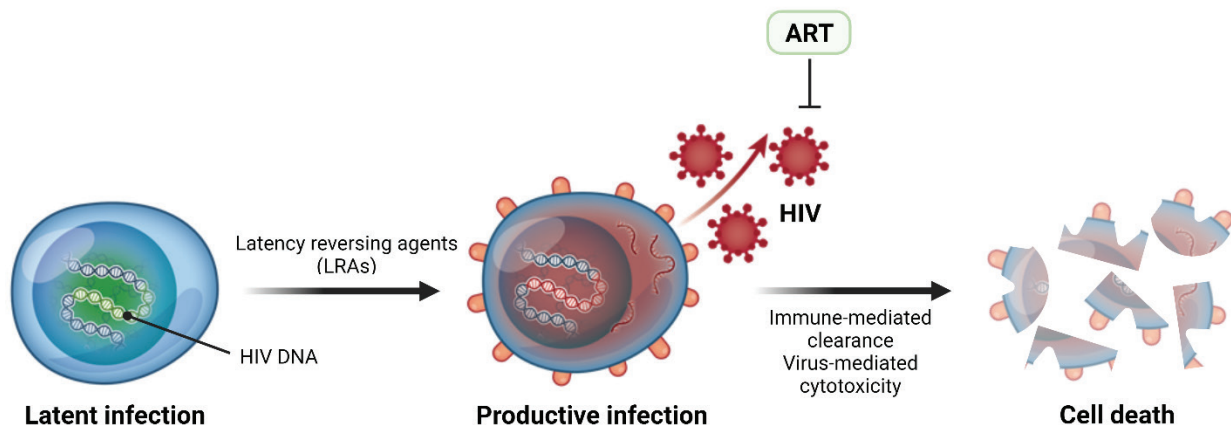


Figure 3. 'Shock and kill' approach to eliminate the latent reservoir. Latent HIV, which is enriched in resting CD4⁺ T cells, is characterized by the presence of integrated HIV provirus but a lack of HIV RNA and protein production. The 'shock and kill' approach aims to use latency-reversing agents (LRAs) to induce viral transcription and translation, potentially resulting in the release of new HIV virions. The reactivated cell would subsequently be cleared through immune recognition or virus-mediated cytotoxicity, leading to a reduction of the latent reservoir. This approach is performed in the continuous presence of antiretroviral therapy (ART) to prevent new infections.

performed in the presence of ongoing ART to prevent *de novo* infection of uninfected cells (Figure 3). The field of ‘shock and kill’ is undergoing rapid innovation and new LRAs are being developed continuously. Multiple subclasses of LRAs exist, including histone deacetylase inhibitors (HDACis), bromodomain inhibitors, protein kinase C (PKC) agonists and second mitochondria-derived activator of caspases (SMAC)-mimetics, which each induce viral gene expression through different, synergistic mechanisms¹¹¹⁻¹¹⁹. The potency of several such LRAs has been assessed in clinical trials, and while most were found to induce viral transcription, none of the LRAs trialed to date has led to a reduction of the latent HIV reservoir¹²⁰⁻¹²⁸. As such, ongoing efforts focus on combining LRAs with pro-apoptotic compounds to sensitize latently infected cells to apoptosis^{129,130} or using immunomodulatory LRAs that concurrently reverse latency and enhance immune-mediated killing¹³¹⁻¹³⁹. Yet, a major limitation of the current generation of LRAs is the dose-limiting toxicities associated with their administration and the lack of specificity to the HIV provirus^{140,141}. In this regard, several nanoparticle systems have been explored as strategies for more targeted delivery of small molecule LRAs to the T cell reservoir¹⁴²⁻¹⁴⁵. Furthermore, nanomedicine may provide avenues for the delivery of more potent LRAs, such as the “CRISPR activation” (CRISPRa) system (Figure 4), which has been shown to specifically reactivate transcription of the HIV provirus without affecting host cell transcription¹⁴⁶⁻¹⁵². Initial reports furthermore suggested that in contrast to first-generation LRAs, this CRISPRa-mediated latency reversal results in viral protein accumulation and subsequent viral cytopathic effect^{149,153}.

In contrast to the strategies discussed above, which ultimately aim to reduce or eradicate the latent reservoirs, the ‘block and lock’ approach aims to enforce or promote latency by blocking HIV transcription and locking the HIV promotor in a deep state of viral latency. Such an approach would prevent viral rebound upon cessation of ART, despite the continued presence of proviral DNA. Several therapeutics have been explored in this regard, which are primarily nucleic acid-based. Didehydro-Cortistatin A (dCA) and NullBasic have been shown to inhibit viral RNA levels *in vivo* and *ex vivo* by inhibiting the transcription-activating role of HIV Tat¹⁵⁴⁻¹⁵⁶. While dCA is a small molecule drug administered systemically, NullBasic is delivered as plasmid DNA or mRNA encoding the mutant version of the HIV Tat protein. Further, siRNA-based approaches have received extensive attention for their ability to induce epigenetic silencing of the provirus by targeting the HIV LTR¹⁵⁷. Such epigenetic silencing was also shown to be induced through CRISPR inhibition, which in analogy to CRISPR activation, employs a catalytically inactive Cas9 fused to transcriptional repressor domains^{158,159}. Lastly, several variants of the CRISPR-Cas13 machinery

have been successfully used to destroy viral RNA and suppress latency reversal in cell line models of HIV^{160,161}.

In summary, multiple new strategies towards long-term HIV remission are nucleic acid-based, making their success dependent on a delivery vehicle. Other approaches suffer from off-target toxicities, which could be overcome by more specific delivery to the latent reservoir. As such, the development of an efficient T-cell targeting nanoparticle delivery method could greatly expedite the clinical progression of multiple HIV cure strategies.

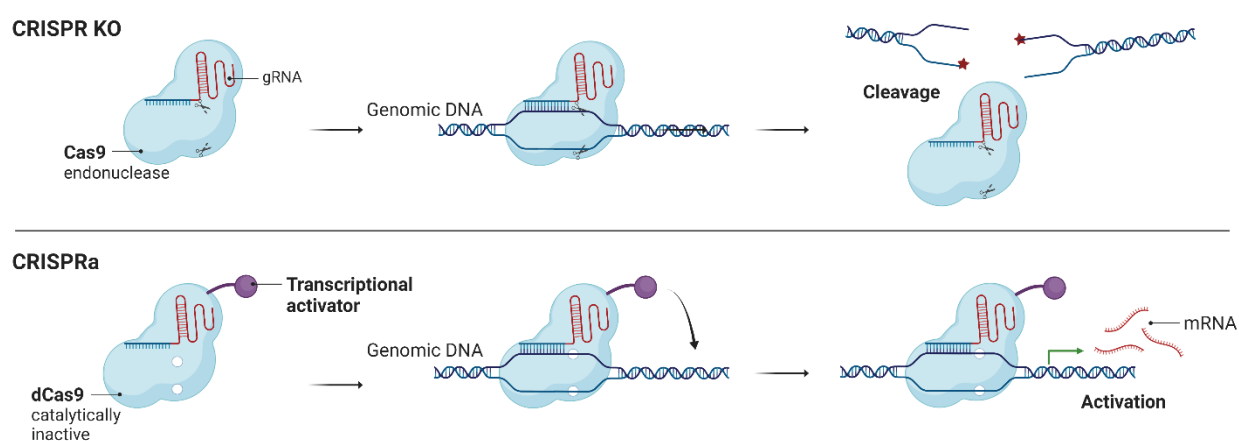


Figure 4. Mechanism of action of conventional CRISPR and CRISPR activation. (Top) wildtype Cas9 uses RNA-guided targeting to generate a double-stranded break in genomic DNA, usually leading to a knock-out of the gene of interest. (Bottom) CRISPR activation (CRISPRa) is based on the same gRNA-mediated genome targeting as conventional CRISPR technology. However, CRISPRa uses a catalytically inactive Cas9 (deadCas9 or dCas9) combined with transcriptional activators to enhance the transcription of a certain gene, rather than induce a knock-out. CRISPR, clustered regularly interspaced short palindromic repeats; CRISPRa, CRISPR activation; dCas9, deadCas9; gRNA, guideRNA; KO, knock-out.

3. Nanoparticle design: exploring the options

A plethora of nanoparticle systems is being developed for therapeutic delivery, diagnostic, and imaging applications^{28,162}. Based on their chemical composition, nanoparticle systems can be divided into several broad categories: polymer-based nanoparticles, lipid-based nanoparticles, and inorganic nanoparticles. Each system carries its benefits and limitations in terms of drug loading, stability, biocompatibility, and biodegradability. The physicochemical properties of these nanoparticles, including size and surface charge, are important parameters known to influence their interaction with cells and downstream delivery efficacy¹⁶³.

3.1. Influence of drug type on the choice of nanomaterial

The main types of therapeutics delivered to T cells are small hydrophobic molecules, proteins, and nucleic acids. Although most nanoparticle systems are highly versatile, not all types of therapeutics can be loaded onto or into every type of nanoparticle. As such, the type of therapeutic cargo is a major determinant in the choice of nanomaterial (Figure 5).

Many chemotherapeutic agents, as well as some of the therapeutics studied to eradicate latent HIV, are small hydrophobic molecules. For these types of drugs, lipid-based materials and oil-based nanoemulsions allow drug loading by complexing with lipids or encapsulation in an oily core, respectively¹⁶⁴⁻¹⁶⁶. Amphiphilic polymeric materials, including poly(lactic-co-glycolic acid) (PLGA)-based nanoparticles, also allow encapsulation of hydrophobic drugs via hydrophobic interactions with the water-insoluble portion of the polymer during nanoparticle assembly¹⁶⁷. In addition, small drug molecules have been conjugated to the surface of gold and other inorganic nanoparticles¹⁶⁸.

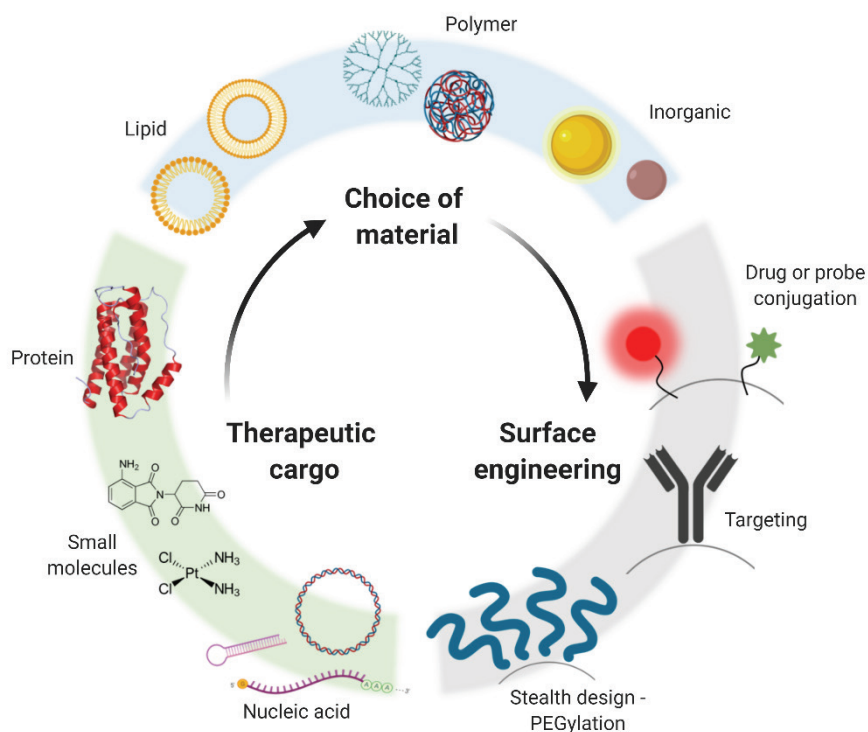


Figure 5. Therapeutic cargo informs the choice of the nanoparticle material that in turn influences the surface modification approach employed. Different classes of drugs, including proteins, small molecule drugs, and nucleic acids, can be delivered using nanoparticles. Not all nanoparticle types are equally suitable for the delivery of each of these drug classes. The possibilities for surface engineering are subsequently dependent on the material chosen and the presence of functional groups in both the nanoparticle and the ligand (*i.e.*, drug, probe, targeting moiety, or stealth molecules).

Proteins such as diphtheria toxin, ricin A, and the Cas9 protein of the CRISPR toolbox can be loaded into various types of nanoparticles by physical entrapment or chemical conjugation. For instance, using a water-in-oil-in-water double emulsion technique, proteins can be entrapped in hydrophobic polymer-based nanoparticles such as PLGA¹⁶⁷. Proteins can also be entrapped in liposomes¹⁶⁴, mesoporous silica nanoparticles¹⁶⁹, CaCO₃ particles¹⁷⁰, and metal-phenolic capsules¹⁷¹, or incorporated into the caveosphere outer membrane via fusion to caveolin-1¹⁷². Additionally, the thiol (–SH) groups present in most proteins can be exploited to covalently bind protein therapeutics to the surface of gold nanoparticles^{173,174}. In general, however, forming stable protein nanoformulations is challenging because of the potential occurrence of protein degradation^{167,175}. To overcome this, a strategy was proposed recently, in which a variety of nanomaterials can be coated with protein-phenolic network assemblies that retain the structure and function of the protein¹⁷⁵.

For certain immunotherapeutic strategies, as well as strategies to engineer CAR-T cells or perform CRISPR-mediated genome editing in T cells *in vivo*, the therapeutic agents are based on nucleic acids, including siRNA, mRNA and plasmid DNA. Nucleic acids are innately hydrophilic and negatively charged. Traditionally, cationic polymers such as polyethylenimine (PEI)^{176,177}, chitosan or poly-β-amino esters (PBAE)^{178,179} have been used to deliver nucleic acids. The attachment of nucleic acids on solid nanoparticles has also been studied, *via* electrostatic interactions or covalent attachment using nucleic acids with added functional end groups; the solid nanoparticles include inorganic nanoparticles such as gold, silica, and iron oxide¹⁸⁰. However, more recently, most attention has shifted towards the use of lipids and, in particular, lipid nanoparticles (LNPs) for the delivery of siRNA and mRNA therapeutics or vaccines. Lipid-based nucleic acid delivery is initially mediated by lipids with cationic or ionizable head groups. Their positive charge, either permanent or induced by a low pH, induces the complexation with the negatively charged nucleic acid cargo, as well as mediates endosomal membrane destabilization that results in endosomal escape of the nanoparticles. In addition, LNPs commonly contain phospholipids to aid particle stability¹⁸¹, sterols to modulate particle rigidity¹⁸² and polyethylene glycol (PEG)-lipids to control the particle size, decrease aggregation and prolong the blood circulation time^{183,184}. Varying the individual lipids used within these four classes or their relative ratios allows for a high degree of LNP tunability, rendering LNPs a powerful tool for a range of therapeutic applications¹⁸⁵⁻¹⁸⁷.

3.2. Influence of the nanoparticle material on surface engineering options

In addition to the inherent physicochemical properties of nanoparticles, the nanoparticle surface can be modified to tether or conjugate ligands, including targeting molecules, drugs, detection probes, biotin, and stealth macromolecules¹⁸⁸. Surface modification of nanoparticles can be achieved by using one or more chemical methods to either form a noncovalent interaction or covalent bonds between the nanoparticle surface and ligand (reviewed therein^{188,189}). Noncovalent interactions include electrostatic, hydrophobic, van der Waals forces, hydrogen bonding, and ligand exchange^{190,191}, whereas covalent attachment involves chemical conjugation between the nanoparticle surface and functional end groups on the drug or ligand, with or without the aid of a cross-linker. The choice of the modification strategy is influenced by the functional groups available on the nanoparticles and ligands, and whether the reaction conditions can maintain the stability of the nano- particles and the function of the conjugated drug or ligand (Figure 5).

In summary, the choice of nanoparticle material is often dictated by the type of cargo to be delivered *i.e.*, the physicochemical properties of the drug, and in turn, the chosen material dictates subsequent surface modifications. The ability to scale up nanoparticle production at a reasonable cost plays a key role in determining the suitability of the nanoparticles for clinical application.

4. The bio–nano interface: improving nanoparticle fate and nanoparticle–T cell interactions

As most nanoparticles are prepared from exogenous materials (*i.e.*, originating from outside the organism), they face multiple effective clearance mechanisms. First, upon entering the bloodstream, synthetic nanoparticles will attract and adsorb proteins and other plasma components to form the so-called protein corona (or biomolecular corona)^{192,193}. The presence of immunoglobulins and other opsonins in this protein corona makes these nanoparticles prone to sequestration by phagocytes in the mononuclear phagocyte system (MPS; also called the reticuloendothelial system) including Kupffer cells of the liver^{194,195}. In addition, nanoparticles with a diameter of less than ~6 nm are efficiently excreted via the kidneys^{196,197}. Typically, less than 5% of the administered nanoparticles reach their target tissue^{194,198}.

The physicochemical properties of a nanoparticle, including size, shape, composition, and surface properties, can influence how it interacts at the bio–nano interface¹⁶³. As such, rational

nanoparticle design is essential to control the fate of nanoparticles in the complex *in vivo* environment. There has been limited work on the effect of physicochemical properties on nanoparticle-T-cell interactions and nanoparticle uptake by T cells¹⁹⁹. We can, however, learn from what is known about bio-nano interactions in the various organs and tissues where T cell targeting for therapeutic purposes is desired. This section describes four core aspects of T cell targeting and discusses strategies to improve association with and uptake by T cells *in vivo*, by reducing nonspecific interactions and improving targeting to T cells specifically.

4.1. Bio-nano interactions in whole blood: the importance of stealth and protein corona

Although only an estimated 2–3% of all T cells are found in the blood¹, circulating T cells are an important target for nanoparticle-based drug delivery. In the case of T cell leukemia or HIV infection, circulating T cells are the direct subject of disease^{20,200}, whereas for CAR-T therapy or other immunotherapies, circulating T cells could simply provide the most accessible T cell pool to target³¹. Even when circulating T cells are not the intended target T cell subset, many therapeutics are dependent on the bloodstream for circulation throughout the body.

Studying the behaviour of nanoparticles in this physiological compartment has been achieved *in vitro* by incubating nanoparticles with whole blood and assessing nanoparticle association with various blood cell populations^{201,202}. Although only a few such studies have specifically examined differential association with T cells, the available data suggest that in the absence of targeting strategies, T cells demonstrate considerably lower nanoparticle association compared with other immune cells^{172,203–205}. Controlling interactions between nano- particles and off-target blood cells through surface engineering strategies is thus essential to achieve efficient nanoparticle-based drug delivery to T cells.

Avoiding immune recognition and clearance by off-target cells is commonly referred to as “stealth”^{206,207}. The design of stealth nanoparticles is a major field of ongoing investigation. Conjugation with the hydrophilic and neutrally charged PEG, referred to as PEGylation, is well known to significantly increase blood circulation time, and multiple PEGylated nanoformulations are commercially and clinically available²⁸. However, as PEG is an exogenous compound, administration of PEGylated formulations can induce an immunoglobulin M (IgM) response by the spleen, which results in accelerated blood clearance (referred to as the “ABC phenomenon”) upon repeated dosing of PEGylated nanomedicine^{208,209}. Other nonexogenous stealth strategies have also been explored including the use of proline-alanine-serine (PAS) (PASylation)^{210,211} and CD47,

a marker of “self” overexpressed on red blood cell membranes to prolong blood circulation²¹². Further research is warranted to progress these to nanoformulations for clinical use.

While the above-described strategies aim to generate low-fouling (low-binding) nanoparticles with minimal formation of a protein corona, it has been suggested that the protein corona can be controlled and exploited rather than being considered a hindrance to achieving the desired bio-nano response²¹³. For instance, the recruitment of certain types of proteins from blood (e.g., albumin, apolipoprotein E (ApoE)) on the nanoparticle surface can help improve the biodistribution profile of nanoparticles, overcome biological barriers, and direct them to targeted regions and tissues²¹⁴. In this regard, the concept of forming a personalized protein corona using a patient’s specific serum composition is being explored²¹⁵.

4.2. Targeting tissue-resident T cells

When T cells in the bloodstream are not the intended targets, further biological barriers and tissue-specific clearance mechanisms can provide additional challenges to efficient nanoparticle delivery. As discussed above, T cells circulate in the lymphatic and vascular systems and reside in secondary lymphoid tissues and other organs including the liver, lungs, brain, and gut (Figure 1a). Within these tissues, they perform specialized roles and functions in immunity and disease that may be of specific interest for therapeutic interventions. The following sections discuss nanoparticle delivery to these target sites.

Delivery to lymph nodes can be particularly relevant in the context of T cell lymphoma and HIV, where the lymph nodes form major reservoirs of malignant and latently infected T cells, respectively²¹⁶. Additionally, the lymph nodes are of interest for immunomodulatory interventions that directly act on naive T cells to regulate their activation and differentiation upon antigen recognition²¹⁷. Delivery to the lymph node paracortex, where T cells mainly reside, can be achieved via intramuscular injection and subsequent uptake by the lymphatic system or via blood circulation and subsequent migration through the walls of high endothelial venules²¹⁷. For the former strategy, a nanoparticle size range of 10–100 nm is considered to be an ideal size range for the efficient internalization of nanoparticles by the lymphatics²¹⁷. Extravasation can be enhanced via targeting of the peripheral node addressin on the capillary wall, thereby mimicking the natural homing process of T cells into the lymph node²¹⁸. PEGylation of polymer nanoparticles has also been shown to enhance lymph node targeting *in vivo*²¹⁹.

The liver is an immunologically complex organ that is subjected to cancers, inflammatory diseases, such as liver fibrosis, and viral or parasitic infections such as hepatitis B virus and malaria infection²²⁰. Liver tissue-resident T cells have been suggested as targets for immunotherapeutic strategies, for example targeting liver fibrosis²²¹. While a majority of nanoparticles localize in the liver following intravenous injection, most are internalized by Kupffer cells (macrophages), limiting access to liver-resident T cells²²². Strategies to prevent uptake by Kupffer cells include presaturation of Kupffer cells by nontherapeutic particles²²³ and transient depletion of Kupffer cells²²⁴, although this might be challenging to achieve clinically.

Tissue-resident T cells in the lungs can be involved in the defence against respiratory pathogens and lung cancers. Alternatively, they can play a role in airway inflammation in response to allergens and acute cellular rejection following lung transplantation²²⁵. In the lungs, mucus presents a physical barrier to the delivery of nanoparticles via inhalation in addition to the cellular barrier posed by the alveolar macrophages that roam the pulmonary tissue^{214,226}. To enter the alveolar region where most T cells reside, nanoparticles can be injected systemically or inhaled in liquid droplets or powders²²⁷. Recent studies showed that the deposition of metal-phenolic capsules could be tuned precisely to the alveolar region *versus* the bronchi by controlling the aerodynamic diameter of the capsules²²⁸. The physicochemical properties of nanoparticles that affect their fate in the lungs are reviewed by Liu *et al*²²⁹.

Targeting T cells within the central nervous system (CNS) is of interest for immunotherapy against brain tumours or to reduce the pathogenesis of several neurodegenerative disorders to which T cells have been linked^{230,231}. However, nanoparticle delivery to the CNS is complicated by the blood-brain barrier (BBB), which prevents access of many biomolecules and nanoparticles²³². The strategies used to overcome or bypass the BBB to facilitate nanomaterial delivery to the brain are discussed therein²³². In addition to manipulating the BBB, *e.g.*, by focused ultrasound or microwaves to make it susceptible to drug extravasation, the surface modification of nanoparticles with cationic and amphiphilic components or receptor-targeting ligands has been investigated to exploit transport mechanisms across the BBB²³². To bypass the BBB completely, nanoparticles have been administered via alternative routes, including intratympanic or inner ear delivery²³³, intranasal delivery^{234,235}, or direct tumour delivery through minimally invasive surgery²³⁶.

The gut presents an important drug target in strategies to eradicate HIV, as it is considered a major reservoir of HIV-infected T cells⁸⁰. Gut-residing T cells are furthermore known to play a role in

inflammatory bowel disease pathogenesis, the antitumor response against gastrointestinal cancers and maintaining tolerance against gut microbiota and food antigens²³⁷. Targeting T cells in the mucosal intestinal tissue can be attempted through oral administration; however, the wide variation in pH, the presence of mucus, and the inherent physiological functions of the gastrointestinal tract complicate efficient delivery²³⁸. Various nanoparticle assemblies that seek to address these challenges have been investigated for oral delivery²³⁸. For example, to achieve targeting to the T cell-rich small intestine epithelium²³⁹ and gut-associated lymphatic tissue, nanoparticles must withstand the acidic conditions in the stomach. This can be achieved by using materials that are insensitive to acidic pH (e.g., polyanionic polymers and enteric polymers). In addition, the nanoparticles must overcome mucus when targeting the underlying cells in the intestine. Surface modification of nanoparticles with either PEG²⁴⁰ or mucus-degrading enzymes^{241,242} has been shown to improve mucus penetration, although nanoparticle size, charge, and composition also play a role²⁴³. Surface modification of nanoparticles to increase interaction with target cells or increase residence time in the small intestine using various ligands, including lectins, Claudin 4, Fc regions of antibodies, and vitamin B12, has been described and is summarized therein²³⁸. Lipid-based nanocarriers have been attractive for targeting the intestinal lymphatic system because of their role in the oral adsorption of dietary lipids²⁴⁴⁻²⁴⁶. Lopinavir, a hydrophobic antiretroviral drug, formulated in solid lipid nanoparticles showed almost a fivefold higher intestinal lymphatic transport compared with lopinavir suspension²⁴⁷.

In summary, targeting T cells present in different sites in the body presents a major technological challenge – specifically, realizing an optimized nanoparticle system capable of reaching multiple T cell target sites via one route of administration is challenging. Alternatively, targeting T cells one site at a time is likely more feasible and the fate of the nanoparticle rests on the ability of the nanoparticle to overcome or exploit the physical and biological barriers present at each target site, which can be facilitated by precise nanoparticle engineering.

4.3. Targeting nanoparticles through ligand–receptor interactions

Advantages of targeted nanomedicines include the increased specificity of drug delivery and concurrent reduction of off-target effects and toxicity. Multiple targeting molecules or ligands have been explored as surface decorations of nanomedicines including antibodies or antibody fragments, proteins, and aptamers^{248,249}.

The choice of a target receptor for nanoparticle-mediated drug delivery is influenced by (i) the

level of specificity of that receptor to the intended target population and (ii) the receptor density on the cell surface²⁴⁸. In T cell targeting studies, the most commonly chosen receptors to date are general T cell markers *i.e.*, CD3, CD4, CD7, and CD8,^{14,19,250-253} likely because of their high expression levels and (mostly) exclusivity to T cells. However, as has been observed with immune checkpoint inhibitors, such general targeting to all T cell subsets can result in dose-limiting toxicities¹⁶. As such, if possible, markers of specific cell subsets or markers overexpressed by malignancies are chosen, *e.g.*, PD-1, CD25/IL-2 receptor, or protein tyrosine kinase 7 (PTK7)^{254,255}. An alternative targeting strategy could be to use antibodies that target a foreign antigen expressed on the diseased target cells. For example, in HIV infection, HIV envelope-specific antibodies such as bispecific T cell-engaging (BiTE) antibodies or dual affinity retargeting proteins (DARTs) that attract killer cells have been explored as an antiviral strategy^{256,257}. Attaching such antigen-specific antibodies to nanoparticles has the potential to achieve highly targeted drug delivery to HIV envelope-expressing cells. However, a singular surface biomarker of disease is not always available or known⁶⁸. In addition, it is important to note that when antibodies are used as the targeting ligands on nanoparticles, receptor targeting via antibody binding *in vivo* can induce receptor signalling, potentially triggering cellular responses. For example, CD3 stimulation leads to immunosuppression and T cell anergy²⁵⁸, a desirable effect in an organ transplant setting but highly undesirable otherwise.

Of note, targeting and stealth are highly interrelated concepts and efficient targeting is unlikely to occur in the absence of stealth. However, stealth strategies can compromise targeting abilities and *vice versa*. Therefore, a balance between stealth and targeting is required to create biologically effective nanoparticles²⁰⁶.

4.4. Nanoparticle internalization and endosomal escape

Upon reaching the target tissue and T cell subset, most nanoparticle systems require internalization for drug release²⁵⁹. The main routes of internalization are phagocytosis, macropinocytosis, and receptor-mediated endocytosis (RME). The mechanism of internalization depends on the size of the nanoparticle that is to be internalized²⁶⁰ (Figure 6). The availability of these endocytic pathways differs between cell types and phagocytosis, in particular, is restricted to certain cell types (professional phagocytes), such as macrophages and other antigen-presenting cells, as well as a limited number of nonprofessional phagocytes (fibroblasts and epithelial and endothelial cells)^{194,261}.

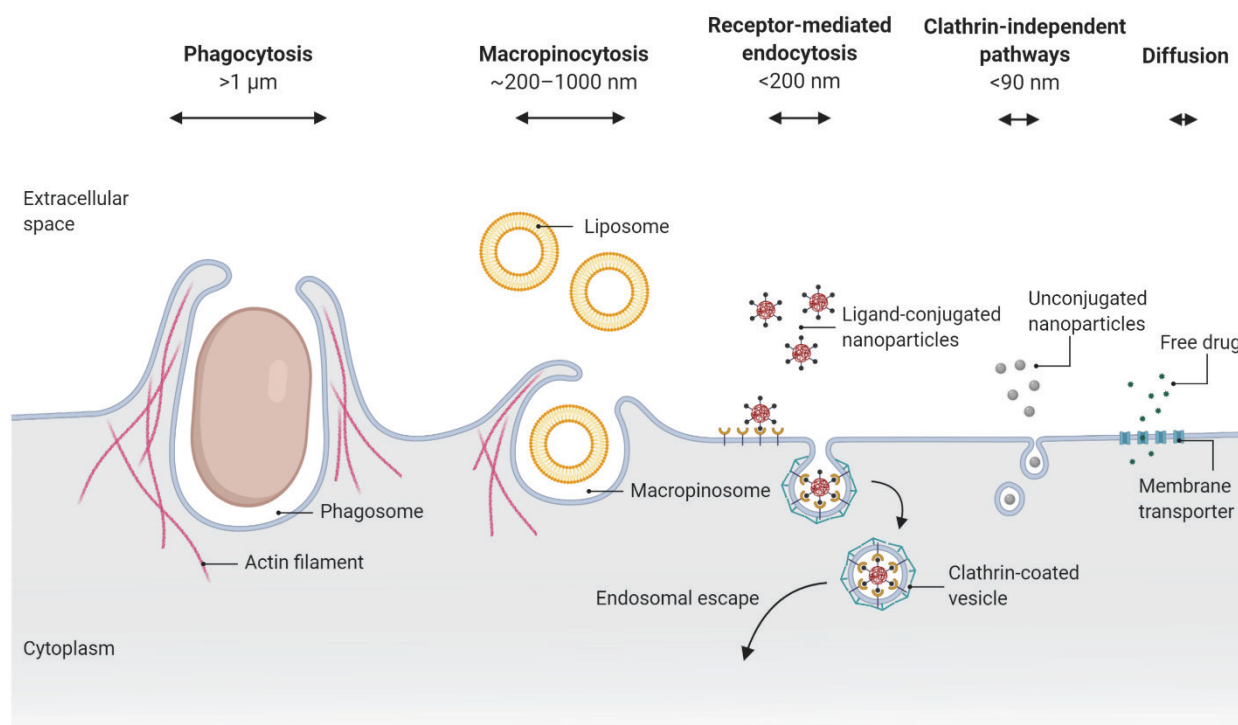


Figure 6. Routes of nanoparticle endocytosis. Four main routes of endocytosis can be distinguished depending on the size of the nanoparticle being taken up. Phagocytosis: phagocytes can engulf large particles of $>1 \mu\text{m}$, but this process is restricted to certain cell types. Macropinocytosis: macropinosomes can vary in diameter, but the efficiency of nanoparticle uptake decreases with increasing particle size. RME and clathrin-independent pathways are the main routes of uptake for smaller particles of $<200 \text{ nm}$. In contrast, small-molecule drugs can diffuse through the plasma membrane or internalize through membrane transporters. RME is induced by receptor binding of ligand-conjugated nanoparticles. Upon internalization into a clathrin-coated vesicle, the nanoparticle needs to escape endosomal compartments to release its cargo into the cytoplasm.

Nanoparticle-mediated drug delivery to T cells is complicated by the non-phagocytic nature of T cells. Nanoparticle internalization into these cells is thus limited to macropinocytosis, RME, and a few other, less relevant pathways (Figure 6). Nanoparticle uptake through macropinocytosis is nonspecific and the vesicles formed during macropinocytosis are heterogeneous in size but generally $0.2\text{--}1 \mu\text{m}$ ^{260,262,263}.

While professional phagocytes undergo constitutive macropinocytosis²⁶², this endocytic pathway seems restricted to activated T cells²⁶⁴ and is generally considered as inefficient. In contrast, RME is a highly specific mechanism of uptake that is induced by receptor binding²⁶⁵. Classic examples of receptors known to internalize through RME include the transferrin receptor^{266,267} and chemokine receptors²⁶⁸. The efficiency of RME is strictly dependent on the internalization kinetics of the target receptor, the activation state of the cell, and the size of the particle to be taken up¹⁹⁷. Studies on receptor internalization kinetics have been reported^{269,270}, and chemokine receptors

are generally considered to be rapidly recycling²⁶⁸. However, comparative studies in T cells are lacking. For RME, which is mediated by clathrin-coated vesicles, an optimum nanoparticle diameter of ~50 nm has been suggested and the efficiency of endocytosis is known to decrease with increasing particle size^{197,232,265,271-273}. Altogether, these restrictions pose significant challenges to nanoparticle delivery to T cells.

Once a nanoparticle is internalized, controlling its intracellular trafficking and subsequent drug release poses a further challenge²⁵⁹. Entrapment in acidic endosomal or lysosomal compartments can result in drug degradation and recycling to the cell membrane (exocytosis), leading to a loss of therapeutic efficacy. Several strategies for endosomal escape have been reported, and nanomedicines have been designed accordingly: (i) cationic polymers and lipids can interact with the negatively charged endosomal membrane and cause membrane flipping and destabilization (“flip-flop” mechanism); (ii) polymers containing protonatable functional groups can induce endosomal swelling and rupture (“proton-sponge effect”); (iii) cationic lipid carriers can induce membrane fusion; and (iv) pore-forming peptides can self-assemble into drug-releasing pores in the endosomal membrane^{259,274}. Studies in this context often approach nanoparticle endosomal escape from a nanoscience perspective and may disregard cell-specific effects. However, it has recently been suggested that T cells may present additional barriers to endosomal escape compared to the commonly used HeLa cells in the form of slower endosomal acidification, enhanced autophagy, and the presence of immune-sensing proteins²⁷⁵.

Of note, nanoparticle internalization is not required for all drug delivery systems. Other strategies for drug delivery include fusion of liposomal formulations with the cell membrane and extracellular release of hydrophobic drugs in the microenvironment of the target cell^{14,144,145,276-278}. The latter strategy, although highly promising, is limited to small molecule drugs that can permeate the cell membrane without any vectors and may be less effective for nucleic acid or protein delivery.

5. Nanoparticle-mediated drug delivery to T cells: successes to date

Despite the importance of T cells in various diseases, there are relatively few studies exploring direct T cell-targeted drug delivery using nanoparticles. Most reports focus on drug delivery to the T cell surface, thus not requiring nanoparticle internalization^{14,144,145,277,279}. Although this is a valuable approach for drugs that can be delivered to the cell surface, the following discussion will

focus on strategies for nanoparticle-mediated intracellular drug delivery to T cells, noting that most reported studies originate from the field of oncology^{19,25,251,255,280-285} and HIV^{142,143,286-289}. For a detailed listing of the studies described in this section, see Table 1.

5.1. Material characterization

In the T cell field, efforts have revolved around the delivery of a plethora of different therapeutic compounds ranging from siRNA and plasmid DNA to peptides and small hydrophobic drugs. Hydrophobic polymer- and lipid-based nanoparticles are most commonly used and these have been exploited for the delivery of both nucleic acids^{19,250,286,290,291} and hydrophobic drugs^{143,281,282,292}. Gold nanoparticles have been used for the delivery of nucleic acids¹⁷⁴; however, to our knowledge, there are no reports of this strategy in a T cell context. Specifically, gold nanoparticles have only been used for intracellular delivery of small hydrophobic drugs to T cells *in vitro*^{251,283,284}. It is challenging to derive guidance from these few reports in terms of the optimal nanomaterial for delivery to T cells. Studies have indicated that the conjugation ability of the nanoparticle system to a targeting molecule is a major rationale for the choice of material^{19,172,283,284}. Irvine and co-workers initially performed studies on liposomal delivery of hydrophobic drugs but subsequently used gold-based nanoparticles because of their higher drug loading capacities^{251,282,293}. The ease of synthesis was also provided as a rationale for the use of gold nanoparticles^{283,284}.

The average nanoparticle size used for intracellular delivery to T cells is around 100 nm (range from 14.9 to 354 nm), with gold nanoparticles being significantly smaller than the lipid- based or polymeric nanoparticles used (Table 1). The surface charge of the nanoparticles used is mostly negative, although not all studies report a ζ -potential measurement (Table 1). Further studies are needed to assess whether a negative nanoparticle surface charge effectively increases drug delivery to T cells. Only a selection of these studies discusses the mechanism of drug release from the nanomaterial. Most of the studies report a sustained drug release over time^{142,278,281,282,285}, which in one study is shown to be further triggered after nanoparticle internalization²⁸¹. Drug release from gold nanoparticles has been described to be pH-dependent^{283,284} or triggered by endosomal cleavage of the nanoparticle shell²⁵¹.

5.2. Nanoparticle targeting

The rationale behind the use of antibodies for nanoparticle targeting is twofold: (i) targeted

nanoparticles increase the delivered drug dose to target cells through enhanced nanoparticle–cell interactions and (ii) the off-target effects of the therapeutic cargo are reduced. Several studies have consistently shown that conjugation of nanoparticles with a targeting ligand increases the nanoparticle–cell association^{19,143,172,250–252,280,292,293} or drug delivery^{290,291} to target cells in a monoculture. *In vitro* analyses of selective nanoparticle association have shown an enhanced association with target cells *versus* nontarget cells in a mixed cell culture^{143,172,251}. To date, studies that specifically assess selective nanoparticle internalization, as opposed to association, by target T cells are lacking.

Table 1. Overview of T cell-targeted nanomedicines for intracellular drug delivery.

nanoparticle	size (nm)	ζ-potential (mV)	cargo	targeting ligand	Application	<i>In vivo</i> ^a	ref
<i>polymeric</i>							
chitosan	305–354	15.2–17.5	siRNA	anti-CD7	fundamental, RNAi	X	252
PLGA	200	–23	no cargo	cIBR peptide targeting LFA-1	T-ALL treatment	X	280
PLGA-lipid-PEG	~110	~–25	imatinib	tLyp1 peptide targeting Nrp1	cancer immunotherapy	✓	281
PBAE–PGA	155 ± 40	–7.8 ± 2.1	plasmid DNA	anti-CD3e	CAR T cell therapy	✓	19
carbosilane	43.8	not reported	doxorubicin	aptamer targeting PTK7	T-ALL treatment	X	255
PEI-melittin	~70–200	~–2–7	siRNA	transferrin	Pulmonary delivery; asthma	✓	294,295
<i>lipid-based</i>							
caveospheres	40	not reported	no cargo	anti-CCR5	fundamental, targeted delivery	X	172
LNP	129 ± 5	–10	siRNA	anti-CD4	fundamental, RNAi	✓	250
LNP	88.4 ± 4.2	not reported	mRNA	anti-CD4	CAR T cell therapy	✓	291
LNP	~80	not reported	mRNA	anti-CD5	CAR T cell therapy	✓	290
liposomes	173 ± 13	not reported	no cargo	anti-CD90 or anti-IL-2R	fundamental, targeted delivery	✓	293
liposomes	83 ± 11	not reported	TGFβ inhibitor	anti-CD90 or anti-CD45	cancer immunotherapy	✓	282
liposomes	141 ± 25	–9.2	siRNA	anti-LFA-1	RNAi for anti-HIV prophylaxis	✓	286
liposomes	219	not reported	bryostatin-2, nelfinavir	anti-CD4	HIV cure	X	143
PEG-liposomes	195.4 ± 3	–41.1	methotrexate	peptide targeting CD40L	autoimmune diseases	✓	292
<i>inorganic</i>							
gold	~23	not reported	TGFβ inhibitor	anti-CD8	cancer immunotherapy	✓	251
gold	14.9 ± 0.5	–32.4 ± 1.6	daunorubicin	aptamers targeting PTK7 and nucleolin	T-ALL treatment	X	17,283

^aWhere applicable, *in vivo* studies have been performed in mice. CAR, chimeric antigen receptor; cIBR, cyclo(1,12)-Pen-PRGGSVLVTGC-OH; fundamental, fundamental research (*i.e.*, no clinical application specified); LFA-1, lymphocyte function-associated antigen-1; LNP, lipid nanoparticle; Nrp1, neuropilin 1; PBAE, poly-β-aminoester; PEG, polyethylene glycol; PEI, polyethylenimine; PGA, polyglutamic acid; PLGA, poly(lactic-co-glycolic acid); PTK7, protein tyrosine kinase 7; RNAi, RNA interference; T-ALL, T cell acute lymphoblastic leukemia; TGFβ, transforming growth factor beta.

In vivo data generally showed increased colocalization of antibody-conjugated nanoparticles with their target tissue or target cell^{19,250,251,281,282,293}. In a study using polymeric nanoparticles consisting of PBAE and polyglutamic acid, antibody functionalization resulted in a different biodistribution in mice with CD3-targeted nanoparticles accumulating in lymphoid organs¹⁹, whereas nontargeted

nanoparticles accumulated in the liver. A separate study showed that in the absence of antibody conjugation, PEGylation of poly(methacrylic acid)-based nanoparticles improved targeting to lymph nodes, including the T cell-rich paracortex²¹⁹.

In some of the studies listed in Table 1, the choice of the targeting ligand was based specifically on the efficiency of the receptor to induce RME^{19,172,252,282,293-295}. Based on these studies, CD3¹⁹, CD7²⁵², CCR5¹⁷², transferrin receptor^{294,295}, CD90^{282,293}, and IL-2R²⁹³ would make good candidates for inducing nanoparticle internalization in T cells. However, transferrin receptor expression is not limited to T cells, thus potentially limiting its targeting specificity^{294,295}. CD4 and CD45 are shown to be noninternalizing surface receptors^{172,282}, although two studies successfully used CD4-targeted lipid nanoparticles to deliver nucleic acid-based therapeutics intracellularly^{250,291}. Following nanoparticle association and/or internalization, the surface expression of the targeted CD4 receptor was reduced, possibly due to receptor sequestering²⁵⁰. Some of these findings were confirmed in a study that sought to identify a surface anchor, rather than a cycling receptor, and found that CD45 internalized more slowly than CD2, CD8, CD11 α , or CD90²⁷⁷.

5.3. Rational design of T cell-targeting nanoparticles

Based on the literature published to date, the following features are suggested to be important for targeting T cells *in vivo* (Figure 7).

- (i) For efficient T cell entry, nanoparticles should ideally be <200 nm in diameter to be compatible with RME. An optimum size of ~50 nm is suggested^{197,265}. However, nanoparticles should remain larger than ~10 nm to avoid renal clearance via glomerular filtration^{197,296}.
- (ii) Nanoparticles should preferably interact with specific receptors on the T cell surface that, upon ligation, induce their own endocytosis (RME) through clathrin-coated vesicles, as this is the most efficient route of uptake for T cells. Therefore, for T cell targeting, nanoparticles should be conjugatable to antibodies or other RME ligands^{264,265}. The intracellular signalling that arises from this receptor ligation should be nontoxic to the cells.
- (iii) For compounds that are unable to cross a cellular membrane (lipid bilayer) – *i.e.*, large compounds and hydrophilic compounds – nanoparticles are required to induce endosomal escape and subsequent cytosolic delivery to prevent endosomal entrapment, recycling to the plasma membrane, or transport to lysosomes (with potential degradation)²⁵⁹.

(iv) Nanoparticles should be immune evasive *i.e.*, avoid engulfment (phagocytosis) by macrophage-like cells in both the blood as well as the mononuclear phagocyte system that is triggered by nanoparticle opsonization. This also generates a further restriction in the ideal nanoparticle size to <100 nm, which promotes escape from mononuclear phagocyte system scavenging^{248,297,298}.

In summary, the use of nanomedicines for *in vivo* targeting of T cells is a promising but still nascent field and studies to date are mostly proof of concept. More fundamental, comparative studies of nanoparticle internalization kinetics into T cells are needed. These will validate and potentially expand the design features illustrated in Figure 7, which are likely to advance translation of *in vitro* T cell-targeting nanoparticles to clinically approved formulations.

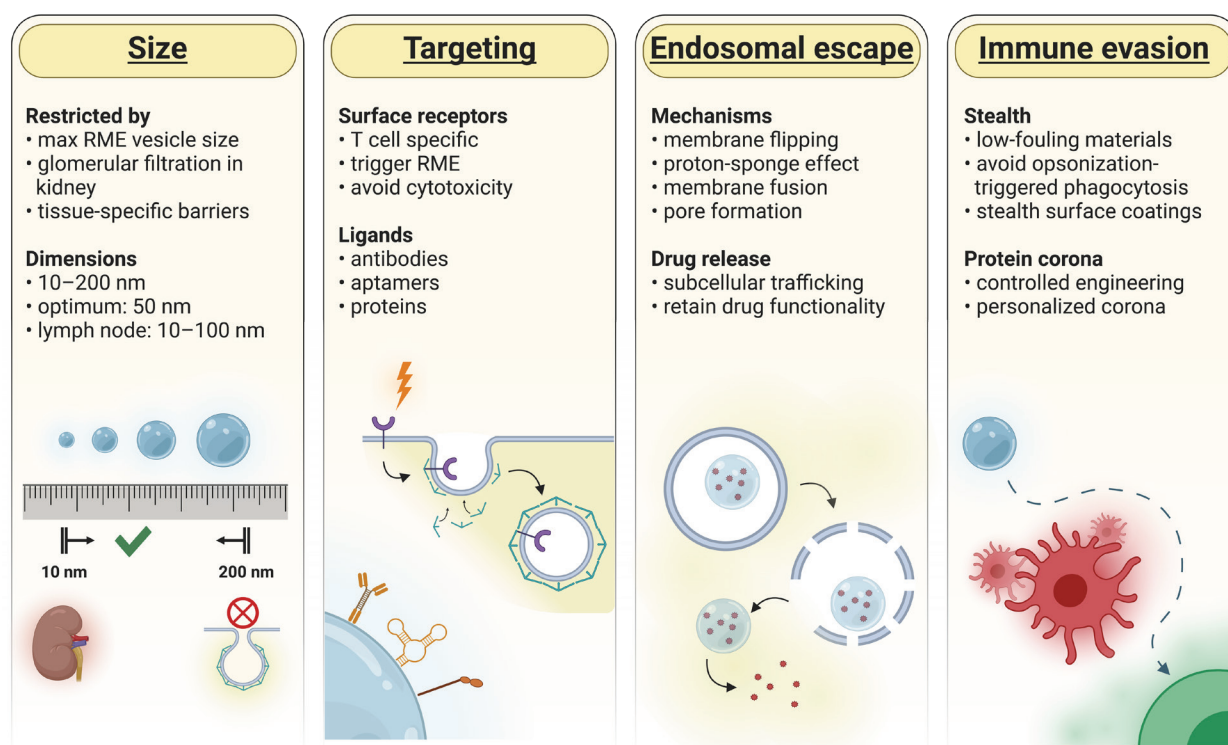


Figure 7. Important features for future rational design of T cell-targeting nanoparticles. *In vivo* drug delivery to T cells using nanoparticles remains a technological challenge. The nature of T cells, their *in vivo* biodistribution, and complex cellular environment pose barriers that require specific and rational material engineering solutions to address those challenges. RME, receptor-mediated endocytosis.

