

Pooled CAR-T cell screening in nonhuman primates to inform therapeutic design for cancer and HIV

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Abstract

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Chimeric antigen receptor (CAR)-T cell therapy represents a major milestone in immunotherapy, with the potential to durably treat, and in some cases cure, a range of hematologic malignancies, chronic infections, and autoimmune diseases. As powerful living drugs, CAR-T cells can recognize antigen-bearing cells with precision, expand upon engagement, and exert potent cytotoxic effects. Yet despite rapid innovation and the development of thousands of CAR constructs across diverse indications, relatively few have translated into clinical success. Many candidates fail due to insufficient persistence, poor efficacy in complex tissue environments, or immune-mediated toxicities such as cytokine release syndrome (CRS) and neurotoxicity—limitations that are often not predicted by preclinical models. These challenges reflect shortcomings not only in CAR design, but in the systems used to evaluate them. Large animal models, such as nonhuman primates (NHPs), may help bridge the gap between murine models and humans by offering greater immunological and physiological similarity, and by enabling long-term studies of CAR-T cell persistence, trafficking, and safety. By combining next-generation CAR designs with rigorous and reproducible testing in more predictive, human-relevant systems, we can begin to address longstanding barriers to translation and advance the development of safer, more durable, and clinically effective cell therapies.

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Chapter 1: Large animal models for CAR-T cell therapy

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Abstract

CAR-T cell therapies have revolutionized the treatment of certain cancers and are being actively investigated for chronic viral infections such as HIV. Despite promising clinical outcomes, challenges related to persistence, toxicity, immunogenicity, and long-term efficacy continue to limit broader application. While murine models, particularly immunodeficient mice, have been instrumental in early-stage development, they frequently fall short in modeling key aspects of CAR-T biology, including immune responses, tissue trafficking, and durability. In contrast, large animal models, particularly NHPs, offer a more clinically relevant platform due to their physiological, anatomical, and immunological similarity to humans. NHPs enable longitudinal monitoring, clinically scaled dosing, and in-depth analysis of CAR-T kinetics and function *in vivo*. Here, we review progress to date in the use of NHP models to evaluate CAR-T cell therapies, with a focus on oncology and HIV, and highlight how these studies are advancing our understanding of CAR-T cell biology and informing the design of next-generation therapies.

Introduction

Animal models are indispensable tools in translational research, providing critical insights into disease biology and therapeutic development. In the context of engineered cell therapeutics, including CAR-T cells, rodent models have enabled rapid early-stage innovation but often fail to capture the complexity of human immune responses, tissue environments, and treatment-related toxicities.¹ These limitations pose a major barrier to developing therapies with sustained efficacy, manageable safety profiles, and reliable clinical translation. As CAR-T therapies move into increasingly complex indications, including solid tumors and chronic infections such as HIV, there is a growing need for preclinical systems that better reflect human physiology and immunobiology.

Advantages of NHP models

NHPs offer a uniquely valuable platform for evaluating CAR-T cell therapies in a setting that more closely recapitulates human biology. Compared to rodents, NHPs share greater similarity in human immune system architecture, anatomy, and physiology (**Table 1**).²⁻⁶

They are also genetically diverse and immunocompetent, enabling the study of antigen-specific T cell responses, immune cell trafficking, and host interactions under conditions that are difficult to replicate in murine models.⁷

Beyond biological relevance, NHPs provide important practical advantages for translational research. Their larger body size supports longitudinal sampling of larger blood volumes and biopsies across multiple tissue compartments, enabling high-resolution tracking of CAR-T cell biodistribution. Clinically relevant routes of intravenous administration can be directly evaluated in NHPs, in contrast to rodent models which typically rely on less-representative methods such as tail vein or intraperitoneal injection. In addition, NHP studies enable evaluation of manufacturing feasibility and scalability, including testing of clinically aligned protocols for cell collection, transduction, expansion, and dosing, which are difficult to model at scale in rodents. Finally, NHPs are particularly well suited for long-term studies. Their extended lifespan allows for evaluation of CAR-T cell durability over clinically relevant timescales, which is essential for addressing issues around CAR-T persistence that currently limit long-term efficacy.⁸ Together, these features position NHP models as a powerful bridge between preclinical discovery and clinical translation in the development of next-generation CAR-T cell therapies.