Aims and hypothesis

The works described in this thesis address the goal of developing a T cell-targeted nanoparticle platform that can deliver therapeutics towards an HIV cure. Based on the challenges in nanoparticle-based drug delivery to T cells and the lack of comparative nanoparticle studies, we believe such a platform requires thorough rational design as well as improved methods to quantify nanoparticle performance. As such, the specific objectives of this thesis were to:

- a) to establish a method to evaluate the interactions between nanoparticles and cells through absolute quantitation, to allow rational, inter- and intra-class comparisons of nanoparticles;
- b) to enhance nanoparticle internalization by inducing receptor-mediated endocytosis, through the use of a novel nanoparticle internalization assay;
- c) to investigate the use of lipid nanoparticles for the delivery of a more potent, HIV-specific latency reversal agent based on CRISPR activation; and
- d) to improve the delivery of lipid nanoparticle-based therapeutics to primary CD4⁺ T cells through the conjugation of targeting antibodies to the lipid nanoparticle surface.

Together, these works aim to contribute to a rational design of novel nanomedicine, and the improved, comparative assessments thereof. We aim to strengthen the concept that nanomedicine can play an important role in future therapeutic strategies towards an HIV cure as well as other T cell-targeted therapeutic strategies, as a result of improved nanoparticle delivery to hard-to-transfect T cells.

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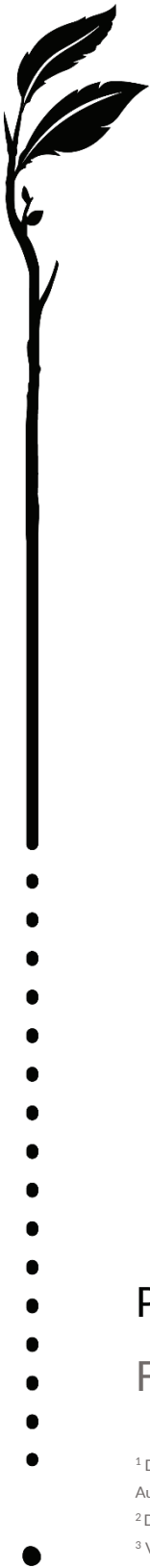
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Chapter 2

Experimental quantification of interactions between drug delivery systems and cells *in vitro*: a guide for preclinical nanomedicine evaluation

J. Vis. Exp. (187), e64259, doi:10.3791/64259 (2022).

P.M. Ceva¹, M. Roche², S.R. Lewin^{2,3,4}, F. Caruso⁵, M. Faria⁶

¹ Department of Microbiology and Immunology, The University of Melbourne at The Peter Doherty Institute for Infection and Immunity, Melbourne, VIC, Australia

² Department of Infectious Diseases, The University of Melbourne at The Peter Doherty Institute for Infection and Immunity, Melbourne, VIC, Australia

³ Victorian Infectious Diseases Service, Royal Melbourne Hospital at The Peter Doherty Institute for Infection and Immunity, Melbourne, VIC, Australia

⁴ Department of Infectious Diseases, Alfred Hospital and Monash University, Melbourne, VIC, Australia

⁵ Department of Chemical Engineering, The University of Melbourne, Melbourne, VIC, Australia

⁶ Department of Biomedical Engineering, The University of Melbourne, Melbourne, VIC, Australia

Abstract

A major component of designing drug delivery systems concerns how to amplify or attenuate interactions with specific cell types. For instance, a chemotherapeutic might be functionalized with an antibody to enhance binding to cancer cells ("targeting") or functionalized with polyethylene glycol to help evade immune cell recognition ("stealth"). Even at a cellular level, optimizing the binding and uptake of a drug carrier is a complex biological design problem. Thus, it is valuable to separate how strongly a new carrier interacts with a cell from the functional efficacy of a carrier's cargo once delivered to that cell. To continue the chemotherapeutic example, "how well it binds to a cancer cell" is a separate problem from "how well it kills a cancer cell". Quantitative in vitro assays for the latter are well established and usually rely on measuring viability. However, most published research on cell-carrier interactions is qualitative or semiquantitative. Generally, these measurements rely on fluorescent labeling of the carrier and, consequently, report interactions with cells in relative or arbitrary units. However, this work can be standardized and be made absolutely quantitative with a small number of characterization experiments. Such absolute quantification is valuable, as it facilitates rational, inter- and intra-class comparisons of various drug delivery systems-nanoparticles, microparticles, viruses, antibody-drug conjugates, engineered therapeutic cells, or extracellular vesicles. Furthermore, quantification is a prerequisite for subsequent meta-analyses or in silico modeling approaches. In this article, video guides, as well as a decision tree for how to achieve in vitro quantification for carrier drug delivery systems, are presented, which take into account differences in carrier size and labeling modality. Additionally, further considerations for the quantitative assessment of advanced drug delivery systems are discussed. This is intended to serve as a valuable resource to improve rational evaluation and design for the next generation of medicine.

Introduction

The design of drug delivery constructs that exhibit specific, designed behavior depending on what cell type they encounter has attracted substantial research interest. Potential drug delivery constructs or "carriers" include lipid formulations, nano-grown inorganics, polymeric assemblies, extracellular vesicles, functionalized bacterial cells, or modified viruses. All of these can exhibit organ, tissue, or cell specificity due to physical properties, surface properties, or engineered chemical functionalizations such as antibody attachment^{1,2}.

A nearly ubiquitous step in *in vitro* carrier evaluation is to incubate cells with a suspension containing said drug-loaded carrier. Post incubation, carrier performance is measured *via* a functional readout of the drug cargo's performance, for example, transfection efficiency or toxicity. Functional readouts are useful, as they are a downstream measure of carrier effectiveness. However, for more complex drug delivery constructs, it is increasingly important to move beyond functional readouts and separately quantify the degree of carrier interaction with the cell of interest. There are a few reasons for this.

First, there is increasing interest in discovering (and iteratively improving) "platform" carrier technologies, which can carry a variety of cargo. For example, lipid nanoparticles (LNPs) designed to encapsulate RNA can exchange one RNA sequence for another with few caveats³. Thus, to iteratively improve the carrier technology, it is critical to quantify its performance independent of the cargo functionality. Second, functional readouts may not be straightforward for the cargo of interest, compromising the ability to rapidly iterate and evaluate carrier formulations. While one could perform *in vitro* optimization using a model cargo with a straightforward functional readout (for instance, fluorescence), changing the cargo can change the biological response to a carrier⁴ and may, thus, not yield representative results. Third, many carriers are designed to interact with and be taken up by a specific cell type. Such *targeting capability* of a carrier can and should be differentiated from the *performance of its therapeutic cargo* post targeting. To continue the LNP example, an RNA cargo might be extremely potent, but if the LNP is unable to bind to the cell, be internalized, and release the RNA, no downstream functional effect will be observed. This can be an issue particularly for carriers intended to target hard-to-transfect cell types, such as T cells⁵. Conversely, an LNP could target extremely effectively, but the RNA cargo might not function. A downstream assay that just measures cargo functionality will be unable to differentiate between

these two situations, thus complicating the development and optimization of carrier drug delivery systems.

In this work, how to absolutely quantify carrier *association* is discussed. Association is a term that refers to the experimentally measured degree of interaction between a carrier and a cell. Association does not differentiate between membrane binding and internalization – a carrier may be associated because it is bound to the cell surface or because the cell has internalized it. Association is commonly measured as part of cell-carrier incubation experiments. Historically, association has been reported either in arbitrary fluorescent units (typically "median fluorescence intensity" or MFI) or as "percent association," metrics whose limitations have been previously discussed⁶. In short, these measurements are not comparable between experiments, laboratories, and drug carriers due to differences in experimental protocols, flow cytometer settings, and the labeling intensities of different carriers. Efforts have been made to overcome the former by calibrating the cytometer, thereby converting the relative measure of MFI into an absolutely quantitative measure of fluorescence⁷. However, this method does not account for the variability in the labeling intensity of various carriers and, thus, does not allow the rational comparison of various carrier performances in a target cell of choice⁸.

Here, how to practically convert from relative, arbitrary fluorescent units to the absolute quantitative metric of the "number of carriers per cell" is demonstrated by performing a small number of additional characterization experiments. If another metric of carrier concentration is desired (e.g., carrier mass per cell or carrier volume per cell), it is straightforward to convert from carriers per cell, provided carrier characterization has been done. For brevity and to avoid jargon, the word "carrier" is used within this work to refer to the vast assortment of drug delivery constructs. These quantification techniques are equally applicable, whether applied to a nano-engineered gold particle or a bio-engineered bacteria.

A few facts enable the conversion from arbitrary fluorescent units to carriers per cell. First, the measured fluorescence intensity is proportional to the concentration of a fluorophore⁹ (or a fluorescently labeled carrier), assuming the fluorescence is within the detection limits of the instrument and the instrumentation settings are the same. Thus, if the fluorescence of a carrier and the fluorescence of a sample are known, one can determine how many carriers are present in that sample if all the measurements were performed under the same settings and conditions. However, especially for smaller carriers, it may not be possible to measure carrier fluorescence,

cell autofluorescence, and cell-associated-with-carriers fluorescence on the same instrument with the same settings. In this case, there is a second requirement to make it possible to convert between measured fluorescence on one instrument and measured fluorescence on another. To do so, a standard curve of fluorophore concentration can be established to measure the fluorescence intensity on both instruments, taking advantage of the Molecules of Equivalent Soluble Fluorochrome (MESF) standard⁹. This then allows measurement of the carrier fluorescence in bulk on a non-cytometer, a measurement that can be done on carriers of any size or characteristic. When such bulk quantification is done on a carrier suspension of known concentration, the number of carriers per cell of a sample can, once again, be calculated.

While this work demonstrates the process for measuring carrier association (as determined by measured fluorescence intensity), an analogous protocol could be performed for other measures of cell-carrier interaction (e.g., an experimental protocol that differentiates internalized and membrane-bound carriers). Additionally, this protocol would be largely the same if association was measured through a non-fluorescent assay (for instance, through mass cytometry).

Protocol

1. Choosing the appropriate stream

- 1.1. Follow the decision tree outlined in Figure 1 to determine the best workflow (stream) (Figure 2) for the experimental setup used. Refer to the discussion for further comments on this choice of stream.
- 1.2. If following the Cytometer Stream, continue with steps 2.1.1-2.2.7. If following the Bulk Stream, continue with steps 3.1.1.1-3.3.7.

2. The Cytometer Stream

2.1. Carrier counting

NOTE: Any flow cytometer can be used for this measurement, provided the flow rate ($\mu\text{L/s}$) is known. If the flow rate is unknown and cannot be determined, do not proceed with this step. Instead, proceed with step 3.1.1.1. Counting the carriers in suspension allows accurate and reproducible determination of the number of carriers incubated in each cell experiment.

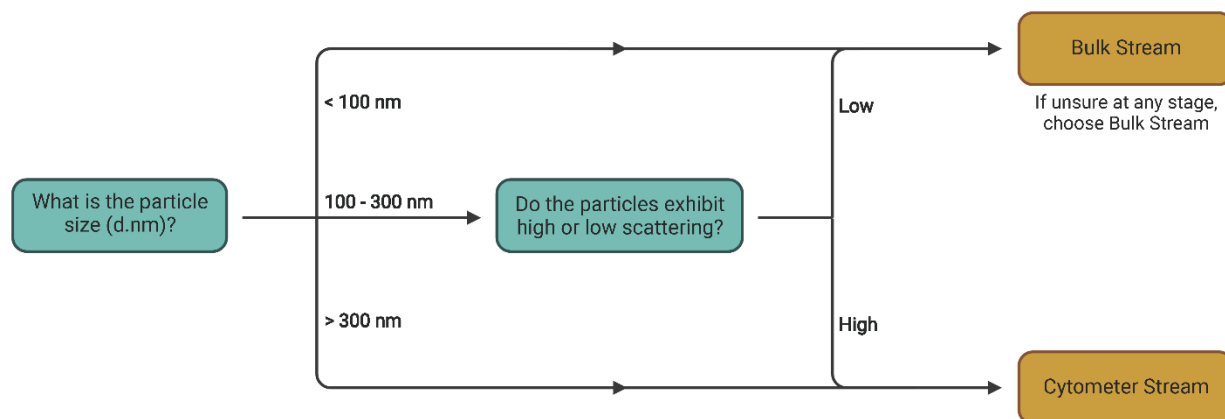


Figure 1. Workstream decision tree. The decision as to which Stream to use depends primarily on the carrier type of interest. Larger carriers and carriers with high scattering properties can more easily be detected individually on cytometers, thus making them suitable for quantification using the Cytometer Stream. The Bulk Stream is suitable for all other carrier types.

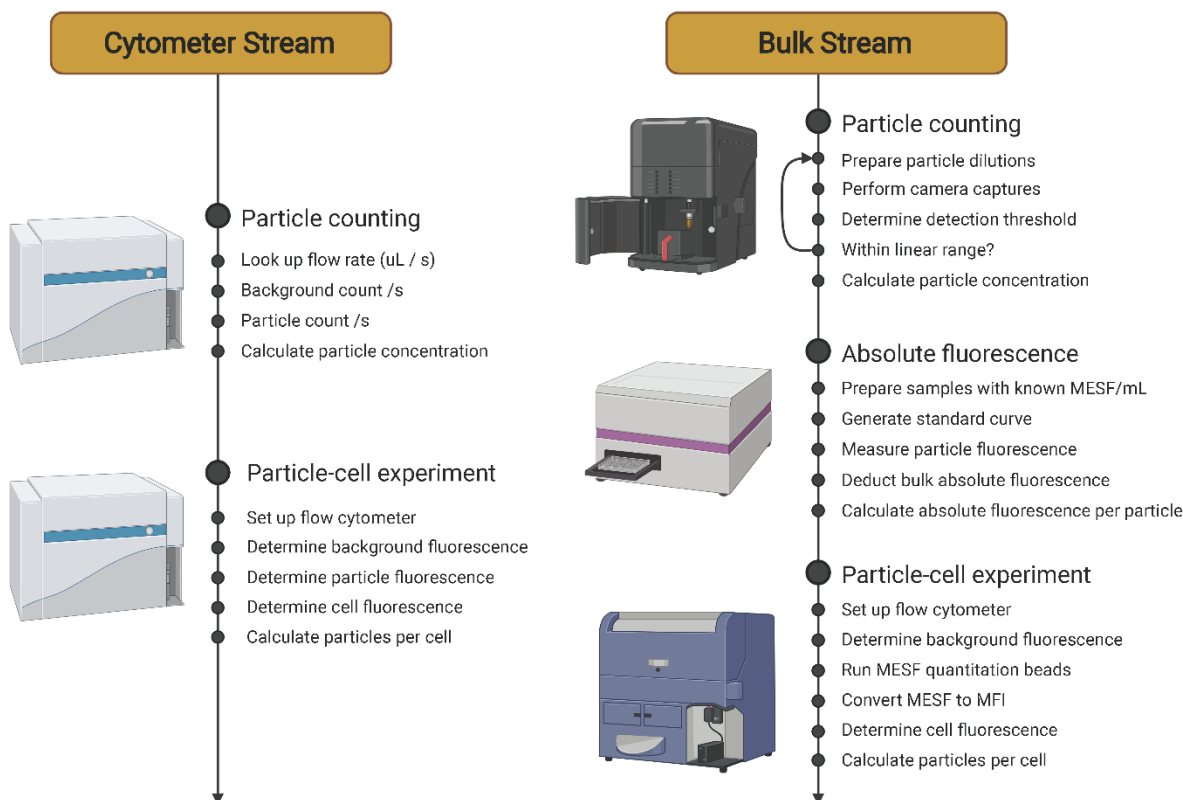


Figure 2. Overview of workstreams. This protocol is split into two different Streams. The Cytometer Stream uses a sensitive cytometer to count the carriers in suspension, measure their individual fluorescence, and then determine the fluorescence of cells incubated with carriers. The Bulk Stream uses non-cytometry-based techniques, such as Nanoparticle Tracking Analysis, to count the carriers in suspension. The individual carrier fluorescence is then quantified using a microplate reader or spectrofluorometer. The use of the flow cytometer is, therefore, restricted to measuring the final fluorescence of cells incubated with carriers, a measurement that can be done on a wider range of cytometers and that is independent of the carrier type used. MESF, Molecules of Equivalent Soluble Fluorochrome; MFI, median fluorescence intensity.

2.1.1. Set up the cytometer to detect carriers, both by an optical scattering channel (typically side scatter [SSC]) and fluorescence. Make sure to adjust the threshold to allow detection of the carriers.

NOTE: Iteration through different optical scattering channels may be required if SSC does not provide a clear signal (e.g., forward scatter [FSC]).

2.1.2. Run a diluent-only sample to quantify the background event count in both the SSC and fluorescent channels.

NOTE: Ideal background event counts are <100 events/s.

2.1.3. Prepare the carriers for flow cytometry.

2.1.3.1. Ensure the carriers are well suspended by vortexing or sonication, depending on the carrier system involved.

2.1.3.2. If possible, ensure the carrier concentration is between 1,000 carriers/ μ L and 10,000 carriers/ μ L. An event count of one to two orders of magnitude higher than the background is a good start. If the order of magnitude of the carrier concentration is unknown, a good start is to prepare a 1:1,000 dilution from stock. Use the initial results as feedback to inform future sample dilutions.

NOTE: A cloudy suspension is generally too concentrated.

2.1.4. Load the first carrier sample onto the cytometer and start recording.

2.1.5. Compare event counts resulting from both the SSC and fluorescence channels; these should be approximately equal (<10% difference). If not, check the cytometer settings, e.g., photomultiplier tube (PMT) settings and laser intensity for the fluorescent channel. Alternatively, use other methods such as confocal microscopy to validate that the fluorescent labeling of the carriers is present and uniform.

2.1.6. Repeat steps 2.1.3-2.1.5 two or more additional times with different dilutions from the stock. Ensure that the event count in each sample is at least one order of magnitude higher than the background event count.

2.1.7. Verify that three or more samples show a linear trend, that is, a two-fold sample dilution should result in a corresponding two-fold reduction in the measured carrier concentration.

2.1.8. Use the samples within the linear range, corresponding dilution factors, and the known cytometer flow rate to calculate the stock carrier concentration according to equation (1):

$$C = \frac{\text{event count}}{\text{flow rate}} \cdot 1000 \quad (1)$$

where C is the stock carrier concentration in carriers/ mL. It is recommended to use the event count derived from optical scattering detection rather than fluorescence.

2.2. Flow cytometry readout of the carrier-cell experiment, including determination of fluorescence intensity per carrier.

NOTE: Ideally, the fluorescent intensity per carrier will be determined as close as possible to the carrier-cell experiment. This is to ensure that the MFIs obtained for individual carriers can directly be compared to the MFIs of cells associated with the carriers. In practice, a cytometer will usually generate similar results when used on consecutive days using the same PMT voltages, but this cannot be guaranteed.

2.2.1. Design the carrier-cell experiment. Use the carrier concentration determined in step 2.1 to administer the desired dose of carriers.

2.2.2. Set up the flow cytometer for the final carrier-cell experiment by determining optimal PMT voltage settings in the relevant channels. Set the thresholds to allow carrier detection.

2.2.3. Run the carriers in suspension to determine the fluorescence intensity per carrier under the current PMT settings.

2.2.4. If needed, change the cytometer thresholds to detect the cells and not the carriers.

2.2.5. Run a negative control sample – cells not incubated with carriers – to determine the background fluorescence (autofluorescence) of the cells.

2.2.6. Run the carrier-cell samples to determine the fluorescence intensity per cell. This fluorescence is a linear combination of cellular autofluorescence and the presence of fluorescent carriers.

2.2.7. Calculate the number of carriers per cell using the following equation (2):

$$P_{assoc} = \frac{(FI_{cell} - FI_{background})}{FI_{carrier}} \quad (2)$$

where P_{assoc} is the number of carriers associated per cell, FI_{cell} is the MFI of cells incubated with carriers, $FI_{background}$ is the MFI of cells not incubated with carriers, and $FI_{carrier}$ is the MFI of carriers in suspension.

3. The Bulk Stream

3.1. Carrier counting: nanoparticle tracking analysis

NOTE: In the Bulk Stream, carrier counting is a necessary step to quantify the absolute fluorescence intensity per carrier (see step 3.2.5). In addition, counting the carriers in suspension allows the accurate and reproducible determination of the number of carriers incubated in each cell experiment.

3.1.1. Preparation

3.1.1.1. Mount the flow cell onto the laser module and lock the entire laser module in place inside the instrument.

3.1.1.2. Slowly (not faster than 0.1 mL/s) flush the flow cell with ~1 mL of distilled water. If bubbles form within the flow cell, retract the suspension partially to merge the bubble with the air-liquid interface before proceeding.

3.1.1.3. Start the camera roughly halfway through flushing; be sure to confirm that carrier debris is washed out. Select **Capture** to open the **Capture Settings** tab and click **Start Camera**.

3.1.1.4. Dry the system with 1 mL of air. If any static carriers are visible on the screen,

clean the flow cell according to the manufacturer's instructions.

3.1.1.5. Prepare the carriers for Nanoparticle Tracking Analysis by ensuring the carriers are well suspended via vortexing or sonication, depending on the carrier system involved. If the order of magnitude of the stock concentration is unknown, prepare a 1:100 dilution from the stock and use the initial results as feedback to inform future sample dilutions. Dilute the carriers in water and not phosphate-buffered saline (PBS) to prepare at least ~0.6-1 mL of each sample with a carrier concentration between 1×10^7 carriers/mL and 1×10^9 carriers/mL.

NOTE: A cloudy suspension is generally too concentrated. Buffers and salts can generate high background noise.

3.1.2. Measurement

3.1.2.1. Take out the laser module and place it upright.

3.1.2.2. Draw up the first carrier sample into a 1 mL syringe. Attach the syringe to the tube inlet and carefully load the sample into the flow cell. If bubbles form within the flow cell, retract the suspension partially to merge the bubble with the air-liquid interface before proceeding. Ensure the entire flow cell is filled with liquid, then pause.

3.1.2.3. Adjust the camera focus if needed to visualize individual carriers. Make coarse focus adjustments with the rotating knob on the right-hand side of the instrument. Make finer adjustments by selecting the **Hardware** tab | **Pumps/Stage**. Change the focus by adjusting the **Focus** slider.

3.1.2.4. Adjust the camera level to make sure there is no oversaturation. Within the **Capture** tab, select the optimal **Camera Level** by adjusting the slider.

3.1.2.5. If the instrument is equipped with this accessory, load the syringe containing the carrier sample into the **Syringe Pump** to ensure continuous sample flow during measurements.

3.1.2.6. Under the **SOP** tab, select **Standard Measurement** to take five captures of 30

s each. Enter **Base filename** and, if desired, add additional sample information by clicking the **Advanced** button (which opens a modal dialogue with a variety of choices).

NOTE: If a dilution factor is entered, the final carrier concentration measurement will be adjusted automatically by the software. Entering this factor is advised against. Instead, perform the adjustment manually, which facilitates the analysis and allows the assessment of whether each dilution falls within the dynamic range of the instrument (step 3.1.3.4).

3.1.2.7. Press **Create** and **Run Script** and wait for a pop-up to appear asking to **Please advance sample**.

3.1.2.8. If using the **Syringe Pump**, select the **Hardware** tab | **Syringe Pump** tab | set the **Infusion Rate** at **30-80** and press **Infuse**. If not using the **Syringe Pump**, manually advance the sample.

3.1.2.9. In the pop-up window, select **OK** to start capturing. After each of the five captures, when the **Please advance sample** pop-up reappears, check that the sample is still moving through the flow cell, either manually or via the **Syringe Pump**. Then, select **OK** to proceed with the next capture.

NOTE: After five captures, the software automatically opens the Process tab and opens a pop-up asking to adjust the process settings.

3.1.3. Analysis

3.1.3.1. In the **Process** tab, adjust the **Detection Threshold** slider (between **4** and **8**) to correctly identify distinct carriers visible on the screen. Adjust the **Screen Gain** too to aid visualization; it will not affect the downstream analysis. Use the slider under the capture screen to scroll through multiple frames of the video to aid detection threshold setting.

NOTE: The detection threshold should be set once and, subsequently, should not be altered between measurements or samples.

- 3.1.3.2. In the pop-up (note after step 3.1.2.9), press **OK** to initiate tracking analysis. Monitor the progress of the analysis by clicking on the **Analysis** tab | **Single Analysis** tab.
- 3.1.3.3. Once the analysis is finished, look for an **Export Settings** prompt to appear, in which **Include PDF** and **Include Experiment Summary** should be selected by default. Select any other export formats as desired.
- 3.1.3.4. In the **Results** section of the **PDF data export**, to ensure the concentration measured is reliable, verify that the measured carrier concentration is between 1×10^7 carriers/mL and 1×10^9 carriers/mL – the dynamic range of the instrument – and check for any error messages or messages of caution underneath the concentration measurement result.
- 3.1.3.5. Repeat steps 3.1.2.1-3.1.3.4 two or more times with different dilutions from the stock. Ensure that the concentration of each sample falls within the linear range of the instrument.
- 3.1.3.6. Select three or more samples that show a linear trend, that is, a two-fold sample dilution should result in a corresponding two-fold reduction in the measured carrier concentration. Use the selected samples and corresponding dilution factors to calculate the stock carrier concentration.

3.2. Determination of the absolute fluorescence intensity per carrier

NOTE: Since the fluorescence of individual carriers in this stream cannot be characterized directly, the fluorescence intensity is quantified in bulk. This method relies on the fact that fluorescence intensity is linearly related to the fluorochrome concentration according to the Lambert-Beer law. When such bulk quantification of carriers in suspension is done on a suspension of known carrier concentration (see step 3.1), the fluorescence per carrier can be derived. This step can be done on either a fluorescence plate reader or a spectrofluorometer. The fluorescence intensity is compared to a standard curve of samples with known absolute fluorescence, given in number of MESFs.

- 3.2.1. Use a solution of the free fluorochrome to label the carrier: resuspend the dye in the appropriate buffer (e.g., DMSO) and perform further dilutions in the same buffer as the

carrier diluent. Alternatively, use a solution of an antibody conjugated to the fluorochrome. Calculate the concentration of the stock solution (MESF/mL) from the concentration in mg/mL, the molecular weight in mg/mole, and Avogadro's number using equation (3). Perform a serial dilution in the carrier diluent to generate standard curve samples.

$$M = C \cdot (MW)^{-1} \cdot 6.022e23 \quad (3)$$

NOTE: Use a fluorochrome-conjugated antibody only if the degree of labeling, *i.e.*, the molar ratio between the fluorochrome and antibody in the solution, is known. Initially, generate a standard curve with a wide range, as the fluorescence intensity of the carrier sample is still unknown. From here, narrow it down to include the range required.

3.2.2. Prepare the carrier samples.

NOTE: The best practice is to test two or more carrier dilutions to validate that the measurements are linear and fall within the range of the standard curve.

3.2.3. Measure the fluorescence of equal volumes of each sample, *i.e.*, both carrier and standard curves.

3.2.4. Generate a standard curve and deduct the bulk absolute fluorescence intensity in MESF/mL for the carrier samples measured.

3.2.5. Calculate the absolute fluorescence intensity per carrier (MESF/carrier) by dividing the bulk fluorescence (MESF/mL) by the carrier concentration (carriers/mL) as in equation (4):

$$FI_{particle} = \frac{FI_{sample}}{C_{carrier}} \quad (4)$$

3.3. Cell experiment (including the determination of the equivalent fluorescence intensity per carrier)

NOTE: In this step, flow cytometry quantitation beads are used to generate a standard curve of the relationship between MESF and MFI. These quantitation beads consist of multiple bead populations with a known number of MESF per bead, and these individual beads can be

detected by any cytometer. Ideally, the MESF standard curve is determined at the same time as the readout of the carrier-cell experiments. This is to ensure that the MFI values calculated for individual carriers can directly be compared to the MFI of cells associated with carriers. In practice, a cytometer will usually generate similar results when used on consecutive days using the same PMT voltages, but this cannot be guaranteed.

3.3.1. Design the carrier-cell experiment. Use the carrier concentration determined in section 3.1.3 to administer the desired dose of carriers.

3.3.2. Set up the flow cytometer for the final carrier-cell experiment by determining optimal PMT voltage settings in the relevant channels.

3.3.3. Run a negative control sample, that is, cells not incubated with carriers, to determine the background fluorescence.

3.3.4. Prepare and resuspend the flow cytometry quantitation beads. Use the same buffer as used for the cell samples (e.g., PBS). If the bead populations are provided separately, pool them together.

3.3.5. Run the flow cytometry quantitation bead sample.

3.3.6. Run the carrier-cell samples to determine the fluorescence intensity per cell.

3.3.7. Use the quantitation bead sample to generate a standard curve converting the absolute fluorescence intensity (MESF) into MFI. Use this standard curve and the results from step 3.2 to calculate the theoretical MFI of the carriers. Calculate the number of carriers per cell using equation (5):

$$P_{assoc} = \frac{(FI_{cell} - FI_{background})}{FI_{carrier}} \quad (5)$$

Where P_{assoc} is the number of carriers associated per cell, FI_{cell} is the MFI of cells incubated with carriers, $FI_{background}$ is the MFI of cells not incubated with carriers, and $FI_{carrier}$ is the calculated MFI of carriers in suspension (step 3.2).

Representative Results

As discussed previously, different drug carrier types require the use of different techniques for the absolute quantification of cell-carrier association. For example, 633 nm disulfide-stabilized poly(methacrylic acid) (PMA_{SH}) core-shell particles are large and dense enough for detection using a sensitive flow cytometer. As such, these particles were labeled fluorescently, then gated and counted using small-angle light scattering (SALS, analogous to SSC), as well as the appropriate fluorescent channel (Figure 3). The difference in event count in both channels was 1.98%, well within the acceptable range.

In contrast, 100 nm superparamagnetic iron oxide nanoparticles are too small to detect individually and were, thus, analyzed using the Bulk Stream. These nanoparticles were counted and characterized using Nanoparticle Tracking Analysis (Figure 4). The mean nanoparticle size of 136 nm reflects the hydrodynamic diameter of the nanoparticle in water. The nanoparticle concentration measured – still uncorrected for the dilution performed – falls within the dynamic range of the instrument, suggesting successful and accurate determination of the nanoparticle concentration.

Continuing in the Bulk Stream, the absolute fluorescence intensity of a carrier suspension is to be converted into an MFI value on the flow cytometer used for the final cell-carrier incubation. In this experiment, quantitation beads labeled with the same fluorescent dye as the carrier were used on

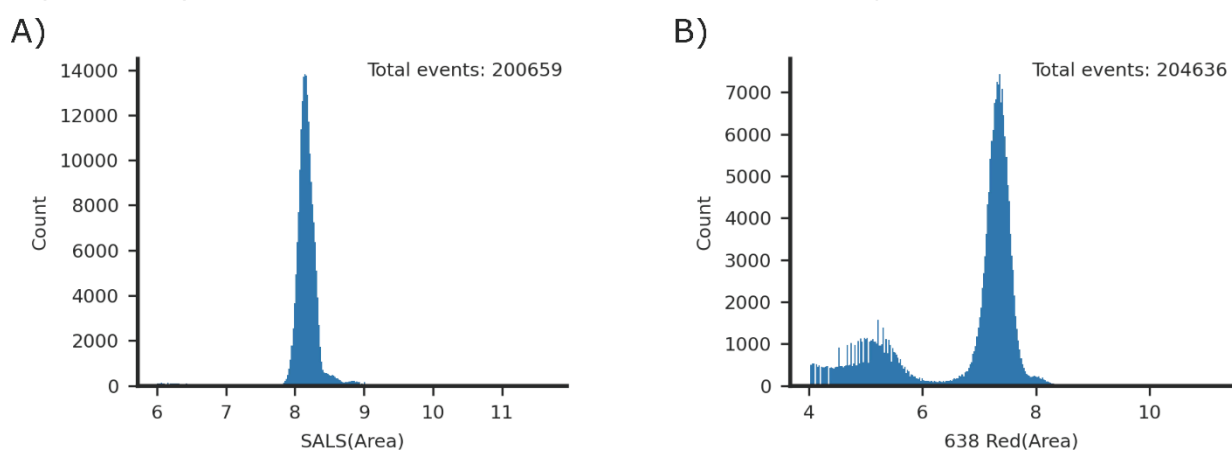


Figure 3. Counting particles using a flow cytometer (Cytometer Stream). An Apogee flow cytometer was used. (A) Particle counting using an optical channel (SALS), resulting in an event count of 200,659. (B) Carrier counting using the carrier's fluorescent channel, resulting in an event count of 204,636. In both cases, an arcsinh transform followed by gating was applied (at 6 and 4, respectively). Figures created from raw data of 633 nm core-shell particles first published in Faria *et al.*¹⁰. SALS, small-angle light scatter.

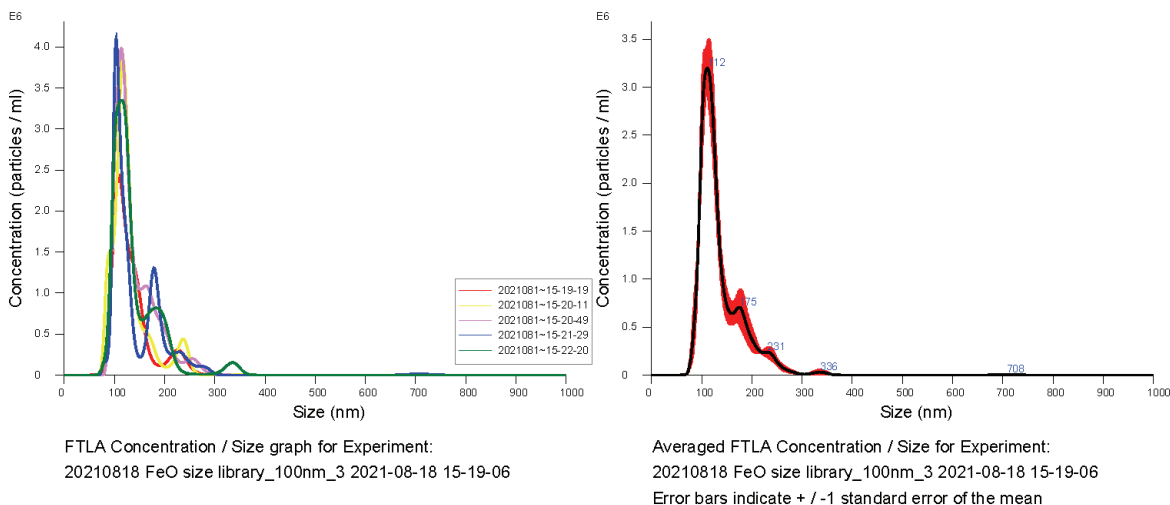


Figure 4. Counting carriers using Nanoparticle Tracking Analysis (Bulk Stream). Superparamagnetic iron oxide nanoparticles of 100 nm in size were characterized and counted using Nanoparticle Tracking Analysis. Left, concentration/ size histogram of individual results of five replicate measurements. Right, average concentration/size distribution $\pm$ SEM of five replicates. In this particular sample, a 1:2,000 dilution from stock was performed to obtain a concentration within the dynamic range of the instrument.

multiple days to generate standard curves on a flow cytometer (Figure 5). The correlation between the measured MFI and the MESF value of the quantitation beads is linear and largely similar between the dates measured. However, slight differences between dates can be observed, and, as such, it is recommended to regenerate a standard curve as part of the readout of the carrier-cell experiments.

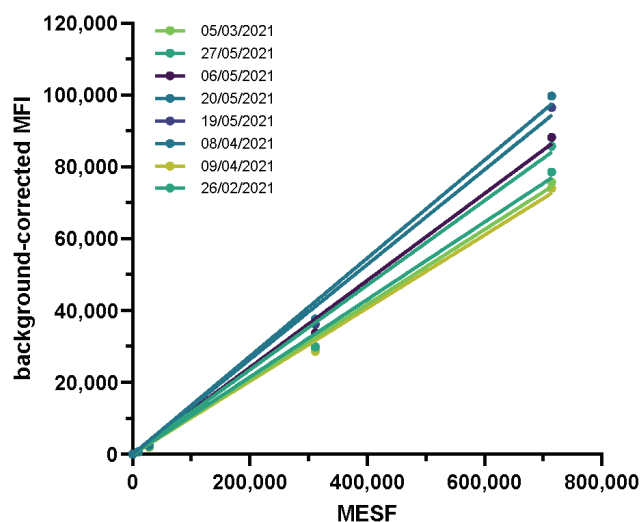


Figure 5. Converting MESF to MFI using quantitation beads on a flow cytometer (Bulk Stream). Fluorescent beads with four different MESF values were analyzed on a flow cytometer on various days, and standard curves were drawn. MESF, Molecules of Equivalent Soluble Fluorochrome; MFI, median fluorescence intensity.

Once the MFI of individual carriers has been determined, the cell-carrier association data can be absolutely quantified and more accurately interpreted. Performing time course experiments, e.g., incubating HeLa cells fluorescently labeled with 235 nm PMA_{SH} capsules, for various timepoints between 0 h and 24 h (Figure 6) is recommended. As expected, median cell fluorescence increases over time, indicating the capsules are associating with HeLa cells. While such experiments can be used to compare the relative carrier performance at various timepoints, these results are not absolutely quantitative.

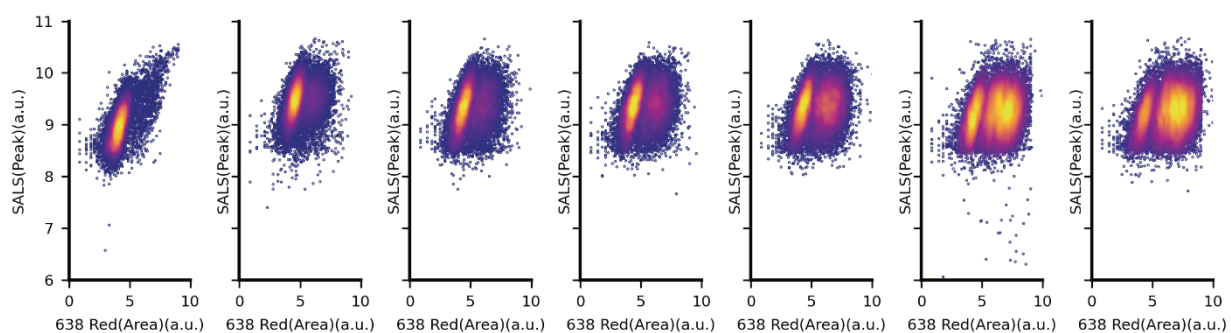


Figure 6. Final cell experiment on flow cytometer-time course series (Cytometer Stream and Bulk Stream). Representative images of flow cytometry data from a time course experiment. THP-1 cells were incubated with a 235 nm polymeric capsule for 0 h, 1 h, 2 h, 4 h, 8 h, 16 h, and 24 h (left to right). Carrier fluorescence is shown on the x-axis, while an optical channel is shown on the y-axis. In all cases, an arcsinh transform was performed. No gating was performed (other than choosing the limits of the graph). Created from raw data in Faria *et al.*¹⁰. SALS, small-angle light scatter.

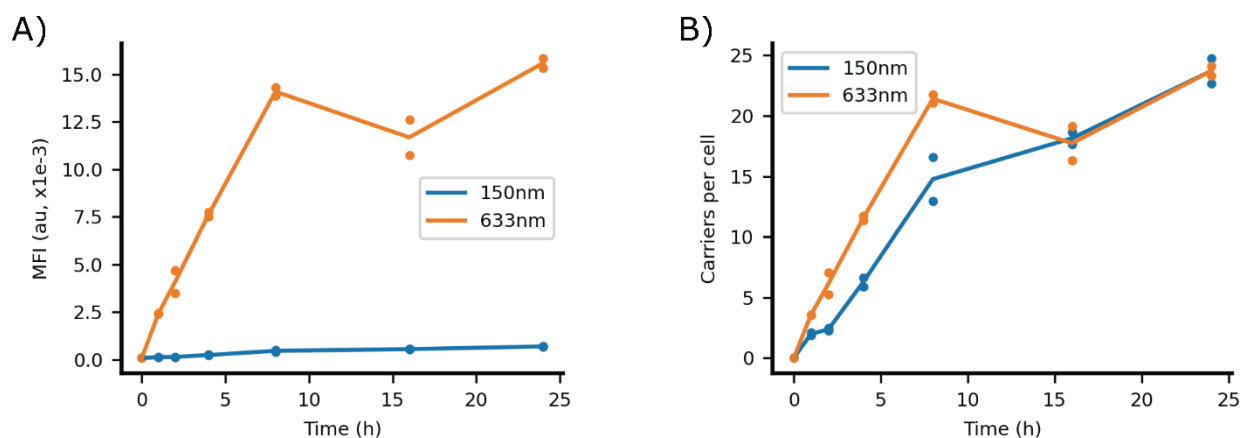


Figure 7. Relative versus absolute quantification of carrier-cell association (Cytometer Stream and Bulk Stream). Both figures visualize data from the same two experiments, a 24 h incubation between RAW264.7 macrophages and 150 nm (blue) or 633 nm core-shell carriers (orange), created from raw data in Faria *et al.*¹⁰. The medians of both technical replicates are plotted. (A) Relative quantification, reported as MFI in a.u. (B) Absolute quantification, reported as the number of carriers per cell. MFI, median fluorescence intensity; a.u., arbitrary units.

The importance of absolute quantitation, whether done *via* the Cytometer Stream or Bulk Stream, becomes clear when comparing the association of two carrier types. Figure 7 depicts the same two experiments, analyzed by either relative quantitation (Figure 7A) or absolute quantitation. The difference in the apparent cellular response to carriers is stark, depending on the analysis performed; carriers should *not* be directly compared when using relative quantification (Figure 7A), whereas absolute quantification is independent of labeling intensity and, thus, more comparable (Figure 7B).

Discussion

Characterizing the interactions between drug carriers and cells is becoming increasingly important in the development of novel drug delivery systems. Specifically, to allow the rational evaluation and comparison of various carrier constructs, absolute quantification of the performance of said carrier to interact with target and off-target cells is critical. This protocol describes a two-stream methodology that allows any researcher working with a drug carrier to convert relative, semiquantitative flow cytometry data on cell-carrier association into absolute quantitative results. The outlined process is applicable to any type of carrier – small, large, organic, inorganic – provided they are fluorescently labeled. However, different carriers require different approaches to calculate the carriers per cell. This is due to the limits of various instrumentation used for the required characterization measurements.

As such, this protocol is split into two Streams: the Cytometer Stream and the Bulk Stream. The first, the Cytometer Stream, is the more straightforward approach and requires only a flow cytometer, but this cytometer needs to be sensitive enough to detect the individual carriers used, or, in other words, the drug carrier of interest needs to be large and dense enough to be detected. The Bulk Stream is compatible with any carrier type, as the need to detect individual carriers is circumvented through the use of alternative instrumentation. However, the Bulk Stream requires more characterization experiments to be done.

To assist with choosing the appropriate Stream (and as such, the appropriate technologies), the above considerations have been summarized into a decision tree (Figure 1). To reiterate, the Bulk Stream is suitable for all researchers irrespective of the carrier type used and can, thus, always be reverted to as a backup. The workflows of the two Streams are outlined in Figure 2. Notably, with

the ongoing advances in the sensitivity of flow cytometers, it is possible that the Cytometer Stream can be used for more <300 nm carriers.

A few notes should be made on this procedure. First, the described strategy for converting the cell fluorescence into an absolute number of carriers per cell relies on the measurement of the individual carrier fluorescence in suspension. However, fluorescence is influenced by the immediate chemical environment of the fluorochrome (e.g., the carrier diluent, the cell surface, or various intracellular compartments). In particular, the acidic environment of the endosomal compartment is known to affect the fluorescence intensity of certain primarily protein-based dyes¹¹. As such, irrespective of the carrier type studied, it is recommended to use pH-insensitive and highly photostable dye ranges for the labeling of drug carriers (see the Table of Materials for a recommendation). The additional benefit of this dye range is its general brightness, which enhances the sensitivity of drug carrier detection.

Second, the methods discussed above are intended to provide guidance but by no means form an exclusive list of techniques that can be used to perform the various steps in the quantitation workflow. In particular, a variety of techniques exist to count smaller or low-scattering carriers (as those in the Bulk Stream). These include other instruments able to perform the same Nanoparticle Tracking Analysis¹², as well as other methods altogether, such as multi-angle dynamic light scattering, mass spectroscopy, electron microscopy¹³, gravimetry, or optical density measurements (further reviewed by Shang and Gao¹⁴). Furthermore, this experimental quantification – moving from arbitrary fluorescence units to an absolute number of carriers per cell – is only part of what might be termed a "quantitative biology workflow". Absolute experimental quantification is *necessary* but not *sufficient*. Merely quantifying and reporting the carrier association does not account for differences in particle association due to the experimental setup, such as the incubation time, dose, and concentration of drug carrier added, or the number of cells used per experimental container.

Additionally, carriers with different physicochemical properties may have very different dosages delivered to cells, even when the experimental setup is identical¹⁵. Carrier performance – even *in vitro* – cannot be determined without accounting for all these factors. In other words, merely quantifying the carrier-cell association does not, in general, allow for an unbiased comparison of carriers between researchers and laboratories. In this light, there has been prior discussion of the importance of performing time-course experiments of carrier-cell association and subsequent *in*

silico mathematical modeling to derive kinetic parameters (*i.e.*, rate constants) as an unbiased and quantitative measure of the affinity between a particular carrier-cell pair¹⁰. Experimental quantification is also a prerequisite for other *in silico* techniques to be applied, such as identifying potential sources of biological heterogeneity¹⁶ or determining penetration kinetics in a complex biological model¹⁷. Combined with the Minimum Information Reporting in Bio-Nano Experimental Literature guidelines for standardized reporting¹⁸, the quantitative evaluation of carrier performance can accelerate the development of novel nanomedicine.

Disclosures

The authors have no conflicts of interest to disclose.

Acknowledgments

This work was supported by the Australian National Health and Medical Research Council (NHMRC; Program Grant No. GNT1149990), the Australian Centre for HIV and Hepatitis Virology Research (ACH2), as well a gift from the estate of Réjane Louise Langlois. F.C. acknowledges the award of a National Health and Medical Research Council (NHMRC) Senior Principal Research Fellowship (GNT1135806). **Figure 1** and **Figure 2** were created with BioRender.com.