In the sections that follow, we examine how NHP models have been applied to both cancer- and HIV-targeted CAR-T cell therapies, and highlight their value in advancing CAR design, functional assessment, and translational readiness.

	Rodent models	NHP models
Sampling	Limited blood volumes; poor access to secondary organs, feasibility of survival surgeries	Larger blood and tissue volumes support longitudinal sampling across multiple tissues
Relevant cellular subsets	Profound immunodeficiency in xenograft models; Incomplete humanization in humanized mice (e.g., limited myeloid and erythroid lineage reconstitution) ⁹	Immune cell subsets are often highly conserved; hematopoiesis closely mirrors human biology
Relevant anatomical & immunological systems	Small size; limited structural comparability to humans	Close resemblance to human immune system

Lifespan	Short lifespan limits long-term safety or durability studies	Longer lifespan enables long-term studies (years to decades)
Cost	Low	High
Manufacturing feasibility	Small size supports low-cost, small-scale production, but workflows often don't scale to clinical applications.	Larger size enables evaluation of scalable, clinically relevant manufacturing and dosing
Route of administration	Frequently not relevant to humans (e.g., intraperitoneal or tail vein injections)	Human-relevant routes (e.g., intravenous, intramuscular, central venous access)
Infectious disease models	Fewer human pathogens capable of infecting rodents	Broader susceptibility to human or closely related pathogens (e.g., SHIV, SIV) ^{4,10}

Table 1. Benefits of NHP models.

Limitations of NHP models

While NHP models offer unparalleled immunologic and physiologic relevance for translational research, their use is limited by several key challenges. High costs and restricted accessibility, typically within the seven National Primate Research Centers (NPRCs) in the U.S., hamper widespread use and often limit studies to small sample sizes, reducing statistical power. The lack of robust, genetically tractable tumorigenesis models also limits the use of NHPs in oncology research. Ethical considerations surrounding the use of cognitively advanced species also warrant careful deliberation, particularly for invasive or long-duration studies. In addition, NHP models are logistically demanding: experimental timelines are long, and species-specific reagents for flow cytometry, cytokine detection, and gene expression analysis remain incomplete. These factors, combined with high inter-animal variability, can complicate both experimental design and data interpretation.

NHP models for cancer-targeted CAR-T cell therapy

The most widely used preclinical models for CAR-T cell therapy are immunodeficient mice, particularly NOD/SCID/IL2R γ -knockout (NSG) mice engrafted with human tumors.¹ While valuable for early-stage research, these models are limited by their severely compromised immune systems. NSG mice lack functional T cells, B cells, and natural killer (NK) cells, and also exhibit deficiencies in the myeloid compartment, including reduced macrophage phagocytosis and impaired dendritic cell maturation.¹¹ As a result, they do not accurately model antigen presentation or key immune-mediated toxicities such as cytokine release

syndrome (CRS), which is driven primarily by monocyte- and macrophage-derived cytokines.^{12–14} These toxicities continue to pose a major obstacle to effective CAR-T cell therapy, with up to one-third of treated patients experiencing CRS or immune effector cell-associated neurotoxicity syndrome (ICANS) following infusion.¹⁵ Therefore, it is important to complement murine preclinical studies with preclinical models capable of recapitulating these toxicities, to better understand their mechanisms and evaluate mitigation strategies.

In contrast, NHPs provide a more physiologically relevant platform for evaluating CAR-T cell activity and toxicity, while also enabling assessment of clinically relevant dosing strategies and lymphodepletion regimens. Although tumor-bearing NHP models are not readily available, healthy NHPs have served as valuable systems for assessing pharmacodynamics and on-target effects. For example, CD20-targeted CAR-T cells designed to treat B cell malignancies expanded robustly and induced CD20⁺ B cell aplasia both peripherally and within lymphoid follicles in healthy, lymphodepleted rhesus macaques (**Table 2**).^{16–18} Importantly, NHPs also recapitulated hallmark CAR-T-associated toxicities, including CRS and ICANS, with clear evidence of CAR-T cell infiltration into the cerebral spinal fluid (CSF). Because these immune-related adverse events (IRAEs) remain major barriers to safe and effective CAR-T administration, the ability to study their onset, associated biomarkers such as IL-6 and IL-1, and management strategies in NHPs represents a critical advantage.

NHP models also enable evaluation of on-target, off-tumor toxicities, which are a major concern when antigens are overexpressed on tumors but also expressed at lower levels on normal tissues. This challenge is particularly relevant in solid tumor applications, where truly tumor-specific antigens are rare.¹⁹ Several preclinical studies in NHPs have helped de-risk targets with this expression profile. One example is ROR1, a tumor-associated antigen found in several hematologic and epithelial cancers, but also present at lower levels in healthy tissues such as adipocytes, pancreas, and lung.^{20,21} In a study by Berger et al., ROR1-targeted CAR-T cells administered to healthy rhesus macaques trafficked to lymph nodes and bone marrow where ROR1⁺ B cells reside, but did not induce detectable toxicity in normal tissues, even at high doses.²² Similarly, Ephrin type-B receptor 4 (EPHB4) is a promising target since it is overexpressed in multiple cancers, including rhabdomyosarcoma,²³ lung cancer,²⁴ colorectal cancer,²⁵ breast cancer,²⁶ esophageal cancer,²⁷ and melanoma,²⁸ among others. Despite its low-level expression in normal tissues, EPHB4-targeted CAR-T cells were well tolerated in cynomolgus macaques following single-dose infusion into lymphodepleted animals.²⁹ Notably, the cells expanded *in vivo* without inducing detectable off-tissue toxicity. A third example is the granulocyte-

macrophage colony-stimulating factor receptor (GMR), a candidate target for subsets of acute myeloid leukemia (AML). Because GMR is also expressed on monocytes, macrophages, hematopoietic progenitors, and vascular endothelial cells, off-tumor/off-tissue effects are a serious concern. However, in a study by Morokawa et al., GMR-targeted CAR-T cells infused into cynomolgus macaques displayed no evidence of monocytopenia, vasculitis, or bone marrow suppression, suggesting a favorable safety profile.³⁰ These findings ultimately supported the initiation of a first-in-human clinical trial evaluating GMR-directed CAR-T cells in patients with relapsed or refractory myeloid malignancies.³¹ Collectively, these studies underscore the translational value of NHPs for evaluating pharmacology, safety, and tissue-specific effects of cancer-directed CAR-T cells in a large animal immunocompetent setting.

NHP models for HIV-targeted CAR-T cell therapy

NHPs are widely regarded as the gold standard model for studying HIV pathogenesis and therapeutic interventions.^{4,6} Because HIV exclusively infects human cells, traditional murine models must first be humanized with human peripheral blood and/or hematopoietic stem cells (HSCs), engrafted into an immunodeficient background in order to create an HIV-permissive immune system. Humanization protocols are technically demanding, labor-intensive, and often result in incomplete or skewed development of immune cell lineages.⁹ In contrast, although HIV-1 does not efficiently infect macaque cells,³² NHPs are naturally susceptible to closely related lentiviruses such as simian immunodeficiency virus (SIV) and engineered chimeric viruses such as simian/human immunodeficiency virus (SHIV).⁶ Each faithfully recapitulates key features of human HIV infection and pathogenesis, including CD4⁺ T cell depletion, progression to AIDS-like disease, and establishment of reactivatable latent reservoirs, making NHPs a valuable model for both mechanistic and therapeutic HIV research.³³