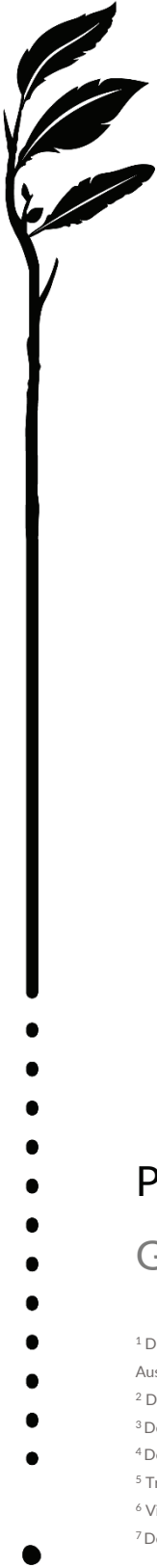
Supplementary material Chapter 2

Table S1. Table of Materials.

Name of Material/Equipment	Company	Catalog Number	Comments/Description
Alexa Fluor™ 647 C2 Maleimide	Invitrogen	A20347	pH-stable dye used to label 150 nm, 235 nm or 633 nm PMA _{SH} carriers; example of good dye to use in cell-carrier association studies
Apogee flow cytometer	Apogee		Sensitive flow cytometer capable of detecting small carriers for counting
CytoFLEX S Flow Cytometer	Beckman Coulter		Sensitive flow cytometer capable of detecting small carriers for counting as well as read out final cell-barrier experiments
FCS Express	De Novo Software		Software used to analyze flow cytometry data, <i>i.e.</i> , perform gating and derive median fluorescent intensity values of populations of choice. Alternatives include FlowJo, OMIQ, Python
Infinite 200 PRO	Tecan Lifesciences		Standard microplate reader instrument used for bulk fluorescence measurements of carriers in solution
LSRFortessa™ Cell Analyzer	BD Biosciences		Less sensitive flow cytometer, but one more generally available to researchers. Can be used to read out final cell-carrier experiment
NanoSight NS300	Malvern Panalytical		Instrument used for Nanoparticle Tracking Analysis
Prism 8	GraphPad		Software used to graph and calculate standard curves. Alternatives include Microsoft Excel, Origin, Minitab, Python amongst many others
Quantum™ MESF kits Alexa Fluor 647	Bangs Laboratories	647	Absolute quantitation beads for flow cytometry. Used to convert fluorescence intensities measured in bulk on a microplate reader to fluorescence intensities measured on a flow cytometer using the MESF standard

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Chapter 3

Targeting the HIV reservoir: utilizing receptor-mediated endocytosis to enhance nanoparticle delivery to T cells

(In preparation)

P.M. Ceva¹, C. Cortez-Jugo², A. Tan³, M. Faria⁴, C.
Gubser³, F. Caruso², M. Roche³, J. Symons^{3,5}, S.R. Lewin^{3,6,7}

¹ Department of Microbiology and Immunology, The University of Melbourne at The Peter Doherty Institute for Infection and Immunity, Melbourne, VIC, Australia

² Department of Chemical Engineering, The University of Melbourne, Melbourne, VIC, Australia

³ Department of Infectious Diseases, The University of Melbourne at The Peter Doherty Institute for Infection and Immunity, Melbourne, VIC, Australia

⁴ Department of Biomedical Engineering, The University of Melbourne, Melbourne, VIC, Australia

⁵ Translational Virology, Department of Medical Microbiology, University Medical Center, Utrecht, the Netherlands.

⁶ Victorian Infectious Diseases Service, Royal Melbourne Hospital at The Peter Doherty Institute for Infection and Immunity, Melbourne, VIC, Australia

⁷ Department of Infectious Diseases, Alfred Hospital and Monash University, Melbourne, VIC, Australia

Introduction

A critical challenge in the development of effective nanomedicine is delivering a sufficiently high dose of the therapeutic agent to the intended cell *in vivo*¹. Targeting approaches are generally split into two categories: 'active targeting', where the surface of the nanoparticle is decorated with targeting moieties such as antibodies, aptamers or peptides that specifically bind receptors on the target cell surface; and 'passive targeting', which relies on distinctive biological features of the target cell microenvironment that allow or induce local accumulation of nanoparticles. Designing such targeting properties is inherently tied to engineering stealth – the ability of nanoparticles to avoid interactions with off-target cells². In this regard, much effort has been put into enhancing the fundamental understanding of bio-nano interactions; specifically the role of the protein corona or biomolecular corona³. Nevertheless, a literature survey showed that for hundreds of nanomedicine tested, a median of only 0.7% of the administered nanoparticle dose reached the intended cells¹, indicating a gap in our understanding of the processes involved in nanoparticle targeting.

Successful intracellular delivery of a nanoparticle-based therapeutic agent requires the nanoparticle to reach the anatomical site of the target cell, bind to the target cell surface, be taken up by that cell and the therapeutic agent to be released into the desired cellular compartment. Most works studying various targeting strategies focus on the first two steps. For example, *in vivo* targeting studies usually report a single metric of percentage injected dose (% ID), sometimes combined with measurements of the percentage of target cells that have nanoparticles associated (% nanoparticle association). Concerns have been raised about the use of either as single metrics^{4,5} and indeed, the focus on these first two steps towards successful delivery overlooks the critical barriers that exist in certain hard-to-transfect cell types when it comes to nanoparticle uptake and subsequent endosomal escape⁶.

An excellent example of a hard-to-transfect cell type is T cells, which form a critical part of the adaptive immune response against viruses and cancers. In addition, T cells can be subject to disease themselves, in the form of haematological malignancies or T cell-tropic viral infections. Indeed, human immunodeficiency virus (HIV) primarily infects CD4⁺ T cells and integrates its genome into the host cell DNA, thereby establishing a life-long infection^{7,8}. An important barrier to finding a cure for HIV is the persistence of latently infected, resting CD4⁺ T cells harbouring replication-competent virus^{7,8}, which can rebound upon activation and re-establish viremia in the

absence of antiretroviral treatment. Attempts to eradicate this latent reservoir with systemic, small-molecule therapeutics have suffered from dose-limiting toxicities in off-target cells⁹. In this regard, it has been suggested that the use of targeted nanoparticle-based drug delivery could provide more potent and less toxic avenues towards an HIV cure^{6,10-13}.

A major barrier to successful nanoparticle delivery to T cells is their non-phagocytic nature and low rates of macropinocytosis in the absence of activation¹⁴. Clathrin-mediated endocytosis, in contrast, is used by resting cells to recycle cell-surface receptors¹⁵. While certain receptors are endocytosed constitutively, the internalization of others can be stimulated by binding specific ligands¹⁶. The degree of internalization, however, is expected to vary between receptors. As such, we hypothesized that active targeting of nanoparticles to surface receptors with particularly strong internalization kinetics could provide an avenue for enhancing uptake into T cells. We defined the ideal target receptor by three parameters; (i) receptor density on the target cell surface, (ii) specificity of the receptor expression to the target cell, and (iii) speed and degree of receptor internalization when bound by the targeting moiety.

Here, we established a library of target receptors on the T cell surface within the context of the latent HIV reservoir. We developed a flow cytometry-based, high-throughput assay that could universally and quantitatively assess the degree of receptor and nanoparticle internalization into target cells. Using these assays, we first studied the expression, specificity and internalization kinetics of potential target receptors in T cell lines as well as unstimulated primary CD4⁺ T cells and found vastly varying degrees of internalization. Combining our internalization assay with a phenotypic panel, we found that the activation state of T cells influenced the degree of receptor internalization. We subsequently validated our approach by demonstrating greater nanoparticle uptake, including substantial uptake in resting CD4⁺ T cells, when targeting the highly expressed and highly endocytosed receptors CD2 and CD7. Lastly, we showed that targeting these receptors enhanced the association of nanoparticles to T cells in the presence of off-target cells, thus enhancing the specificity of nanoparticle delivery. Taken together, these results demonstrate a promising method of optimizing targeting efficiency to hard-to-transfect cell types.

Methods

Antibodies

Primary antibodies targeting the various receptors of interest were all purchased as mouse-anti-

human Alexa Fluor 647-conjugated monoclonal antibodies. Anti-CCR4 (1G1, 557863), anti-CCR6 (11A9, 560466), anti-CCR7 (150503, 560816), anti-CD3 (UCHT1, 557706), anti-CD4 (RPA-T4, 557707), anti-PD-1 (AH12.1, 560838) and anti-transferrin receptor (OKT9, 566724) antibodies were purchased from BD Biosciences. Anti-CD25 (BC96, 302618), anti-CD5 (UCHT2, 300616), anti-CXCR3 (G025H7, 353712), anti-CXCR5 (J252D4, 356906) and anti-OX40 (OX40, 350018) antibodies were purchased from Biolegend. Anti-CCR5 (45529, FAB184R), anti-CD2 (299812, FAB18561R), anti-CD7 (848438, FAB7579R), anti-CXCR4 (44716, FAB172R) and isotype control (133303, IC0041R) antibodies were purchased from R&D Systems. Primary antibodies that were stored in a buffer containing sodium azide were buffer-exchanged into Phosphate Buffered Saline (PBS) using Zeba™ Spin Desalting Columns, 7K MWCO (89882, Thermo Fisher Scientific). The secondary, PE-conjugated goat-anti-mouse antibody (P-852) was purchased from Thermo Fisher Scientific.

Cell culture

The human Jurkat T lymphocyte cell line (clone JE6.1) was cultured in RPMI 1640 medium containing 10% heat-inactivated fetal calf serum (FCS), supplemented with 100 U/mL penicillin, 100 µg/mL streptomycin and 2 mM L-glutamine (RF10) in a humidified 37°C, 5% CO₂ incubator. For primary cell experiments, peripheral blood mononuclear cells (PBMC) were isolated from buffy coats obtained from the Australian Red Cross Blood Service *via* Ficoll-Paque (GE Healthcare, Champaign, IL) density centrifugation. CD4⁺ T cells were subsequently isolated through negative magnetic-activated cell sorting using a CD4⁺ T cell isolation kit (130-096-533, Miltenyi Biotec). PBMC and isolated total CD4⁺ T cells were frozen in FCS supplemented with 10% DMSO and stored in liquid nitrogen until use. Prior to usage, total CD4⁺ T cells were thawed and rested overnight in RF10 supplemented with 2 U/mL IL-2. Subsequent experimental incubations of total CD4⁺ T cells were performed in the continued presence of 2 U/mL IL-2.

Receptor internalization assay

To assess T cell surface receptor internalization kinetics, a dual antibody staining approach was used based on prior work¹⁷. Jurkat T cells or primary total CD4⁺ T cells were harvested, counted and washed once in cold PBS with 1% FCS and 1 µM EDTA (FACS wash buffer) prior to staining with titrated volumes of the respective primary antibodies for 30min, on ice, in the dark. Cells were kept on ice to inhibit any active endocytic processes during the binding of the primary antibodies. Next, excess primary antibody was washed off and cells were resuspended in cold culture media

prior to incubation at 37°C for 0, 15, 30, 60, 90 or 180min. After incubation, cells were placed back on ice and kept cold throughout subsequent handling to inhibit further receptor internalization. A secondary antibody stain with PE-conjugated goat-anti-mouse antibody was then performed for 30min, on ice, in the dark to dually stain any receptor remaining on the cell surface, while any internalized receptors remained singly stained. All cells were stained with LIVE/DEAD™ Fixable Aqua Dead Cell Stain (L34957, Thermo Fisher Scientific) to allow gating on live cells during subsequent analysis. In primary cell experiments, cells were subsequently incubated with unlabelled IgG from murine serum (I5381, Sigma) to block any free anti-mouse epitope binding sites of the goat-anti-mouse secondary antibody, followed by staining against the activation markers HLA-DR (564364, BD Biosciences) and CD69 (564364, BD Biosciences) with mouse-anti-human antibodies. Cells were then fixed in PBS with 1% paraformaldehyde (PFA) prior to flow cytometry analysis using an LSR Fortessa II (BD Biosciences) or microscopy analysis (Zeiss LSM780 Confocal).

Nanoparticle preparation

100 nm Absolute Mag™ Protein G Magnetic Particles (WHM-X035, Creative Diagnostics) (1 mg/mL, 1.3×10^{11} particles / mL) were functionalized with Alexa Fluor 647-labelled primary antibodies to generate fluorescently labelled, targeted nanoparticles (Ab@SPIONs). In brief, 10 µg of nanoparticles was mixed with 1 µg of primary antibody in 100 µL of 10mM phosphate buffer (PB), pH 7.4, supplemented with 0.05 mg/mL bovine serum albumin (BSA), vortexed briefly to mix, then incubated for 30min at room temperature. Excess unbound antibody was removed by immobilizing the nanoparticles using a magnet (EasyEights™ EasySep™ Magnet, Stemcell) and washing twice with 10 mM PB, pH 7.4, 0.5 mg/mL BSA, before resuspending in equal volumes of that same buffer to ensure equal nanoparticle concentration across all Ab@SPIONs. Antibody-functionalized nanoparticle solutions were then sonicated briefly using an ultrasonic bath. The hydrodynamic diameter and polydispersity index (PDI) of the resulting solutions, as measures of nanoparticle size and uniformity, respectively, were subsequently analysed using dynamic light scattering (DLS) using a Zetasizer (Nano-ZS, Malvern Analytical). Immediately prior to cell experiments, nanoparticle solutions were sonicated again to ensure a single-particle suspension.

Nanoparticle internalization assay

Jurkat T cells or primary total CD4⁺ T cells were harvested, counted and resuspended in RF10. Cells were then plated at 1.25×10^6 cells / mL in 200 µL in round-bottom 96-well culture plate,

after which respective targeted nanoparticles were added at a nanoparticle-to-cell ratio of 100 : 1. Cells were incubated for 24 or 48 hours at 37°C. To allow for simultaneous harvesting and analysis, the remaining timepoints were started “in reverse”; at 8, 4, 2 and 1 hours before harvesting in separate wells. For primary CD4⁺ T cells, all timepoints were run using this reverse timecourse set-up. After incubation, cells were immediately pelleted (400 rcf, 4min) in a pre-cooled centrifuge (4°C) and kept cold throughout subsequent handling to inhibit further nanoparticle internalization. Unbound nanoparticles were washed off through three washes with cold PBS, followed by cell staining with LIVE/DEAD™ Fixable Aqua Dead Cell Stain and a secondary PE-conjugated goat-anti-mouse antibody as per previous. Cells were fixed in PBS with 1% paraformaldehyde (PFA) prior to flow cytometry analysis using an LSR Fortessa II (BD Biosciences).

Nanoparticle association assays

The interaction of the various targeted nanoparticles with fresh whole blood cells and PBMC was studied as previously described¹⁸. Whole blood was collected from healthy volunteers after informed consent through microbleeds into NH sodium heparin collection tubes (454051, Greiner Bio-One). Fluorescently labelled, targeted nanoparticles were added at a ratio of 5×10^7 particles per 200 μ L of whole blood and incubated for 1 hour at 37°C. Cells were then transferred to ice and kept cold throughout subsequent handling to inhibit further nanoparticle association. Red blood cells were lysed through incubation with 1X Pharm Lyse™ solution (BD Biosciences) for 15min on ice, after which leukocytes were stained with LIVE/DEAD™ Fixable Blue Dead Cell Stain (L23105, Thermo Fisher Scientific) for 30min on ice, in the dark, followed by surface staining with anti-CD56-AF488 (B159, BD Biosciences), anti-CD19-PE (HIB19, BD Biosciences), anti-CD66b-PE/Dazzle594 (G10FS, Biolegend), anti-HLA-DR-Pacific Blue (L243, Pharmingen), anti-CD3-Brilliant Violet 510 (UCHT1, BD Biosciences) and anti-CD14-Brilliant Violet 785 (M5E2, Biolegend) for 30min on ice, in the dark. Cells were fixed in PBS with 1% paraformaldehyde (PFA) prior to flow cytometry analysis using an LSR Fortessa II (BD Biosciences). The following cell subsets were identified: granulocytes (CD66b⁺), T cells (CD66b⁻/CD3⁺), monocytes (CD66b⁻/CD14⁺), NK cells (CD66b⁻/CD3⁻/CD14⁻/CD56⁺), B cells (CD66b⁻/CD3⁻/CD14⁻/CD19⁺), dendritic cells (DC) ((CD66b⁻/CD3⁻/CD14⁻/CD56⁻/CD19⁻/HLA-DR⁺). PBMC were obtained as described above, thawed and plated at 4×10^5 cells per well in 100 μ L in flat-bottom 96-well culture plates. Subsequently, respective targeted nanoparticles were added at a nanoparticle to cell ratio of 100:1 and cells were incubated for 1 hour at 37°C. Cells were then phenotyped and analysed as described above.

Absolute quantitation of fluorescence

Conversion of median fluorescent intensity (MFI) values of Alexa Fluor 647 fluorescence into absolute number of nanoparticles was performed as published previously¹⁹ and described in Chapter 2. In brief, the absolute fluorescent intensity (in molecules of equivalent soluble fluorochrome (MESF)) per nanoparticle was determined by comparing the fluorescence of 100 μ L of each fluorescently labelled, targeted nanoparticle solution with known concentration of nanoparticles / mL to a standard curve of 100 μ L samples with known MESF Alexa Fluor 647 using a microplate reader (FLUOstar Omega, BMG Labtech). Using fluorescence quantitation beads (Quantum™ MESF 647 beads, Bangs laboratories), these absolute fluorescent intensities per nanoparticle were then converted into a theoretical MFI value per carrier; a measure of the contribution of one individual nanoparticle to the overall MFI of the cell sample as measured by flow cytometry. The number of associated nanoparticles per cell was then calculated using Equation 1:

$$P_{assoc} = \frac{(FI_{cell} - FI_{background})}{FI_{nanoparticle}} \quad (1)$$

Where P_{assoc} is the number of nanoparticles associated per cell, FI_{cell} is the MFI of a given sample of cells incubated with nanoparticles, $FI_{background}$ is the MFI of cells not incubated with nanoparticles (background fluorescence), and $FI_{nanoparticle}$ is the calculated MFI of carriers in suspension.

Conversions of MFI values of Alexa Fluor 647 fluorescence representing receptor expression levels were converted into an absolute number of antibodies bound per cell (antibody binding capacity, ABC) using a similar approach. Fluorescence quantitation beads were used as above to calculate the MESF per cell as a result of receptor staining with an Alexa Fluor 647-labelled antibody. Of each of these antibodies, the MESF per antibody (degree of labelling) was determined using fluorescence spectroscopy (Fluorolog 3, HORIBA) by comparing fluorescence values at the peak wavelength a standard curve of samples with known MESF Alexa Fluor 647. The degree of labelling was then calculated by dividing the molar concentration of dye (MESF/mL) to the molar concentration of antibody. The antibody binding capacity was finally calculated by dividing the MESF Alexa Fluor 647 per cell by the degree of labelling of the corresponding antibody.

Analysis

Flow cytometry analysis, compensation and cell subset gating was performed using FCS Express

7. Further analysis including conversion of relative fluorescence into absolute number of nanoparticles per cell and graphing was performed in Python, using the Pandas, Numpy, Matplotlib, Sklearn and Seaborn packages. Confocal images were processed using ImageJ.

To calculate the receptor or nanoparticle internalization scores, cells were gated on single cells, live cells, then cells expressing the receptor of interest (for calculation of receptor internalization) or positive for nanoparticle association (for calculation of nanoparticle internalization). Within FCS Express, a new parameter was then computed (“Ratio primary (1°) to secondary (2°) antibody”), which was defined as the fluorescent intensity of surface receptor expression or nanoparticle surface binding divided by the fluorescent intensity of total receptor expression or nanoparticle association. The mean of this new parameter was then extracted and normalized to the first timepoint analysed (baseline) to calculate the receptor or nanoparticle internalization scores using Equation 2:

$$Internalization\ score = 1 - \frac{Ratio\ 1^{\circ}\ to\ 2^{\circ}\ antibody}{Baseline\ ratio\ 1^{\circ}\ to\ 2^{\circ}\ antibody} * 100 \quad (2)$$

Results

Low expression precludes assessment of potential target receptors

The most obvious receptor targets that one could choose for nanoparticle delivery to the HIV reservoir are CD4, CXCR4 and CCR5 – the primary and co-receptors respectively that HIV itself exploits to bind and enter a target cell. Indeed, CD4 and CCR5 have both been investigated previously to enhance nanoparticle delivery to T cells^{20,21}. CCR5, however, demonstrated poor selectivity towards T cells and may therefore not pose an ideal receptor for *in vivo* T cell targeting²². While CD4 demonstrated excellent selectivity of expression on T cells, it has been described to exhibit negligible receptor internalization^{23,24}. As such, we first conducted a literature search to identify a library of potential target receptors (Table 1). Receptors were included if they exhibited one or more of the following characteristics: (i) high expression on T cells, (ii) selective expression on T cells and not phagocytic cells, (iii) prior indication of good receptor cycling kinetics, (iv) associated with enrichment of the latent HIV reservoir, (v) prior usage for targeting nanoparticles to T cells. The resulting library consisted of a range of pan-T cell markers and chemokine receptors, as receptor internalization has previously been described for G protein-coupled chemokine

Table 1. Overview of potential target receptors and their rationale for inclusion.

Category	Marker	Rationale	Reference
General T cell markers	CD2	Pan-T cell marker; shown to enrich for HIV in PLWH on ART	25,26
	CD3	Pan-T cell marker; known to undergo receptor-mediated endocytosis and used previously to target nanoparticles to T cells	27,28
	CD4	HIV entry receptor; used previously to target nanoparticles to T cells	20
	CD5	Pan-T cell marker; used previously to target nanoparticles to T cells	29
	CD7	Pan-T cell marker; used previously to target nanoparticles to T cells	30,31
Chemokine receptors	CXCR3	Shown to enrich for integrated HIV DNA in memory T cells of PLWH on ART	32
	CXCR4	HIV co-receptor	-
	CXCR5	Follicular T-helper cell (T _{fh}) marker; T _{fh} are enriched in HIV DNA	33
	CXCR6	Primarily expressed in various T cell subsets	-
	CCR4	Primarily expressed in various T cell subsets	-
	CCR5	HIV co-receptor; used previously to target nanoparticles to T cells	21
	CCR6	Shown to enrich for integrated HIV DNA in memory T cells of PLWH on ART	32,34
	CCR7	Central memory T cell (T _{cm}) marker; T _{cm} form a major reservoir of HIV in PLWH on ART	35
	CCR9	Primarily expressed in lymphocytes	-
Immune checkpoint markers	OX40	Member of TNF receptor super family; shown to enrich for HIV as part of a larger panel of markers	36
	CTLA-4	Immune checkpoint inhibitor; shown to enrich for SIV in ART-treated rhesus macaques and known to undergo receptor-mediated endocytosis	17,37
	PD-1	Marker of immune exhaustion; repeatedly shown to enrich for (translation-competent) HIV in PLWH both on and off ART	38-42
Cytokine receptor	CD25 / IL-2R	Activation marker; associated with HIV latency. Known to undergo receptor-mediated endocytosis upon stimulation, used previously to target nanoparticles to T cells	36,43,44

ART, antiretroviral therapy; CD, cluster of differentiation; CCR, C-C chemokine receptor; CTLA-4, cytotoxic T-lymphocyte-associated antigen 4; CXCR, C-X-C chemokine receptor; HIV, human immunodeficiency virus; IL-2R, interleukin-2 receptor; PD-1, Programmed cell death protein 1; PLWH, people living with HIV; SIV, simian immunodeficiency virus; TNF, tumour necrosis factor

receptors⁴⁵ and several chemokine receptors have been described to enrich for HIV³²⁻³⁵. Immune checkpoint and activation markers have also been shown to enrich for HIV^{17,36-44}, and were thus included.

For each receptor, we then determined the level of expression and antibody binding capacity as a measure of receptor density on total CD4⁺ T cells (Figure 1) and found that CTLA-4, CCR9 and CXCR6 exhibited particularly low levels of expression (3%, 3% and 9% of CD4⁺ T cells expressing, respectively) and low receptor density (antibody binding capacity per cell (ABC) < 300) in those cells that did express the receptor in question. As such, these receptors were deemed unsuitable targets and therefore excluded from further analysis.

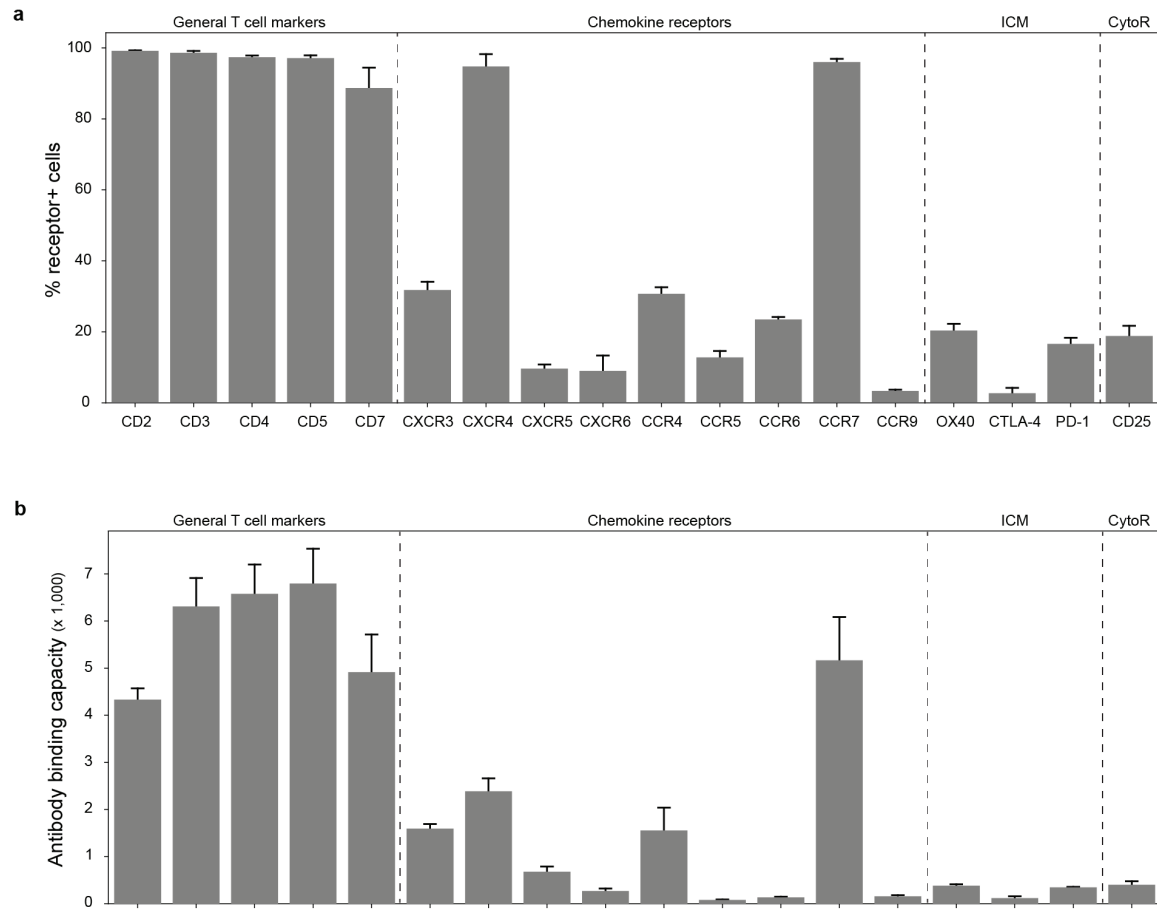


Figure 1. Expression patterns on primary CD4⁺ T cells of surface receptors included in screening library. (a) Breadth of receptor expression measured as the percentage of live CD4⁺ T cells expressing the receptor of interest. (b) Receptor density on cells positive for receptor expression, represented by the absolute number of anti-receptor antibodies bound per cell (antibody binding capacity). Values shown as mean $\pm$ SEM of $n=4$ donors. ICM, immune checkpoint markers; CytoR, cytokine receptors.

Receptor internalization can be measured using a dual staining approach on flow cytometry

To assess the ability of the receptors in our library to undergo receptor-mediated internalization upon binding with a targeting antibody, we designed a high-throughput, flow cytometry-based internalization assay based on previous work by Qureshi *et al*¹⁷. Initial assay development was performed using Jurkat T cells, an acute T cell leukemia-derived cell line, using CD71 (transferrin receptor, TfR – known to cycle extensively^{46,47}) and CD4 (known to exhibit minimal endocytosis^{23,24}) as positive and negative controls for receptor internalization, respectively. We found that receptor internalization could be tracked by staining a surface receptor of interest with a pH-stable, fluorescently labelled primary (1°) antibody (Figure 2a, red) on ice, which is endocytosed along with the receptor during the subsequent incubation period at 37°C. Any receptor-antibody complexes present on the cell surface after incubation could be co-stained with

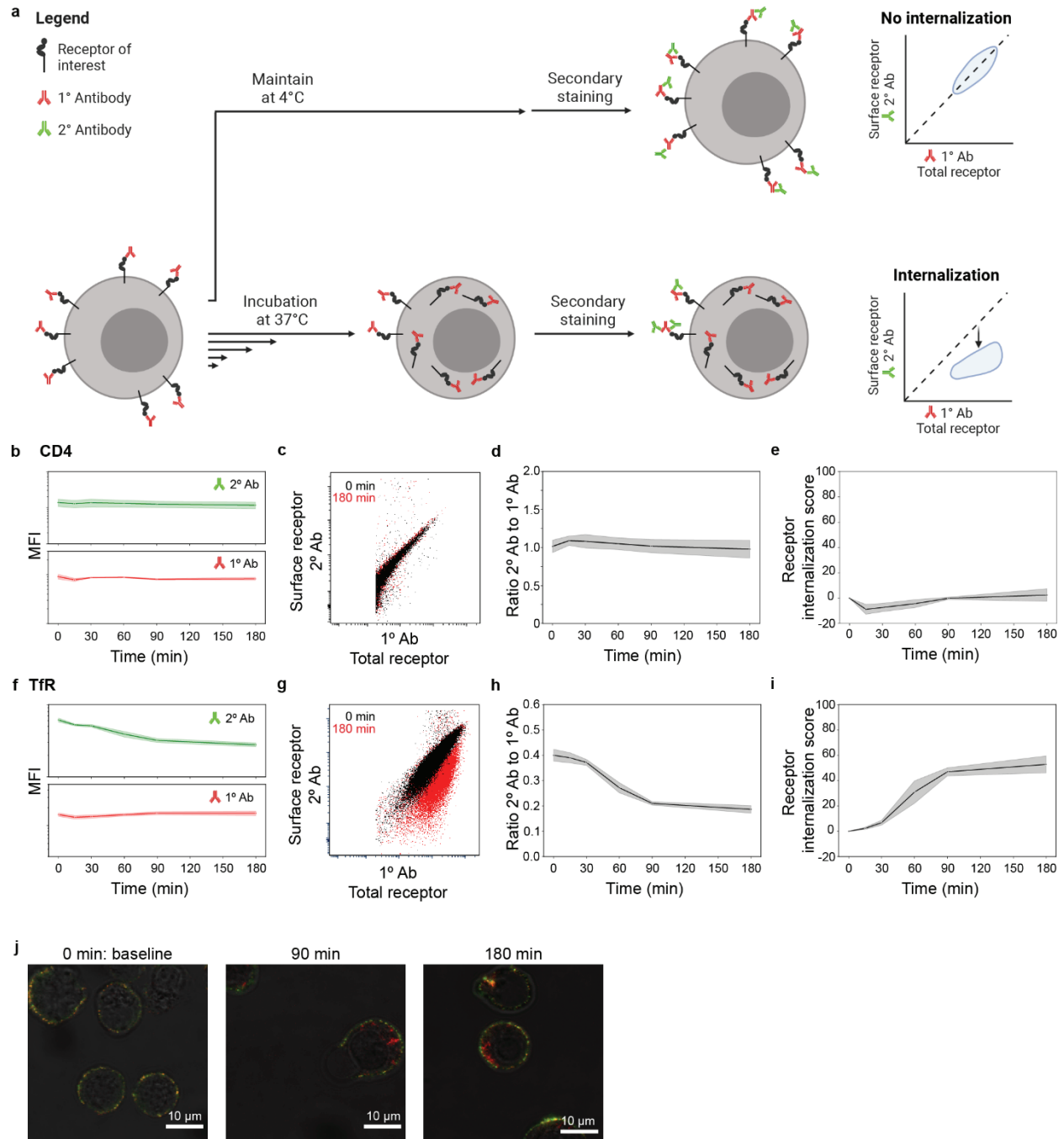


Figure 2. Receptor internalization kinetics can be assessed using a dual staining approach. (a) Schematic overview of the receptor internalization assay. The receptor of interest is stained with a fluorescent primary antibody (red), then kept on ice or incubated at 37°C to allow receptor internalization. Receptors present on the cell surface are then dually labelled with a secondary antibody, upon which receptor internalization can be detected by a downward shift in the fluorescent correlation between the primary (x-axis) and secondary (y-axis) fluorescent signals on flow cytometry. (b-i) Representative results of the receptor internalization assay for a non-internalizing (CD4, b-e) and strongly internalizing (transferrin receptor, TfR, f-i) receptor as negative and positive controls, respectively. (b, f) Raw fluorescent signals of primary and secondary antibody staining over time. The secondary antibody signal decreases over time in the presence of receptor internalization, while the primary antibody signal remains constant irrespective of receptor internalization kinetics. (c, g) Overlay of representative dot plots of the primary and secondary antibody signals at baseline (0 min, black) and 180 min (red), showing a downward shift over time in the presence of receptor internalization. (d, h) Ratio of secondary antibody signal to primary antibody signal over time as a newly computed parameter per cell, quantifying any receptor internalization. (f, i) Receptor internalization score over time, calculated by normalizing the values in (d) or (h) to the corresponding baseline (0 min timepoint). $n=3$, values shown as

mean $\pm$ SEM. (j) Representative confocal microscopy images of dual receptor staining at various timepoints. At baseline (0 min), no internalization of the primary antibody-receptor complexes (red) is observed, and all receptors are dually stained with the secondary antibody (green). At later timepoints, primary antibody-receptor complexes can be observed within the cytoplasm, while the secondary antibody selectively co-stains receptors present on the cell surface.

a fluorescently labelled secondary (2°) antibody (Figure 2a, green). In the absence of receptor internalization, a linear relationship between the primary and secondary antibody was observed, as all receptors labelled with the primary antibody remained available for labelling with the secondary antibody. Receptor internalization, in contrast, resulted in a reduced secondary antibody signal and as such, a reduced ratio of the secondary antibody signal to the primary antibody signal (Figure 2a). Indeed, we observed these phenotypes for CD4 and TfR (Figure 2b-d and Figure 2f-h, respectively) and quantified these results by calculating a receptor internalization score of 2.5 ± 8.5 (CD4) and 52.8 ± 6.8 (TfR) (Figure 2e,i). Using confocal microscopy, we confirmed that the primary antibody signal could be internalized, whereas the secondary signal selectively co-stained receptor-antibody complexes that were present on the cell surface after incubation (Figure 2j).

Degree and kinetics of receptor internalization vary substantially in Jurkat T cells

We then measured the receptor internalization kinetics in Jurkat T cells of the subset of receptors that were expressed in this cell line (Figure S1). CD2, CD3, CD7 and CXCR4 were expressed on >80% of Jurkat T cells and CXCR4, in particular, exhibited a high receptor density (ABC >12,400), making these promising candidates for targeting. The pan-T cell markers CD2, CD3, CD5 and CD7 were found to display significantly stronger internalization after 3 hours than CD4 ($p=0.0003$, $p=0.0086$, $p=0.0002$, $p=0.001$, respectively), while the chemokine receptor CXCR4 only showed a modest increase in internalization (internalization score (IS) of 15.2 ± 12.4) compared to CD4 (IS 2.5 ± 8.5) that did not reach statistical significance ($p=0.59$) (Figure 3a). We moreover found that the kinetics of receptor internalization differ, with CD5 exhibiting more rapid internalization (IS of 31.6 ± 3.8 in 15 min) (Figure 3b), further underlining the importance of studying receptor internalization kinetics.

CD2 and CD7 are highly expressed and internalized in resting and activated primary CD4⁺ T cells

We hypothesized that the immortal nature of Jurkat T cells could influence cell biological processes such as endocytosis and therefore next compared findings in Jurkat T cells and total CD4⁺ T cells. Indeed, CD5 receptor internalization was not observed at all while CD3 receptor internalization was substantially reduced in total CD4⁺ T cells (IS of 24.1 ± 3.3) compared to Jurkat

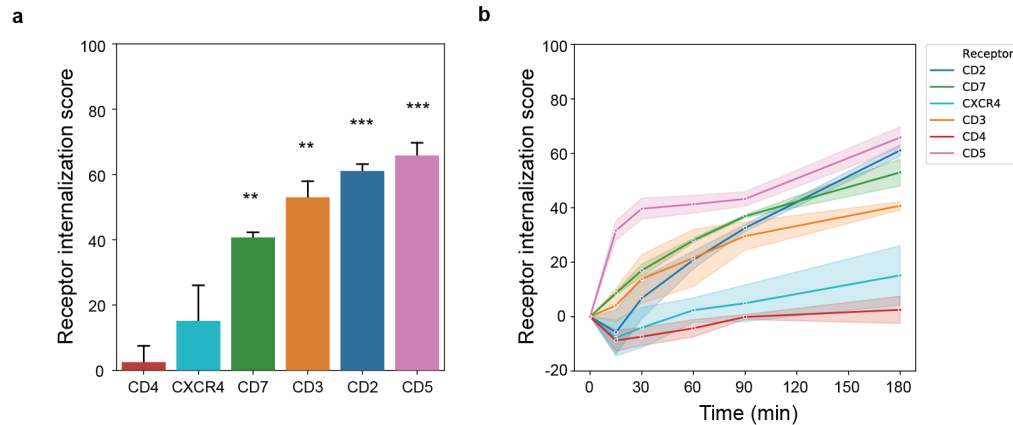


Figure 3. Receptor internalization kinetics in Jurkat T cells vary significantly. (a-b) Internalization score of a selection of receptors expressed on Jurkat T cells after 180 min (a) and kinetics over time (b). $n=3$, values shown as mean $\pm$ SEM. SEM is shown as either error bars (a) or shading (b). Significance was determined through one-way ANOVA and Dunnett's multiple comparisons test using CD4 as reference. ns $p > 0.05$, * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$.

T cells (IS of 40.3 ± 1.6) (Figure 4a). In contrast, CD2 and CD7 internalization was maintained in both cell models, with an IS of 56.6 ± 4.3 and 61.8 ± 4.0 , respectively, in CD4⁺ T cells after 180 min. CCR5 was identified as a receptor with potent receptor internalization kinetics with an IS of 71.3 ± 7.6 (Figure 4a). Of these, CD7 and CCR5 internalized more rapidly than CD2 (Figure 4b). Interestingly, programmed cell death protein 1 (PD-1), a marker repeatedly shown to enrich for latent HIV in individuals both on and off antiretroviral treatment³⁸⁻⁴², also showed potent internalization with an IS of 46.3 ± 7.2 after 180 min. CD2 and CD7, however, exhibited the highest expression and receptor density (Figure 1) of the receptors that were found to internalize most potently.

To confirm that the observed receptor internalization also occurred in resting CD4⁺ T cells, we included phenotypic staining of the activation markers CD69 and HLA-DR in the receptor internalization assay. On average, 94% of total CD4⁺ T cells were found to be resting (CD69-/HLA-DR-) across all timepoints (data not shown). While activated cells exhibited stronger receptor internalization than resting cells for both CD2 and CD7, substantial internalization was still observed in resting CD4⁺ T cells, as can be seen by a stark reduction in the ratio of 2° antibody signal over 1° antibody signal compared to baseline (0 min) (Figure S2).

Receptor targeting enhances nanoparticle association and internalization in Jurkat T cells

We then aimed to examine whether the receptors identified as strongly internalizing could be exploited to enhance internalization of targeted nanoparticles. To this end, we used 100 nm superparamagnetic iron oxide nanoparticles (SPIONs) coated in protein G for facile surface decoration with fluorescently labelled targeting antibodies (Figure S3). We adapted the receptor

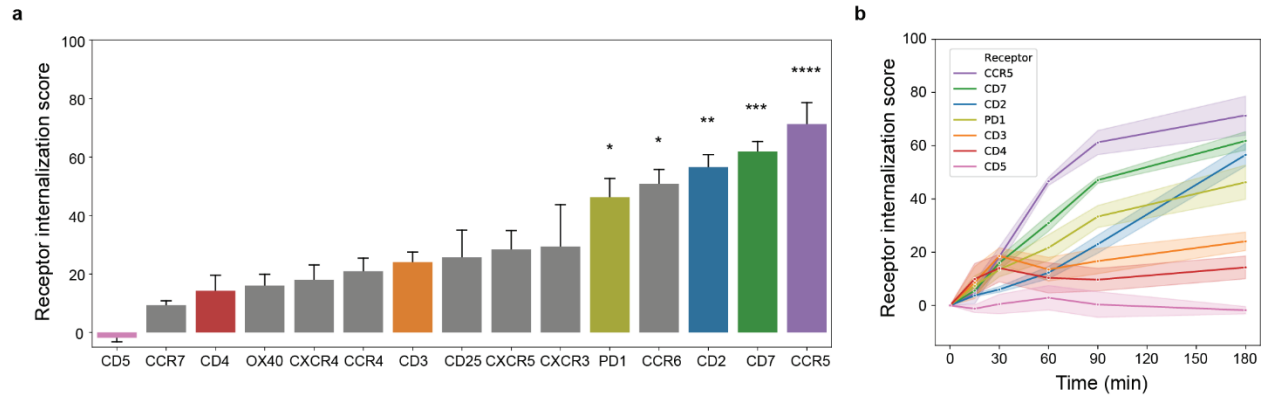


Figure 4. CCR5, CD7 and CD2 exhibit potent receptor internalization in CD4⁺ T cells. (a-b) Internalization score of the selection of receptors expressed sufficiently on CD4⁺ T cells after 180 min (a) and kinetics over time (b). n=4, values shown as mean $\pm$ SEM as either error bars (a) or shading (b). Significance was determined through one-way ANOVA and Dunnett's multiple comparisons test using CD4 as reference. ns $p > 0.05$ (not shown), * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$

internalization assay to allow assessment of targeted nanoparticle association as well as internalization over time (Figure 5). In brief, cells were incubated with SPIONs coated with the same fluorescently labelled antibody clones as used previously (Ab@SPIONs) for between 1 and 48 hours, to allow nanoparticle binding and internalization to cells. As the Ab@SPIONs remained present in the culture, nanoparticle association or dissociation continued over time. We first determined the percentage of cells exhibiting nanoparticle association at each timepoint (Figure 5a, 1). This provided a measure of the breadth of the association that can be achieved in the target cell population. Within this subset of cells exhibiting nanoparticle association, we then assessed the kinetics of the absolute number of nanoparticles these cells had associated with (Figure 5a, 2). Simultaneously, nanoparticles that were present at the cell surface were co-stained with a secondary antibody, allowing for quantitation of nanoparticle internalization (Figure 5a, 3) analogous to the receptor internalization assay described above. We expressed changes in internalization relative to the first baseline timepoint.

We found that in Jurkat T cells, nanoparticle targeting to any of the receptors tested resulted in enhanced nanoparticle association after 48 hours compared to untargeted nanoparticles, although we observed strong differences in the percentage association between the various Ab@SPIONs (Figure 5b). As the fluorescence intensity – and thus, the percentage association – of cell-nanoparticle complexes is a function of the number of nanoparticles associated and the individual fluorescent intensity of the various Ab@SPIONs tested, we proceeded to quantify the absolute number of nanoparticles associated per cell for those cells found positive for nanoparticle association (Figure 5c and Table S1). Again, we observed that targeting any of the receptors tested,

resulted in a higher number of nanoparticles per cell compared to untargeted nanoparticles. The most efficient delivery of nanoparticles was achieved upon targeting CD3 (mean $\pm$ SEM 119 $\pm$ 8.1 nanoparticles per cell), CD5 (67 $\pm$ 1) and CD2 (52.3 $\pm$ 6.7) (Figure 5c), despite both CD7 and CXCR4 exhibiting receptor expression in a larger proportion of Jurkat T cells as well as at a higher receptor density (Figure S1). These findings potentially reflect the higher degree of internalization of CD3-, CD5- and CD2@SPIONs (Figure 5d) compared to their CD7 and CXCR4 counterparts resulting in particle accumulation within the intracellular compartments as well as on the cell surface.