While CAR-T cell therapies are most often associated with cancer, similar CAR-targeted strategies have been developed to deplete HIV-infected cells. HIV-targeted CAR-T cells are typically engineered to recognize the viral envelope glycoprotein (Env) on the surface of infected cells, often using a single-chain variable fragment (scFv) specific for Env as the extracellular binding domain.³⁴ Several groups have evaluated anti-Env CAR-T constructs in NHPs infected with SHIV or SIV, primarily in the setting of suppressive antiretroviral therapy (ART).^{35,36} In some studies, additional modifications such as overexpression of CXCR5 have been introduced to enhance lymphoid tissue trafficking and improve *in vivo* persistence.^{37,38} These therapies have generally been well tolerated, which is an important consideration for people living with HIV (PLWH) who are otherwise healthy and virally suppressed on

antiretroviral therapy (ART). However, in most cases, CAR-T cell expansion following infusion has been limited, and viral rebound typically occurred after ART interruption, suggesting minimal antiviral activity. Some studies have also explored prophylactic administration of Env-targeted CAR-T cells prior to viral challenge, but this strategy failed to prevent acquisition.³⁵ Additionally, several groups detected anti-CAR and anti-SIV antibodies in the plasma of treated NHPs,^{36,38} and these antibodies were found to interfere with CAR-T cell function in *ex vivo* cytotoxicity assays, suggesting that humoral immune responses may play a role in limiting therapeutic efficacy. These findings underscore the utility of NHP models for evaluating CAR-T cell immunogenicity and anti-drug antibody (ADA) responses, both of which are critical considerations for human clinical translation.³⁹

Rather than scFv-based targeting, our group and others have developed a distinct anti-HIV CAR design that leverages the virus's natural entry mechanism by incorporating the CD4 protein as the extracellular antigen recognition domain, resulting in so-called CD4CAR-T cells. This approach takes advantage of the essential interaction between HIV Env and the CD4 receptor, allowing CD4CAR-T cells to selectively target Env-expressing cells. By engaging a highly conserved viral entry mechanism, CD4-based CARs may offer additional advantages over scFv-based designs, including reduced susceptibility to Env escape.⁴⁰ Preclinical studies with CD4CAR-T cells in SHIV- or SIV-infected, ART-suppressed NHPs have yielded mixed results, with some groups reporting limited CAR-T expansion and minimal impact on viral control.³⁵ PLWH who are durably suppressed on ART typically exhibit very low, often undetectable, levels of Env antigen on the surface of infected cells.⁴¹ This presents a major challenge for Env-targeted CAR-T strategies, which rely on antigen engagement for activation. We hypothesized that the low-antigen environment created by suppressive ART could be overcome by an "antigen-boosting" strategy, in which exogenous antigen is provided in *trans*. In a proof-of-concept study, we administered CD4CAR-T cells into ART-suppressed, SHIV-infected NHPs, followed 19 days later by infusion of K562 cells engineered to stably express the HIV Env protein.⁴² After ART interruption, we observed robust CAR-T expansion, and in a subset of animals, this expansion was associated with transient viral control. To our knowledge, this study represents the first demonstration of substantial *in vivo* expansion of HIV-targeted CAR-T cells in an NHP model of HIV. Despite initial promise, further experiments described in Chapter 3 highlight important limitations of this previous study.

To lessen the requirement for sparsely expressed surface antigens, HIV-targeted CAR-T cells may need to be redirected toward alternative, more abundant markers that also enable effective clearance of the HIV reservoir. One well-characterized reservoir site is within secondary lymphoid tissues, where HIV persists in T follicular helper (T_{FH}) cells that

reside in B cell follicles.⁴³ These are generally immune-privileged sites that are largely inaccessible to cytotoxic T cells. Given the success of B cell-directed CAR-T therapies in depleting B cells in NHPs, we hypothesized that CD20-targeted CAR-T cells could disrupt B cell follicles in lymph nodes, potentially exposing T_{FH} cells to endogenous HIV-specific cytotoxic T cells. In SHIV-infected, ART-suppressed nonhuman primates, infusion of CD20-targeted CAR-T cells led to robust expansion, efficient depletion of peripheral B cells, and transient ablation of B cell follicles.¹⁷ Despite this, no measurable impact on viremia or the HIV reservoir was observed. Similarly, pre-treating SIV-infected NHPs with an anti-CD20 antibody prior to CD4CAR-T cell infusion, intended to disrupt B-cell follicles and expose HIV-infected cells for clearance, also failed to produce any measurable reduction in viral load.⁴⁴ A related strategy aimed to deplete HIV-infected cells based on their expression of the immune checkpoint marker PD-1, which is commonly upregulated in HIV-infected cells.⁴⁵ Eichholz et al. tested PD-1-targeted CAR-T cells in SIV-infected, ART-suppressed NHPs and observed robust CAR-T expansion and effective depletion of PD-1⁺ cells in 3 of 6 treated NHPs.⁴⁶ However, following ART interruption, plasma viremia paradoxically increased, suggesting that PD-1⁺ cells may play a more complex role in reservoir dynamics and immune regulation in the context of HIV. Together, these studies highlight the major barriers that continue to impede effective clearance of the HIV reservoir, including low antigen levels, immune-privileged sites, and complex host-virus dynamics.