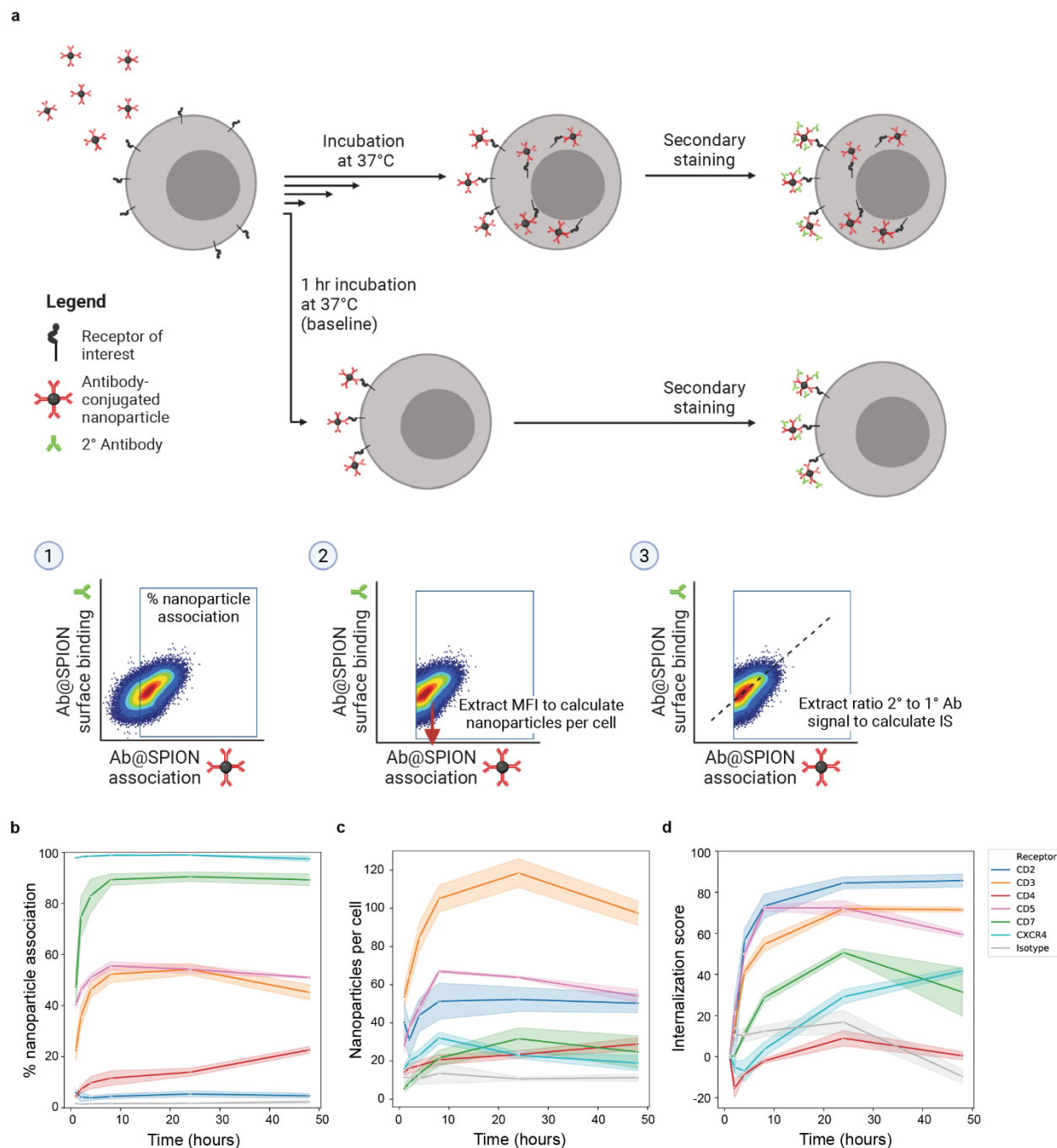


Figure 5. (Previous page) Dual staining of targeted nanoparticles allows for simultaneous assessment of association and internalization. (a) Schematic overview of the nanoparticle internalization assay. Nanoparticles conjugated with fluorescently labelled, primary antibodies are added to target cells and incubated for various durations, after which nanoparticles on the cell surface are co-stained using a secondary antibody. After harvest, the percentage nanoparticle association is first determined (1). Within cells found positive for nanoparticle association, the median fluorescent intensity (MFI) of the Ab@SPION signal was then extracted and converted into a measure of the average absolute number of nanoparticles associated per cell (2). Simultaneously, the ratio of secondary (2°) to primary (1°) antibody signal, representing surface-bound nanoparticles and total nanoparticles, respectively, was extracted to calculate a nanoparticle internalization score (3). The first timepoint harvested was used as baseline for nanoparticle internalization. (b-d) Jurkat T cells were incubated with each of six targeting Ab@SPIONs or untargeted (isotype) control particles and nanoparticle association and internalization were assessed over time. (b) Percentage of live cells demonstrate association of either Ab@SPION at each timepoint. (c) Absolute number of each Ab@SPIONs associated per cell of cells found positive for nanoparticle association in (b). (d) Internalization score of each Ab@SPIONs over time in cells found positive for nanoparticle association in (a), calculated by normalizing the ratio of secondary to primary antibody signal to the first timepoint assessed (1 hour). n=3, values shown as mean ± SEM.

Targeting CD2 induced the highest as well as most rapid internalization of SPIONs into Jurkat T cells (maximal IS of 85.7 with an IS of 56.6 reached within 4 hours) while CD4 targeting resulted in a negligible IS of 0.42 after 48 hours (Figure 5d). These findings are consistent with the internalization kinetics for these two receptors (Figure 2b), suggesting that targeting strongly internalizing receptors could indeed serve as a strategy towards enhancing the uptake of nanoparticles.

CD2 and CD7, but not CCR5 targeting enhances nanoparticle delivery to CD4⁺ T cells

To examine whether nanoparticle uptake could be triggered similarly in total CD4⁺ T cells, we generated Ab@SPIONs targeting CD2, CD7 and CCR5 – the receptors that exhibited the most potent receptor internalization (Figure 4). CD3@SPIONs were included as a control, as CD3 internalization remains one of the most well-described examples of receptor-mediated endocytosis in primary T cells²⁸. Surprisingly, CCR5 targeting only resulted in nanoparticle association in, at best, 2.3% of CD4⁺ T cells (1 hour), compared to 1.5% for untargeted control particles (Figure 6a), and a modest increase from 2.0±0.86 to 13.8±4.7 nanoparticles per cell (Figure 6b). This low breadth of T cell targeting using CCR5 is reflective of the low breadth of CCR5 expression in total CD4⁺ T cells (Figure 1a), and the minimal increase in nanoparticles associated per cell may reflect the low receptor expression in CCR5⁺/CD4⁺ T cells (Figure 1b). Furthermore, in those cells that associated with CCR5@SPIONs, minimal to no internalization was observed (Figure 6c). The discrepancy between CCR5 receptor internalization and CCR5@SPION internalization could mean that in our studies, CCR5 was internalized into endocytic vesicles that are too small to engulf a 100-120 nm nanoparticle.

Targeting CD2 and CD7, in contrast, resulted in a broad nanoparticle association in 98% and 68% of CD4⁺ T cells, respectively, after one hour, which increased further to 99% and 91%, respectively, at 48 hours (Figure 6a). The behaviour of CD2@SPIONs differed strongly from that observed in

Jurkat T cells, where CD2@SPION association at any timepoint did not exceed 5.9% (Figure 6a). These findings potentially reflect the higher CD2 receptor density on primary CD4⁺ compared to Jurkat T cells (Figure 1b and Figure S1b), suggesting the expression level on Jurkat T cells may be insufficient to recruit CD2@SPIONs. While targeting CD2 resulted in a higher dose of nanoparticles per cell (99.6 ± 14.4) compared to CD7 (21.1 ± 7.2) (Figure 6b), both receptors triggered high internalization of those nanoparticles associated with IS of 78.4 ± 2.8 and 66.0 ± 5.1 for CD2 and CD7, respectively, both exceeding internalization achieved upon CD3 targeting (Figure 6c). Nanoparticle internalization of untargeted control nanoparticles could not reliably be determined due to the low percentage of CD4⁺ T cells that exhibited nanoparticle association (Figure 6a) and was as such excluded from further analysis. Overall, these findings corresponded closely to the internalization behaviour observed in our receptor internalization assay, suggesting that CD2 and CD7 are promising candidates for T cell targeting.

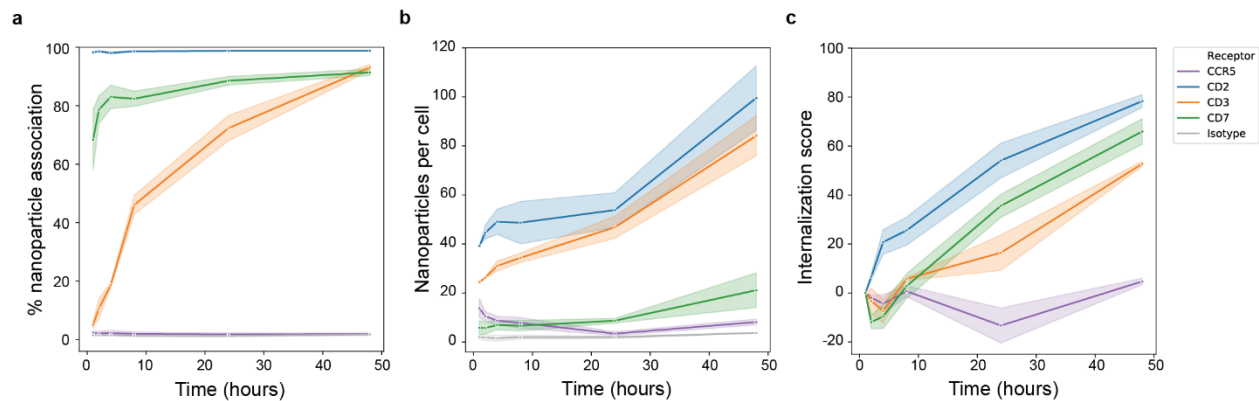


Figure 6. Targeting of CD2 and CD7 induces nanoparticle uptake in total CD4⁺ T cells. Primary CD4⁺ T cells were incubated with nanoparticles targeting CCR5, CD2, CD3 or CD7, or untargeted (isotype) control particles and nanoparticle association and internalization were assessed over time. (a) Percentage of live cells demonstrate association of either Ab@SPION at each timepoint. (b) Absolute number of each Ab@SPIONs associated per cell of cells found positive for nanoparticle association in (a). (c) Internalization score of each Ab@SPIONs over time in cells found positive for nanoparticle association in (a), calculated by normalizing the ratio of secondary to primary antibody signal to the first timepoint assessed (1 hour). An internalization score for isotype@SPIONs could not reliably be determined due to the negligible percentage of cells found positive for nanoparticle association, and was therefore excluded from analysis. n=4, values shown as mean $\pm$ SEM.

CD2 and CD7 targeting enhances specific association of nanoparticles with T cells in the presence of bystander cells

Targeting a receptor that enhances nanoparticle internalization in T cells will benefit T cell targeting *in vivo* specifically when the chosen receptor exhibits a degree of specificity to the T cell compartment. As expected, we found that untargeted control particles exhibited strong association with phagocytic cells such as monocytes, B cells and DCs (79%, 44% and 23%, respectively, compared to 3% association with T cells) (Figure 7a), despite T cells comprising the

largest proportion of PBMCs (Figure S5a). Targeting CD3 enhanced the proportion of T cells that associated with nanoparticles to 16% as well as increased the achieved dose from <1 to 10 ± 1.7 nanoparticles per cell (Figure 7b), although neither reached statistical significance (Figure 7f). Nonetheless, CD3 targeting significantly reduced the percentage of monocytes ($p < 0.0001$) and B cells ($p = 0.0006$) that exhibited nanoparticle association compared to untargeted control particles.

We next determined the expression of CCR5, CD2 and CD7 in PBMC and subsequently assessed whether targeting these receptors could enhance nanoparticle association to T cells during a 1-hour co-culture with PBMC. As observed previously, CCR5 was only expressed on a small subset

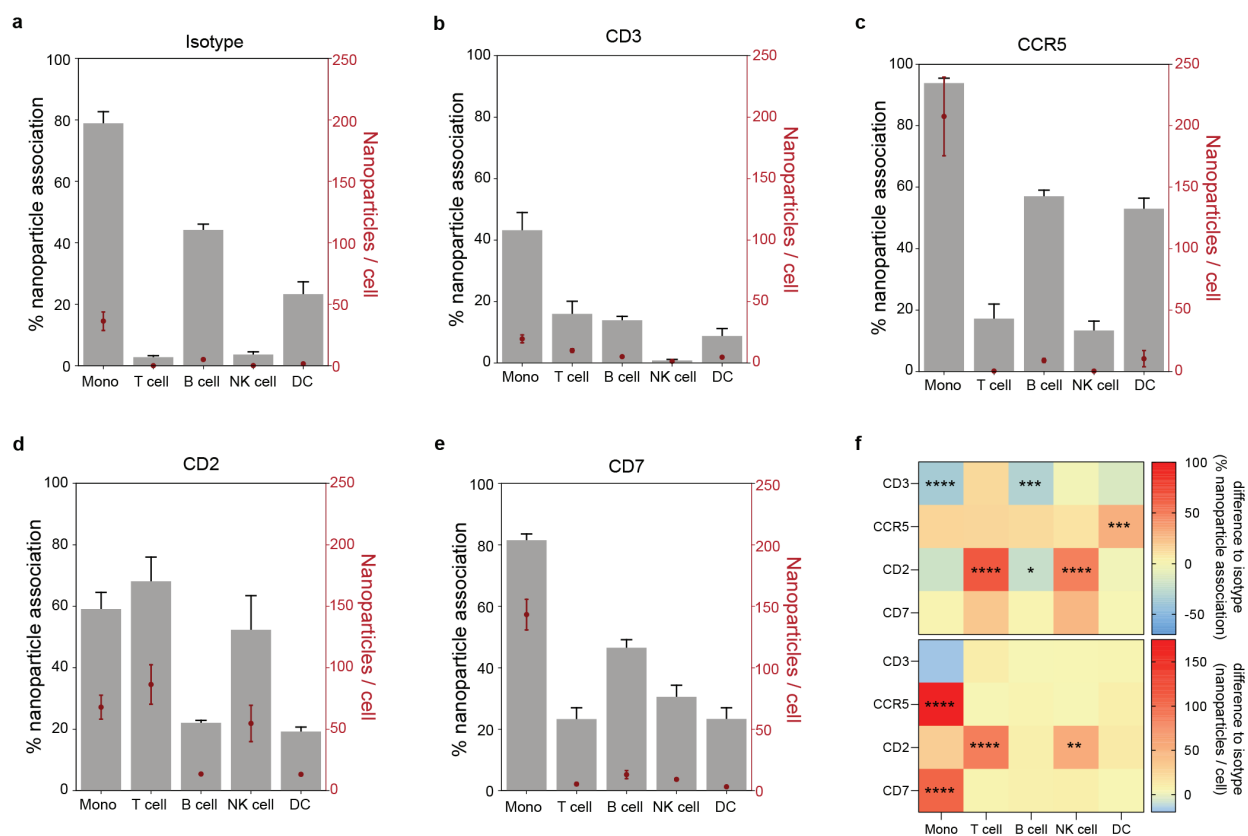


Figure 7. Receptor targeting can enhance nanoparticle association to T cells in the presence of off-target cells. (a-e) PBMC were incubated with untargeted control nanoparticles (isotype) (a) or nanoparticles targeted to CD3 (b), CCR5 (c), CD2 (d) or CD7 (e) and nanoparticle-cell interactions with various PBMC subsets were assessed after 1 hour. For each nanoparticle, association was measured as the percentage of live cells that demonstrate association of the Ab@SPION (bars, grey), as well as the absolute number of each Ab@SPIONs associated per cell of cells found positive for nanoparticle association (symbols, red). $n=4$, values shown as mean $\pm$ SEM. (f) Summary of data in a-e, shown as a heat map with differences of means of each Ab@SPION compared to untargeted control particles for each PBMC subset. Top, difference in percentage nanoparticle association; bottom, difference in absolute number of Ab@SPIONs associated per cell. Significance was determined through one-way ANOVA and Bonferroni's multiple comparisons test using untargeted control particles as reference. ns $p > 0.05$ (not shown), * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$. Mono, monocyte; NK, natural killer; DC, dendritic cell.

(8%) of T cells at a low receptor density (antibody binding capacity of 535 per cell) (Figure S4a,d). Conversely, CCR5 was expressed more broadly on phagocytes (monocytes (94%), B cells (43%) and DCs (49%)) (Figure S4a) at higher receptor densities (antibody binding capacities of > 5,000 per cell) than in T cells (Figure S4d). CCR5@SPIONs association was found to correlate closely to receptor expression, with monocytes, B cells and DCs displaying higher proportions of nanoparticle association as well as substantially higher numbers of nanoparticles per cell than T cells (Figure 7c). Compared to untargeted control nanoparticles, CCR5@SPIONs exhibited significantly increased association with DC ($p = 0.0008$) and increased number of nanoparticles associated with monocytes ($p < 0.0001$). CCR5 targeting did not lead to significant increases in the percentage of T cells associated with nanoparticles nor the absolute number of nanoparticles associated (Figure 7f).

In contrast, the pan-T cell markers CD2 and CD7 were expressed more selectively on T cells with negligible expression in phagocytes, although NK cells were found to express both receptors at similar levels and receptor densities to T cells (Figure S4b,c,e,f). Due to their non-phagocytic nature and lower prevalence in PBMC (Figure S5a), we hypothesized NK cell scavenging of nanoparticles to be less of a hurdle towards *in vivo* T cell targeting. Indeed, targeting either CD2 or CD7 resulted in increased nanoparticle association with T cells of 68% ($p < 0.0001$) and 24% ($p = 0.06$), respectively, compared to isotype control particles (Figure 7d,e). Interestingly, these results exceeded the targeting observed using CD3@SPIONs (Figure 7b), which we hypothesized would achieve the greatest targeting specificity towards T cells due to the exclusive expression of CD3 in the T cell compartment.

The absolute number of nanoparticles per cell in T cells was greatest upon CD2 targeting at 86 ± 17 nanoparticles/cell, which exceeded the dose of CD2@SPIONs found to associate with any other cell type (Figure 7d) and was significantly higher than the number of nanoparticles per T cell in the absence of targeting ($p < 0.0001$) (Figure 7f). CD7 targeting did not significantly increase the number of nanoparticles per T cell, yet surprisingly did lead to significantly greater nanoparticles per monocyte ($p < 0.0001$) (Figure 7f). As expected, targeting either CD2 or CD7 led to increases in the percentage of NK cells exhibiting nanoparticle association as well as the number of nanoparticles per NK cell compared to untargeted control particles, yet these increases only reached statistical significance for CD2 targeting ($p < 0.0001$ and $p = 0.0034$, respectively) (Figure 7f). Nonetheless, the targeting effect of CD2@SPIONs was greater towards T cells than towards

NK cells (Figure 7d, f). In summary, these findings indicate the potent targeting capability of CD2- and, to a lesser extent, CD7@SPIONs to T cells in the presence of bystander cells.

T cell targeting capabilities are largely maintained in whole human blood *ex vivo*

When introduced into biological fluids, proteins and other biomacromolecules adsorb onto the nanoparticle surface. This biomolecular corona changes the physicochemical properties of the nanoparticle, which affects both targeting and stealth characteristics. As such, we re-assessed the T cell targeting capabilities of the various Ab@SPIONs in the presence of blood serum by incubating these nanoparticles with whole blood and measuring association with various leukocyte subsets including granulocytes (Figure S5b), as described previously⁴⁸. The distribution of nanoparticle association in the absence of targeting remained largely unchanged, with T cell association being practically absent and phagocytes accounting for the majority of nanoparticle association observed (Figure 8a). Consistent with previous findings, incubating whole blood with CD3@SPIONs resulted in a specific association with T cells (Figure 8b) ($p = 0.0129$) that coincided with a significant reduction in B cell association ($p < 0.0001$) compared to untargeted control (Figure 8f). For CCR5, in contrast, we found that the marginal targeting observed in PBMC was largely abrogated in the presence of blood serum, with only 3% of T cells associating with CCR5@SPIONs and with minimal nanoparticles per cell (Figure 8c).

CD2 and CD7 targeting, on the other hand, were broadly similar to that observed in PBMC. Similar to CD2@SPION targeting in the absence of blood serum, we found that CD2 targeting in whole blood led to association with each leukocyte subtype assessed. However, association with T cells appeared most potent with 98% of T cells associating with an average of 151 ± 31 nanoparticles per cell (Figure 8d), thus yielding significantly higher association breadth ($p < 0.0001$) as well as strength ($p < 0.0001$) with T cells compared to untargeted control particles (Figure 8f). CD2 targeting furthermore significantly reduced nanoparticle association to B cells ($p = 0.0038$). While CD2 targeting significantly increased the percentage of association to NK cells ($p < 0.0001$) as well as the number of nanoparticles per NK cell ($p < 0.0001$), these effects were less pronounced than the targeting effect towards T cells (Figure 8d, f). The T cell targeting capacity of CD7@SPIONs in whole blood showed a similar trend to CD2@SPIONs, significantly increasing the percentage of T cells associated with nanoparticles ($p = 0.0136$). Yet, despite CD2 and CD7 exhibiting similar receptor expression patterns in T cells, CD7@SPION association with T cells reached only 37% (Figure 8e,f). CD7 targeting appeared to more specifically increase NK cell association compared

to untargeted control ($p < 0.0001$). These findings suggest that targeting CD7 and, in particular, CD2 leads to pronounced association, and thus potentially internalization, with T cells in biologically relevant conditions.

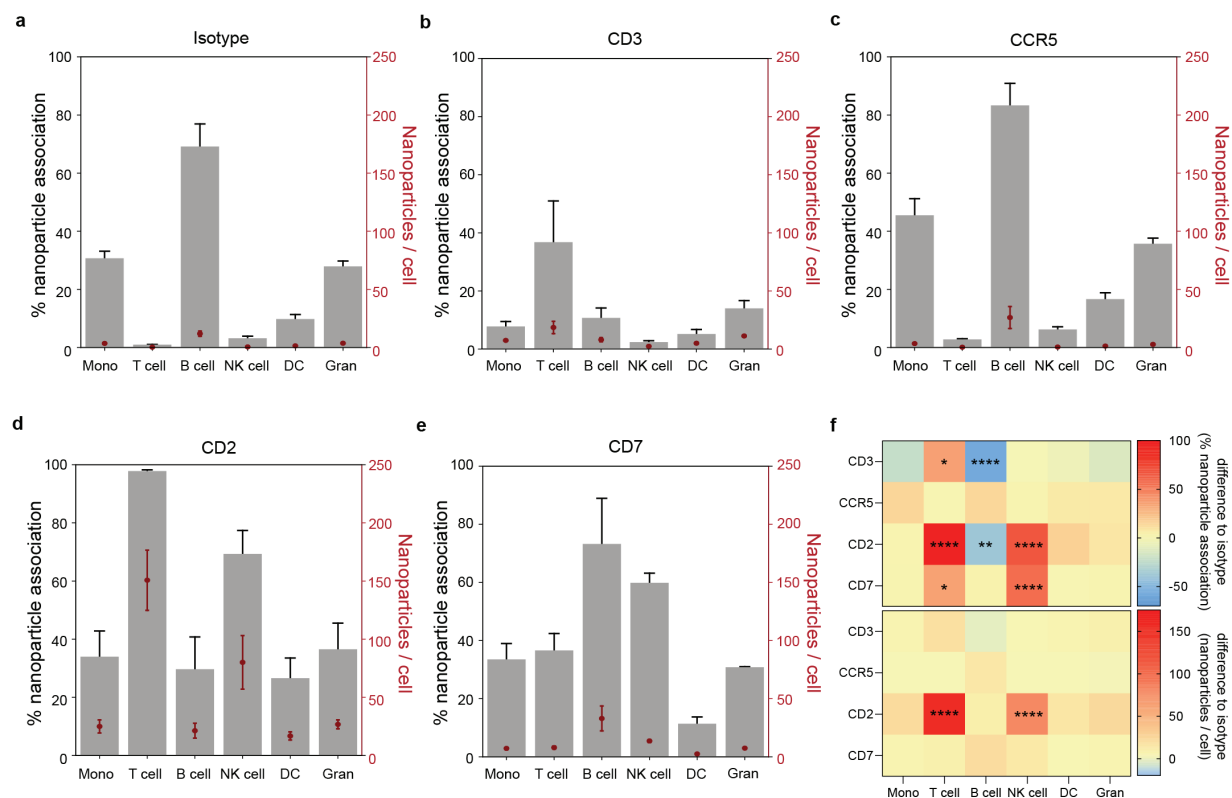


Figure 8. CD2 and CD7-mediated T cell targeting is maintained in whole blood. (a-e) Untargeted control nanoparticles (isotype) (a) or nanoparticles targeted to CD3 (b), CCR5 (c), CD2 (d) or CD7 (e) were incubated with whole blood and nanoparticle-cell interactions with various leukocyte subsets were assessed after 1 hour. For each nanoparticle, association was measured as the percentage of live cells that demonstrate association of the Ab@SPION (bars, grey), as well as the absolute number of each Ab@SPIONs associated per cell of cells found positive for nanoparticle association (symbols, red). $n=4$, values shown as mean $\pm$ SEM. (f) Heat map showing a summary of data in a-e, presented as differences of means of each Ab@SPION compared to untargeted control particles for each WBC subset. Top, difference in percentage nanoparticle association; bottom, difference in absolute number of Ab@SPIONs associated per cell. Significance was determined through one-way ANOVA and Bonferroni's multiple comparisons test using untargeted control particles as reference. ns $p > 0.05$ (not shown), * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$. Mono, monocyte; NK, natural killer; DC, dendritic cell; Gran, granulocyte.

Table 2. Summary T cell targeting potential of key receptors based on all parameters assessed. The performance of the key receptors in this study – CD2, CD3, CD7, and CCR5 – across the various assays performed was scored to identify the most promising candidate for successful T cell targeting. For each parameter, scoring indicates absence (-) or presence (+) of beneficial effect of the respective receptor towards T cell targeting, with (++) or (+++) indicating stronger effects than (+).

Parameter assessed	CD2	CD3	CD7	CCR5
Receptor expression (CD4 ⁺ T cells)	+++	+++	+++	+
Receptor density (CD4 ⁺ T cells)	+++	+++	+++	+
Receptor specificity to target cell	++	+++	++	-
Receptor internalization in target cell	+++	+	+++	+++
Targeted nanoparticle association in monoculture	+++	++	++	+
Targeted nanoparticle internalization in monoculture	+++	+++	+++	-
Specificity of nanoparticle association	+++	+	+	-
Specificity of nanoparticle association (in serum)	+++	+	+	-

Overall, using a library of potential receptor targets, we demonstrated that CD2 and CD7 are the most promising candidates to enhance nanoparticle association to and internalization by T cells *in vitro*, based on the range of parameters (Table 2).

Discussion

Successful targeting of nanomedicine to the intended cell *in vivo* requires nanoparticle association as well as internalization, however, the latter remains vastly understudied. We here describe evidence that active targeting towards receptors that undergo extensive receptor cycling can be exploited to enhance nanoparticle internalization in primary CD4⁺ T cells *in vitro*, a cell type generally considered as hard to transfect⁴⁹.

In this work, we employed a novel assay that simultaneously quantified nanoparticle association as well as the degree of internalization. Several historic and recent ‘nanoparticle internalization assays’ have been described, which are typically based on quenching of membrane-bound nanoparticles⁵⁰⁻⁵⁶. These approaches, however, suffer from a lack of quenching specificity that precludes multiplexing⁵⁰⁻⁵² or use specific conjugation strategies that are not compatible with all nanoparticle types⁵³⁻⁵⁶. Furthermore, none of these described assays allow for concurrent assessment of nanoparticle association. The nanoparticle internalization assay developed here is universal, in that the methodology can be applied to any type of fluorescent nanoparticle provided that a secondary antibody exists to the targeting moiety used or any other part of the nanoparticle surface. For example, anti-poly(ethylene) glycol (PEG) antibodies could be used to label any membrane-bound PEGylated nanoparticle even in the absence of active targeting. We furthermore integrated this assay with recently developed methods to absolutely quantify nanoparticle association¹⁹ (presented in Chapter 2), thereby contributing to improved metrics and better reporting in the field of nanomedicine that will accelerate translation and progress towards clinical applications^{5,57}.

Specifically targeting therapeutics to the HIV reservoir towards a potential HIV cure or remission is complicated by the lack of a clear biomarker for latently infected cells^{36,42}. Our findings demonstrate that the most intuitive targets, CD4, CXCR4 and CCR5 as the HIV entry receptors in fact result in poor nanoparticle association and/or internalization in CD4⁺ T cells and as such, are less potent candidates for *in vivo* targeting. Interestingly, CD4-targeted lipid nanoparticles have previously been shown to enhance mRNA delivery to CD4⁺ T cells *in vitro* and *in vivo* compared to

untargeted control particles²⁰. Yet, despite the presence of targeting, less than 1% of particles added to the T cell monoculture were found to associate²⁰, indicating a clear need for improved targeting approaches. The same authors used CD5-targeted particles in a follow-up study and again showed increased T cell targeting compared to untargeted control particles, but did not provide data to support their choice of target receptor⁵⁸. In our studies, we were unable to detect any internalization of the CD5 receptor on CD4⁺ T cells, suggesting other receptor targets may lead to improved nanoparticle delivery to T cells *in vivo*. We confirm earlier findings of CD3 exhibiting receptor internalization²⁸ and as such forming a potential candidate for nanoparticle targeting⁵⁹, yet aimed to find alternative receptor targets that are not associated with systemic T cell activation⁵⁹.

We present evidence that CD2 and CD7 are the most potent enablers of enhanced nanoparticle delivery to CD4⁺ T cells. CD2 and CD7 exhibited broad receptor expression within CD4⁺ T cells, high receptor density and strong receptor internalization. Targeting of either receptor led to strongly increased nanoparticle association and internalization in T cell monocultures, and targeting CD2, in particular, resulted in specific targeting of T cells in the presence of bystander cells. Three important caveats need to be made in relation to our findings. First, the calculation of the nanoparticle internalization score relied on normalization of nanoparticle internalization to the earliest timepoint assessed. In the absence of targeting, nanoparticle internalization was expected to be minimal and slow; as such, in the current study, a 1-hour timepoint was chosen as baseline. Our findings, however, that targeting strongly internalizing receptors can potently and rapidly trigger nanoparticle internalization suggested that in these instances, internalization could already be occurring within the first hour. Therefore, our method of analysis could lead to underestimation of the internalization score of targeted nanoparticles with very rapid internalization. Control conditions in which cells are kept on ice throughout the incubation, are fixed prior to nanoparticle addition, or are cultured in the presence of inhibitors of endocytosis⁶⁰ could provide better baseline measurements. Second, our assay was unable to discriminate nanoparticle recycling – *i.e.*, internalization followed by exocytosis without subsequent dissociation from the cell – from retention on the cell membrane. The reduction observed in the number of nanoparticles associated per cell for certain Ab@SPIONs suggests that exocytosis with subsequent dissociation does indeed occur. The importance of this distinction is questionable though, as such rapidly recycled nanoparticles are unlikely to contribute to effective delivery of a therapeutic dose of the nanoparticle cargo. Third, while we aimed to assess internalization kinetics of various receptors,

the measured kinetics will, to an extent, be tied to the targeting moiety employed in this study. For most commercially available antibodies, the exact binding epitope on the target receptor has not been described. Therefore, it is unknown whether these antibodies ligate the target receptor and trigger intracellular signalling processes. Extrapolating our results beyond the constraints of the antibody-receptor pair used should thus be done with caution, and further studies that compare different monoclonal antibody clones are needed to prove if the receptor targeting assessed in this study is consistent across different target epitopes. Nonetheless, the poor and strong internalization kinetics of the CD4 and CD7 receptors respectively that we found in the current study correspond closely to prior literature^{23,24,31}, strongly supporting the validity of our approach.

Both CD2 and CD7 are transmembrane glycoproteins of the immunoglobulin supergene family that have been shown to function as co-stimulatory molecules, as well as regulate cellular adhesion⁶¹⁻⁶³. Through cofilin-mediated modulation and hijacking of the actin cytoskeleton – required for viral nuclear migration⁶⁴ – CD2 stimulation was shown to potently inhibit latent infection of resting CD4⁺ T cells⁶⁵. CD2 has also been proposed as a target for the elimination of latently infected CD4⁺ T cells²⁶, as CD2^{high}/CD4⁺ resting memory T cells harboured higher HIV DNA copy numbers than other CD4⁺ T cell subsets⁶⁶. Both CD2 and CD7 are expressed exclusively on T and NK cells. Indeed, we observe an enhanced specificity of association with these subsets in the presence of bystander cells. However, it is important to note that in our studies, neither CD2 nor CD7 targeting resulted in an abrogation or even reduction of association with phagocytes – monocytes, B cells and DC. As avoiding off-target cells is as critical to successful *in vivo* targeting as reaching the target cells, these findings highlight the need of including specific measurements to understand uptake in the context of multiple cell types in *in vitro* assays, rather than solely focussing the target cell population. We speculate that the observed association of CD2/CD7@SPIONs with non-expressing cells could be explained by non-specific interactions or the engagement of Fc receptors, although the protein G-based conjugation method employed in this study should minimize Fc region exposure. Follow-up research is needed to assess whether using single-chain variable fragments (scFv), aptamers or natural ligands to the receptor of interest could enhance the targeting specificity further.

The current study does not provide insight into the nanoparticle internalization kinetics of the various blood cell subsets. However, it can be hypothesized that the route and, therefore, kinetics

of nanoparticle internalization will differ between cells expressing and non-expressing the targeted surface receptor. Residual nanoparticle association with off-target cells is thus, if not receptor-mediated, likely linked to a lower level of nanoparticle internalization and subsequent off-target drug delivery. In other words, nanoparticle internalization can be thought of as a competitive kinetic process between target and off-target cells, in which active receptor targeting can be used to tip this balance towards enhanced uptake in target cells. The importance of finetuning this balance will need to be addressed for each potential therapeutic cargo, as it strongly correlates to the specificity of the cargo itself towards the target cell. Furthermore, internalization *via* receptor-mediated endocytosis is restricted by the size of the clathrin-coated vesicles that encapsulate any endocytosed cargo. Much debate exists around the exact cut-off and thus maximum diameter of nanoparticles able to be taken up through receptor-mediated endocytosis, with reported diameters of clathrin-coated vesicles ranging from 50-200 nm^{15,67-69}. Nevertheless, we expect that the rapid nanoparticle internalization kinetics observed in the current study do not extend to particles of larger diameters.

While targeting may provide avenues towards the enhanced uptake of nanomedicine into T cells, it does not address the subsequent barrier of endosomal escape. Such nanoescapology is generally addressed from a materials science perspective; the ability of a nanoparticle to induce endosomal escape is attributed to characteristics of the engineered nanomaterial⁷⁰. This approach, however, disregards any cell-specific parameters involved⁷¹ and indeed, it has been suggested that T cells may present additional barriers to endosomal escape in the form of slower endosomal acidification, enhanced autophagy, and the presence of immune-sensing proteins⁷² – all warranting further investigation.

In conclusion, to our knowledge, this is the first study identifying CD2 as a potent receptor target towards nanoparticle delivery to T cells. Our findings demonstrate that understanding the internalization kinetics of potential target receptors can aid greatly in the rational design of nanomedicine aimed to deliver therapeutics to harder to transfect cell types, including CD4⁺ T cells that comprise the HIV reservoir. Our nanoparticle internalization assay will be a valuable tool in such undertakings and will contribute to the accelerated development of novel nanomedicine.

Supplementary material Chapter 3

Table S1. Fluorescent intensities of Ab@SPIONs.

Nanoparticle	Experiment			
	Jurkat T cells	CD4 ⁺ T cells	PBMC/WBC	
TfR@SPION	305	-	-	(MESF/nanoparticle)
CD2@SPION	41.8	41.7	32.1	(MESF/nanoparticle)
CD3@SPION	38.7	48.1	48.1	(MESF/nanoparticle)
CD4@SPION	122	-	-	(MESF/nanoparticle)
CD5@SPION	158	-	-	(MESF/nanoparticle)
CD7@SPION	105	233	97.6	(MESF/nanoparticle)
CXCR4@SPION	339	-	-	(MESF/nanoparticle)
CCR5@SPION	-	373	243	(MESF/nanoparticle)
Isotype@SPION	190	358	175	(MESF/nanoparticle)

CD, cluster of differentiation; CCR, C-C chemokine receptor; CTLA-4, cytotoxic T-lymphocyte-associated antigen 4; CXCR, C-X-C chemokine receptor; MESF, molecules of equivalent soluble fluorochrome; PBMC, peripheral blood mononuclear cells; SPION, superparamagnetic iron oxide nanoparticles; TfR, transferrin receptor; WBC, whole blood cells.

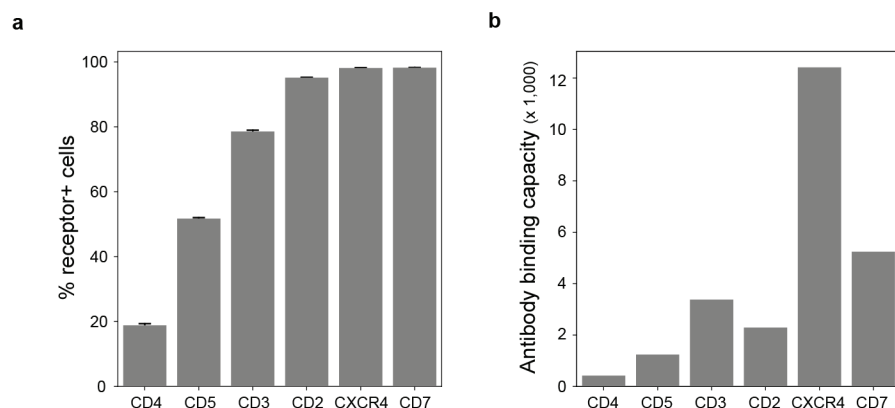


Figure S1. Expression patterns on Jurkat T cells of a subset of the receptor library. (a) Breadth of receptor expression measured as the percentage of live Jurkat T cells expressing the receptor of interest. Values shown as mean $\pm$ SEM of $n=3$. (b) Receptor density on cells positive for receptor expression, represented by the absolute number of anti-receptor antibodies bound per cell (antibody binding capacity). For receptors not shown in this graph, no expression was detected.

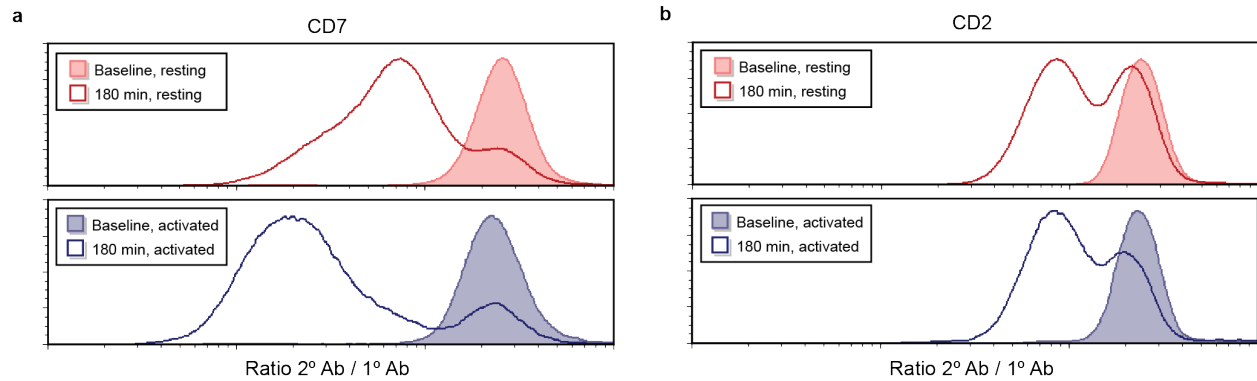


Figure S2. Receptor internalization occurs in both resting and activated CD4⁺ T cells. (a-b) Activated CD4⁺ T cells were identified by flow cytometry through by staining for CD69 and HLA-DR after the dual staining procedure to detect receptor internalization. Representative histograms showing the distribution of the ratio of secondary (2°) to primary (1°) antibody (Ab) signals per cell, as a raw measure of CD7 (a) or CD2 (b) internalization, for resting (top, red) and activated (bottom, blue) cells. Overlays depict the baseline ratio (filled curve) *versus* the ratio observed at the final timepoint of 180 min. Note that a lower ratio of secondary to primary antibody compared to baseline indicates receptor internalization has occurred.

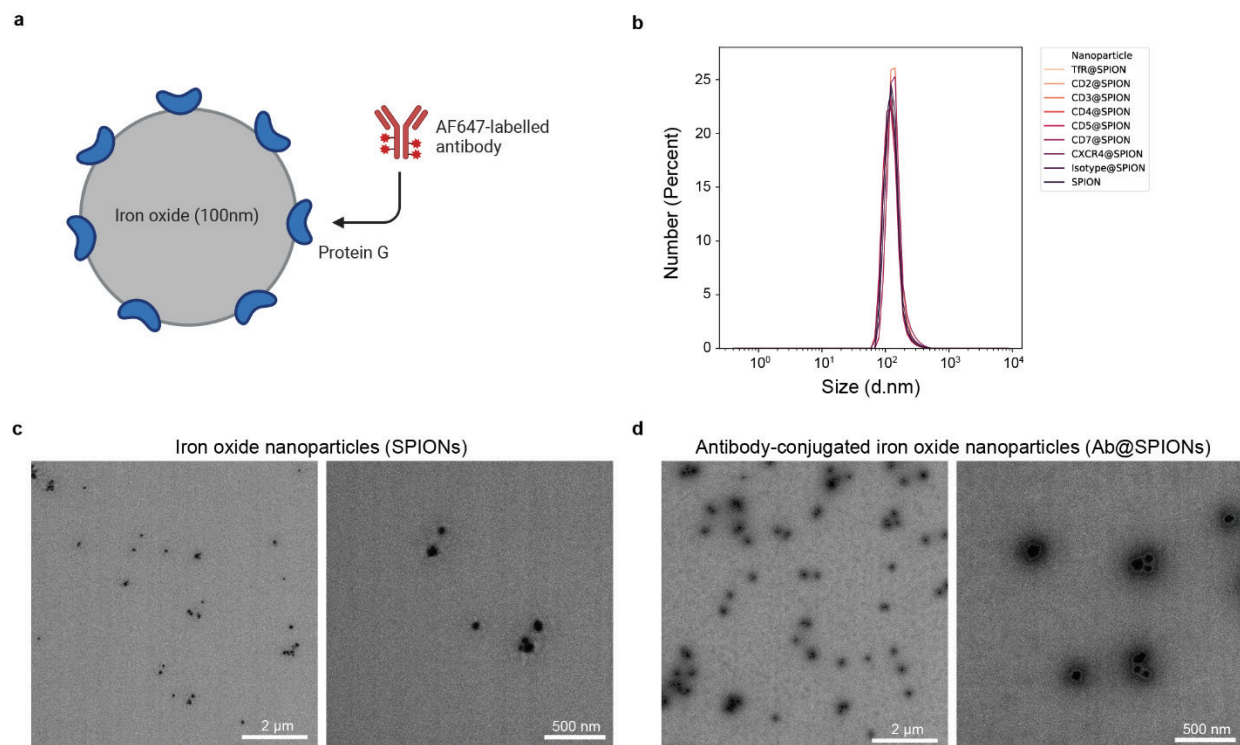


Figure S3. Generation and characterization of Ab@SPIONs. (a) 100 nm superparamagnetic iron oxide particles (SPIONs) coated in protein G were decorated with Alexa Fluor 647-labelled targeting antibodies through interaction between protein G and the Fc region of the antibodies. Excess unbound antibody was washed away and Ab@SPIONs were sonicated prior to use. (b) Size measurement of resulting Ab@SPIONs and unconjugated SPIONs as determined through dynamic light scattering. (c-d) Visualization of antibody conjugation to SPIONs through cryo-EM; the lighter area surrounding the SPIONs in (d) but not (c) indicates the addition of a protein layer.

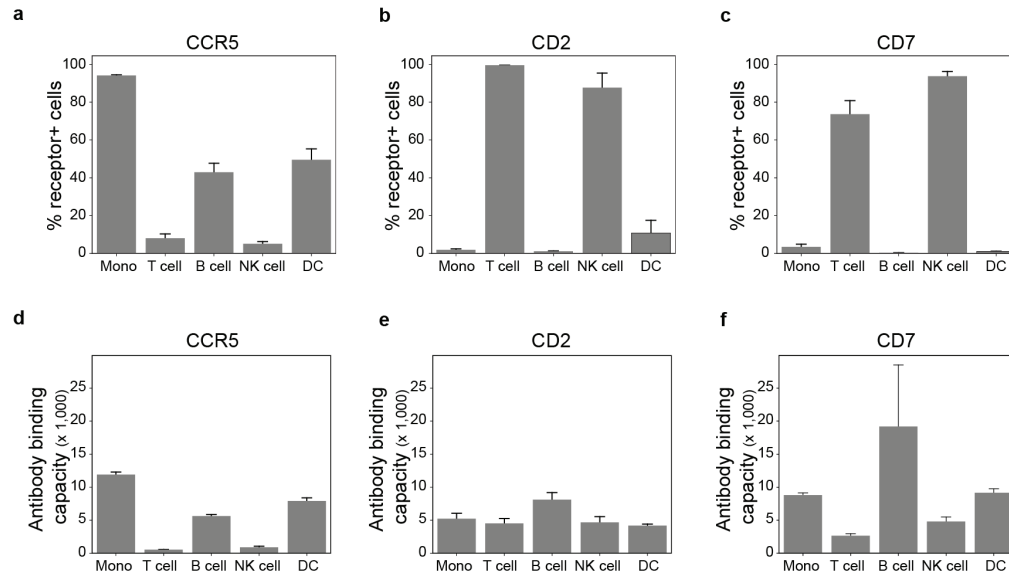


Figure S4. Potential target receptors express various levels of specificity towards T cells. Expression of CCR5, CD2 and CD7 was determined in the various PBMC subsets studied in association assays. (a-c) Breadth of receptor expression measured as the percentage of live cells expressing CCR5 (a), CD2 (b) or CD7 (c). (d-f) Receptor density on cells positive for receptor expression, represented by the absolute number of anti-CCR5 (d), anti-CD2 (e) or anti-CD7 (f) antibodies bound per cell (antibody binding capacity). Values shown as mean $\pm$ SEM of n=4 donors. Mono, monocyte; NK, natural killer; DC, dendritic cell.

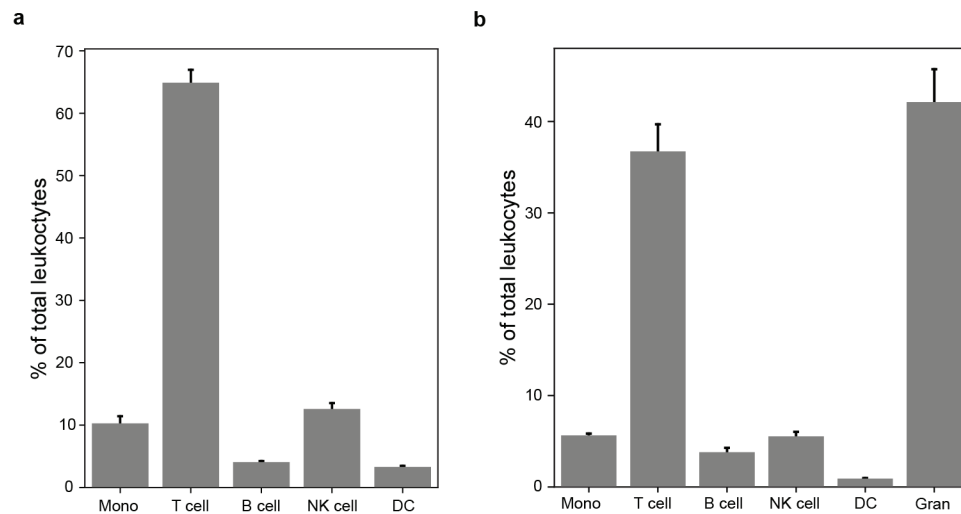


Figure S5. Distribution of cell subsets in PBMC and whole blood. In all nanoparticle association studies, the proportion of monocytes, T cells, B cells, NK cells and dendritic cells of total leukocytes was determined. Cell subsets were identified as follows within single, live cells: granulocytes (CD66b⁺), T cells (CD66b⁺/CD3⁺), monocytes (CD66b⁺/CD14⁺), NK cells (CD66b⁺/CD3⁺/CD14⁺/CD56⁺), B cells (CD66b⁺/CD3⁺/CD14⁺/CD19⁺), dendritic cells (DC) ((CD66b⁺/CD3⁺/CD14⁺/CD56⁺/CD19⁺/HLA-DR⁺). Values shown as mean $\pm$ SEM of n=4 donors. Mono, monocyte; NK, natural killer; DC, dendritic cell.

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Chapter 4

Towards HIV-specific latency reversal: lipid nanoparticle-based delivery of HIV-reactivating RNAs to eradicate the latent reservoir

P.M. Ceva¹, S. Kan², A. Tan², M. Holz¹, D.L. Furtado³, D. Purcell¹, A. Johnston⁴, C. Pouton⁴, T. Payne⁴, W. Zhao², F. Caruso³, J. Symons^{2,5}, M. Roche², S.R. Lewin^{2,6,7}

¹ Department of Microbiology and Immunology, The University of Melbourne at The Peter Doherty Institute for Infection and Immunity, Melbourne, VIC, Australia

² Department of Infectious Diseases, The University of Melbourne at The Peter Doherty Institute for Infection and Immunity, Melbourne, VIC, Australia

³ Department of Chemical Engineering, The University of Melbourne, Melbourne, VIC, Australia

⁴ Monash Institute of Pharmaceutical Sciences, Monash University, Parkville, VIC, Australia

⁵ Translational Virology, Department of Medical Microbiology, University Medical Center, Utrecht, the Netherlands.

⁶ Victorian Infectious Diseases Service, Royal Melbourne Hospital at The Peter Doherty Institute for Infection and Immunity, Melbourne, VIC, Australia

⁷ Department of Infectious Diseases, Alfred Hospital and Monash University, Melbourne, VIC, Australia

Introduction

The HIV pandemic has proven to be one of the major challenges of global health. A key barrier to finding a cure for HIV is the persistence of latently infected CD4⁺ T cells harbouring replication-competent virus^{1,2}. One approach towards an HIV cure is the 'shock and kill' approach, which aims to reactivate HIV transcription using latency-reversing agents (LRAs), with the goal of subsequently inducing death through viral cytopathic effects or immune-mediated clearance of the infected cell^{3,4}. Traditionally, the 'shock' is attempted through small, hydrophobic compounds, that target different cellular pathways to activate transcription (reviewed in^{5,6}). These first-generation LRAs can be administered systemically and will passively cross the plasma membranes to exert their effect intracellularly. Several LRAs have demonstrated potent induction of unspliced HIV RNA *in vitro*, *ex vivo* and in clinical trials, yet to date, no clinical trial of an LRA alone has shown a reduction in the size of the latent HIV reservoir⁷⁻¹⁴.

There are multiple reasons why LRAs alone are unable to induce clearance of infected cells. First, the use of unspliced HIV RNA as a biomarker for efficient latency reversal only measures transcriptional initiation¹⁵. Given that multiple blocks exist to viral RNA elongation, splicing and protein expression, these steps must all be overcome to efficiently reverse HIV latency³. Second, persistent immune exhaustion in PLWH on ART limits clearance of virus-producing cells¹⁶⁻¹⁸. Lastly, first-generation LRAs activate transcription in a non-HIV-specific manner, meaning their potency to reactivate HIV is unavoidably coupled to activation of host gene transcription. Indeed, certain LRAs have been shown to directly inhibit CD8⁺ T cell and natural killer (NK) cell function *in vitro*, further hampering immune-mediated clearance of reactivated cells^{19,20}. In conclusion, new LRAs or combinations of LRAs are needed that have greater potency, lower toxicity, and greater specificity for the HIV provirus.

Clustered regularly interspaced short palindromic repeats (CRISPR) is traditionally associated with the recognition of specific target sequences of DNA and gene editing by Cas9^{21,22}. However, CRISPR can have other applications including sequence-specific activation of transcription without inducing DNA cleavage of the target site (CRISPR activation or CRISPRa)²³. CRISPRa uses a catalytically inactive Cas9 (deadCas9 or dCas9) combined with transcriptional activators to enhance transcription of a certain gene, rather than induce a knock-out. This provides a promising new avenue towards latency elimination by targeting this system to the HIV LTR, allowing for greater specificity and potency leading to reduced toxicity, compared to first-generation LRAs

currently in clinical trials^{3,24}.

Traditionally, CRISPRa systems employ fusion proteins of dCas9 and activation domains, such as the multimer of C-terminal herpes virus transcriptional activation domain 16 (VP64), the histone acetyltransferase p300, or hybrid fusions of VP64, p65 and Rta (VPR). Further engineering led to the development of dCas9-SunTag, in which multiple copies of VP64 are recruited to the target sequence; or the Synergistic Activation Mediator (SAM) system, in which the gRNA is modified to contain minimal hairpin aptamers that allow the recruitment of p65 and heat shock factor 1 (HSF1) trans-activators through binding of the bacteriophage protein MS2. This recruitment of multiple copies of the MS2-p65-HSF1 fusion protein that act synergistically with dCas9-VP64 has been shown to greatly enhance the potency of CRISPRa²³.

Multiple CRISPRa systems have been shown to potently activate HIV transcription in latently infected cell lines²⁵⁻³⁰. The dCas9-SAM, dCas9-SunTag and dCas9-VP64 systems can induce robust, HIV-specific latency reversal in various cell line models of HIV latency^{25,27,28}, although dCas9-SAM was shown to have significantly higher potency than dCas9-VP64²⁸. A later study demonstrated that dCas9-SAM exhibited superior potency both to dCas9-SunTag and first-generation LRAs²⁶. Compared to first-generation LRAs, latency reversal was found to be more consistent across different cell line models of HIV latency and induced less off-target gene dysregulation^{29,31}. Notably, dCas9-SAM-mediated latency reversal resulted in viral protein accumulation and subsequent viral cytopathic effect^{25,26}, suggesting CRISPRa-mediated latency reversal could bypass the hurdle of immune exhaustion that persists in PLWH on ART.

Thus far, CRISPRa has only been assessed as an HIV-specific LRA using plasmids encoding the various CRISPRa components²⁵⁻³⁰. Plasmid transfection was performed either through nucleofection^{27,29,30}, lentiviral transduction²⁵, or using *in vitro* transfection reagents^{26,27} - none of which are viable *in vivo* delivery strategies. Adeno-associated viruses (AAVs) have shown promise as an *in vivo* delivery vector for CRISPR but are limited by packaging constraints, immunogenicity and reports of hepatic oncogenicity in animal models³². Thus, the main hurdle to exploring the full potency of HIV-specific CRISPRa in *ex vivo* and *in vivo* models of HIV latency is the lack of an efficient delivery vehicle of the CRISPRa machinery.

Recent advances in the field of mRNA technology have coincided with the progression of lipid nanoparticles (LNPs) into the clinic. In 2018, Patisiran was approved as the first LNP-based

therapeutic for the delivery of siRNA for transthyretin-mediated amyloidosis³³. The same platform has since been used to deliver mRNA to generate two of the most effective vaccines against COVID-19 (Pfizer/BioNTech and Moderna). More recently, LNPs have been used to deliver mRNA for CRISPR-Cas9 gene editing *in vivo*, which was found to be safe in human clinical trials³⁴. Delivery of CRISPRa *via* mRNA-LNPs has the further advantage of inducing transient expression, reducing the potential immunogenicity, toxicity and off-target effects of CRISPRa-based therapeutics.

In this study, we explore the potential of LNPs to deliver the dCas9-SAM CRISPRa machinery to T cell lines as a novel strategy towards an optimized, HIV-specific LRA. We show that mRNA-LNPs exhibit excellent transfection efficiencies in various cell lines, including T cell lines, rendering them promising delivery vehicles for mRNA-based LRAs. We then show, to the first of our knowledge, that the three RNA components of the dCas9-SAM CRISPRa can be co-encapsulated into one LNP to potentially reactivate LTR-mediated transcription in cell line models of HIV latency. For simplicity, we will refer to the dCas9-SAM CRISPRa machinery as CRISPRa in subsequent discussions, unless noted otherwise.

Methods

DNA template preparation for mRNA synthesis

Plasmids encoding mCherry (pLenti6-mCherry, kindly provided by Prof. Damian Purcell) or HIV Tat exon 1 (NL4-3 ΔEnv EGFP, NIH HIV Reagent Program) were used for DNA template generation for subsequent *in vitro* transcription. In brief, the coding regions were amplified and a T7 promotor sequence (5'-GAAATTAATACGACTCACTATAGG-3') and a 37 nt poly-A sequence were added through overhang PCR using Platinum Taq High Fidelity polymerase (Life Technologies) (Table S1). Resulting reaction products were cleaned up using a QIAquick PCR purification kit (Qiagen) as per the manufacturer's instructions. For second generation mRNA synthesis of mCherry, HIV Tat, dCas9-VP64 and MS2-p65-HSF1, coding regions of interest were cloned into a backbone harbouring the T7 promotor sequence, 5' UTR, 3' UTR and 2 x 30 nt segmented poly-A tail (pMH, designed by and kind gift from Dr. Marvin Holz) (Figure S1). Inserts were amplified using overhang PCR to add *ScaI* or *FspI* (5') and *EcoRI* or *XbaI* (3') restriction sites (Table S1) and resulting reaction products were cleaned up as above, then digested with the respective restriction enzymes (all NEB) to generate the required overhangs. pMH backbone was

digested using the corresponding 5' and 3' restriction and simultaneously dephosphorylated to prevent self-ligation, then gel-extracted and cleaned up as above. Digested inserts were then ligated into the pMH backbone using T4 DNA ligase (NEB). Circular plasmids were propagated through transformation of Top10 *E. coli* competent bacteria using kanamycin resistance-selection and isolated using ZymoPURE Plasmid Miniprep or Maxiprep Kits (Zymo Research) as per the manufacturer's protocol. Successful cloning was confirmed through Sanger sequencing (Australian Genome Research Facility (AGRF), Melbourne, Australia). pMH plasmids were then linearized through overnight restriction digest immediately downstream of the segmented poly-A sequence using 250 units AseI (NEB) per 10 µg of pDNA to generate the template for subsequent *in vitro* transcription.

mRNA synthesis

In vitro transcription was performed using the mMESSAGE mMACHINE T7 Transcription kit (Thermo Fisher Scientific) as per the manufacturer's instructions, which includes a GG Cap0 for co-transcriptional mRNA capping. For first-generation mRNA constructs, 400-600 ng of PCR product was loaded into each 20 µL reaction. For second-generation mRNA constructs, 1 µg of linearized plasmid DNA template was loaded into each 20 µL reaction. Reactions were left for 4 hours to maximize mRNA yield. For second-generation mRNA constructs, an AG Cap1 structure (CleanCap Reagent AG, Trilink) – compatible with the pMH T7 promotor sequences – was added to the reaction mixture at a final concentration of 6 mM. mRNA was cleaned up using lithium chloride precipitation and resuspended in RNase-free water. Quality control of the synthesized mRNA was performed in two ways: mRNA size, integrity and purity were assessed through gel electrophoresis using a TapeStation instrument (Agilent) after which the concentration was determined through fluorescence detection using the Quant-iT RiboGreen RNA Assay kit (Thermo Fisher Scientific) as per the manufacturer's low range protocol. mRNA was aliquoted and stored at 1000 ng/µL at -80 °C until further use.

gRNA sequences

Modified single gRNA (msgRNA, gRNA hereafter) sequences were selected from previous works by Zhang *et al.* and Tantale *et al.*^{25,35} (Table S2). A scrambled (scr, non-targeting) gRNA sequence was included as negative control. Custom targeting and scr gRNA constructs harbouring 2' O-methyl bases and phosphorothioate bonds at the first and last two nucleotides were obtained from IDT, resuspended in RNase-free water and stored at 1000 ng/µL at -80 °C until further use.

LNP synthesis and characterization

Lipid nanoparticles encapsulating one or more mRNA constructs and/or gRNA were synthesized through microfluidic mixing using a NanoAssemblr Spark (Precision Nanosystems). In brief, ionizable lipids (D-Lin-MC3-DMA (MC3), Selleckchem, or SM102 (8-[(2-hydroxyethyl)[6-oxo-6-(undecyloxy)hexyl]amino]-octanoic acid, 1-octylnonyl ester, Sapphire Bioscience)), DSPC (1,2-distearoyl-sn-glycero-3-phosphocholine, Avanti Polar Lipids), sterols (cholesterol, Sigma-Aldrich, or β -sitosterol, Sigma-Aldrich) and DMG-PEG2000 (1,2-Dimyristoyl-rac-glycero-3-methoxypolyethylene glycol-2000, Avanti Polar Lipids) were resuspended in 100% ethanol at 10 mM and stored at -30°C until further use. Lipids were mixed at a molar ratio of 50:10:38.5:1.5 of MC3:DSPC:cholesterol:DMG-PEG2000 or SM102:DSPC: β -sitosterol:DMG-PEG2000 for standard and modified LNPs, respectively. For fluorescently labelled LNPs, DiD (1,1'-dioctadecyl-3,3,3',3'-tetramethylindodicarbocyanine, 4-chlorobenzenesulfonate salt, Invitrogen) was added at a molar ratio of 0.2:100 of DiD:total lipids. Immediately prior to microfluidic mixing, RNA constructs were diluted to 150 ng/ μ L in 30 mM RNase-free sodium acetate buffer, pH 4.0. Aqueous and organic phases were then mixed at an empirically determined optimum flow rate ratio of 1.8 (aqueous):1 (organic) (N/P ratio of 6:1) and resulting LNPs were diluted 4-fold into PBS to neutralize the pH. LNP size and polydispersity were determined using dynamic light scattering (Zetasizer Ultra, Malvern Panalytical). RNA encapsulation efficiency and total RNA concentration of the LNP solution were determined using a modified RiboGreen assay. In brief, RNA concentration measurements were performed on LNP samples as per the manufacturer's low-range assay protocol after 30-minute incubation in either standard TE buffer (for free RNA measurement) or in TE buffer with 1% Triton X-100 to release encapsulated RNA (for total RNA measurement). The RNA encapsulation efficiency was subsequently determined using Equation 1. LNPs were either stored at 4 °C for a maximum of 2 weeks or frozen at -80 °C in the presence of 20% w/v sucrose for cryoprotection.

$$\text{Encapsulation Efficiency (EE)} = \frac{[\text{total RNA}] - [\text{free RNA}]}{[\text{total RNA}]} * 100\% \quad (1)$$

Cell culture

The human Jurkat T lymphocyte cell line (clone JE6.1) and daughter cell line J-Lat LTR-Tat-IRES-GFP clone A2 (ARP-9854, NIH HIV Reagent Program) were cultured in RPMI 1640 medium containing 10% heat-inactivated fetal calf serum (FCS), supplemented with 100 U/mL penicillin,

100 µg/mL streptomycin and 2 mM L-glutamine (RF10) in a humidified 37°C, 5% CO₂ incubator. HEK293T cells and the HeLa-derived TZM-bl LTR-luciferase reporter cell line (ARP-8129, NIH HIV Reagent Program) were cultured in DMEM containing 10% heat-inactivated fetal calf serum (FCS), supplemented with 100 U/mL penicillin, 100 µg/mL streptomycin and 2 mM L-glutamine (RF10) in a humidified 37°C, 5% CO₂ incubator.

Transfections

For LNP treatment of suspension T cell lines, 1×10^5 suspension cells were plated in duplicate in 100 µL of RF10 in 96-well round-bottom plates on the day of treatment. LNPs were dosed according to the total RNA concentration as per the RiboGreen assay, then added to cells and mixed through to ensure even distribution of cells and LNPs in suspension. Cells were harvested after 24 hours, then stained with LIVE/DEAD™ Fixable Violet Dead Cell Stain (L34955, Thermo Fisher Scientific) to allow gating on live cells during subsequent analysis and fixed in PBS with 1% paraformaldehyde (PFA) prior to flow cytometry analysis using an LSR Fortessa II (BD Biosciences).

For LNP treatment of adherent TZM-bl cells, 1×10^4 cells were plated in triplicate in 100 µL of DMEM10 in 96-well flat-bottom plates one day prior to LNP treatment. LNPs were added to cells through replacement of the culture media with 100 µL DMEM10 containing the appropriate LNP dose, after which cells were incubated for 24 hours prior to harvest. For CRISPR activation RNA transfections of TZM-bl cells using lipofectamine, 2×10^4 cells were plated in triplicate in 100 µL of DMEM10 in 96-well flat-bottom plates one day prior to transfection. dCas9-VP64 mRNA, MS2-p65-HSF1 mRNA and various gRNAs were then complexed with Lipofectamine 3000 (Thermo Fisher Scientific) as per the manufacturer's protocol at a ratio of 100 ng RNA per 0.4 µL of P3000 reagent and 0.3 µL of Lipofectamine 3000 reagent. Complexed solutions were added dropwise to the corresponding wells, after which cells were incubated for 24 hours prior to harvest. A colorimetric cell proliferation assay (MTS assay, CellTiter 96® AQueous One Solution, Promega) was used as per the manufacturer's protocol to determine cytotoxicity resulting from LNP treatment. To measure the induction of LTR-mediated transcription, cells were either lysed in 50 µL 1X Luciferase Cell Culture Lysis Reagent (Promega), after which 50µL luciferase substrate (Promega) was added to 10µL of lysate to determine the activity of the luciferase reporter construct. Alternatively, luciferase activity was determined using the Bright-Glo Luciferase Assay System (Promega) as per the manufacturer's protocol. Luminescence was measured using a FLUOstar Omega microplate reader (BMG Labtech).

Western Blots

To confirm expression of the dCas9-VP64 and MS2-p65-HSF1 proteins, TZM-bl or HEK293T cells were transfected with plasmids (pLenti_dCAS-VP64_Blast and pLenti_MS2-P65-HSF1_GFP (GenScript)) or second-generation, *in vitro* transcribed mRNA using lipofectamine and protein expression was detected using Western Blot. In brief, 1×10^5 cells were plated in 500 μ L in 12-well plates one day prior to transfection. Cells were then transfected with plasmid DNA or mRNA or treated with mRNA-LNP encoding either CRISPR activation protein as above. After 24 hours, cells were harvested, and washed pellets were frozen until further use. Cells were then lysed by resuspension in radioimmunoprecipitation assay (RIPA) buffer (50 mM Tris-HCl pH7.4, 150 mM NaCl, 1% NP-40, 0.1% SDS, 50 mM NaF, 2 mM EDTA) with added 1X Halt Protease Inhibitor Cocktail (Thermo Fisher Scientific) followed by brief sonication. DNA was then pelleted through centrifugation at 18,000 rcf for 10 min at 4°C and protein concentrations of the supernatants were determined through colorimetric detection using a Bradford assay (Bio-Rad). Samples were then mixed with 2X Laemmli sample buffer (Bio-Rad) containing 200 mM dithiothreitol (DTT) and denatured at 95°C for 5 min. Proteins were separated through SDS-PAGE using 8% or 10% polyacrylamide gels that were run for 20 min, 100V then 60 min, 120V, after which proteins were transferred onto PVDF membranes for 90 min, 140A using a wet electroblotting system (Bio-Rad). Antibodies used for detection were anti-p65 (ab16502, Abcam), anti-Cas9 (MA1-201, Thermo Fisher Scientific) and anti-GAPDH (2118, Cell Signalling). Anti-mouse-IgG-HRP (31430, Cell Signalling) and anti-rabbit-IgG-HRP (21360, Cell Signalling) were used as secondary antibodies. Blots were incubated Western Lighting Plus Chemiluminescent Substrate (Perkin Elmer) prior to imaging.

Analysis

Flow cytometry analysis, compensation and cell subset gating was performed using FCS Express 7. Western Blot image analysis was performed in Image Lab (Bio-Rad). Data were further analysed and graphed using GraphPad Prism 9.

Results

Lipid nanoparticles can efficiently and reproducibly encapsulate mRNA encoding mCherry and HIV Tat

We first aimed to validate the LNPs as a platform for encapsulating mRNA cargo. As such, we designed two reporter mRNA constructs that could be used for proof-of-concept experiments of LNP encapsulation and subsequent functional delivery. The first reporter construct encoded for mCherry, a fluorescent protein that can be detected using flow cytometry. The second reporter construct was a short mRNA (256 bp) that encodes for the first exon of the HIV protein Tat, which binds to early HIV transcripts produced from the HIV LTR promotor and potentially enhances transcriptional elongation (Figure 1a). This could be detected using reporter cell lines that express a reporter protein (e.g., GFP or Firefly Luciferase (FLuc)) under the control of the HIV LTR promotor (Figure 1b,c). We generated DNA templates containing a T7 promotor and poly-A tail of each reporter construct through overhang PCR (Figure 2a). Using these templates, we were able to synthesize uniform, *in vitro* transcribed mRNA of ~800 nt (mCherry) and ~300 nt (Tat exon 1), as confirmed through RNA gel electrophoresis (Figure 2a, b).

We then formulated LNPs encapsulating these mRNA constructs. LNPs typically consist of four lipid components: (i) an ionizable lipid, (ii) a helper lipid, (iii) a sterol, and (iv) a polyethylene glycol (PEG)-lipid (Figure 3a). For initial experiments, we formulated LNPs with D-Lin-MC3-DMA, DSPC, Cholesterol and DMG-PEG2000 (MC3 LNP) as per the Patisiran formulation³³. We found that both mRNA-loaded and empty control LNPs could be formulated at sub-100 nm size with high reproducibility (Figure 3b-d). Empty control LNPs exhibited a smaller size of 36.1 ± 1.3 d.nm compared to mRNA-loaded LNPs (62.7 ± 1.0 d.nm and 77.2 ± 5.2 d.nm for mCherry- and Tat-LNPs, respectively). All LNP formulations showed high uniformity as measured by a polydispersity index (PDI) of < 0.2 (Figure 3b-d). Using a modified RiboGreen assay to quantify the mRNA encapsulation efficiency (EE), we found that both mCherry- and Tat-LNPs encapsulate a high percentage of the loaded mRNA, with mean encapsulation efficiencies of 90% and 83%, respectively (Figure 3c-d).

mRNA-LNPs potentially reactivate LTR-mediated transcription in TZM-bl cells

We next assessed whether the mRNA encapsulated in these LNPs remained functional and whether the LNPs were capable of efficiently delivering this payload to cells. HeLa cells are commonly used to study nanoparticle uptake due to their ease of transfection. Therefore, we

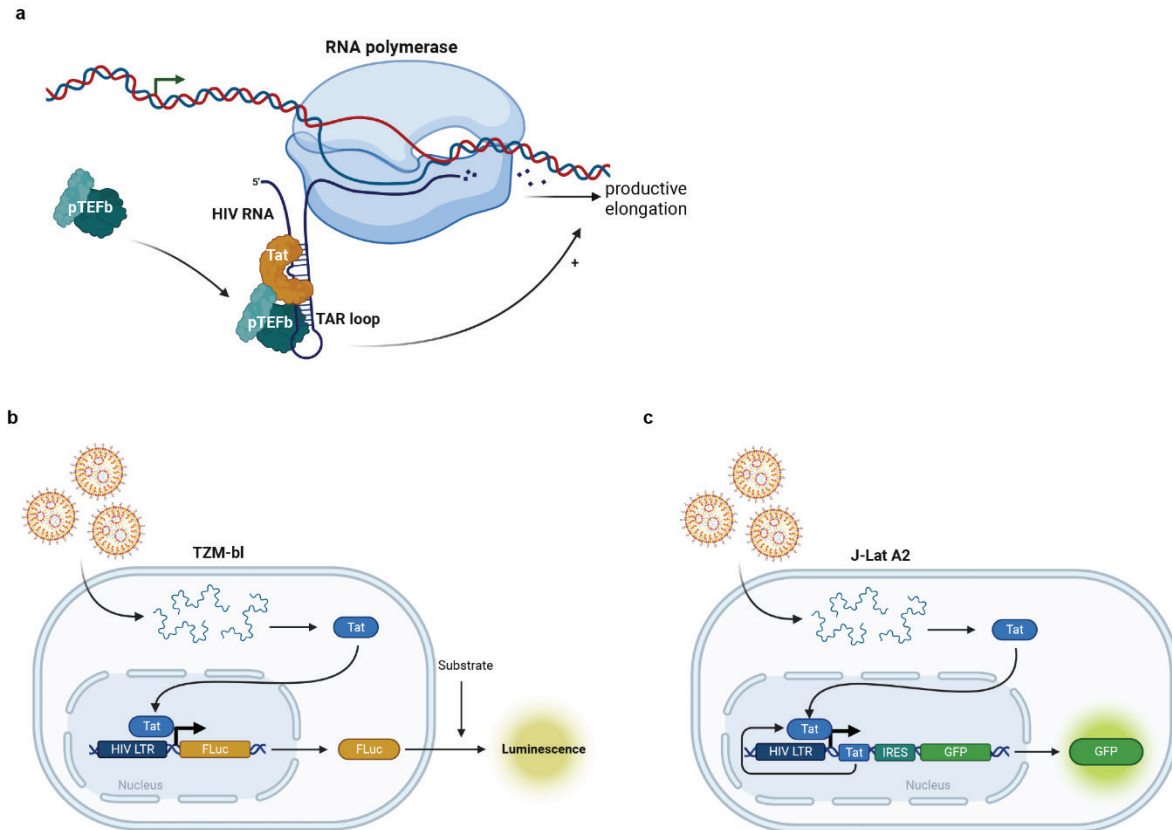


Figure 1. HIV Tat reporter systems. (a) Schematic overview of the mechanism of action of HIV Tat for promoting transcriptional elongation. Tat binds to the TAR loop in the early HIV RNA transcript and thereby recruits the host-cell transcription factor pTEFb to the RNA polymerase complex, which mediates the transition into productive elongation. (b) TZM-bl cells harbour a Firefly Luciferase (FLuc) reporter gene under the control of the HIV LTR promoter. Upon addition of luciferase substrate, the potency of Tat mRNA-containing LNPs (or other LRAs) can be assessed through measuring luminescence. (c) J-Lat A2 cells have an integrated HIV LTR-Tat-IRES-GFP construct. The integrated *tat* gene induces a positive feedback loop upon activation of LTR-mediated transcription, leading to signal amplification. The potency of Tat mRNA-containing LNPs (or other LRAs) can be assessed through measuring GFP expression.

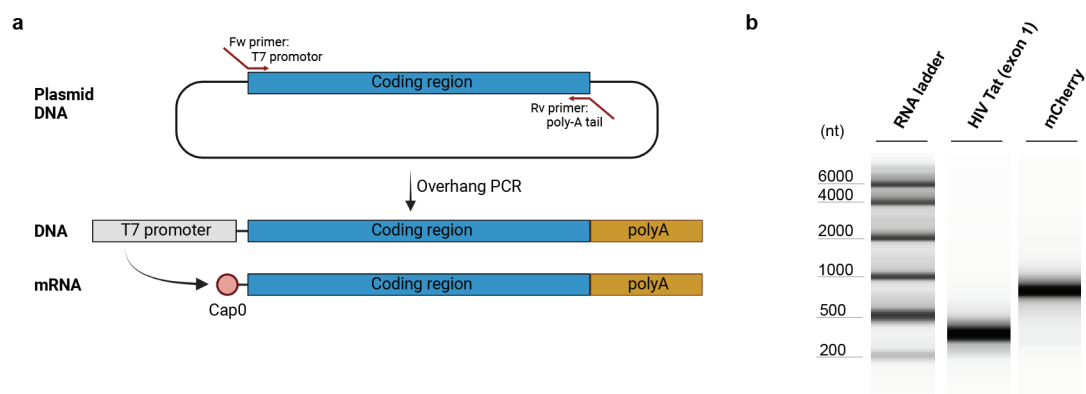


Figure 2. First-generation mRNA synthesis. (a) Schematic overview of linear DNA template generation from plasmid DNA. Overhang PCR was used to amplify the coding region of the construct of interest, using forward (Fw) and reverse (Rv) primers to add on the T7 promoter and poly-A tail sequence on the 5' and 3' end of the coding region, respectively. mRNA was then synthesized through *in vitro* transcription. (b) Representative RNA gel electrophoresis for first-generation *in vitro* transcribed mRNA encoding HIV Tat (exon 1) and mCherry, indicating mRNA purity, integrity, and size.

treated the HeLa-derived TZM-bl cell line with mCherry-LNPs for 24 hours and found a high proportion of cells (> 90%) expressing mCherry (Figure 4a). The mCherry expression was dose-dependent and non-toxic (Figure 4b), indicating that LNPs could indeed deliver intact, functional mRNA payload to cells. We then treated TZM-bl cells with Tat-LNPs and assessed functional Tat mRNA delivery through measuring the LTR-driven reporter FLuc expression. We observed a highly potent reactivation of LTR-mediated transcription of up to 830-fold over untreated control cells, while neither empty control LNPs nor mCherry-LNPs triggered upregulation (< 5-fold increase) of LTR-mediated transcription (Figure 4c). At doses higher than 25 ng, a slight decrease in reactivation was observed following Tat-LNP treatment, consistent with modest toxicity of this treatment at 50 ng or 100 ng doses. mCherry-LNPs or empty control LNPs, in contrast, did not result in clear toxicity at any of the doses tested (Figure 4d).

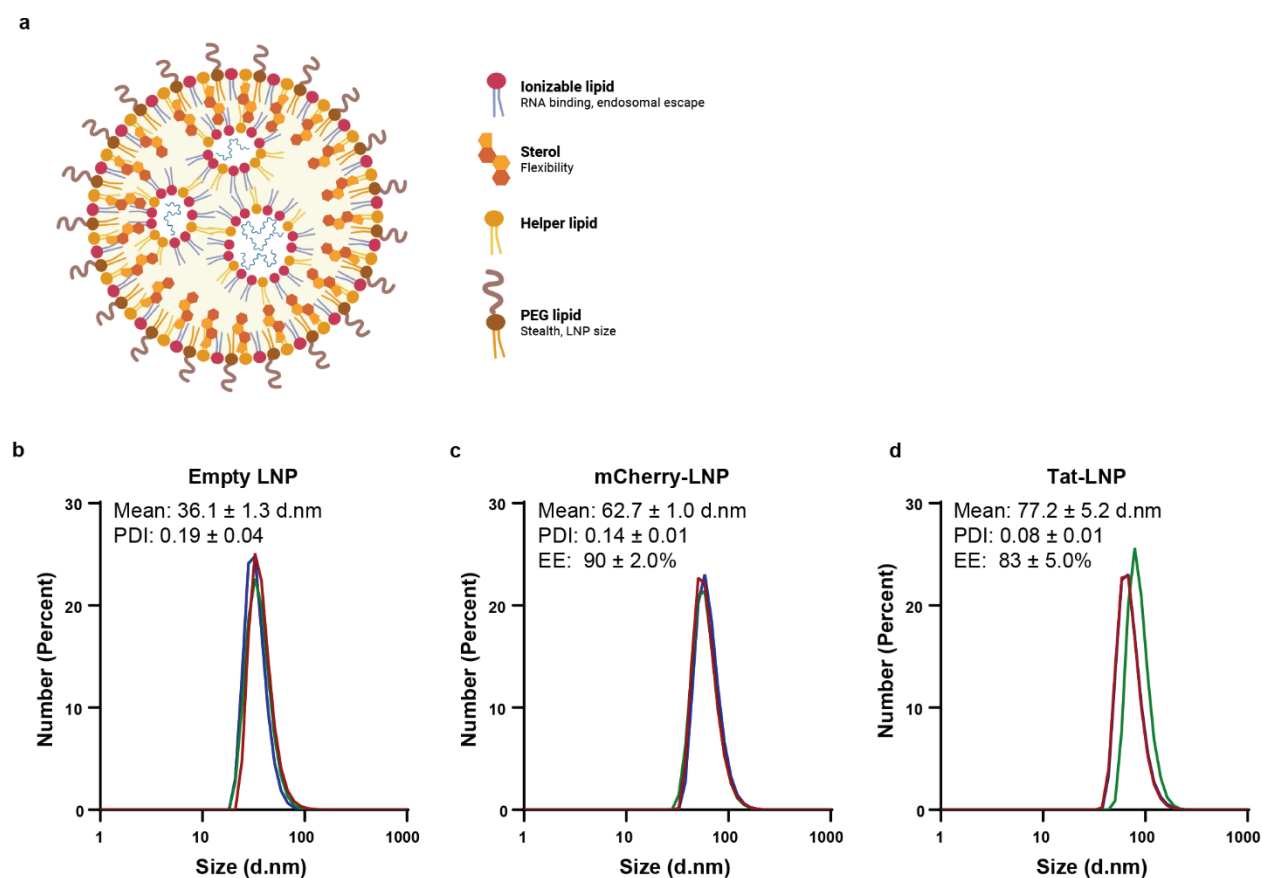


Figure 3. Lipid nanoparticles can be formulated reproducibly at sub-100 nm size and with high uniformity. (a) Schematic representation of a lipid nanoparticle (LNP), consisting of an ionizable lipid for RNA binding and endosomal escape, a sterol for enhanced flexibility, a helper lipid and a poly(ethylene glycol) (PEG) lipid to improve stealth properties and to control the LNP size. (b-d) Representative results of the characterization of empty LNP (b), mCherry-LNP (c) and Tat-LNP (d) using dynamic light scattering to measure LNP size (Number distribution) and polydispersity. Lines on each graph represent three independently prepared LNP formulations. The mean size, polydispersity index (PDI) and encapsulation efficiency (EE, determined by RiboGreen assay) $\pm$ SEM of the three formulations are shown.

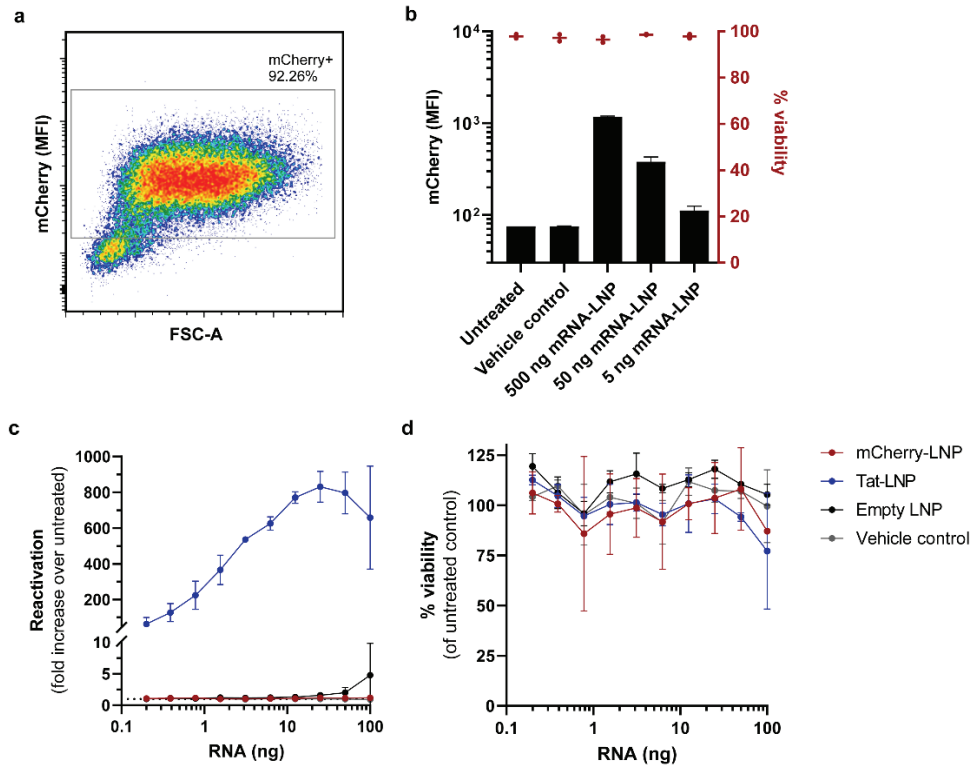


Figure 4. LNP-mediated delivery of mCherry and Tat mRNA to T cell line models of HIV latency is efficient and non-toxic. (a-b) T cell line models were treated with multiple dose-titrations of mCherry-LNPs and mCherry expression was measured by flow cytometry after 24 hours. (a) Representative dot plot of 500 ng mCherry-LNP treatment of T cell line models, showing a high transfection efficiency. (b) Levels of mCherry expression (MFI) (black, left y-axis) and viability (red, right y-axis) of T cell line models after 24 hours incubation with mCherry-LNPs or vehicle control. Data presented as means $\pm$ s.d. (black, bars and error bars) or means and individual measurements (red) of duplicate measurements from a single experiment. (c-d) T cell line models were treated with various doses of Tat-LNP, mCherry-LNP or empty control LNP for 24 hours, after which reactivation of LTR-mediated transcription was measured as a fold change in luciferase expression over untreated control cells (c) and viability was assessed through a colorimetric cell proliferation assay and expressed as a percentage viability compared to untreated control cells (d). Lines represent means $\pm$ SEM of $n=2$ independent experiments.

LNP allow for highly efficient transfection and mRNA delivery to T cell line models of HIV latency

After validating the potency of mCherry- and Tat-LNPs at delivering functional cargo, we assessed their capacity to transfect suspension T cell lines. As discussed in Chapter 3, T cells are generally less susceptible to nanoparticle uptake in the absence of active targeting. Surprisingly, however, we found that treatment of Jurkat T cells with 500 ng mCherry-LNPs resulted in a transfection efficiency of 99% (Figure 5a). Furthermore, treatment with doses as low as 16 ng resulted in a similarly high transfection efficiency, while a dose-response effect was clearly observed at doses of 8 ng and below (Figure 5a). Given these promising results, we next tested the capacity of Tat-LNPs to reactivate HIV LTR-mediated transcription in J-Lat A2 cells – a T cell line model of HIV latency that harbours a GFP reporter gene under the control of the HIV LTR (Figure 1c). Upon treatment of J-Lat A2 cells with Tat-LNPs, we observed potent reactivation of LTR-mediated transcription of up to 87% GFP expression, exceeding the reactivation observed following maximal

stimulation with PMA/ionomycin (positive control). Importantly, treatment with mCherry- or empty control LNPs did not result in GFP expression, indicating that the reactivation observed was mediated by the expression of exogenous Tat exon 1 (Figure 5b). Treatment with Tat- or control LNPs furthermore resulted in negligible toxicity (Figure 5c).

We then used these two reporter cell lines to assess whether LNPs could be frozen without loss of potency. When suspended in PBS, LNPs were shown to lose most of their potency after one freeze/thaw cycle (Figure S2a). In contrast, the addition of sucrose to the LNP solution as a cryoprotectant rescued LNP potency, with a sucrose concentration of 20% resulting in complete retention of potency (Figure S2a). However, even in the presence of this cryoprotectant, the LNP potency declined after multiple freeze/thaw cycles (Figure S2b). As such, for all following experiments, LNPs were either stored at 4°C for up to two weeks or preserved at -80°C in the presence of 20% sucrose until use; LNPs were not re-frozen.

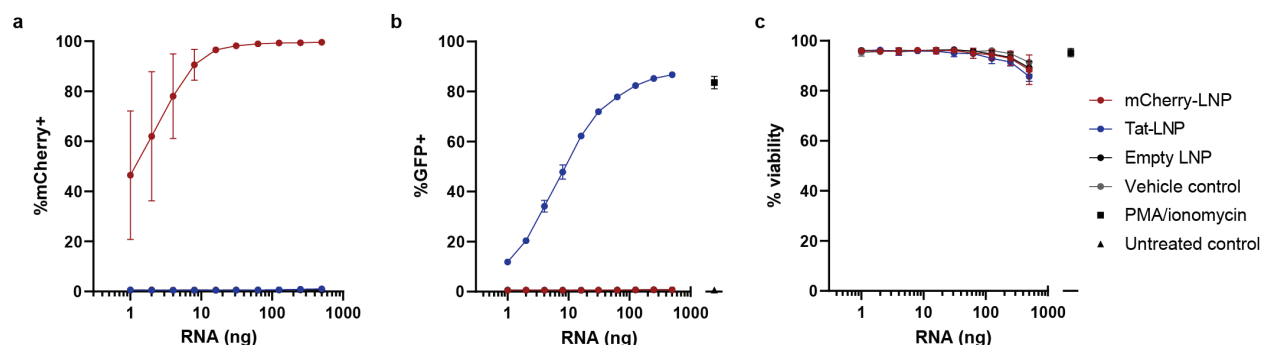


Figure 5. mRNA-LNPs exhibit strong transfection efficiency and latency reversal in T cell lines. (a) Jurkat T cells were treated with different doses of mCherry-LNP or control Tat-LNP and empty LNP. Percentage of mCherry-expressing cells was determined through flow cytometry after 24 hours. (b-c) J-Lat A2 cells were treated with different doses of Tat-LNP, mCherry-LNP or empty control LNP for 24 hours. Reactivation of LTR-mediated transcription as a percentage of cells expressing GFP (b) and viability (c) were measured using flow cytometry. Lines represent means $\pm$ SEM of n=2 independent experiments.

Rational design of mRNA construct leads to higher protein expression

Our findings thus far validate that LNPs are powerful delivery vehicles for mRNA-based reporter constructs to T cell lines. Next, we focused on the mRNA construct itself and aimed to optimize protein expression from the delivered mRNA. To this end, we employed a different method of DNA template synthesis for subsequent *in vitro* transcription, which resulted in a second-generation mRNA construct with 5' and 3' untranslated regions (UTRs) as well as a longer poly-A tail for enhanced mRNA stability. This approach used a plasmid backbone (pMH, kindly provided by Dr. Marvin Holz) that contained the T7 promotor, an optimized minimal 5' and 3' UTR and

segmented poly-A tail (Figure S1). Furthermore, these mRNAs utilized a Cap1 structure to enhance the mRNA translation efficiency, as opposed to the Cap0 incorporated in our first-generation mRNAs. Restriction sites downstream of the 5' UTR and upstream of the 3' UTR allowed for cloning of any gene of interest into this “*in vitro* transcription-ready” plasmid (Figure 6a). The resulting pMH plasmid was then linearized using restriction digest immediately downstream of the segmented poly-A sequence to generate the final template for *in vitro* transcription (Figure 6b). To generate such second-generation mRNA for our mCherry constructs, we amplified the 720 bp coding region and added restriction sites using overhang PCR (Figure 6c), which were digested and subsequently ligated into the digested pMH backbone. The resulting pMH_mCherry plasmid was validated using diagnostic restriction digest (Figure 6d) as well as Sanger sequencing (data not shown). We then used this new DNA template for *in vitro* transcription and showed that we could once more generate a uniform mRNA construct of ~1100 nt, as expected (Figure 6e). The increased size of the second-generation compared to the first-generation mCherry mRNA (Figure 2b) reflects the addition of 5' and 3' UTRs as well as the longer poly-A tail of the second-constructs. LNPs formulated with the first- or second-generation mCherry mRNA demonstrated similar size and PDI (Figure 6f), however, upon their addition to Jurkat T cells, second-generation mCherry-LNPs induced a substantially higher protein expression compared to first-generation mCherry-LNPs at all doses tested (Figure 6g). These data suggest that indeed, the inclusion of 5' and 3' UTRs, Cap1 structure and longer poly-A together enhance the mRNA stability upon intracellular delivery.

CRISPR RNAs can be exploited to reactivate latency in TZM-bl cells

Using these optimized mRNA constructs as well as the validated LNP formulation, we proceeded to synthesize and assemble the CRISPR activation machinery. The dCas9-SAM CRISPR activation system requires the co-expression of two proteins – the catalytically inactive dCas9 protein fused to a VP64 transcriptional activator domain, and an MS2-p65-HSF1 fusion protein – as well as a modified single guide RNA (gRNA) that targets the promoter of interest (Figure 7a). In this work, we employed five previously studied gRNA sequences targeting the HIV LTR^{25,35} (Table S2), mainly spanning the region of the HIV LTR just upstream of the core and enhancer regions (Figure 7b). Second-generation versions of the two mRNA constructs encoding dCas9-VP64 and MS2-p65-HSF1 were synthesized through *in vitro* transcription, resulting in uniform mRNAs of ~4.8 kb and ~1.8 kb, respectively (Figure 7c).

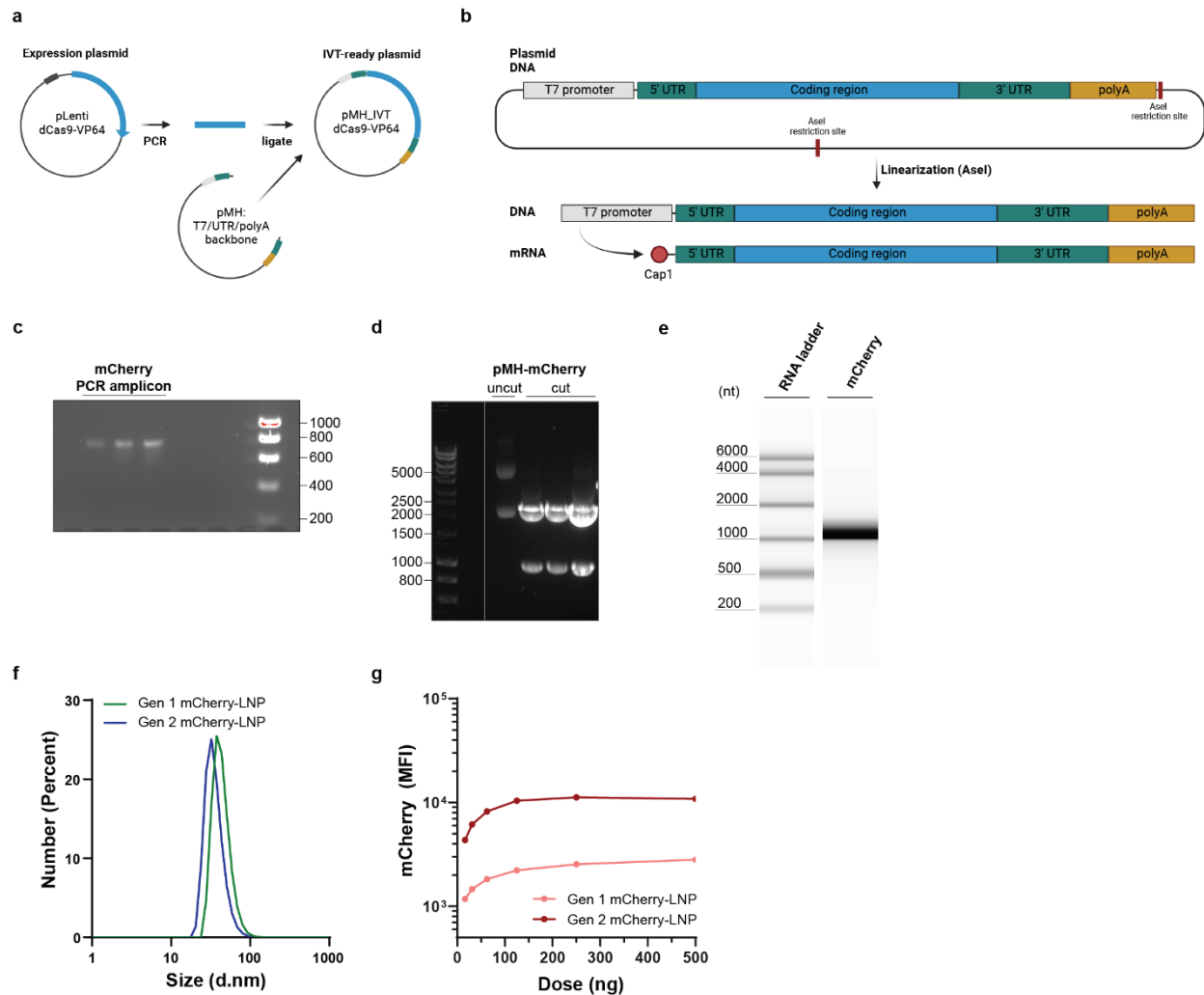


Figure 6. Second-generation mRNA results in enhanced protein expression. Schematic overview of the cloning of coding regions of interest from (lentiviral) expression plasmids into the PMH backbone, containing T7 promoter, untranslated regions (UTRs) and poly-A sequences up- and downstream of the cloning sites. (b) Linear DNA template for subsequent *in vitro* transcription of second-generation mRNA is obtained through restriction digest of pMH plasmids with AseI, cleaving directly downstream of the poly-A sequence. Resulting mRNA is capped with a Cap1 structure and includes 5' and 3' UTRs. (c) Gel electrophoresis result of the PCR amplicon of the mCherry coding region with required restriction sites for subsequent cloning into pMH. (d) Restriction digest of pMH-mCherry to prepare linearized IVT template, showing complete digestion of the pMH plasmid, releasing the expected fragments of ~900 and ~2150 bp. (e) Representative RNA gel electrophoresis result of second-generation mCherry mRNA synthesis, indicating mRNA purity, integrity, and size. (f) Representative results of dynamic light scattering measurements of first and second-generation (Gen 1 and Gen 2) mCherry mRNA-LNPs, demonstrating comparable size and polydispersity between the two LNP formulations. (g) Direct comparison of mCherry protein expression in Jurkat T cells upon treatment with Gen 1 or Gen 2 mCherry mRNA-LNPs for 24 hours. mCherry expression was determined using flow cytometry. Lines and error bars represent mean $\pm$ s.d. of duplicate measurements of a single experiment.