Disease	Animal model	CAR	Lymphodepletion	Reported findings	Ref
Lymphatic & epithelial malignancies	Healthy rhesus macaques (n=3)	ROR1-targeted CAR-T cells	None	No toxicity; depletion of ROR1 ⁺ B-cells; CAR-T expansion and trafficking to bone marrow; limited persistence (1–2 weeks)	²²
B-cell malignancies	Healthy rhesus macaques (n=4)	CD20-targeted CAR-T cells	Cyclophosphamide	CRS and neurotoxicity; depletion of CD20 ⁺ B-cells; CAR-T expansion; trafficking to CNS	^{16,47}
AML	Healthy cynomolgus	GM-CSF receptor-	None	No toxicity; limited CAR-T persistence (~1 week)	³⁰

	macaques (n=3)	targeted CAR-T cells			
Multiple cancers (e.g., AML, RMS, solid tumors)	Healthy cynomolgus macaques (n=6)	EPHB4- targeted CAR-T cells	Cyclopho sphamide & fludarabin e	No toxicity; transient CAR-T expansion; limited persistence (< 1 week)	29
HIV	SHIV- infected, ART- suppressed rhesus macaques (n=4)	CD4CAR-T cells + Env- expressing K562 cells (antigen boost)	None	No toxicity; CAR-T expansion; transient viral control in 2 of 4 NHPs	42
HIV	SIV-infected ART- suppressed rhesus macaques (n=2)	Anti-Env scFv-based CAR-T cells + Env- expressing APCs (antigen boost)	None	No toxicity; minimal CAR-T expansion or viral control; anti-CAR and anti-SIV antibodies detected that inhibited CAR-T function <i>ex vivo</i>	36
HIV	SHIV (n=12) or SIV (n=8)- infected rhesus macaques, +/- ART	Multiple Env- targeted CAR-T cells (CD4CARs, anti-Env scFv-based CARs)	None	No toxicity; minimal CAR-T expansion; no effect on viral acquisition (prevention) or viremia (treatment)	35
HIV	SIV-infected, ART- suppressed rhesus macaques (n=7)	Anti-Env scFv-based CAR-T cells + CXCR5 overexpressi on	None	No toxicity; minimal CAR-T expansion; limited persistence (<4 weeks); some lymphoid trafficking; anti-	37,38

				CAR antibodies detected	
HIV	Healthy (n=4) and SHIV-infected, ART-suppressed pigtail macaques (n=4)	CD20-targeted CAR-T cells	Cyclophosphamide	No toxicity; depletion of peripheral CD20 ⁺ B-cells; transient follicular B cell ablation in lymphoid tissues; CAR-T expansion; minimal HIV reservoir impact	17
HIV	SIV-naïve (n=2) and SIV-infected, ART-suppressed (n=4) rhesus macaques	PD-1-targeted CAR-T cells	None	No toxicity; depletion of PD-1 ⁺ memory T cells; CAR-T expansion and persistence in 3 of 6 NHPs (up to 100 days); post-ART viremia increase	46

Table 2. Overview of CAR-T cell studies in NHPs. AML, acute myelocytic leukemia; RMS, rhabdomyosarcoma; ART, antiretroviral therapy; APC, antigen presenting cells.

Collectively, these studies demonstrate the unique value of NHP models for evaluating the safety, pharmacodynamics, and immunogenicity of CAR-T cell therapies in a physiologically relevant setting. However, despite advances in construct design and preclinical modeling, many CAR-T therapies still fail to achieve durable responses *in vivo*. This underscores the need for systematic approaches to identify and optimize CAR architectures that can overcome barriers to persistence, trafficking, and efficacy. In the following chapters, we describe two such efforts: a pooled *in vivo* screening platform used to identify optimized CAR designs targeting CD20 (Chapter 2), and a series of systematic NHP studies aimed at reproducing and advancing CAR-T cell therapy for HIV (Chapter 3), both of which leverage the unique capabilities of the NHP model to inform next-generation CAR-T cell engineering.

Chapter 2: Pooled CAR-T cell screening in nonhuman primates identifies designs with enhanced proliferation, trafficking and persistence

This chapter is adapted from the following manuscript:

Maynard, Lucy H., et al. "Pooled CAR-T screening in nonhuman primates identifies designs with enhanced proliferation, trafficking, and persistence." *bioRxiv* (2025): 2025-03.

Abstract

Chimeric antigen receptor T (CAR-T) cell therapy has revolutionized treatment for B-cell malignancies, yet over 60% of patients relapse within one year, often due to insufficient CAR-T persistence. While mouse and primary cell models have been instrumental in advancing CAR-T therapy, they frequently fail to predict clinical outcomes, underscoring the need for more translationally relevant models. To address this limitation, we conducted the first systematic evaluation of CAR structure-function relationships in an immunocompetent nonhuman primate (NHP) model. We engineered an array of 20 CD20-targeted CARs with distinct combinations of hinge, transmembrane, and costimulatory domains. Following *ex vivo* characterization, we administered pooled autologous CAR-T arrays to three NHPs and tracked CAR abundance *in vivo* longitudinally using a novel digital droplet PCR assay. *Ex vivo*, CAR-T cells incorporating the MyD88-CD40 costimulatory domain exhibited markedly distinct functional profiles, including increased activation, unique cytokine secretion, tonic signaling, and resistance to exhaustion. *In vivo*, MyD88-CD40 CARs expanded dramatically, comprising up to 100% of peripheral T cells and significantly outperforming canonical CD28- and 4-1BB-based CARs. This expansion was associated with robust B-cell depletion across all animals. MyD88-CD40 CARs, particularly those with a CD28 hinge and transmembrane domain, demonstrated superior trafficking to secondary lymphoid tissues and persistence through study endpoint, unlike other CARs which waned by day 28. Our findings highlight the value of NHP models for screening CAR designs and identify MyD88-CD40 CARs as candidates with unmatched potency. The unique functional attributes conferred by this domain may provide key insights into features that drive enhanced CAR-T cell activity.

Introduction

Chimeric antigen receptor T (CAR-T) cell therapy has transformed the treatment landscape for B-cell malignancies, leading to the approval of seven therapies targeting CD19 and B-cell maturation antigen (BCMA). However, tumor recurrence and patient relapse remain significant challenges. Relapse occurs in 50-70% of patients with diffuse large B-cell lymphoma (DLBCL) and B-acute lymphoblastic leukemia (B-ALL) treated with CD19-targeted CAR-T cells,⁴⁸⁻⁵³ and in over 80% of multiple myeloma (MM) patients receiving BCMA-targeted CAR-T cells.^{54,55} A meta-analysis spanning eight clinical studies across nine B-cell malignancies further underscores this issue, revealing that over 60% of these patients relapse within just 12 months after CAR-T infusion, emphasizing the rapid progression of disease recurrence.⁵⁶

The high rate of relapse highlights a critical need to improve the long-term efficacy of CAR-T cell therapy. While various factors contribute to relapse, mounting evidence suggests that limited *in vivo* persistence of CAR-T cells is a primary challenge.⁵⁷ Relapse of antigen-positive tumor cells is frequently associated with limited CAR-T durability,^{58,59} detected in 33-78% of CD19-targeted,^{48,58-61} and 67-96% of BCMA-targeted CAR-T cell^{54,62,63} relapse cases. Recent studies have further identified CAR-T cell persistence as a crucial determinant of long-term disease-free survival,^{64,65} highlighting the need for strategies to enhance CAR-T durability.