We next aimed to validate protein expression through Western Blot detection of both constructs in TZM-bl cells. We found that expression of dCas9-VP64 in TZM-bl cells could be detected using an anti-(wildtype)Cas9 antibody, as indicated by the clear staining observed around 160 kDa upon transfection of TZM-bl cells with pLenti_dCAS-VP64_Blast (Figure 7d). We then treated TZM-bl cells with dCas9-VP64-LNPs or MS2-p65-HSF1-LNPs as a control, and could again detect dCas9-

VP64 expression (Figure 7e), although expression was less pronounced than upon plasmid transfection. Furthermore, we observed a reverse dose-response effect with the 1 μ g dCas9-VP64-LNP dose resulting in higher expression than the 2 μ g dose. While these preliminary findings could be an artefact caused by slight differences in loading efficiency (Figure 7e, bottom panel (GAPDH)), they possibly indicate cellular toxicity or stress caused by high amounts of mRNA delivered intracellularly.

We repeated these experiments for MS2-p65-HSF1 detection using an antibody staining a domain of the endogenous p65 protein that is incorporated in the MS2-p65-HSF1 fusion construct. This fusion protein contains only the domains of p65 and HSF1 required for transcriptional activation, and as such has – counterintuitively – a smaller molecular weight of 48 kDa compared to the endogenous p65 protein. Upon transfection of TZM-bl cells with pLenti_MS2-P65-HSF1_GFP, we indeed observed a band at ~65 kDa in each condition tested, including non-transfected control cells, representing endogenous p65. Transfection with pLenti_MS2-P65-HSF1_GFP resulted in a second band at a slightly lower molecular weight, representing the fusion protein (Figure 7f). A similar expression pattern could, however, not be detected following treatment of TZM-bl cells with MS2-p65-HSF1-LNPs (Figure 7g). Indeed, when directly comparing expression upon plasmid transfection, mRNA transfection (using Lipofectamine 3000) and mRNA-LNP treatment in 293T cells, expression appeared to be present in either transfected condition, but not in mRNA-LNP treated conditions (Figure 7h). However, the presence of multiple bands, likely reflecting post-translational modifications, complicated the correct identification of MS2-p65-HSF1. Together, these findings suggest that the dCas9-VP64 and, likely, MS2-p65-HSF1 mRNA constructs synthesized are functional, yet the delivery vehicle appears to be an important and potentially limiting factor for subsequent protein expression levels.

Consequently, we first assessed the potency of CRISPR activation using Lipofectamine 3000 transfection of the three CRISPR activation RNAs in TZM-bl cells. To this end, cells were concomitantly transfected with three times 100 ng of separately prepared lipofectamine mixtures of dCas9-VP64 mRNA, MS2-p5-HSF1 mRNA and one of the five targeting gRNAs or a non-targeting (scrambled) control gRNA. All gRNAs were modified with 2' O-methyl bases and phosphorothioate bonds at the first and last two nucleotides for enhanced gRNA stability³⁶. After 24 hours, transfection with the CRISPR activation machinery was found to modestly enhance LTR-mediated transcription for all targeting gRNAs tested (1.5 to 2.0-fold increases over untreated

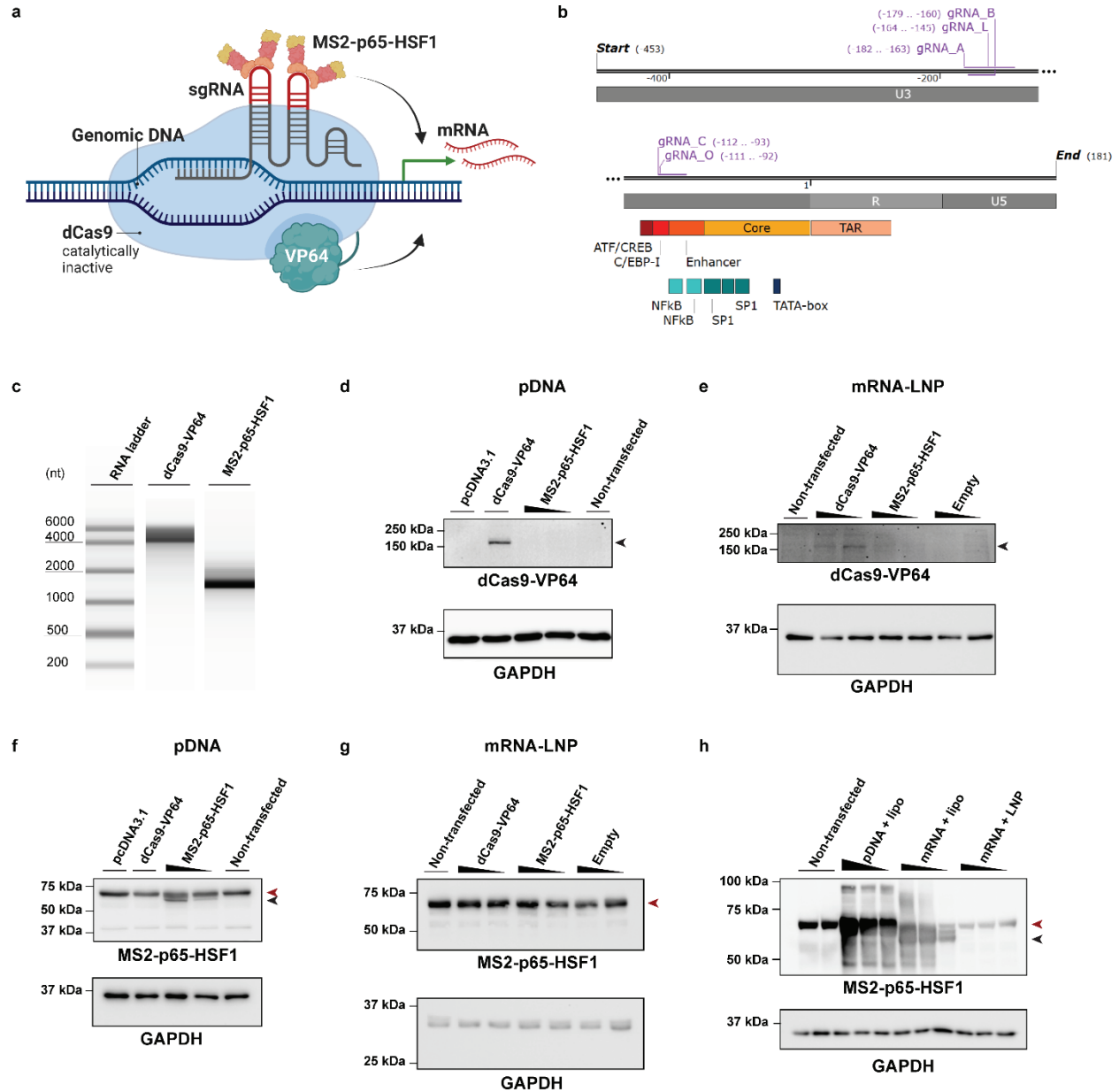


Figure 7. Expression of dCas9-VP64 and MS2-p65-HSF1 can be achieved using mRNA transfection. Schematic overview of the dCas9-SAM CRISPRa system. A catalytically inactive Cas9 (dCas9) is fused to a VP64 transcriptional activation domain. The modified single guide RNA (gRNA) targets a specific region in the host-cell genome and includes minimal hairpin aptamers to allow for the recruitment of the MS2-p65-HSF1 fusion protein, further enhancing transcriptional activation. (b) Schematic overview of the HIV LTR with key regions annotated. The binding sites of the five targeting gRNAs employed in this study are highlighted in purple. (c) Representative RNA gel electrophoresis result of second generation dCas9-VP64 and MS2-p65-HSF1 mRNA synthesis, indicating mRNA purity, integrity, and size. (d-g) T2M-bl cells were transfected with 2 μ g of pLenti_dCas9-VP64_Blast, 4 μ g or 2 μ g of pLenti_MS2-p65-HSF1_GFP or 4 μ g of pcDNA3.1 control plasmid (d, f) or treated with 2 μ g or 1 μ g of dCas9-VP64 mRNA-LNP, MS2-p65-HSF1 mRNA-LNP or empty control LNP (e, g) for 24 hours, after which dCas9-VP64 and MS2-p65-HSF1 expression were measured through Western Blot using anti-(wildtype)Cas9 and anti-p65 antibodies. (h) HEK293T cells were transfected with 6 μ g, 4 μ g or 2 μ g doses of pLenti_MS2-p65-HSF1_GFP (pDNA + lipofectamine (lipo)), 3 μ g, 2 μ g or 1 μ g doses of MS2-p65-HSF1 mRNA (mRNA + lipo) or treated with 3 μ g, 2 μ g or 1 μ g doses of MS2-p65-HSF1 mRNA-LNP for 24 hours. MS2-p65-HSF1 expression was measured through Western Blot. (d-h) GAPDH staining was included as a loading control. Black arrows indicate bands likely representing protein of interest, red arrows indicate bands likely representing endogenous p65.

control) (Figure 8a) at minimal cellular toxicity (Figure 8b). Transfection with the scrambled control gRNA also resulted in modest reactivation, but at lower levels than observed for targeting gRNAs.

We assessed whether these levels of reactivation could be replicated if the delivery mode of one of the three RNA constructs at a time was changed from Lipofectamine to an RNA-LNP. To counter any reduced delivery efficiency of the RNA-LNPs compared to Lipofectamine, small dose-titrations of the RNA-LNPs were performed. Overall, changing the delivery mode for dCas9-VP64 and MS2-p65-HSF1 mRNA reduced the effect of CRISPR activation, even at up to three times increased doses (300 ng) of the mRNA-LNP – yet, reactivation could still be observed using gRNA_L and exceeded that of the scrambled control gRNA (Figure 8c,d). Surprisingly, replacement of the delivery mode of the gRNA itself with a gRNA-LNP increased the reactivation potential (Figure 8e), but the baseline transfection using three times Lipofectamine deviated from results observed previously (Figure 8a), thus warranting caution in drawing conclusions from this pilot experiment. Furthermore, the observed viability in these experiments (Figure 8f-h) was lower than seen previously (Figure 8b), potentially reflecting technical inaccuracies in the experimental set-up that could influence the levels of reactivation measured. Therefore, when we next assessed the reactivation potential of CRISPR activation upon delivery by three RNA-LNPs, a range of different dose combinations of each of the three RNA-LNPs was tested. Promisingly, 1.6-fold reactivation of LTR-mediated transcription over untreated control was observed for gRNA_O in multiple dose-combinations tested (Figure 8i, green highlights), compared to minimal reactivation for scrambled control gRNA. Interestingly, within conditions with the same ratio of dCas9-VP64- to MS2-p65-HSF1- to gRNA-LNPs, higher overall doses appeared to result in less potent reactivation, potentially correlating to earlier findings (Figure 7e) suggesting inverse dose-response effects.

Overall, we demonstrated that CRISPR activation is indeed able to reactivate HIV LTR-mediated transcription using various gRNAs. However, the detected level of transcriptional upregulation was modest, complicating further studies optimizing the delivery vehicle, dosing and dose ratios between the various CRISPR activation RNA constructs.

CRISPRa-LNPs potently reactivate HIV latency in J-Lat A2s

To further investigate the potency of CRISPR activation, we employed two strategies. Firstly, we re-evaluated CRISPRa-LNPs in the J-Lat A2 cell line. Not only is this T cell line more representative of the latent HIV reservoir in PLWH, the reporter construct in J-Lat A2 cells also differs from that in TZM-bl cells by harbouring the *tat* gene upstream of the IRES-GFP cassette (Figure 1c). As a

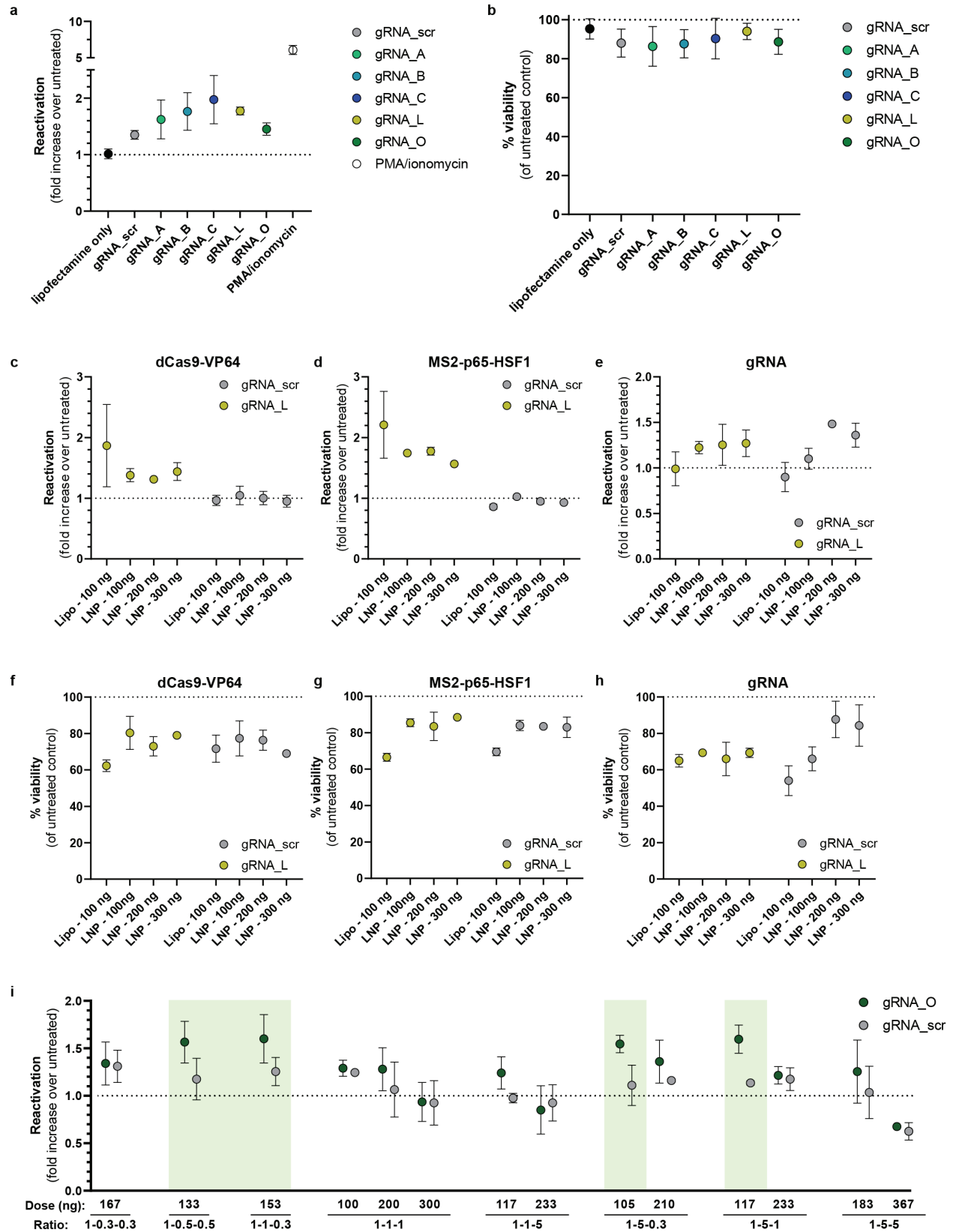


Figure 8. (Previous page). CRISPRa can induce LTR-mediated transcription in TZM-bl cells. (a-b) TZM-bl cells were transfected with dCas9-VP64 mRNA, MS2-p65-HSF1 mRNA and various targeting (A, B, C, L, O) or scrambled (scr) control gRNA using Lipofectamine 3000. PMA/ionomycin treatment was included as a positive control for reactivation of LTR-mediated transcription. After 24 hours, reactivation (a) and viability (b) were assessed. Datapoints indicate mean $\pm$ SEM of n=3 independent experiments. (c-h) TZM-bl cells were transfected with the two CRISPRa mRNAs and gRNA_L or gRNA_scr using three concomitant Lipofectamine 3000 transfections (lipo) or a combination treatment in which either the dCas9-VP64 mRNA (c, f), MS2-p65-HSF1 mRNA (d, g) or gRNA (e, h) delivery was performed using an LNP in three different doses. After 24 hours, reactivation (c-e) and viability (f-h) were assessed. Datapoints indicate mean $\pm$ s.d. of duplicate measurements. (i) TZM-bl cells were treated with different weight ratios of dCas9-VP64-LNP, MS2-p65-HSF1-LNP and gRNA_O- or gRNA_scr-LNP (ratio of the three components is indicated in that respective order) and reactivation was assessed after 24 hours. Datapoints indicate mean $\pm$ SEM of n=2 independent experiments.

result, reactivation of the HIV LTR through latency-reversing agents – e.g., CRISPRa-LNPs – is likely to trigger a positive feedback loop leading to signal amplification. Furthermore, in contrast to TZM-bl cells that exhibit a high level of basal transcription, J-Lat A2 cells exhibit minimal GFP expression at baseline, making latency reversal more pronounced. As such, J-Lat A2 cells are potentially a more sensitive reporter of CRISPR activation function. Secondly, we optimized the LNP formulation used to deliver the CRISPR activation machinery. Two changes were made to the LNP formulation. First, the ionizable lipid D-Lin-MC3-DMA (MC3) was replaced with SM-102, an ionizable lipid shown to lead to greater cytosolic mRNA delivery through enhanced endosomal escape^{37,38}. Second, these SM-102-LNPs were further modified using β -sitosterol, a cholesterol analogue also associated with enhanced endosomal escape³⁸, to create modified SM-102 LNPs (mSM102-LNP).

We treated Jurkat T cells with either MC3 mCherry-LNPs or mSM102 mCherry-LNPs and found that indeed, mSM102 LNPs resulted in a substantially higher (6-fold at the 500 ng dose) mCherry expression compared to MC3 LNPs (Figure 9a), demonstrating that this new LNP formulation could also aid CRISPR activation delivery. We then generated MC3 and mSM102 CRISPRa-LNPs by mixing dCas9-VP64 mRNA, MS2-p65-HSF1 mRNA and gRNA at a 1:1:1 w/w ratio prior to encapsulation into either LNP and added these to J-Lat A2 cells for 24 hours. MC3 CRISPRa-LNP resulted in modest (< 5%) reactivation of HIV LTR-mediated transcription, yet mSM102 CRISPRa-LNP induced GFP expression in up to 95% of J-Lat A2 cells (Figure 9b), confirming the critical importance of a potent delivery vehicle.

To further optimize the CRISPRa-LNP potency, we hypothesized the amount of gRNA delivered to likely be a limiting factor, as the gRNA needs to withstand degradation by cytosolic RNases while the dCas9-VP64 and MS2-p65-HSF1 mRNAs are translated. We therefore formulated mSM102 CRISPRa-LNPs encapsulating increasing ratios of the gRNA to the dCas9-VP64 and MS2-p65-HSF1 mRNAs. However, we found that mSM102 CRISPRa-LNPs with increased gRNA led to lower, instead of higher reactivation of J-Lat A2 cells when administered at sub-optimal

doses of 7.5 ng and 15 ng (Figure 9c). We then used this optimized mSM102 CRISPRa-LNP formulation with equal parts of each CRISPR activation RNA (1:1:1) to compare the potency of all 5 targeting gRNAs and observed a dose-responsive reactivation of HIV LTR-mediated transcription with all targeting, but not the scrambled control gRNA (Figure 9d). The highest reactivation was achieved using gRNA_L, followed by gRNA_O and gRNA_C, which exhibited similar potencies, reflecting their mostly overlapping target region in the HIV LTR (Figure 7b). Treatment of J-Lat A2 cells with targeting or scrambled control gRNA CRISPRa-LNPs resulted in negligible toxicity at any of the doses tested (Figure 9e). These data demonstrate that LNP-mediated delivery of a three-component CRISPR activation system is possible and that CRISPRa-

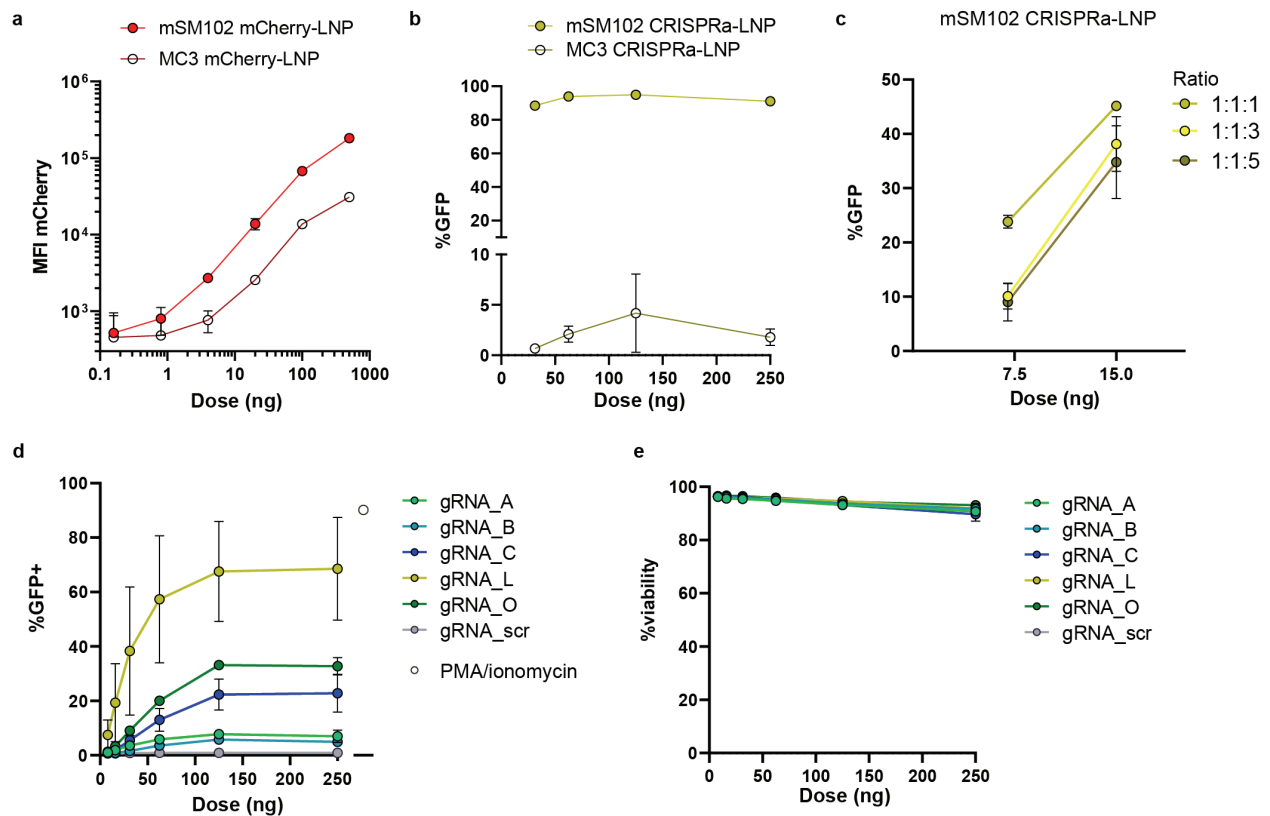


Figure 9. CRISPRa induces potent latency reversal in J-Lat A2 cells at minimal toxicity when delivered using mSM102-LNP. (a) Jurkat T cells were treated with equal doses of mCherry-LNP using either the MC3 (open circles) or mSM102 (closed circles) formulation. Protein expression was determined by flow cytometry after 24 hours. Datapoints indicate mean $\pm$ SEM of $n=2$ independent experiments. (b) dCas9-VP64 mRNA, MS2-p65-HSF1 mRNA and gRNA_L were co-encapsulated into either MC3 (open circles) or mSM102 (closed circles) LNPs at a 1:1:1 w/w ratio and administered to J-Lat A2 cells at various doses. After 24 hours, reactivation of LTR-mediated transcription was determined by measuring GFP expression using flow cytometry. (c) mSM102-LNPs were used to co-encapsulate various ratios of dCas9-VP64 mRNA, MS2-p65-HSF1 mRNA and gRNA_L representing increasing proportions of the gRNA_L. J-Lat A2 cells were dosed with suboptimal doses (7 ng and 15 ng) of these CRISPRa-LNPs and reactivation was assessed after 24 hours. (b, c) Datapoints indicate mean $\pm$ s.d. of duplicate measurements from a single experiment. (d, e) J-Lat A2 cells were treated with increasing doses of mSM102 CRISPRa-LNPs with each of the five targeting gRNAs or control gRNA_scr and reactivation (d) and viability (e) were assessed after 24 hours. PMA/ionomycin treatment was included as a positive control for the reactivation of LTR-mediated transcription. Datapoints indicate mean $\pm$ SEM of $n=2$ independent experiments.

LNPs can induce potent, specific and non-toxic reactivation in the J-Lat A2 cell line model of HIV latency.

Discussion

CRISPR activation forms a promising strategy towards a more potent, HIV-specific latency reversal agent. However, the key bottleneck towards the translation of this potential therapeutic to the clinic is the lack of an efficient delivery vehicle of the CRISPRa machinery *in vivo*. In this study, we describe the first evidence that LNPs can be used to efficiently co-deliver the three RNAs that form the dCas9-SAM CRISPRa system, to potentially reactivate HIV latency in cell line models.

We showed that sub-100 nm LNPs encapsulating two reporter mRNAs – mCherry and HIV Tat – could reproducibly be formulated and demonstrated a high delivery efficiency of these mRNAs to T cell lines in the absence of toxicity. Combined with the relative ease of mRNA and LNP synthesis, these findings are of great interest generally, as they propose mRNA-LNPs as a promising tool for the *in vitro* transfection of genetic material. Such an alternative is much needed, as traditionally used transfection methods exhibit strong drawbacks. Lentiviral transduction methods suffer from a limit in the size of the delivered cargo, complicating their use for the delivery of, for example, CRISPR-based therapeutics due to the high molecular weight of the Cas protein. Nucleofection-based strategies often fail to uncouple transfection efficiency from cellular toxicity, due to the cellular stress caused by the disruption of the cell membrane that is required for transfection. Lastly, while other lipid-based strategies such as Lipofectamine have shown remarkable potency in adherent cell types, their use in suspension cells appears hindered by poor efficiency and toxicity caused by the high concentration of the permanently charged cationic lipids.

For our study, the high delivery efficiency of mRNA-LNPs to T cell lines provided the proof of concept that LNPs could be used for the delivery of the CRISPRa machinery as an HIV-specific LRA. Indeed, we subsequently demonstrated in two different cell line models of HIV latency that functional delivery of the CRISPR machinery can be achieved through RNA-LNP delivery. We found that multiple gRNAs can reactivate LTR-mediated transcription, yet at different potencies, with gRNA_L exhibiting the greatest potency of those gRNAs tested. These findings are in line with several previous studies highlighting the fact that the optimal target region within the HIV LTR for CRISPRa is upstream of the Enhancer region (-106 to -165 bp to transcription start site)³⁰. Indeed, our gRNA_L was the only gRNA tested in this study that falls completely within this region,

and other studies assessing this same gRNA sequence under different names all demonstrated superior potency of this gRNA compared to others tested^{26,27,30}. This optimal target region within the HIV LTR also corresponds to the general optimal target region for CRISPRa (-100 to -200 bp to transcription start site) identified by Konermann *et al.*²³. However, in our study, gRNAs A, B and L targeted overlapping regions in the HIV LTR but demonstrated different reactivating potential in J-Lat A2 cells. This indicates that the target region is not the sole determinant of gRNA function²³, and further research into the role of specific nucleotide content, gRNA orientation and mismatch tolerability in HIV LTR targeting is needed.

We found that the LNP formulation itself played a critical role in CRISPRa functionality upon LNP delivery, as well as the ratio of the three CRISPRa RNAs, albeit to a lesser extent. These findings pose interesting questions about the required dose of each of the three CRISPRa RNAs. Based on previous studies^{37,38} as well as our findings, delivery of RNA with mSM102-LNPs should result in a higher proportion of the RNA reaching the cytosol – a critical step towards the subsequent assembly of the CRISPRa complex and function. The stark contrast observed in CRISPRa functionality in J-Lat A2 cells using the mSM102 *versus* the MC3 LNP formulation thus suggests that the amount of intracellularly delivered CRISPRa RNAs is a key bottleneck towards transfection. Yet, we observed that MC3 LNPs can deliver a reporter mRNA to T cell lines with high efficiency, leading to clearly detectable protein expression in > 90% of T cells at doses as low as 16 ng. In contrast, MC3 CRISPRa LNPs exhibited negligible functionality even at a 250 ng dose. The combined delivered dose, in summary, may not explain our findings fully.

Further answers may lie in the individual delivered doses of each of the three CRISPR RNA constructs – the dCas9-VP64 mRNA, MS2-p65-HSF1 mRNA and gRNA. A recent study by Beyersdorf *et al.* using dCas9-VPR showed that the co-encapsulation of a larger dCas9-fusion mRNA and a smaller gRNA into one LNP is biased towards the gRNA, thus leading to lower than predicted doses of the dCas9-fusion protein delivered to cells³⁹. These findings potentially translate to our attempts to co-encapsulate the larger dCas9-VP64 and MS2-p65-HSF1 mRNAs (~4,800 nt and ~1,800, respectively) with the smaller gRNA (~200 nt). One interpretation is that the delivered doses of specifically dCas9-VP64, or to a lesser extent the MS2-p65-HSF1, are the key bottleneck to CRISPRa functionality – rather than gRNA stability³⁶. This hypothesis would be in line with our findings that increasing the amount of gRNA co-encapsulated into mSM102-LNPs did not further enhance CRISPRa potency – if anything, we observed a slight reduction. No

possible explanation for the biasing of encapsulation towards smaller RNA constructs has yet been proposed; therefore, it remains elusive whether this effect is influenced by different LNP formulations harbouring different ionizable lipids, and thus whether this could prove a further explanation for the differences observed between MC3- and mSM102-LNPs in our study. Further studies optimizing the ratio of co-encapsulating, particularly using an increasing amount of larger RNAs, could therefore be of great interest. The intra-assay variation observed in the potency of mSM102 gRNA_L CRISPRa-LNPs (Figure 9b and d) further suggests that the co-packaging of three CRISPR RNAs will require ongoing optimization. As the encapsulation of single RNA constructs was robust throughout this study, we hypothesize that there may well be a significant effect from the interaction between differently-sized RNA constructs.

This study did not assess the potency of either MC3- or mSM102-LNPs to transfect primary CD4⁺ T cells. Given the strong differences observed in CRISPRa functionality between MC3- and mSM102-LNPs in T cell lines, achieving efficient RNA delivery to the cytosol of primary CD4⁺ T cells is of critical importance for the further development of CRISPRa as an LRA. Additionally, studies into the toxicity, cellular stress and activation caused by RNA-LNP delivery to primary CD4⁺ T cells will have to be performed.

Our findings furthermore highlight the importance of mRNA engineering to achieve high protein expression. The key differences between our first- and second-generation mRNAs were the incorporation of 5' and 3' UTRs, and the use of a Cap1 structure as opposed to a Cap0. While the general importance of the UTRs for mRNA stability is well known, the detailed effects of the exact UTR sequence on mRNA translatability are only starting to be studied⁴⁰. Careful screening and optimization of these UTR sequences could thus be an avenue towards even more potent mRNA-LNP-based therapeutics. Furthermore, codon optimization of the coding region combined with chemical base modifications has been shown to improve mRNA translatability and reduce immune activation through toll-like receptor signalling⁴¹⁻⁴⁶. These steps in mRNA engineering will undoubtedly need to be explored in the further translation of CRISPRa-LNPs towards *in vivo* models of disease.

Further work will be required to validate the CRISPRa-LNPs in near full-length cell line models of HIV latency as well as *ex vivo* CD4⁺ T cells from PLWH on ART. These models will allow the assessment of the proposition that CRISPRa-mediated latency reversal is potent enough to induce the elimination of the intact proviral reservoir through viral cytopathic effects^{25,26}. Initial studies

should focus on using a series of reverse transcriptase droplet digital PCR (RT-ddPCR) assays described by Yukl *et al.* to measure the existing blocks to complete proviral transcription and the ability of CRISPRa to overcome these blocks⁴⁷. Then, elimination of the latent reservoir through viral cytopathic effects can be assessed through the quantification of intact proviral DNA⁴⁸. One strategy towards further enhancing CRISPRa functionality – particularly in *ex vivo* cells from PLWH on ART – that has not been explored in this study, is the use of multiplexed gRNAs. Multiplexing would have several advantages over the use of a single gRNA, as it tackles concerns around HIV LTR sequence heterogeneity^{26,30} as well as potentiates the recruitment of several CRISPRa complexes to the same LTR, thus leading to even greater transcriptional activation.

In conclusion, our study presents very promising first steps towards a clinically translatable CRISPRa-based therapeutic for an HIV cure through the use of LNPs. Further work will be required to assess the potential of this therapeutic in more representative models of HIV latency.

Supplementary material Chapter 4

Table S1. Primer sequences and application.

1 st generation mRNA synthesis; overhang PCR			
Template	Sequence		
pLenti6-mCherry	Fw	5'- TACTGTAATACGACTCACTATAGGGAGAGCCACCATGGTGAGCAAGGGCGAG-3'	
	Rv	5' TTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTCTAGACTCGAGGCTTGTACAG-3'	
NL4-3 ΔEnv EGFP	Fw	5'-TACTGTAATACGACTCACTATAGGGAGAGCCACCATGGAGCCAGTAGATCCTAGACTAG-3'	
	Rv	5'-TTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTACTGCTTTGATAGAGAAG-3'	
2 nd generation mRNA synthesis; cloning into pMH			
Template	Sequence	RE	
pLenti6-mCherry	Fw	5'-CCTGAAAGTACTGCCAAGATGGTGAGCAAGGGCGA-3'	Scal
	Rv	5'-GGGTGAGAATTCTAGACTCGAGGCTTGT-3'	EcoRI
NL4-3 ΔEnv EGFP	Fw	5'-CCTGAAAGTACTGCCAAGATGGAGCCAGTAGATCC-3'	Scal
	Rv	5'-GGGTGAGAATTCTAATTACTGCTTTGATAGAGAAG-3'	EcoRI
pLenti_MS2-P65-USE1_CFB	Fw	5'-CCTGAATGCGCAGCCAAGATGGCTTCAAACCTTTAC-3'	FspI
	Rv	5'-GGGTGATCTAGATTAGGAGACAGTGGGGT-3'	XbaI
pLenti_dCAS-VP64_Blast	Fw	5'-CCTGAATGCGCAGCCAAGATGAAAAGGCCGGCGGC-3'	FspI
	Rv	5'-GGGTGATCTAGATTAGTTAATCAGCATGT-3'	XbaI
Fw, forward primer; RE, restriction enzyme used; Rv, reverse primer			

Fw, forward primer; RE, restriction enzyme used; Rv, reverse primer

Table S2. Overview of gRNA sequences and origin.

gRNA	Sequence	Reference
A	5'-CCGCCTAGCATTTTCATCACA-3'	35
B	5'-CCATGTGATGAAATGCTAGG-3'	35
C	5'-TGCTACAAGGGACTTTCCGC-3'	35
L	5'-CATGGCCCGAGAGCTGCATC-3'	25
O	5'-GCTACAAGGGACTTTCCGCT-3'	25
scr	5'-GCACTACCAGAGCTAACTCA-3'	

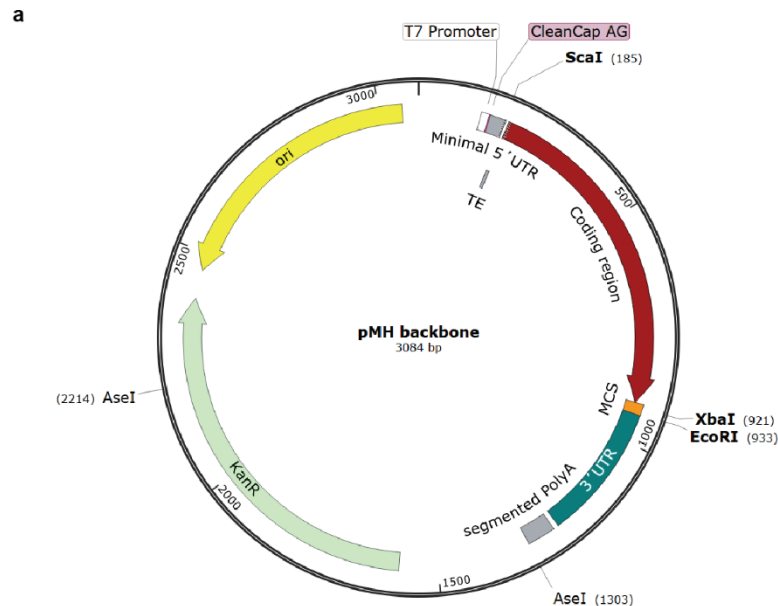


Figure S1. Plasmid map of pMH backbone. T7 promotor, translation enhancer (TE), 5' and 3' UTR, segmented poly-A sequence and ScaI and EcoRI/XbaI restriction sites for cloning are indicated.

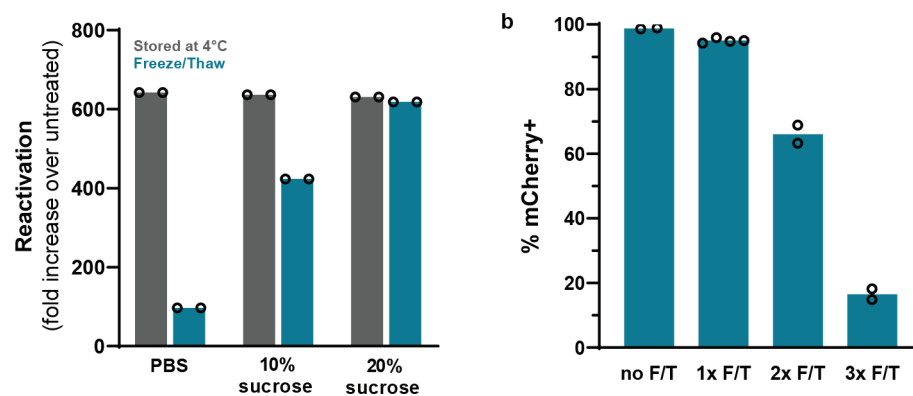


Figure S2. LNP functionality is preserved during one freeze/thaw cycle upon addition of sucrose as a cryoprotectant. (a) HIV Tat-LNPs were prepared in PBS, PBS + 10% sucrose or PBS + 20% sucrose, then stored either at 4°C overnight or frozen and stored at -80°C overnight, then thawed prior to use. LNP functionality after different storage conditions was assessed following treatment of TZM-bl cells with 25 ng of each LNP formulation for 24 hours. Reactivation of LTR-mediated transcription was quantified by luciferase expression. (b) mCherry-LNPs were prepared in PBS + 20% sucrose and maintained at 4°C or exposed to one, two or three freeze/thaw (F/T) cycles. LNP functionality was then determined by treating Jurkat T cells with 125 ng of each LNP formulation and measuring the percentage of mCherry-expressing cells using flow cytometry. Bars and replicate datapoints shown of a single experiment.

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Chapter 5

Optimizing lipid nanoparticle formulations towards reaching primary T cells *in vivo*

P.M. Ceva¹, S. Kan², A. Tan², M. Holz¹, D. Purcell¹, A. Johnston³, C. Pouton³, T. Payne³, D.L. Furtado⁴, F. Caruso⁴, M. Roche², S.R. Lewin^{2,5,6}

¹ Department of Microbiology and Immunology, The University of Melbourne at The Peter Doherty Institute for Infection and Immunity, Melbourne, VIC, Australia

² Department of Infectious Diseases, The University of Melbourne at The Peter Doherty Institute for Infection and Immunity, Melbourne, VIC, Australia

³ Monash Institute of Pharmaceutical Sciences, Monash University, Parkville, VIC, Australia

⁴ Department of Chemical Engineering, The University of Melbourne, Melbourne, VIC, Australia

⁵ Victorian Infectious Diseases Service, Royal Melbourne Hospital at The Peter Doherty Institute for Infection and Immunity, Melbourne, VIC, Australia

⁶ Department of Infectious Diseases, Alfred Hospital and Monash University, Melbourne, VIC, Australia

Introduction

T cells are of significant interest as an *in vivo* therapeutic target for the management of T-cell malignancies, the development of immunotherapies including CAR-T therapy, or the treatment of T-cell tropic infectious diseases^{1,2}. In each of these, nanoparticle-based delivery of the relevant therapeutics could have high clinical potential. In Chapter 4, we specifically demonstrated proof of concept of the use of lipid nanoparticles (LNPs) to deliver a CRISPR-based therapeutic towards the elimination of the latent HIV reservoir. For such nanoparticle-based therapeutics to be clinically viable, they require rational engineering to maximize successful transfection of T cells *in vivo*.

In this regard, three factors are of importance. First, the low endocytic nature of primary T cells³ necessitates the use of active targeting to induce nanoparticle internalization upon encountering a T cell target. Second, the efficiency of endosomal escape – a pre-requisite to the release of functional therapeutic cargo to the cytoplasm – is to be maximized to reduce the overall required dose, thereby limiting nanoparticle-induced toxicity and immunogenicity⁴. Third, successful targeting is directly linked to the successful avoidance of off-target cells – in other words, the stealth capacity of the nanoparticle delivery system⁵.

Therefore, to further any efforts towards the use of LNPs for the *in vivo* delivery of mRNA to T cells, including the CRISPRa-LNP system discussed in Chapter 4, each of these factors need to be addressed. In this current work, we aimed to assess the role of various lipids in the LNP formulations in endosomal escape and stealth. Furthermore, in Chapter 3, we have shown that targeting CD3, CD2 or CD7 can potently enhance nanoparticle uptake into primary CD4⁺ T cells *in vitro* and improve T cell targeting in the presence of bystander immune cells. We therefore aimed to extrapolate these findings to LNPs by generating and evaluating antibody-conjugated LNPs.

We found that the LNP formulation used had dramatic effects on the T cell transfection efficiency through enhancing endosomal escape and increasing LNP stealth. We furthermore found that conjugation of targeting antibodies using copper-free strain-promoted alkyne-azide cycloaddition (SPAAC) between azide and dibenzocyclooctyne (DBCO) moieties was able to enhance LNP potency, yet further investigations into more robust conjugation approaches are needed.

Methods

Reporter mRNA

The HIV Tat and mCherry reporter mRNAs used were synthesized through *in vitro* transcription, characterized and stored as described in Chapter 4 for first-generation mRNA. For primary CD4⁺ T cell experiments, CleanCap® mCherry mRNA with fully substituted 5-Methoxy-U bases (5moU-mCherry mRNA) (L-7203-1000, TriLink Biotechnologies) was used.

LNP synthesis and characterization

Lipid nanoparticles encapsulating first-generation HIV Tat mRNA, first-generation mCherry mRNA or 5moU-mCherry mRNA were prepared as described previously in Chapter 4. LNPs were either formulated using SM102, DSPC, β -sitosterol and DMG-PEG2000 (mSM102-LNPs); or D-Lin-MC3-DMA (MC3), DSPC, Cholesterol and either DMG-PEG2000 (previously referred to as MC3-LNP, henceforth specified as MC3/DMG-LNPs), DSPE-PEG2000 (MC3/DSPE-LNPs) or DSPE-PEG2000-Azide (MC3/Az-LNPs). For fluorescently labelled LNPs, DiD (1,1'-dioctadecyl-3,3,3',3'-tetramethylindodicarbocyanine, 4-chlorobenzenesulfonate salt, Invitrogen) was added at a molar ratio of 0.2:100 of DiD:total lipids prior to microfluidic mixing. LNPs were characterized using dynamic light scattering and RiboGreen assay as described in Chapter 4, to measure LNP size, polydispersity and mRNA encapsulation efficiency, respectively. Average LNP sizes are reported as Peak 1 Means (nm) by Number ordered by area. LNPs were either stored at 4 °C for a maximum of 2 weeks or frozen at -80 °C in the presence of 20% w/v sucrose for cryoprotection.

Antibodies

Antibodies used for targeting were anti-CD3 (clone OKT3, 317326, Biolegend), anti-CD7 (clone 6B7, 343102, Biolegend) and anti-CD2 (clone 299812, MAB18561, R&D Systems). For optimization experiments, polyclonal mouse IgG (I5381, Sigma-Aldrich) was used. All targeting antibodies were unlabelled. Primary antibodies that were stored in a buffer containing sodium azide were buffer-exchanged into Phosphate Buffered Saline (PBS) using Zeba™ Spin Desalting Columns, 7K MWCO (89882, Thermo Fisher Scientific).

Functionalization of targeting antibodies with DBCO

DBCO-PEG4-NHS ester (BP-22288, Broadpharm) was reconstituted in anhydrous DMSO, aliquoted and stored at -30°C. To functionalize targeting antibodies with DBCO, 5 – 30 μ L aliquots of antibody solution were taken, to which 10% v/v sodium bicarbonate buffer, pH 8.5, was added

to increase the pH. DBCO-PEG4-NHS solution was then added at molar ratios of DBCO-PEG4-NHS to antibody of 100:1, 50:1, 20:1 or 5:1 and incubated for 1 hour at room temperature on a thermoshaker. The percentage of DMSO diluent in the final solution was kept below 10%. DBCO-functionalized antibodies were then purified from unreacted DBCO-PEG4-NHS reagent using pre-equilibrated Zeba™ Spin Desalting Columns, 7K MWCO. For optimization of antibody labelling using NHS-ester derivatives, DBCO-PEG4-NHS was replaced with Alexa Fluor 488 NHS ester (A20000, Invitrogen). Dye-only controls excluding the antibody from the reaction were used to assess any flow-through of unreacted dye in the filtrate upon purification of the antibodies using Zeba™ Spin Desalting Columns. The degree of labelling (DOL) was determined by measuring absorbance at 280 nm (protein) and 488 nm (dye) using a Nanodrop One. If flow-through of unreacted dye in the filtrate was detected, a corrected DOL was calculated by subtracting the absorbance at 488 nm in the dye-only control from that in the corresponding antibody-dye condition prior to DOL calculation.