The structure of a CAR plays a critical role in shaping CAR-T cell function, phenotype, and persistence.⁶⁶ CAR molecules comprise several components, including antigen recognition, hinge, transmembrane, costimulatory, and CD3ζ activation domains. The costimulatory domain is particularly influential in modulating CAR-T cell kinetics and durability.^{67,68} Approved CAR-T therapies incorporate either 4-1BB or CD28 costimulatory domains, which exhibit distinct profiles: 4-1BB promotes greater persistence with slower expansion, while CD28 drives rapid expansion but earlier T-cell exhaustion.^{69,70} Recent studies have identified additional domains, such as Ox40,⁷¹⁻⁷³ BAFF-R,⁷⁴ and MyD88-CD40,⁷⁵⁻⁷⁷ which have demonstrated the potential to further augment CAR-T cell activity. Hinge and transmembrane domains also influence CAR-T cell activity, particularly by modulating sensitivity to antigen levels.⁷⁸⁻⁸² Despite these advances, no universally superior CAR architecture has been identified, suggesting a requirement for disease-specific designs. As such, direct head-to-head comparisons of CAR structures could provide critical insights into designs that improve CAR-T cell activity for specific indications.

Developing and validating novel CAR designs through clinical trials is an exceedingly expensive, time-consuming, and resource-intensive process, with a substantial risk of failure. Moreover, commonly used mouse models frequently fail to recapitulate complex human immunology and CAR-T-mediated toxicities, such as cytokine release syndrome (CRS), limiting their utility in predicting clinical outcomes.^{12,13} To address these challenges, the nonhuman primate (NHP) model has emerged as a valuable tool for studying CAR-T therapy. Unlike immunocompromised mouse models, NHPs possess a functional immune system and can recapitulate drug-associated toxicities observed in humans. Furthermore, the longer lifespan of NHPs allows for extended safety and efficacy studies, and their larger size enables longitudinal biopsies and testing of clinical-scale CAR-T manufacturing, which are not feasible in mice.⁸³ Our research group and others have previously developed and validated a NHP model for B-cell targeted CAR-T cell therapy.^{16,17,47,84} This model has demonstrated its utility in recapitulating key clinical findings, including potent B-cell depletion in blood and tissues, transient ablation of B-cell follicles, and management of CAR-T cell-mediated toxicities. Here, we hypothesized that the NHP model could be leveraged in a pooled screening format to simultaneously evaluate multiple CAR structures and identify designs with superior *in vivo* activity. Our findings underscore the importance of CAR design in shaping CAR-T cell proliferation, persistence, and trafficking, and highlight the value of immunocompetent NHP models for advancing next-generation CAR-T therapies.

Results

Design and characterization of a combinatorial CD20-targeted CAR array

To systematically assess how CAR structure impacts CAR-T cell activity, we designed an array of CD20-directed CARs, termed V1-V20, including bespoke combinations of established hinge (CD8, CD28), transmembrane (CD8, CD28), and costimulatory domains (4-1BB, CD28, BAFF-R, MyD88-CD40, OX40) (**Figure 1A and Table 1**). To enable direct comparison, we retained the same validated CD20 scFv and CD3ζ activation domains across all designs,^{16,17} and included a co-expressed epidermal growth factor receptor (EGFRt) to detect CAR-transduced cells.⁸⁵ We manufactured the CAR-T cell array using established methods,^{16,17} including separate isolation and activation of CD4⁺ and CD8⁺ cells from peripheral blood mononuclear cells (PBMCs), transduction with lentiviral vectors, and expansion in flasks designed to maximize gas and nutrient convection (**Figure 1B**).