Assessment of degree of labelling of functionalized antibodies

To measure the DOL of DBCO-functionalized targeting antibodies, antibodies were incubated overnight at 4°C with Alexa Fluor 647 Azide, Triethylammonium Salt (A10277, Invitrogen), reconstituted in anhydrous DMSO, at a molar ratio of 5:1 Azide to antibody. Labelled antibodies were purified from unreacted dye using Zeba™ Spin Desalting Columns, 7K MWCO. Dye-only controls excluding the antibody from the reaction were used to assess any flow-through of unreacted dye in the filtrate upon purification of the antibodies using Zeba™ Spin Desalting Columns. The degree of labelling (DOL) was determined by measuring absorbance at 280 nm (protein) and 633 nm (dye) using a Nanodrop One, as a measure of the degree of DBCO labelling per antibody. If flow-through of unreacted dye in the filtrate was detected, a corrected DOL was calculated by subtracting the absorbance at 633 nm in the dye-only control from that in the corresponding antibody-dye condition prior to DOL calculation. To assess whether DBCO-functionalization interfered with the antigen-binding capacity, Jurkat T cells were incubated with equal concentrations of functionalized and non-functionalized antibody, then stained with a PE-conjugated anti-mouse secondary antibody (P852, Invitrogen) and analysed using flow cytometry.

Conjugation of targeting antibodies to LNP

To conjugate DBCO-functionalized antibody to LNPs using copper-free click chemistry, MC3/Az-LNPs were incubated with DBCO-functionalized antibody at ratios of 1:200, 1:2,000 or 1:20,000

LNP : Ab for 1 hour at room temperature on a thermoshaker, then overnight at 4°C. After antibody conjugation, LNPs were always stored at 4°C and used within one week of conjugation. To assess conjugation of the DBCO-functionalized antibody to the LNP, antibody retention was assessed using SDS-PAGE. To this end, antibody/LNP complexes were run on non-denaturing 6% polyacrylamide gel for 20 min at 100V, then 60 min at 120V before staining with Coomassie blue to visualize unbound antibody. A reduction in the unbound antibody is indicative of antibody retention through binding to the LNP, which cannot migrate into the gel; thus suggesting conjugation to the LNP occurred.

Cell culture

The human Jurkat T lymphocyte cell line (clone JE6.1) and daughter cell line J-Lat LTR-Tat-IRES-GFP clone A2 (NIH HIV Reagent Program) were cultured in RPMI 1640 medium containing 10% heat-inactivated fetal calf serum (FCS), supplemented with 100 U/mL penicillin, 100 µg/mL streptomycin and 2 mM L-glutamine (RF10) in a humidified 37°C, 5% CO₂ incubator.

For primary CD4⁺ T cell experiments, peripheral blood mononuclear cells (PBMC) were isolated from buffy coats obtained from the Australian Red Cross Blood Service *via* Ficoll-Paque (GE Healthcare, Champaign, Illinois) density centrifugation. CD4⁺ T cells were subsequently isolated through negative magnetic-activated cell sorting using a CD4⁺ T cell isolation kit (130-096-533, Miltenyi Biotec). PBMC and isolated total CD4⁺ T cells were frozen in FCS supplemented with 10% DMSO and stored in liquid nitrogen until use. Prior to usage, total CD4⁺ T cells were thawed, then either activated using 3 µg/mL plate-bound anti-CD3 (317326, Biolegend) and 2 µg/mL soluble anti-CD28 (302934, Biolegend) in RF10 supplemented with 10 U/mL IL-2 for three days or rested in RF10 supplemented with IL-2. For incubations of primary CD4⁺ T cells with mSM102-LNPs, cells were rested overnight instead of for 72 hours. Subsequent experimental incubations with LNPs were performed in the continued presence of 10 U/mL IL-2.

Transfections

For LNP treatment of suspension T cell lines and primary CD4⁺ T cells, 1 × 10⁵ cells were plated in 100 µL of RF10 or RF10 supplemented with 10 U/mL IL-2 in 96-well round-bottom plates on the day of treatment. LNPs were dosed according to the total RNA concentration as per the RiboGreen assay, then added to cells and mixed through to ensure an even distribution of cells and LNPs in suspension. Cells were harvested after 24 or 72 hours, then stained with LIVE/DEAD™ Fixable

Violet Dead Cell Stain (L34955, Thermo Fisher Scientific) after harvest to allow gating on live cells during subsequent analysis and fixed in PBS with 1% paraformaldehyde (PFA) prior to flow cytometry analysis using an LSR Fortessa II (BD Biosciences). All cell line transfections were performed with LNPs encapsulating first-generation HIV Tat or mCherry mRNA; all primary cell experiments were performed with LNPs encapsulating 5moU-mCherry mRNA.

Analysis

Flow cytometry analysis, compensation and cell subset gating was performed using FCS Express 7. Data were further analysed and graphed using GraphPad Prism 9.

Results

Pre-activation of primary CD4⁺ T cells is required for successful mCherry mRNA delivery using MC3/DMG-LNPs

We first assessed whether standard MC3/DMG-LNPs were able to transfect non-stimulated primary CD4⁺ T cells. To this end, CD4⁺ T cells were isolated through negative isolation from PBMCs and incubated with mCherry MC3/DMG-LNPs for 72 hours (Figure 1a, top). At the highest dose tested of 500 ng, mCherry expression was only detected in 2% of live cells (Figure 1b), despite clearly detectable association of mCherry and empty control MC3/DMG-LNPs (Figure 1c). At the lower dose of 125 ng, we interestingly found a higher level of association despite an even lower transfection efficiency of < 1%. The toxicity associated with MC3/DMG-LNP treatment was modest, with both empty and mCherry MC3/DMG-LNPs yielding viabilities of 62%-68% compared to a baseline viability of 84% (untreated control cells) or 81%-82% (vehicle control-treated cells) (Figure 1d).

We next asked if the transfection efficiency would increase upon pre-stimulation of the CD4⁺ T cells with anti-CD3/anti-CD28 antibodies (Figure 1a, bottom). We found substantially higher transfection efficiencies of 22% and 28% for the 125 ng and 500 ng dose, respectively (Figure 1e), compared to non-stimulated T cells. These higher transfection efficiencies coincided with stronger LNP association, as indicated by a >10-fold higher MFI of the DiD dye (Figure 1f) compared to that observed in non-stimulated cells (Figure 1c). However, treatment of pre-stimulated CD4⁺ T cells with MC3/DMG-LNPs resulted in greater toxicity than in non-stimulated cells (Figure 1g). This toxicity appeared to be both dose-responsive as well as correlate with mRNA delivery or

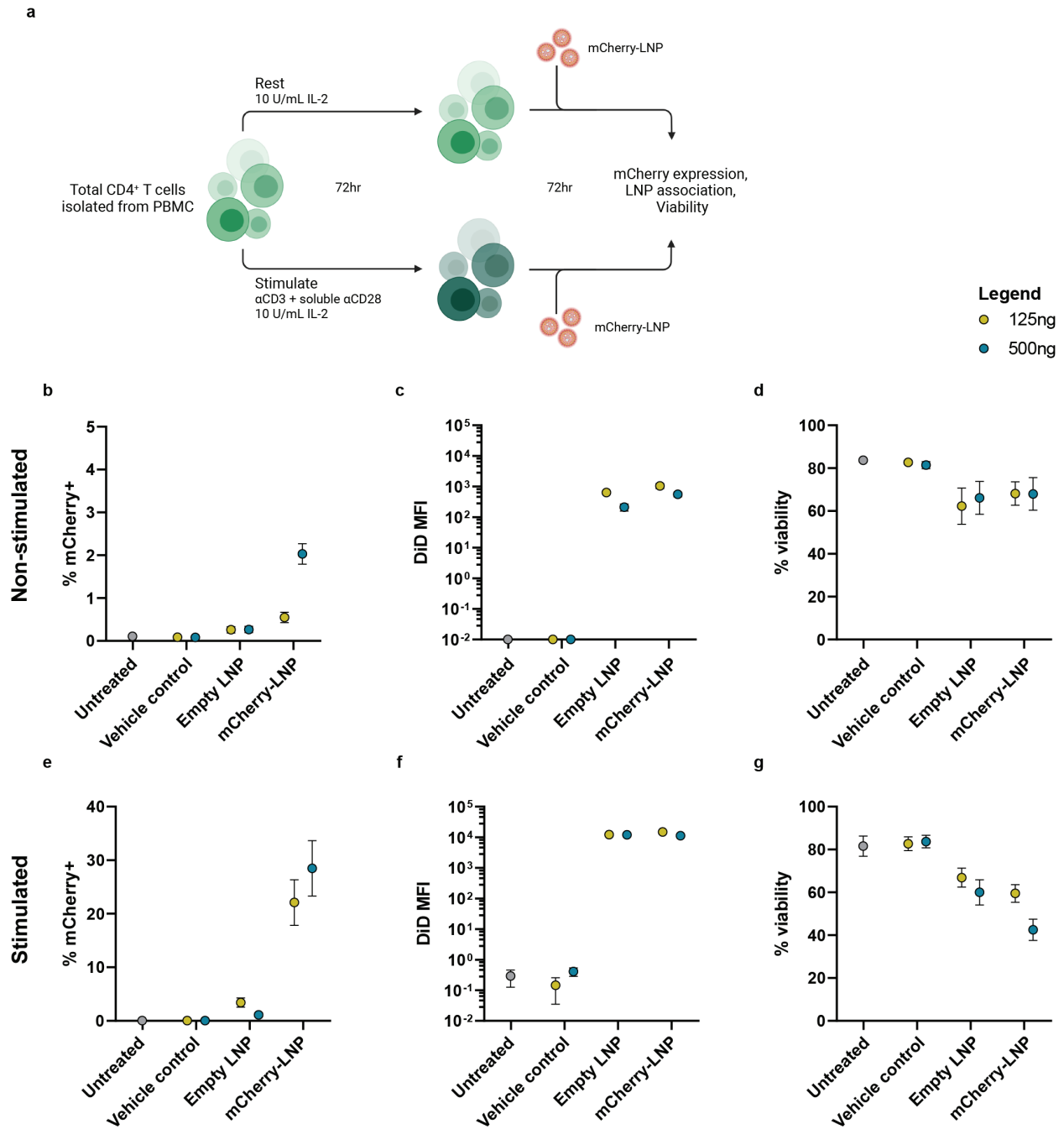


Figure 1. MC3/DMG-LNPs can deliver mRNA to stimulated, but not unstimulated CD4⁺ T cells. (a) Schematic overview of experimental set-up. Total CD4⁺ T cells were isolated from PBMC, then either stimulated with plate-bound anti-CD3 (αCD3) and soluble anti-CD28 (αCD28) or rested for 72 hours, both in the presence of 10 U/mL IL-2. Then, cells were incubated with mCherry MC3/DMG-LNPs or control LNPs for another 72 hours prior to harvest and read-out of mCherry expression, LNP association and viability using flow cytometry. (b-g) Transfection efficiency (b, e), LNP association (c, f) and viability (d, g) measurements of stimulated (b-d) or non-stimulated (e-g) CD4⁺ T cells after 72 hours with mCherry MC3/DMG-LNPs. Note the different scale on the y-axes in (b) and (e). Cells treated with empty MC3/DMG-LNPs, vehicle control-treated cells and untreated cells were included as controls. Datapoints represent means ± SEM of n=4 independent donors.

subsequent protein expression, as suggested by the lower viability observed after mCherry MC3/DMG-LNP treatment compared to empty control MC3/DMG-LNP treatment (42% *cf.* 60%, respectively, at 500 ng doses). Overall, these data show that successful mRNA delivery using MC3/DMG-LNPs to primary CD4⁺ T cells is dependent on the activation state of the T cells.

mSM102-LNPs are able to transfect primary CD4⁺ T cells in the absence of pre-stimulation at high potency and minimal toxicity

The successful delivery of functional mRNA to pre-stimulated primary CD4⁺ T cells is encouraging and of great interest for many biomedical applications. However, a 'shock-and-kill'-based therapeutic towards an HIV cure needs to be delivered to the latent HIV reservoir, the majority of which consists of resting CD4⁺ T cells^{6,7}. As demonstrated and discussed in Chapter 4, mSM102-LNPs harbouring the SM102 ionizable lipid and the cholesterol analogue β -sitosterol exhibited superior potency over MC3/DMG-LNPs in T cell lines which we hypothesised was due to greater endosomal escape. As such, we aimed to assess the potency of mSM102-LNPs in non-stimulated primary CD4⁺ T cells. First, we treated non-stimulated CD4⁺ T cells with mCherry mSM102-LNPs at doses between 100 – 500 ng. After 72 hours, we found an astonishing transfection efficiency of up to 95% of live cells (Figure 2a). All doses tested resulted in transfection efficiencies of > 87%, with 400 ng and 500 ng of mCherry mSM102-LNPs yielding 92% and 87% transfection efficiencies compared to 94-95% for doses of 300 ng and below. This inverse relationship between transfection efficiency and dose was potentially related to toxicity at the higher doses, given that cells treated with mSM102-LNPs had viabilities between 67 – 75% compared to a viability of 83% for untreated control cells (Figure 2b).

To assess the potency of mSM102-LNPs further, we treated non-stimulated CD4⁺ T cells with a reduced dose of LNPs. At lower doses, a dose-titration effect could clearly be observed, with the transfection efficiency dropping to 60% at 25 ng and further to 6% at 3.1 ng mCherry mSM102-LNPs (Figure 2c). Moreover, toxicity was negligible at any of the doses tested compared to untreated control cells (Figure 2d). These findings indicate the importance of endosomal escape for the delivery of therapeutic mRNA to primary T cells. To our knowledge, this is the first demonstration of a transfection method exhibiting such potency and tolerability in non-stimulated primary CD4⁺ T cells. Our findings are currently being assessed by our business development office to establish a patent on this discovery.

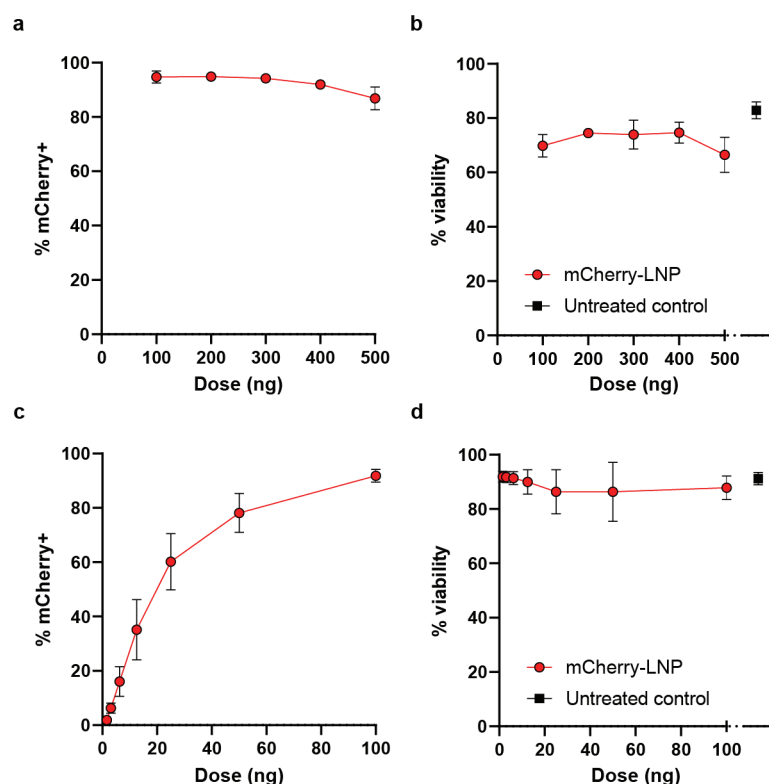


Figure 2. mSM102-LNPs potentially deliver mRNA to non-stimulated T cells with minimal toxicity. (a-d) Non-stimulated CD4⁺ T cells were treated with doses of 100 ng to 500 ng (a, b) or 1.6 ng to 100 ng (c, d) mCherry mSM102-LNPs and mCherry expression (a, c) and viability compared to untreated cells (b, d) was assessed after 72 hours. Datapoints represent mean $\pm$ SEM of $n=2$ independent donors. Data in (a, b) and (c, d) represent independent experiments, but were performed using the same $n=2$ donors.

Incorporation of DSPE-PEG2000 lipid reduces potency of MC3-LNPs

To potentiate successful transfection of CD4⁺ T cells *in vivo*, we next investigated further changes to the LNP formulation, for two reasons. First, to avoid scavenging of LNPs by bystander immune cells in the bloodstream, enhancing stealth characteristics is of great interest. The PEG lipid in the LNP formulation is the key determinant of LNP stealth characteristics⁸. In this regard, the DMG-PEG2000 lipid present in both MC3/DMG-LNPs and mSM102-LNPs has been shown to rapidly diffuse out of the LNP when introduced into biological fluids⁹. In contrast, the incorporation of PEG lipids with longer acyl chains, including DSPE-PEG2000, leads to stronger anchoring of the PEG lipid in the LNP, thus preventing such PEG-shedding and increasing stealth characteristics of the LNP⁹. Second, conjugation of targeting ligands is traditionally done through click-chemistry reactions between the ligand and a functionalized DSPE-PEG2000 lipid. For example, targeting antibodies can be functionalized with a PEG4-linker and DBCO group through NHS chemistry, followed by conjugation of said antibody to a DSPE-PEG2000-Azide-containing LNP through copper-free strain-promoted alkyne-azide cycloaddition (SPAAC)¹⁰. As such, to explore the effect

of exchanging DMG-PEG2000 with DSPE-PEG2000, we prepared MC3/DMG- and MC3/DSPE-LNPs. We found that while empty MC3/DSPE-LNPs exhibited a slightly larger size than MC3/DMG-LNPs, the sizes of both Tat mRNA-loaded LNPs exhibited great similarity (Figure 3a,b). The mRNA encapsulation efficiency was also consistent between both, with average encapsulation efficiencies of 97% and 96% for MC3/DMG- and MC3/DSPE-LNPs, respectively. However, when treating J-Lat A2 cells with equal doses of both Tat-loaded LNPs, much lower reactivation was observed upon MC3/DSPE-LNP compared to MC3/DMG-LNP treatment (Figure 3c). Cellular toxicity was largely absent for both LNP formulations (Figure 3d), indicating a difference in the potency of mRNA delivery between MC3/DMG- and MC3/DSPE-LNPs. We interpret these findings as indicative of the higher stealth characteristics of DSPE-PEG2000-containing LNPs, although specific data on LNP association would further back up these findings. Thus, while not desirable for *in vitro* transfections of T cell lines or primary CD4⁺ T cells, incorporating DSPE-PEG2000 into LNP formulations designed for *in vivo* mRNA delivery to T cells could prove advantageous.

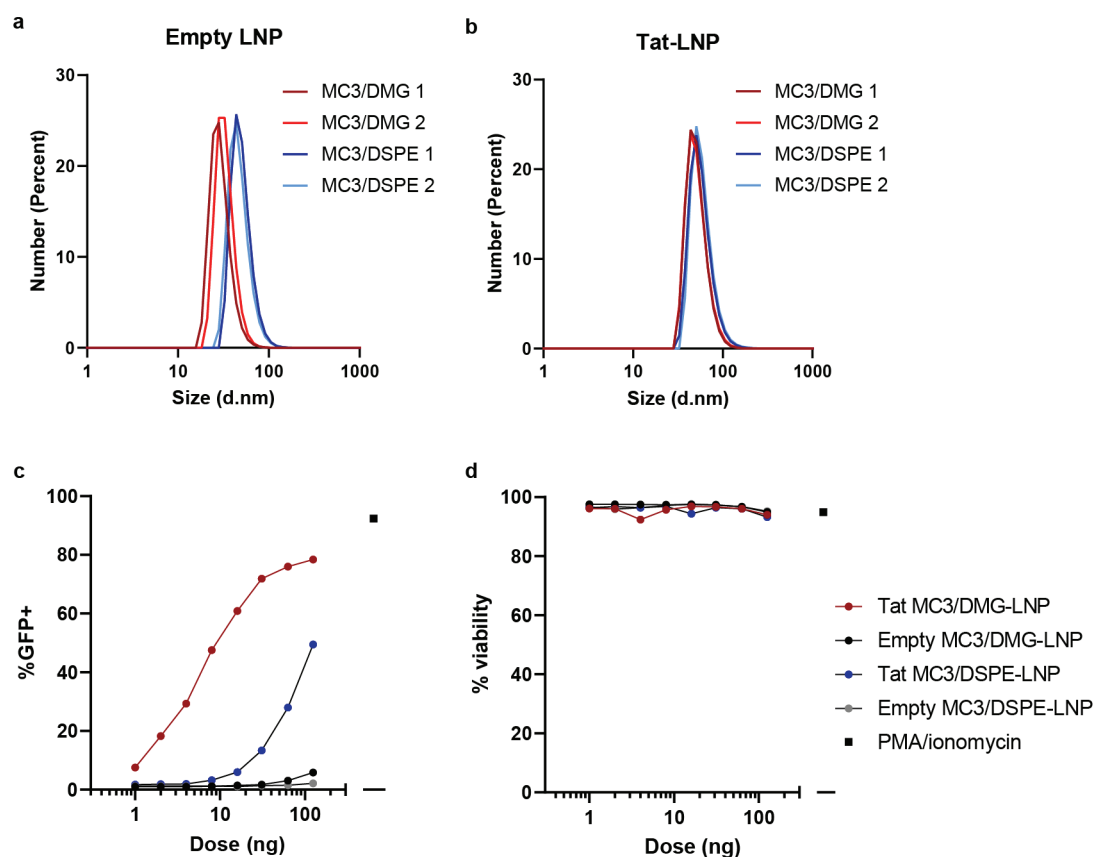


Figure 3. MC3/DSPE-LNPs exhibit similar size but distinct potency to MC3/DMG-LNPs. (a-b) Dynamic light scattering results of two independently formulated batches of empty (a) and Tat (b) MC3/DMG-LNPs and MC3/DSPE-LNPs. (c, d) J-Lat A2 cells were treated with various doses of empty or Tat MC3/DMG-LNPs or MC3/DSPE-LNPs. PMA/ionomycin-treated cells were included as a control. After 24 hours, GFP expression (c) and viability (d) were assessed using flow cytometry. Results from a single experiment are shown.

Targeting antibodies can be functionalized with DBCO without loss of function

We next aimed to functionalize potential targeting antibodies with the clickable DBCO functional group. To this end, targeting antibodies were incubated with the amine-reactive NHS-PEG4-DBCO, yielding covalent conjugation of the DBCO group on a PEG4 linker (Figure 4). To optimize this NHS-activated crosslinking reaction, the NHS-PEG4-DBCO reagent was replaced with Alexa Fluor 488 NHS, allowing subsequent assessments of the degree of labelling (DOL) through UV-Vis spectroscopy (Figure 5a). The DOL measurements were corrected for any residual flow-through of unreacted dye into the antibody fraction. We found that incubation of antibody with NHS reagent at a molar ratio of 1:20 yielded a corrected DOL of just above one (Figure 5b), which was deemed optimal; a DOL below one would require the addition of excess antibody to the LNP as not all antibodies would be DBCO-functionalized, whereas a high DOL would increase the risk of inducing LNP-crosslinking. We then proceeded to functionalize antibodies with NHS-PEG4-DBCO, after which the functional degree of labelling was assessed using an Alexa Fluor 647 Azide dye (Figure 5c,d).

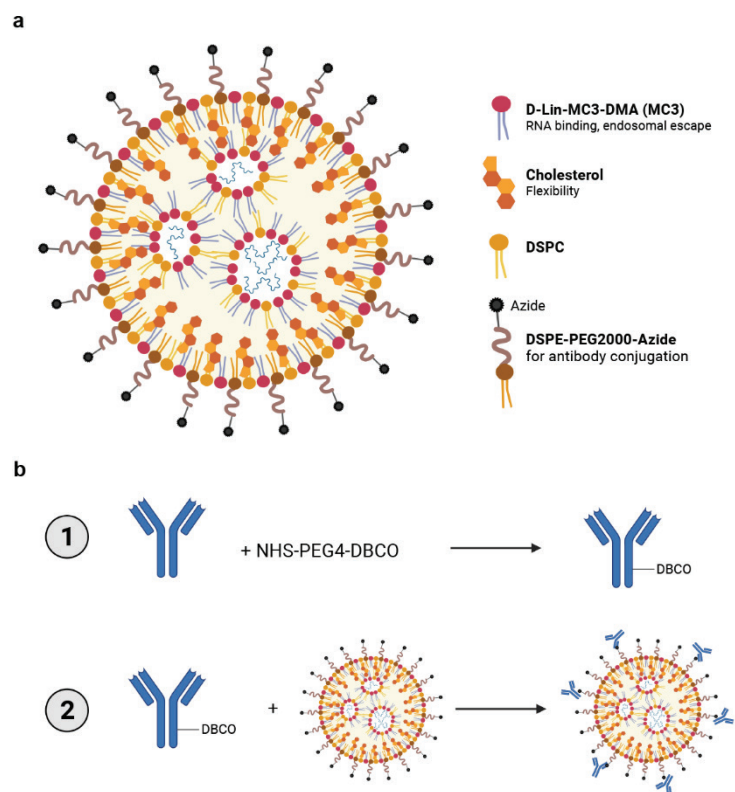


Figure 4. Antibody-LNP conjugation strategy using copper-free click chemistry. (a) Schematic overview of LNP formulation for antibody conjugation. To prepare LNPs with functional azide groups for click chemistry, the DMG-PEG2000 lipid was replaced with DSPE-PEG2000-Azide (MC3/Az-LNP). (b) Schematic overview of antibody functionalization with DBCO groups (1) and subsequent click chemistry reaction of DBCO-functionalized antibody with MC3/Az-LNPs (2).

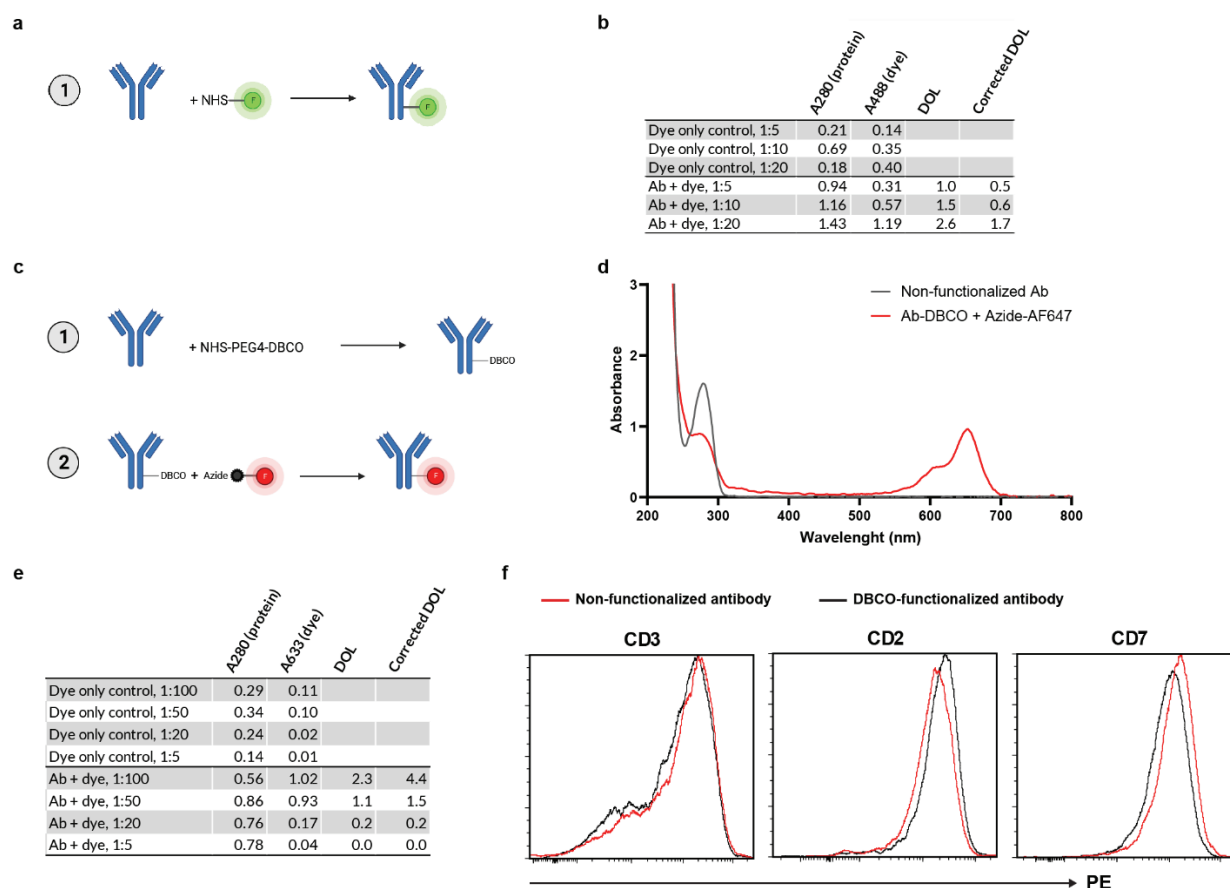


Figure 5. Optimization of DBCO-labelling of targeting antibodies. (a, b) Schematic overview of experimental set-up to optimize antibody-NHS chemistry conditions (a). Polyclonal mouse IgG was incubated with Alexa Fluor 488 NHS ester at various molar ratios (1:5, 1:10, 1:20), then purified to remove any unreacted dye. Dye-only solutions containing equivalent amounts of the dye in PBS were included as controls. The degree of antibody labelling (DOL) and corrected DOL were then determined by measuring absorbance at 280 nm (protein) and 488 nm (dye) using UV-Vis spectroscopy (b). (c-e) Schematic overview of experimental set-up to determine the degree of antibody functionalization with the functional DBCO group (c). Polyclonal mouse IgG was incubated with NHS-PEG4-DBCO at various molar ratios (1:5, 1:20, 1:50, 1:100), then purified to remove any unreacted reagent. Then, the DBCO-functionalized antibody was incubated with Alexa Fluor 647 Azide, followed by a second round of purification to remove unreacted dye. Dye-only solutions containing equivalent amounts of the dye in PBS were included as controls. The degree of antibody labelling (DOL) and corrected DOL were then determined by measuring absorbance at 280 nm (protein) and 633 nm (dye) using UV-Vis spectroscopy (d, e). (f) Validation of antigen-binding capacity of DBCO-functionalized antibodies. Jurkat T cells were stained with 2 µL of 1:20 DBCO-functionalized (black) or non-functionalized (red) CD3, CD2 or CD7 antibody, washed, then labelled with a PE-labelled anti-mouse secondary antibody. PE signal was measured using flow cytometry. Graphs depict representative flow plots from duplicate measurements.

This measurement provides an indication of the DBCO groups accessible for subsequent click-chemistry. We found that incubation of antibody with NHS-PEG4-DBCO at a molar ratio of 1:20 only yielded a corrected DOL of 0.2, prompting us to increase the molar ratio of NHS ester to antibody. Indeed, a corrected DOL of just above one was achieved using a molar ratio of antibody : NHS-PEG4-DBCO of 1:50 (Figure 5e). The potency of DBCO-functionalized targeting antibodies to stain target cells was found to be comparable to non-functionalized antibodies, suggesting the DBCO-functionalization did not significantly interfere with antigen binding (Figure 5f). The

discrepancy in optimal antibody : NHS-reagent ratio between NHS-dye and NHS-PEG4-DBCO could either be due to the NHS-reagent (Figure 5c, step 1), or be a consequence of the click chemistry reaction between DBCO and Azide (Figure 5c, step 2). As such, various molar ratios of Ab:DBCO labelling were taken forward for further testing.

Conjugation of antibodies to LNP through copper-free click chemistry leads to an increase in LNP size

To assess whether DBCO-functionalized antibodies could subsequently be conjugated to MC3/Az-LNPs, antibody retention was measured using SDS-PAGE. 10 µg of DBCO-functionalized mouse IgG (Ab:DBCO ratio of 1:50) was conjugated to empty MC3/Az-LNPs corresponding to an Ab:LNP ratio of 20,000:1. While free non-functionalized and DBCO-functionalized antibody could clearly be visualized through bands around 160 kDa, this band was not visible when equal amounts of the DBCO-functionalized mouse IgG were incubated with MC3/Az-LNPs, suggesting that the antibody was captured by the LNP, which could not migrate into the gel (Figure 6a). As SDS-PAGE is not quantitative and has limited sensitivity, this method did not allow for optimization of the Ab:LNP ratio, nor for further assessment of the optimal Ab:DBCO ratio for conjugation.

We next used dynamic light scattering to qualitatively assess antibody conjugation through detecting an increase in LNP size upon antibody decoration of the LNP surface. Indeed, antibody-conjugated LNPs exhibited an increased average size of 169 nm compared to 55 nm for untargeted control LNPs, suggesting an effect of the addition of DBCO-functionalized antibody (Figure 6b). However, the size increase was greater than the ~30 nm that is reasonably expected from the addition of an antibody monolayer, raising concerns about the DBCO-functionalized antibodies inducing LNP crosslinking. However, the polydispersity index was reduced upon antibody conjugation, suggesting the observed size increase was not due to non-specific LNP aggregation (Figure 6b).

We then performed a screen of antibody-LNP conjugations using empty MC3/Az-LNPs, wherein both the Ab:DBCO molar ratio as well as the Ab:LNP ratio was varied. Antibody-LNP conjugations using non-functionalized antibodies were included to assess the role of non-specific absorption of antibodies onto the LNP surface. We found that at the lowest Ab:LNP ratio of 200:1, no increase in LNP could be observed upon antibody addition, irrespective of the presence and degree of DBCO functionalization (Figure 6c). Increasing the Ab:LNP ratio to 2,000:1 or back to 20,000:1 rescued

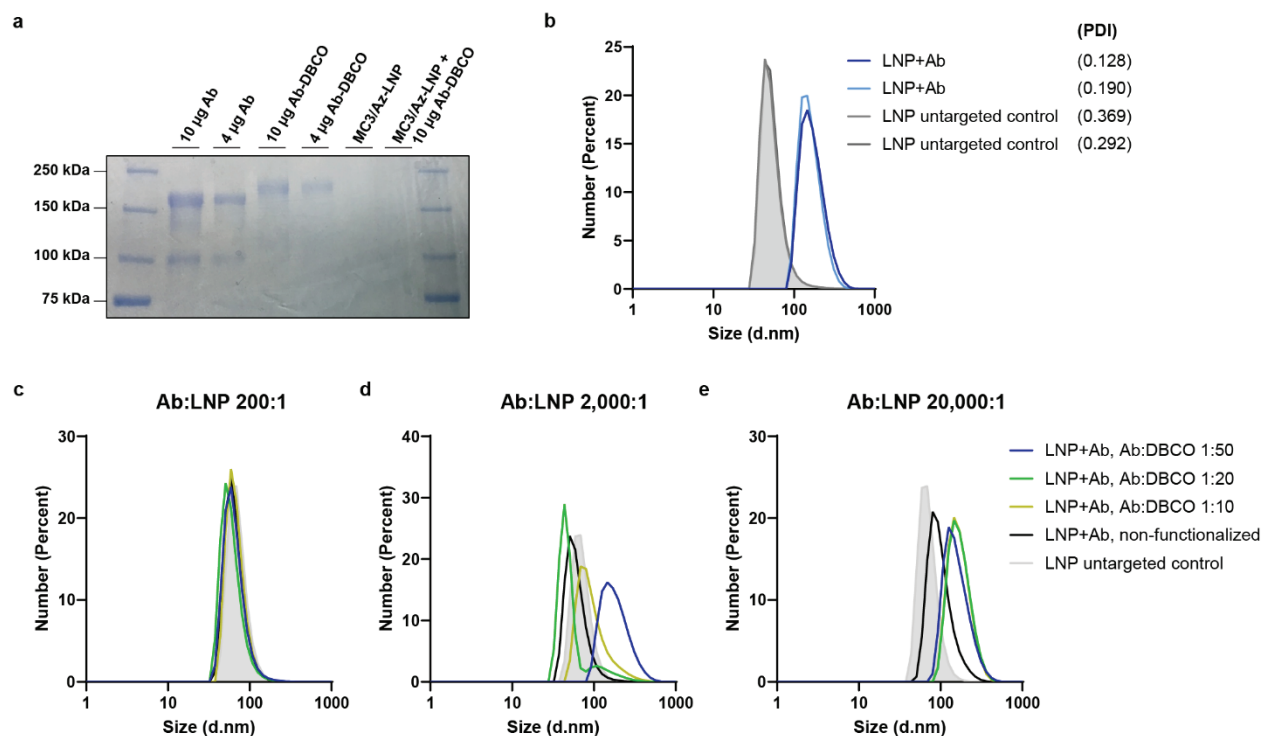


Figure 6. Antibody conjugation onto LNP surface can be detected through protein retention and size increase. (a) Successful conjugation of polyclonal mouse IgG onto MC3/Az-LNPs was assessed through detecting protein retention of the 160 kDa IgG molecule using SDS-PAGE. Free non-functionalized antibody (Ab), DBCO-functionalized antibody (Ab-DBCO) or MC3/Az-LNP alone were included as controls. (b) MC3/Az-LNPs were incubated with DBCO-functionalized mouse IgG (Ab:DBCO 1:50, Ab:LNP 1:20,000) in duplicate and the size of resulting LNP was compared to duplicate untargeted control LNPs using dynamic light scattering. (c-e) MC3/Az-LNPs were incubated with mouse IgG functionalized with DBCO at different molar ratios (1:50, blue; 1:20, green; 1:10, yellow) or non-functionalized mouse IgG (black) at antibody(Ab):LNP ratios of 200:1 (c), 2,000:1 (d) or 20,000:1 (e) and the size of resulting LNP was compared to untargeted control LNPs using dynamic light scattering.

the observed size increase (Figure 6d,e). Yet at the highest ratio, passive absorption of antibody appeared to occur, as indicated by an observed LNP size of 102 nm compared to 72 nm for untargeted control LNPs (Figure 6e). This effect was not detected at an Ab:LNP ratio of 2,000:1 (Figure 6d), which was therefore deemed optimal. At this Ab:LNP ratio, the size of the resulting antibody-conjugated LNPs correlated with the degree of DBCO labelling, with LNP sizes of 101 nm, 143 nm and 189 nm reported for Ab:DBCO ratios of 1:10, 1:20 and 1:50, respectively. Polydispersity indexes were constant at values between 0.19 – 0.23. Combining these with previous DOL findings, we selected an Ab:LNP ratio of 2,000:1 combined with Ab:DBCO ratios of 1:20 and 1:50 for further assessment.

CD3- and CD7-targeted LNPs exhibit greater potency in T cell lines, but protocol is not robust

We used the optimized conditions for antibody-conjugation to LNPs to generate Tat MC3/Az-LNPs targeted to CD3 or CD7, then assessed their transfection efficiency in J-Lat A2 cells. Cell line models were chosen over primary T cells for these initial experiments, as they provide a facile

and high-throughput read-out system. We found that targeting either receptor can enhance the potency of MC3/Az-LNPs by ~2.5-fold (Figure 7a,b). When using CD3-targeting antibodies, the enhanced potency was only observed when functionalizing the antibody at an Ab:DBCO ratio of 1:20, resulting in 54% GFP+ cells (Figure 7a), whereas for CD7 targeting, both 1:20 and 1:50 Ab:DBCO ratios resulted in potent GFP expression (58% and 60%, respectively) (Figure 7b). These increased transfection efficiencies coincided with increases in the LNP association of CD3- or CD7-targeted LNPs compared to untargeted control MC3/Az-LNPs (Figure 7c,d). The effect of CD3- and CD7-targeting on the transfection efficiency was, however, reduced to ~1.8-fold over untargeted control MC3/Az-LNPs when the DBCO-functionalized antibody was stored at 4°C for 1-2 weeks prior to conjugation to LNPs (Figure 7e,f), indicating low stability of these DBCO-antibody formulations.

To test the robustness of the antibody-conjugation protocol, we repeated the DBCO-functionalizations of CD3- and CD7-targeting antibodies (Ab:DBCO ratio of 1:20), subsequent conjugations to MC3/Az-LNPs and incubations with J-Lat A2 cells. We found that enhanced potency through CD3-targeting could be reproduced, albeit at different levels, on three

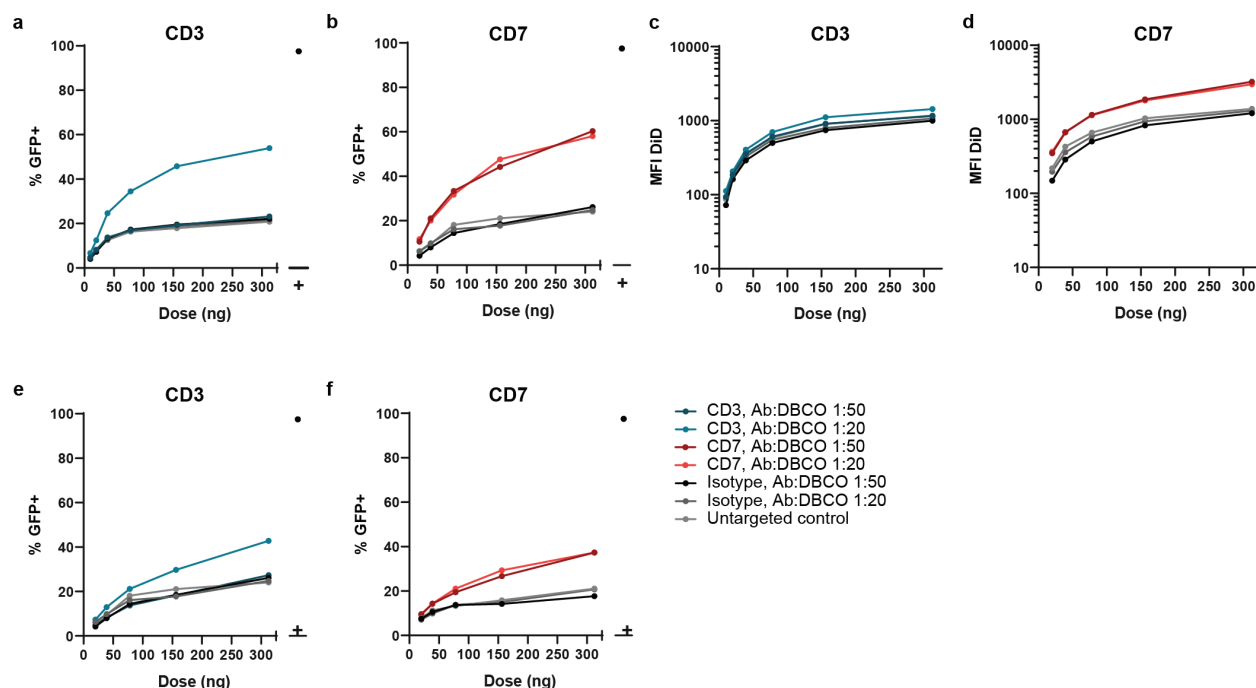


Figure 7. Targeting of LNPs to CD3 or CD7 can potentially increase reporter mRNA delivery to T cell lines. (a-d) J-Lat A2 cells were incubated with various doses of Tat MC3/Az-LNPs incubated with anti-CD3 (a, c) or anti-CD7 (b, d) antibodies functionalized with DBCO at molar ratios of 1:50 or 1:20. Tat MC3/Az-LNPs incubated with isotype control antibodies and untargeted Tat MC3/Az-LNPs were included as controls. PMA/ionomycin treatment was included as a positive control for the induction of GFP expression (+). After 24 hours, transfection efficiency (a, b) and LNP association (c, d) were measured using flow cytometry. (e, f) J-Lat A2 cells were incubated with various doses of CD3- (e) or CD7-modified (f) MC3/Az-LNPs or controls as in (a, b), however, DBCO-functionalized antibodies were stored at 4°C for two weeks prior to conjugation to LNPs. Data from single experiments are shown.

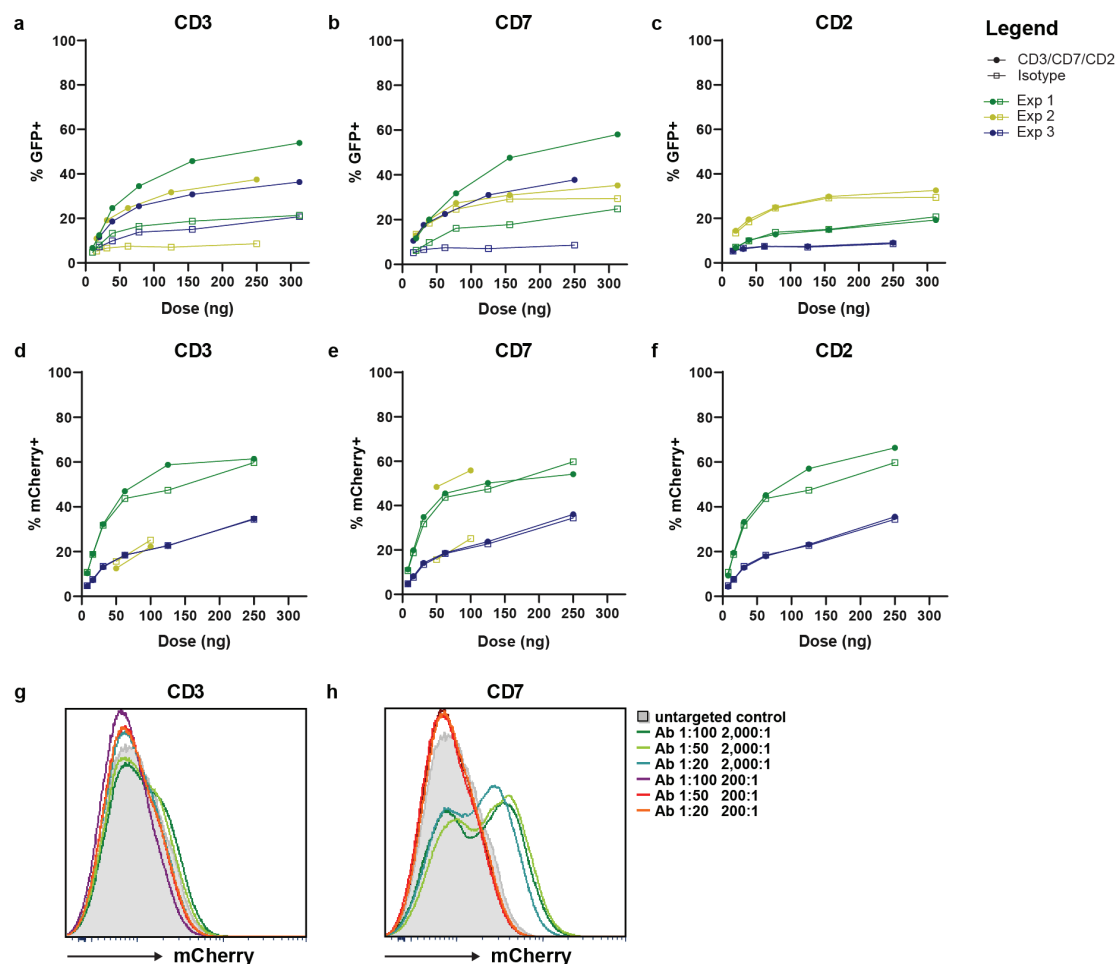


Figure 8. Protocol for antibody conjugation to LNPs using DBCO-Azide click chemistry suffers from lack of reproducibility. (a-c) J-Lat A2 cells were incubated with various doses of Tat MC3/Az-LNPs incubated with DBCO-functionalized (molar ratio 1:20) anti-CD3 (a) anti-CD7 (b) or anti-CD2 (c) targeting antibodies (closed circles), and isotype control antibodies (open squares). Colours represent independent conjugation attempts and subsequent experiments. After 24 hours, transfection efficiency was measured using flow cytometry. (d-f) Jurkat T cells were incubated with various doses of mCherry MC3/Az-LNPs incubated with DBCO-functionalized (molar ratio 1:20) anti-CD3 (d) anti-CD7 (e) or anti-CD2 (f) targeting antibodies (closed circles), and isotype control antibodies (open squares). Colours represent independent conjugation attempts and subsequent experiments (Exp 1, 2, 3). After 24 hours, transfection efficiency was measured using flow cytometry. (g, h) Jurkat T cells were incubated with 100 ng of mCherry MC3/Az-LNPs incubated with anti-CD3 (g) anti-CD7 (h) antibody. DBCO-functionalization of antibodies was performed at different Ab:DBCO ratios (1:100, 1:50, 1:20) and subsequent conjugation to LNPs was performed at different Ab:LNP ratios (2,000:1, 200:1). Untargeted control MC3/Az-LNPs were included as controls. After 24 hours, transfection efficiency was measured using flow cytometry. Results from a single experiment are shown.

consecutive occasions (Figure 8a). Enhanced potency with CD7-targeting was observed on two, but not the third attempt (Figure 8b). We also attempted to target the CD2 receptor but found no difference in transfection efficiency between CD2-targeted LNPs and untargeted control MC3/Az-LNPs on all three occasions (Figure 8c). To exclude the possibility that the inter-assay variations were related to the Tat mRNA or Tat LNP formulation, we first aimed to reproduce the targeting using mCherry MC3/Az-LNPs. We furthermore used fresh NHS-PEG4-DBCO and

DSPE-PEG2000-Azide reagents. Between all three receptors assessed on multiple independent occasions, enhanced potency was only observed once, using anti-CD7 antibodies (Figure 8d-f).

We finally asked whether the chosen ratios of Ab:DBCO and Ab:LNP, despite resulting from extensive optimization, perhaps contributed to the lack of reproducibility observed. To achieve this, we DBCO-functionalized anti-CD3 and anti-CD7 antibodies at Ab:DBCO ratios of 1:20, 1:50 and 1:100 to increase the degree of labelling, then conjugated these to MC3/Az-LNPs at Ab:LNP ratios of 2,000:1 as previously, or 200:1. Here, we failed to observe an effect of CD3-targeting in any of the conditions tested (Figure 8g), while enhanced potency through CD7-targeting could be reproduced in all conditions using an Ab:LNP ratio of 2,000:1, but not 200:1 (Figure 8h). These findings imply that the former Ab:LNP ratio is, indeed, suitable for antibody-conjugation to the LNP surface, and that the degree of DBCO functionalization is of lesser importance. The absence of increases in potency using anti-CD3 antibodies was striking, as an effect of CD3-targeting was observed in previous experiments and the concurrent conjugation of anti-CD7 antibodies was successful on this occasion. These findings further indicate that while targeting LNPs to CD3 or CD7 can enhance their potency, there is a clear need for a more robust conjugation protocol to eliminate inter-assay variability.

Discussion

To potentiate the delivery of mRNA therapeutics to T cells *in vivo* using LNPs, high stealth characteristics need to be combined with specific, active targeting and strong endosomal escape. In this work, we aimed to assess the role of various lipid compositions of the LNPs on these parameters and studied the effect of targeting LNPs to the highly-endocytic receptors CD2, CD3 and CD7.

We found that while standard MC3/DMG-LNPs exhibited high potency in T cell lines, their delivery of mRNA cargo to primary CD⁺ T cells *in vitro* required pre-stimulation of the primary T cells. These findings are in line with recent studies on LNP delivery to primary T cells *in vitro*¹¹⁻¹⁵, as well as Precision Nanosystem's own patented GlenVoy-ILM™ kit, all of which involve activating the T cells prior to LNP treatment. Patel *et al.* specifically claimed that pre-stimulation was necessary for LNP-mediated delivery of mRNA to primary T cells *in vitro*¹². In stark contrast, we here show that mSM102-LNPs demonstrated clear capability to transfect non-stimulated CD4⁺ T

cells *in vitro*. To the best of our knowledge, these findings are the first demonstration of such potent, LNP-mediated delivery of genetic cargo to non-stimulated primary T cells in the absence of toxicity.

mSM102-LNPs differed from MC3/DMG-LNPs by their inclusion of the SM102 ionizable lipid instead of MC3, and β -sitosterol instead of cholesterol. Both SM102 and β -sitosterol were identified as potent transfection enhancers, through their role in improving endosomal escape¹⁶⁻¹⁸. Indeed, endosomal escape has been proposed as a key bottleneck towards nanoparticle-mediated drug delivery in primary T cells, due to their slower acidification of the endosomal compartment¹⁹. To our knowledge, studies into the endosomal escape of LNPs in the context of primary T cells, the role of slower endosomal acidification and the pKa of various LNP formulations are lacking. Yet such efforts are highly warranted, as they could greatly enhance our fundamental understanding of the bottlenecks to efficient drug delivery to resting primary T cells, and subsequent solutions.

We showed that exchanging the PEG lipid in MC3/DMG-LNPs for DSPE-PEG2000 strongly reduced the transfection efficiency in T cell lines with no effect on viability. Due to its longer hydrophobic acyl chain, DSPE-PEG2000 has been shown to exhibit stronger anchoring into the LNP surface compared to DMG-PEG2000^{9,18}, thus resulting in more stable LNP PEGylation. While excess PEGylation can induce accelerated blood clearance of nanomedicine due to anti-PEG-antibodies^{20,21}, greater PEG retention enhances the stealth characteristics of the LNP. For *in vitro* or *ex vivo* transfection of T cell monocultures, higher stealth is non-desirable, as it reduces the transfection efficiency. Yet, *in vivo*, a degree of stealth is essential to avoid scavenging of the LNPs by bystander immune cells. The lower transfection efficiencies of MC3/DSPE- compared to MC3/DMG-LNPs observed in our studies are in accordance with higher stealth of the former, although studies specifically assessing the LNP association and transfection efficiency concurrently and in the presence of PBMC would provide further insights. Likewise, confirming the effect of DSPE-PEG2000 incorporation using other LNP formulations, such as mSM102-LNPs, would strengthen our findings. Nonetheless, our findings demonstrate that incorporating DSPE-PEG2000 in LNP formulations for *in vivo* T cell targeting, including mSM102-LNPs, is certainly of interest.

High stealth and endosomal escape capacities alone are insufficient to yield efficient mRNA delivery to T cells *in vivo*. Our findings showed that targeting LNPs to CD3 or CD7 using antibodies

conjugated to the LNP surface can potentially enhance mRNA delivery to T cell lines. While both CD3- and CD7-targeting have previously been used to target polymeric nanoparticles^{22,23}, only the use of CD3-targeting has been explored to enhance mRNA-LNP delivery to T cells *in vivo*²⁴. The use of anti-CD3 antibodies, however, resulted in systemic T cell activation and temporal loss of CD3 expression²⁴, suggesting targeting T cells through the CD7 or CD2 receptor may be preferable. As discussed in Chapter 3, CD2 targeting has not been studied in the context of nanoparticle-mediated drug delivery to T cells. As such, data on whether CD2 targeting enhances the delivery of functional (mRNA) cargo to T cells are highly anticipated. In the present study, however, we were unable to observe an effect of CD2 conjugation; yet we cannot exclude that this was due to technical failure to conjugate the antibody and, therefore, further optimisation is still required.

The conjugation approach exploited in this work utilized copper-free click chemistry between DBCO groups on the targeting antibody and azide groups on the LNP surface. While this type of click chemistry is well-established and its use in liposome targeting has precedent^{25,26}, this approach has not been used for the conjugation of targeting moieties to LNPs²⁷. In our hands, this approach worked but with a high degree of variability between experiments. We screened a range of reaction conditions, including the Ab:DBCO and Ab:LNP ratios, to identify suitable parameters for antibody conjugation, but were unable to identify conditions that yielded consistent findings. We did, however, confirm that the shelf-life of key reagents, the chosen mRNA construct and the cell line used for read-out were not the cause of the lack of reproducibility.

A more commonly used approach towards antibody-conjugation onto the LNP surface uses thiol-maleimide chemistry²⁷, in which NHS-chemistry is first used to thiolate the targeting antibody, which is then coupled to an LNP harbouring DSPE-PEG2000-maleimide. Indeed, this approach was successfully used in two recent publications, showing successful mRNA-LNP delivery to primary T cells could be enhanced through CD4- or CD5-targeting^{11,13}. In principle, this approach is not too dissimilar to the DBCO-azide click chemistry used in our work; the same NHS-chemistry is used to functionalize the targeting antibody *via* a PEG4 linker, which occurs in a non-site-controlled manner, and a similar incubation subsequently follows to conjugate the functionalized antibody to the functionalized LNP. One difference is the use of a post-insertion technique for the functionalization of the LNP, so that the functional PEG lipid is added to pre-formed, non-functionalized LNPs to allow insertion into the outer leaflet of the LNP^{11,13}. This approach

potentially leads to a more stable expression of the clickable group on the LNP surface.

To better control the orientation of the targeting antibody, Ramishetti *et al.* reduced disulfide bonds within anti-CD4 targeting antibodies with dithiothreitol (DTT) to expose thiol groups in the hinge region, rather than thiolating an intact antibody²⁸. Using reduced antibodies for conjugation resulted in an angled, but controlled orientation of the half-antibody fragments on the LNP surface. Controlling the antibody orientation improved the availability of receptor-binding sites, while simultaneously reducing the exposure of Fc-domains that can induce unwanted interactions with Fc-receptor-expressing bystander immune cells^{29,30}. However, achieving preferential reduction of the disulfide bonds in the antibody hinge region is challenging³¹, thus risking further degradation of the antibody into non-functional fragments. Furthermore, in contrast to methods using NHS chemistry to DBCO-functionalize or thiolate intact antibodies, conjugation of reduced half-antibodies does not allow for incorporation of a PEG linker, which could reduce the targeting efficiency³². Other approaches towards controlled, upright antibody conjugation involve engineering of the targeting moiety to include either a C-terminal peptide recognition sequence for sortase A transpeptidation^{27,30,33}, or a synthetic amino acid at the desired conjugation site within the protein^{29,32}. While potent strategies, these all require knowledge of the exact protein sequence of the targeting antibody in question, and the facilities to re-engineer and synthesize these. Lastly, Kedmi *et al.* published a modular platform for LNP targeting, which utilized a membrane-anchored scFv (ASSET) that binds to the Fc tail of a targeting antibody of choice, thus ensuring an upright orientation of the antibody³⁴. This platform allows for a facile exchange of the targeting antibody with no further optimization required, yet is dependent on the bacterial expression of the ASSET lipoprotein and subsequent purification, complicating widespread usage. In summary, many conjugation methods exist, with each their specific benefits and drawbacks²⁷.

Irrespective of the conjugation method employed, a major challenge in reproducibly generating targeted LNPs is the lack of effective detection methods to quantify and characterize the antibodies on the LNP surface. Indeed, in our own study, our data was not conclusive in indicating the presence, let alone the amount of a targeting antibody on the LNP surface. Gel electrophoresis and dynamic light scattering techniques used in the current study as well as others^{13,24,28,34} provide an indication of an interaction occurring between the antibody and LNP, yet are not quantitative. Similarly, 2-(p-toluidino)-6-naphthalene sulfonic acid (TNS) fluorescent titration assays commonly used to compare the pKa of various LNP formulations^{15,16,35-38} could provide qualitative, but not

quantitative evidence of antibody presence on a nanoparticle surface³⁹. Methods to quantify the presence of protein in the LNP solution, such as UV-Vis spectroscopy or the BCA assay, suffer from a lack of sensitivity or interference by lipids in the solution. Imaging approaches, including structured illumination microscopy (SIM), could be used to visualize and relatively quantify antibody coating on individual LNPs through staining with fluorescent secondary antibodies, but depend on careful blocking of non-specific binding of the secondary antibodies to the LNP surface. In summary, efforts towards improving and standardizing characterization methods for antibody-conjugated LNPs are highly warranted and will benefit both pre-clinical investigations as well as their clinical translation.

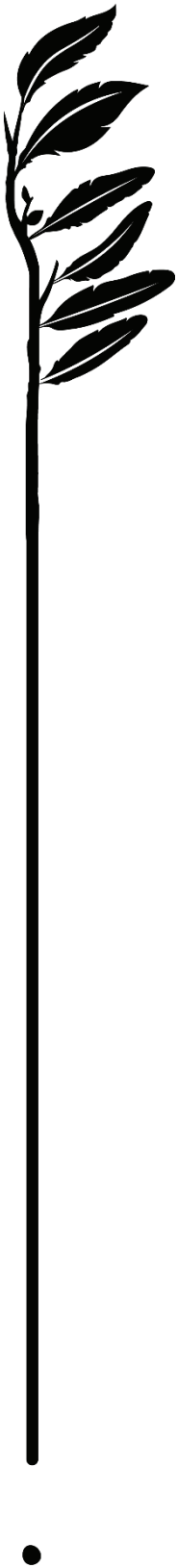
In this study, we chose to use a cell line model to assess the effect of receptor targeting on the transfection efficiency of mRNA-LNPs, due to the ease of high-throughput assaying. Our findings indeed show that targeting CD3 or CD7 can enhance the potency of mRNA-LNPs in the T cell lines used, thus validating this model system. Nevertheless, the full effect of receptor targeting may only be illuminated in (resting) primary CD4⁺ T cells, which exhibit a lower baseline susceptibility to LNP uptake. Furthermore, receptor targeting can also exert a beneficial effect *in vivo* through inducing preferential association of the LNP to T cells *versus* off-target cells. As such, studies investigating mRNA delivery to T cells in the context of PBMCs will allow for a more comprehensive characterization of antibody-conjugated LNPs for the *in vivo* targeting of T cells.

Overall, in this work we took the first steps towards a rationally designed LNP formulation, highlighting the importance of targeting, stealth and endosomal escape properties to overcome key bottlenecks to therapeutic mRNA delivery to T cells *in vivo*. Further work focussing on more robust antibody-conjugation and characterization of subsequent targeted LNPs in primary cell models will be needed to progress these formulations into the clinic.

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Chapter 6

Integrated discussion

T cells are of high interest as therapeutic targets for immunotherapies or for the treatment of T cell-implicated diseases¹. Indeed, T cells are subject to HIV infection and form a key reservoir of latent HIV that forms the major barrier to an HIV cure^{2,3}. Many of the strategies towards such a cure, or long-term remission, are hindered by dose-limiting off-target toxicities or the lack of an efficient delivery vehicle^{4,5}.

As such, we here aimed to develop a nanoparticle-based delivery vehicle targeted to T cells, to further explore nucleic-acid-based, HIV-specific latency reversal agents towards an HIV cure. A key challenge to efficient nanoparticle delivery to T cells is their non-phagocytic nature and their general low rate of endocytosis⁶. We therefore hypothesized that rational nanoparticle design and improved, comparative metrics of nanoparticle performance were keys to success, and consequently addressed the following objectives in the four chapters of this thesis:

- e) to establish a method to evaluate the interactions between nanoparticles and cells through absolute quantitation, to allow rational, inter- and intra-class comparisons of nanoparticles;
- f) to enhance nanoparticle internalization by inducing receptor-mediated endocytosis, through the use of a novel nanoparticle internalization assay;
- g) to investigate the use of lipid nanoparticles for the delivery of a more potent, HIV-specific latency reversal agent based on CRISPR activation; and
- h) to improve the delivery of lipid nanoparticle-based therapeutics to primary CD4⁺ T cells through the conjugation of targeting antibodies to the lipid nanoparticle surface.

Summary of key findings

In **Chapter 2**, we focussed on the challenge of generating quantitative, comparable data on nanoparticle-cell interactions. In particular, we tackled the issue of differential degrees of labelling of different nanoparticles, making the historical measures of nanoparticle association non-comparable between nanoparticle types, repeat experiments or laboratories⁷. We developed and described a methodology to practically convert relative, arbitrary measurements of nanoparticle association into absolutely quantitative numbers of nanoparticles associated per cell. This method is essentially independent of the material type and labelling strategy used, rendering it a powerful tool for both intra- and inter-class nanoparticle comparisons.

This method of absolute quantitation proved key to the work done in **Chapter 3**. By calculating the absolute number of associated nanoparticles and specifically determining the efficiency of nanoparticle internalization for a library of receptor-targeting nanoparticles, we identified CD2 and CD7 as T cell surface receptors capable of triggering substantially enhanced nanoparticle uptake in primary T cells. These findings provided evidence for our hypothesis that receptor-mediated endocytosis could be exploited to improve the kinetics of nanoparticle internalization in target cells – a shift from the historic paradigm, in which active targeting is used solely to enhance the specificity of nanoparticle binding to target cells *versus* off-target cells.

The work we undertook in these first two Chapters did not involve the delivery of any functional cargo or relevant therapeutic. As such, we next focussed on lipid nanoparticles (LNPs) for the delivery of nucleic acid-based therapeutics to T cells in **Chapters 4 and 5**. In **Chapter 4**, we used these LNPs in the context of an HIV cure to further the development of CRISPR activation as a more potent, HIV-specific latency reversal agent. While LNP-mediated delivery of conventional CRISPR and less potent, two-component CRISPR activation has previously been demonstrated⁸⁻¹⁰, to our knowledge, our findings are the first to show that LNP-mediated delivery of the three-component dCas9-SAM CRISPR activation machinery is possible. Using mSM102-LNPs containing SM102 and β -sitosterol, delivery of the CRISPR activation machinery resulted in potent reactivation of LTR-mediated transcription in J-Lat A2 LTR-Tat-IRES-GFP reporter cells.

Yet, testing CRISPR activation-LNPs in *ex vivo* cells from people living with HIV (PLWH) requires prior validation of their ability to transfect primary resting CD4⁺ T cells. In **Chapter 5**, we showed that mSM102-LNPs exhibited a high transfection efficiency in primary CD4⁺ T cells in the *absence* of pre-simulation, highlighting endosomal escape as a barrier to nanoparticle-mediated drug delivery in primary T cells¹¹. Lastly, we built on our findings in **Chapter 3** and showed preliminary results that receptor targeting can increase LNP uptake into T cells, calling for further investigations using alternative methods of conjugation.

In the Chapters summarized above, we took important first steps towards more rational nanoparticle design and obtained promising preliminary results on the potential of CRISPR activation-LNPs to eliminate latent HIV. Yet, many more steps are to be taken before such a T cell-targeting nanoparticle will meaningfully contribute to a cure for HIV. The following sections will discuss two overarching themes of this thesis – improving nanoscience and developing a cure for HIV – in further detail and provide future perspectives on each.

The future of T cell-targeting nanomedicine

Improving *in vitro* nanoparticle evaluation

Standardized metrics that separately and quantitatively assess nanoparticle binding, internalization and endosomal escape prior to assessing the effectiveness of the encapsulated drug will improve the success of nanomedicine development. The conversion of arbitrary fluorescent values to absolute numbers of nanoparticles per cell, as done in Chapters 2 and 3, is a first step towards the standardization of *in vitro* nanoparticle evaluation. Yet, further work needs to be done¹². To achieve full comparability of data, we need to use metrics that are independent of the experimental design^{7,13}. One such approach is using time-course experiments to derive rate parameters of nanoparticle-cell interactions, rather than reporting a metric of particle performance at a single time point. This idea has been investigated in previous work¹³. With the tools developed in Chapter 2 and Chapter 3, we can envision expanding our absolutely quantitative assessment of NP *association* into a quantitative assessment of nanoparticle *internalization*. These measurements could, in turn, be used to derive separate kinetic parameters for particle binding and subsequent uptake through the development of mathematical models. Such efforts are critical to improving the fundamental understanding of the chemistry and biology behind bio-nano interactions.

Similarly, improved assays to measure endosomal escape could provide further insight into the differential performance of various particle designs. Typically, a nanomaterial's capability to induce endosomal escape is assessed through visualization of the subcellular localization at a single time point using fluorescence microscopy. Here, the absence of co-localization with common endosomal and lysosomal markers such as EEA1, Rab7 and LAMP1 is used to identify successful endosomal escape. Such measurements are, however, low-throughput, subjective and non-quantitative¹⁴. The more recently developed high-throughput assays that use β -galactoside-binding lectins (galectins) as reporters for endosomal membrane rupture¹⁵⁻¹⁸ are potential better options. By fluorescently tagging cytosolic galectins, endosomal escape can be quantified in real-time through live-cell confocal imaging and correlated with nanoparticle association and mRNA expression¹⁵⁻¹⁸. Alternative approaches include the use of 'split-reporters', where one fragment of a reporter protein (e.g., GFP or Luciferase) is attached to the therapeutic cargo, while the other is constitutively expressed in the cytosolic compartment of the target cell^{14,19}. Detection of the reporter protein in these experiments thus functions as a direct measure of cytosolic delivery. The need for stable reporter expression in the target cell, though, limits the applicability of these assays

in primary cell models.

More standardized evaluation of nanoparticle designs *in vitro* is also at the core of any attempt to improve the prediction of *in vivo* efficacy from *in vitro* data (so-called ‘*in vitro* to *in vivo* extrapolation’). Such improved predictions are important, as we currently face great inconsistency in the success of *in vitro* to *in vivo* extrapolation²⁰. Two studies found useful correlations between predictions and *in vivo* results^{21,22}, yet other studies showed a stark disconnect between promising candidates identified *in vitro* and those found efficacious *in vivo*^{23,24}. Many efforts towards improving *in vitro* to *in vivo* extrapolation focus on developing *in vitro* model systems with higher predictability^{25,26}, yet part of the issue lies in understanding not *whether*, but *to what degree* an *in vitro* model is able to predict *in vivo* efficacy. Development and refinement of *in vitro* to *in vivo* extrapolation is critically dependent on the availability of standardized *in vitro* and *in vivo* datasets of nanoparticle performance. Yet, typically, nanoparticle screening solely occurs *in vitro*, after which only a selection of candidates is assessed *in vivo*²³. In this regard, increased implementation of high-throughput *in vivo* screening methods, such as a DNA barcoding technique to screen thousands of lipid nanoparticle formulations at once^{23,27}, is of critical importance.

Improving T cell targeting *in vivo*

In Chapters 3 and 5 of this work, we investigated the role of receptor targeting in enhancing the uptake of nanoparticles after association with T cells. Historically, active targeting has primarily been focused on enhancing the *specificity* of nanoparticle binding to target cells *versus* off-target cells. Whilst both these strategies – maximizing specificity and maximizing uptake – are likely to improve T cell targeting *in vivo*, they do not always go hand in hand. For example, the CD2 and CD7 receptors that we found to internalize most rapidly, do not exhibit the highest specificity for (CD4⁺) T cells and *vice versa*. This raises questions about alternative strategies to increase the specificity of nanomedicine towards T cells *in vivo*.

A first strategy relies on using therapeutics that inherently exhibit specificity to the target cell, such as the HIV LTR-targeted CRISPR activation system we used in Chapter 4. Provided the delivery of the therapeutic to off-target cells in itself induces minimal toxicity, this strategy would overcome the need for stringent selectivity in the delivery to the target cell. Another potential approach towards integrating specificity and uptake is the use of targeting moieties with dual specificity. For example, bispecific bivalent antibodies can be engineered to selectively bind double-positive, but not single-positive target cells. This dual selectivity is achieved by lowering

the affinity of the individual antibody arms while maintaining a high combined avidity mediated by concurrent binding of two surface markers²⁸. In the context of HIV, this has been piloted to specifically target CD4⁺/CCR7⁺ double-positive central memory T cells harbouring latent HIV²⁹. Following on from our findings in Chapter 3, one could envision such dual-targeting of a highly-internalizing receptor with a receptor exhibiting high specificity – for example, CD2 and CD4. A final strategy involves the use of synthetic biology to equip mRNA therapeutics with so-called genetic circuits. Such genetic circuit systems can control the expression – and therefore functionality – of the mRNA therapeutic in response to intracellular biomarkers that differentiate target *versus* off-target cells³⁰. For example, an mRNA can be engineered to contain binding sites for specific microRNAs present in off-target, but not target cells, resulting in selective mRNA degradation in off-target cells³¹. Similarly, mRNA therapeutics can be equipped with splicing sites that are responsive to specific intracellular protein biomarkers³². Such strategies are of particular interest for diseases such as HIV, in which a surface biomarker for latently infected cells is yet to be identified³³.

Furthermore, T cell targeting *in vivo* may be improved by using different classes of targeting moieties. Monoclonal antibodies, such as those used in Chapters 3 and 5, are useful for proof-of-concept studies due to the high availability of off-the-shelf clones. However, the Fc domain present in antibodies can result in sub-optimal *in vivo* targeting, due to non-specific interactions with Fc-receptor-expressing off-target cells^{34,35}. One solution includes the use of antibody fragments (such as antigen-binding fragments (Fab), single-chain antibody fragments (scFv) or nanobodies) that lack Fc domains altogether^{36,37} and can be conjugated to particle surfaces in a controlled, upright orientation^{34,35,38}. Likewise, DNA and RNA aptamers are interesting alternatives to antibodies and nanobodies, particularly due to their low immunogenicity, ease of synthesis and facile method of upright conjugation through the end-attachment of reactive groups³⁹. Of note is the fact that both nanobodies and aptamers are typically generated through the selection of clones with high affinity^{40,41}. As such, building on the concept of antibodies with dual specificity, the purposeful selection of nanobodies and aptamers with lower affinity could be explored for the generation of dual-specific, nanobody- or aptamer-targeted nanoparticles.

Lipid nanoparticles as an *in vitro* T cell transfection tool

While the core focus and future objective of our work was to develop T cell-targeted nanomedicine for *in vivo* use, our findings have direct implications for T cell targeting *in vitro*.

Primary T cells are well-known to be hard to transfect *in vitro*, especially in the absence of pre-stimulation. The high mRNA transfection efficiency and minimal toxicity observed in non-stimulated CD4⁺ T cells using mSM102-LNP suggest that this particular formulation is a viable option for the *in vitro* or *ex vivo* transfection of primary T cells. To fully discover the potential of mSM102-LNP as an *in vitro* transfection tool, follow-up studies assessing the delivery of other nucleic acid cargos, including differently sized mRNA constructs and siRNA are of great interest. If our findings extend to these other cargos, mSM102-LNP transfection would open doors for fundamental as well as translational research involving or requiring protein overexpression, gene knockdowns or gene knock-outs in primary T cells. Assessing the potency of mSM102-LNP formulated through manual rather than microfluidic mixing of the organic and aqueous phases could furthermore provide insights into the accessibility of lipid nanoparticles as an *in vitro* transfection tool.

Towards an HIV cure

Further assessment of CRISPR activation as an LRA

In this thesis, we aimed to use our knowledge of T cell-targeted nanomedicine to further the development of CRISPR activation as a clinically applicable latency-reversing agent. As such, the work presented focuses on the proof-of-concept that the CRISPR activation machinery can be encapsulated and successfully delivered to a T cell line model of HIV latency. Our results, however, warrant further investigation into the potential of CRISPR activation in more relevant cell models. We suggest the following cascade of follow-up experiments.

First, CRISPR activation should be studied in near full-length cell line models of HIV latency, to validate our findings in the J-Lat A2 cell line. Comparing the toxicity profile in these two cell line systems will provide further indication of whether the observed virus-mediated cytotoxicity in earlier studies^{42,43} can be replicated. Our findings that mSM102-LNP can efficiently transfect resting primary T cells (presented in Chapter 5) provide encouraging data for subsequent assessment of CRISPR activation-mediated latency reversal in HIV-infected CD4⁺ T cells from PLWH. Assessment of such latency reversal would initially be performed by measuring supernatant HIV RNA, as an endpoint measure of virion production following successful latency reversal. If virion production cannot be detected, subsequent studies should focus on assessing

the entire “transcription profile” upon CRISPR activation-mediated latency reversal of *ex vivo* CD4⁺ T cells. Such profiling can be done through a series of reverse transcription droplet digital PCR reactions specific for various HIV transcripts that define distinct blocks to successful transcription⁴⁴. Indeed, blocks to proximal elongation, polyadenylation and splicing, rather than initiation of transcription, have been identified as the main blocks to latency reversal using various first-generation latency-reversing agents⁴⁴. The observed virus-mediated cytotoxicity upon CRISPR activation-mediated latency reversal in cell line models^{42,43} is a promising indication that CRISPR activation can overcome various blocks to successful transcription. Findings that all three transcriptional activation domains of the dCas9-SAM system (VP64, p65 and HSF1) activate transcriptional initiation as well as elongation⁴⁵⁻⁴⁹ support this hypothesis, yet these findings remain to be confirmed in the context of HIV latency *ex vivo*. Virus-mediated cytotoxicity *ex vivo* and subsequent elimination of latently infected cells could be assessed by measuring total intact and defective HIV DNA using the intact proviral DNA assay^{50,51}. Furthermore, *ex vivo* models would allow better assessment of the optimal gRNA target region within the HIV LTR given the genetic diversity of persisting proviruses, as well as assessment of the role of gRNA multiplexing in maximizing potency⁵² and preventing viral escape⁵³.

Next, the progression of a CRISPR activation-based therapeutic into clinical trials will require the validation of its specificity to the DNA target site. As CRISPR activation differs from conventional CRISPR by its inability to cleave target DNA, any risk of off-target indel introduction is voided. Furthermore, and in further contrast to conventional CRISPR, off-target binding of CRISPR activation will only have effect when the off-target site is a promoter region⁵⁴. Therefore, off-target effects of CRISPR activation are expected to be less frequent and potentially less deleterious. Indeed, no off-target effects of CRISPR activation on host cell transcription were detected in cell line models of HIV latency⁵⁵, yet re-assessment of the specificity of CRISPR activation in primary cells is required. Off-target activation of transcription can be quantified through bulk or single-cell RNA sequencing of PBMC from HIV-negative donors as well as ART-suppressed PLWH following treatment with LTR-targeted or scrambled control CRISPRa-LNPs. Based on the lack of off-target effects observed in cell line models⁵⁵, the transcriptome of PBMC from HIV-negative donors is expected to remain unchanged following treatment with LTR-targeted compared to scrambled control CRISPRa-LNP. In contrast, LTR-targeted CRISPRa-LNPs are expected to induce significant changes in the cellular transcriptome in PBMC from ART-suppressed individuals, assuming there will be a significant increase in HIV transcription in the

subset of cells that harbour HIV.

Can CRISPR activation-based latency reversal cure HIV?

To begin hypothesizing around such a question, we need to look at the scale of the challenge ahead⁵⁶. It has been predicted that a 99.9% reduction of the frequency of viral reactivation is required to induce an average 20-year remission⁵⁶. While the reduction in the frequency of reactivation does not directly equate to the degree of reduction in the reservoir size^{57,58}, it is clear that a large fraction of the reservoir needs to be eliminated for a potential ‘shock and kill’ approach to be successful. Given what we know of CRISPR activation-LNPs thus far, is this remotely feasible? Three key challenges lay ahead.

First, eradicating such a large portion of the HIV reservoir will require the delivery of the CRISPR activation agent to various tissue sites. Indeed, the majority of the HIV reservoir is believed to reside in tissue, not blood⁵⁹. Tissue reservoirs of HIV include lymph nodes and gut-associated lymphoid tissue (GALT)^{60,61}, rectal tissue^{61,62}, cervical mucosa⁶³ and brain^{64,65}. It is unlikely that a single lipid nanoparticle formulation – or any nanoparticle formulation, for that matter – is capable of reaching all these tissue sites. Rather, a combination of various lipid nanoparticle designs, each penetrating one or multiple tissue sites is likely required. Cheng *et al.* showed that passive targeting of the lung, spleen and liver can be achieved by adding a fifth lipid to the lipid nanoparticle formulation⁹. Others have demonstrated similar successes at passively targeting organs or cell types by exchanging or including specific lipids^{66,67}. It appears, therefore, conceivable that formulations can be found that target various HIV tissue reservoirs. Yet, such a combination lipid nanoparticle therapeutic has no precedent and could face challenges in obtaining regulatory approval^{68,69}.

Second, the latent reservoir exhibits great diversity in the site of proviral integration, and thus, the epigenetic environment of the HIV LTR. In this regard, there is ongoing debate around the possible negative influence of the heterochromatin regions around certain HIV integration sites on CRISPR efficiencies⁷⁰. Multiple studies have shown that heterochromatin can impede the efficacy of mutagenesis of conventional CRISPR-Cas9⁷¹⁻⁷⁴. These concerns theoretically extrapolate to the efficiencies of CRISPR activation, as used in this work. Yet, CRISPR activation has specifically been used to open previously inaccessible regions of chromatin⁷⁵⁻⁷⁷. The binding of Cas9 in itself has furthermore been shown to result in a more open chromatin state⁷⁸. This feature is exploited in the “proxy-CRISPR strategy”, where the nuclease activity of a Cas9 in heterochromatin can be

restored through multiplexing with a second, catalytically inactive Cas9 targeting a proximal location. Such proximal binding renders the target site more accessible and thereby enhances the editing efficiency^{79,80}. These findings raise the hypothesis that heterochromatin may primarily affect Cas9 activity downstream of Cas9 binding. For CRISPR activation, the chromatin state was found to primarily affect the duration of transcriptional upregulation of a gene, as a reflection of the permissiveness of that chromatin region to adopt the new chromatin state⁸¹. In summary, the exact effects of heterochromatin around HIV proviral integration sites remain elusive and further studies are needed to address this. In this regard, it is certainly of interest to explore combining CRISPR activation with histone deacetylase inhibitors (HDACi) that have been explored as first-generation latency-reversing agents. While these have not demonstrated sufficient latency reversing potency *in vivo* to eliminate the viral reservoir, they potentially aid CRISPR activation functionality by relaxing the chromatin environment⁸²⁻⁸⁴.

Third, interventions aimed to eliminate the latent reservoir will likely require repeated dosing. Using predictive modelling, Davenport *et al.* found that, for example, a therapeutic capable of reducing the reactivation frequency by 20% per treatment cycle would require ~30 rounds of administration⁵⁶. As such, not only the initial efficacy, but also the tolerability and retention of efficacy of a therapeutic over subsequent rounds of treatment are of important consideration. With a therapeutic as complex and multi-faceted as lipid nanoparticle-encapsulated CRISPR activation, this warrants further investigation. One aspect of concern is repeated dosing of the lipid nanoparticle itself. Ionizable lipids have been associated with the secretion of pro-inflammatory cytokines and reactive oxygen species⁸⁵⁻⁸⁷. Some of the side effects reported for the Pfizer/BioNTech and Moderna COVID-19 vaccines – myalgia, tiredness, swelling, fever⁸⁸⁻⁹⁰ – have been hypothesized to in fact relate to the inflammatory nature of the lipid nanoparticle delivery vehicle, rather than the vaccine itself⁸⁷. Furthermore, the PEG present in the lipid nanoparticle outer layer can induce or boost anti-PEG antibody levels⁹¹⁻⁹³. Such anti-PEG antibodies are known to induce faster clearance of PEGylated therapeutics^{94,95}, which could thus impact the efficacy of repeated administration of a lipid nanoparticle-based intervention towards an HIV cure. A second aspect of concern is the repeated delivery and intracellular expression of the CRISPR activation machinery, which is composed of mostly exogenous proteins. Multiple recent studies detected pre-existing Cas9-reactive effector T cells in healthy donors⁹⁶⁻⁹⁸. While it remains unknown whether these are present at high enough frequencies to trigger an immune response upon a first-round CRISPR treatment^{96,98}, repeated dosing could induce the expansion of Cas9-specific T cell

pools, compromising and potentially increasing the risks associated with subsequent administrations. Two strategies explored to overcome these issues are the design of engineered (d)Cas9 variants with reduced immunogenicity or the stimulation of Cas9-specific regulatory T cells to induce immune tolerance^{97,99}.

Does this mean that further investigations into CRISPR activation as a strategy towards an HIV cure are unwarranted? We believe not. The HIV cure field appears to be shifting away from the notion that a single intervention will achieve remission. Indeed, promising results were achieved recently in various studies combining toll-like receptor (TLR) agonists – which induce latency reversal as well as enhance innate immune function – with either passive¹⁰⁰⁻¹⁰², active¹⁰³, or combined¹⁰⁴ immunization strategies to enhance immune-mediated killing. Such approaches towards post-reactivation control of viremia could potentially act synergistically with CRISPR activation-mediated latency reversal.

Applications in other HIV cure approaches

The primary focus of our work and discussions thus far have revolved around CRISPR activation. Yet, the results presented in this thesis have implications that extend to several other HIV cure approaches. Firstly, while this was not the primary endpoint of our study, our findings in Chapter 4 suggest that HIV Tat mRNA-LNP is a potent latency-reversing agent in itself. HIV Tat specifically promotes transcriptional elongation of the HIV provirus by binding the TAR element in early HIV transcripts and recruiting P-TEFb to the HIV LTR¹⁰⁵. As the secretion of Tat from infected cells in PLWH has been associated with several toxicities, including HIV-associated neurocognitive disorders (reviewed in^{106,107}), further research is required to carefully assess *in vivo* toxicities of Tat-LNP delivery. Secondly, as reviewed in Chapter 1, multiple other strategies towards an HIV cure are nucleic acid-based and could benefit from an efficient nanoparticle-based delivery system. The high efficiency of T cell transfection of the mSM102-LNP presented in Chapters 4 and 5 could therefore hold great potential for advancing the *in vivo* generation of HIV-specific CAR-T cells^{108,109}, siRNA-based ‘block and lock’ strategies¹¹⁰, or other CRISPR-based strategies towards an HIV cure¹¹¹⁻¹¹⁹.

Concluding remarks

The work summarized in this thesis aimed to contribute to the development of T cell-targeted nanomedicine. Particularly in the context of an HIV cure, such therapeutics are highly desired, yet their development is far from straightforward. As such, we worked on integrating the knowledge from various disciplines – materials science, biology, computational science, biochemistry and medical science – to advance our efforts towards more rational nanoparticle design. Our findings are a compelling justification for further research that will have implications beyond the development of an HIV cure alone.

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Glossary

5moU	5-Methoxy-U base	MHC	major histocompatibility complex
ABC	antibody binding capacity	Mono	monocyte
AIDS	acquired immunodeficiency syndrome	MWCO	molecular weight cut-off
ApoE	apolipoprotein E	NK	natural killer
ART	antiretroviral therapy	NNRTI	non-nucleoside reverse transcriptase inhibitor
a.u.	arbitrary units	Nrp1	neuropilin 1
BBB	blood-brain barrier	NRTI	nucleoside reverse transcriptase inhibitor
BiTE	bispecific T cell-engaging	nt	nucleotide
bNAbs	broadly neutralizing antibody	PAS	proline-alanine-serine
BSA	bovine serum albumin	PBAE	poly- β -amino esters
CAR	chimeric antigen receptor	PBMC	peripheral blood mononuclear cells
CCR	C-C chemokine receptor	PB	phosphate buffer
CD	cluster of differentiation	PBS	phosphate-buffered saline
CNS	central nervous system	PCR	polymerase chain reaction
COVID-19	coronavirus disease	PD-1	programmed cell death protein 1
CRISPR	clustered regularly interspaced short palindromic repeats	PEI	polyethylenimine
CRISPRa	CRISPR activation	PEG	polyethylene glycol
CTLA-4	cytotoxic T-lymphocyte-associated protein 4	PFA	paraformaldehyde
CXCR	C-X-C chemokine receptor	PGA	polyglutamic acid
CytoR	cytokine receptor	PI	protease inhibitor
DARTs	dual affinity retargeting proteins	PKC	protein kinase C
DBCO	dibenzocyclooctyne	PLGA	poly(lactic-co-glycolic acid)
DC	dendritic cells	PLWH	people living with HIV
dCA	didehydro-Cortistatin A	PMA _{SH}	poly(methacrylic acid)
dCas9	deadCas9	PMT	photomultiplier tube
DLS	dynamic light scattering	PTK7	protein tyrosine kinase 7
DMSO	dimethyl sulfoxide	RE	restriction enzyme
DOL	degree of labelling	RIPA	radioimmunoprecipitation assay
DTT	dithiothreitol	RME	receptor-mediated endocytosis
EE	encapsulation efficiency	RNAi	RNA interference
FCS	fetal calf serum	RT	reverse transcriptase
FLuc	firefly luciferase	Rv	reverse primer
FSC	forward scatter	SALS	side-angle light scattering
Fw	forward primer	SAM	synergistic activation mediator
GALT	gut-associated lymphoid tissue	scFv	single-chain variable fragments
GFP	green fluorescent protein	scr	scrambled
gp	glycoprotein	s.d.	standard deviation
Gran	granulocyte	SEM	standard error of the mean
gRNA	guideRNA	SIM	structured illumination microscopy
HDACi	histone deacetylase inhibitors	SIV	simian immunodeficiency virus
HIV	human immunodeficiency virus	SMAC	second mitochondria-derived activator of caspases
HSF1	heat shock factor 1	SPAAC	strain-promoted alkyne-azide cycloaddition
HTLV	Human T-cell lymphotropic virus	SPION	superparamagnetic iron oxide nanoparticles
ICM	immune checkpoint marker	SSC	side scatter
ID	injected dose	T-ALL	T cell acute lymphoblastic leukemia
IFN- γ	interferon- γ	TCR	T cell receptor
IgG/M	immunoglobulin G/M	TfR	transferrin receptor
IL	interleukin	TGF β	transforming growth factor beta
IL-2R	interleukin 2 receptor	T _H	helper T cells
IRES	internal ribosomal entry site	TLR	toll-like receptor
IS	internalization score	TNF	tumour necrosis factor
LFA-1	lymphocyte function-associated antigen-1	T _{RM}	tissue-resident T cells
LNP	lipid nanoparticle	UTR	untranslated regions
LRA	latency-reversing agent	VP16/64	virus transcriptional activation domain 16/64
LTR	long terminal repeat	VPR	VP64, p65 and Rta
MESF	molecules of equivalent soluble fluorochrome	WBC	whole blood cells
MFI	median fluorescent intensity		

Research Output

Publications

Cevaál, PM, Roche, M, Lewin, SR, Caruso, F, Faria, M. (2022) Experimental Quantification of Interactions Between Drug Delivery Systems and Cells In Vitro: A Guide for Preclinical Nanomedicine Evaluation. *J. Vis. Exp.* (187), e64259

Dowling, CV, **Cevaál, PM**, Faria, M, Johnston, ST. (2022) On predicting heterogeneity in nanoparticle dosage. *Mathematical Biosciences.* 354, 108928.

Tanaka, K, **Cevaál, PM**, Pascoe, RD. (2022) An Inspiring Journey of Hope and Persistence: Life Lessons with Françoise Barré-Sinoussi. *Viruses.* 14(5), 1108

Cevaál, PM, Ali, A et al. (2021) In vivo T cell-targeting nanoparticle drug delivery systems: considerations for rational design. *ACS Nano.* 15, 3, 3736-3752.

Ramesan, S, Rezk, AR, **Cevaál, PM**, et al. (2021) Acoustofection: High-frequency vibrational membrane permeabilization for intracellular siRNA delivery into nonadherent cells. *ACS Applied Bio Materials.* 4, 3, 2781-2789.

Cevaál, PM, Bekker, LG, Hermans, S (2019) TB-IRIS pathogenesis and new strategies for intervention: Insights from related inflammatory disorders. *Tuberculosis.* 118:101863.

Cevaál, PM, Cortez-Jugo, C, Tan, A, Faria, M, Gubser, C, Caruso, F, Roche, M, Symons, J, Lewin, SR. Targeting the HIV reservoir: utilizing receptor-mediated endocytosis to enhance nanoparticle delivery to T cells. *In preparation*

Zhao, W, Rossello, F, Singh, K, Sozzi, T, **Cevaál, PM**, Martelotto, L, Zerbato, JM, Rhodes, A, Powell, DR, Chiu, C, Anderson, J, Tumpach, C, Fong, D, Revill, P, Crane, M, Roche, M, Audsley, J, Lewin, SR. HIV-1 Tat induces hepatitis B surface antigen expression in hepatocytes co-infected with HIV and hepatitis B virus. *In preparation*

Scientific grants

The Foundation of AIDS Research; TARGET grant

Lewin (PI) Role: Co-Investigator

Oct 2022 – Sept 2024

Lipid nanoparticle-based delivery of HIV-reactivating RNAs to eradicate the latent reservoir

The goal of this project was to investigate lipid nanoparticles for the delivery of the mRNA-based CRISPR activation system as a HIV-specific, more potent latency reversal agent in ex vivo and in vitro models of HIV latency

Australian Center for HIV & Hepatitis Virology Research; Project Grant

Lewin (PI) Role: Co-Investigator

Dec 2021 - Sept 2022

Lipid nanoparticle-based delivery of HIV-reactivating RNAs to eradicate the latent reservoir

The goal of this project was to lay the groundwork for delivering HIV Tat mRNA as a model HIV-specific LRA to latently infected cell lines using lipid nanoparticles.

Australian Center for HIV & Hepatitis Virology Research; Project Grant

Cortez-Jugo (PI) Role: Co-Investigator

Dec 2019 - Mar 2021

Targeted delivery of nanoparticles to CD4+ T cells to combat the viral reservoir

The goal of this project was to investigate strategies to enhance nanoparticle delivery to T cells.

Awards and honours

- | | |
|------------------|--|
| 2022 | Winner of Blackbird Venture's inaugural WildFutures start-up contest with team Gerulus |
| 2022 | Young Achiever Award for best HIV oral presentation, ACH2 Annual Scientific Conference |
| 2021 | Feature in Pod of Science podcast series of Australia's Pint of Science festival for science communication |
| 2021 | People's Choice and Judge's Choice presentation awards for a radio-style interview on my research, Frontiers of Bio-Nano Symposium |
| 2019-Now | Melbourne Research Scholarship |
| 2016-2018 | U/Select excellence programme participant, Graduate School of Life Sciences, The Netherlands |
| 2016 | Poster & Pitch award, Infection and Immunity Master Symposium, The Netherlands |
| 2016 | Multiple personal travel grants (cumul. EUR8,500), The Netherlands |