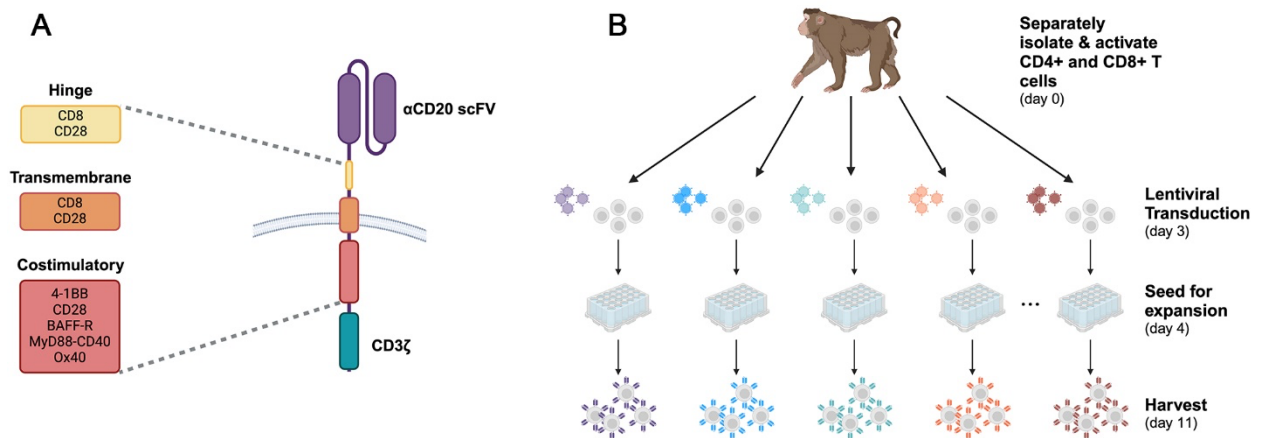


Figure 1. Design and generation of a combinatorial array of CD20-targeted CAR-T cells.

(A) An array of chimeric antigen receptor (CAR) constructs was engineered to systematically explore the impact of 20 distinct CAR architectures in the same animal. The array spans combinations of hinge (CD8 or CD28), transmembrane (CD8 or CD28) and costimulatory (4-1BB, CD28, BAFF-R, MyD88-CD40, Ox40) domains. (B) CAR-T cells were manufactured *ex vivo*: autologous CD4⁺ and CD8⁺ cells from pigtail macaques (*M. nemestrina*) were isolated, activated, transduced with CAR-encoding lentiviral vectors, and expanded before harvesting for infusion and functional assays.

		Hinge	TM	Costim
	V1	CD8	CD8	4-1BB
	V2	CD28	CD28	
	V3	CD8	CD28	
	V4	CD28	CD8	
	V5	CD8	CD8	CD28
	V6	CD28	CD28	
	V7	CD8	CD28	
	V8	CD28	CD8	
	V9	CD8	CD8	BAFF-R
	V10	CD28	CD28	
	V11	CD8	CD28	
	V12	CD28	CD8	
	V13	CD8	CD8	MyD88-CD40
	V14	CD28	CD28	
	V15	CD8	CD28	
	V16	CD28	CD8	
	V17	CD8	CD8	Ox40
	V18	CD28	CD28	
	V19	CD8	CD28	
	V20	CD28	CD8	

Table 1. Domain structures for the combinatorial CAR array. Overview of the 20-plex CAR array, categorized by hinge, transmembrane (TM), and costimulatory (Costim) domains. Symbols represent individual CAR variants and are used consistently throughout the study to denote specific constructs.

All CAR variants were successfully manufactured *ex vivo*, with MyD88-CD40 CAR-T cells exhibiting enhanced expansion during manufacturing (**Figure 2A**). Each variant demonstrated robust CAR expression (**Figure 2B**), balanced CD4:CD8 ratios (**Figure 2C**), and a predominant central memory phenotype (CD28⁺ CD95⁺; **Figure 2D**).¹⁶ Among these, BAFF-R and MyD88-CD40 CAR-T cells displayed higher CD4:CD8 ratios and a greater proportion of central memory T-cells compared to 4-1BB or CD28 CAR-T cells. Notably, MyD88-CD40 CAR-T cells showed significantly increased expression of CD25 (**Figure 2E**, $P < 0.0005$) and HLA-DR (**Figure 2F**, $P < 0.00005$) activation markers,⁸⁶ suggesting that this domain confers a hyperactivation phenotype. These data validate our arrayed anti-CD20 CAR-T cell manufacturing process and highlight CAR-dependent phenotypes that emerge during *ex vivo* manufacturing.

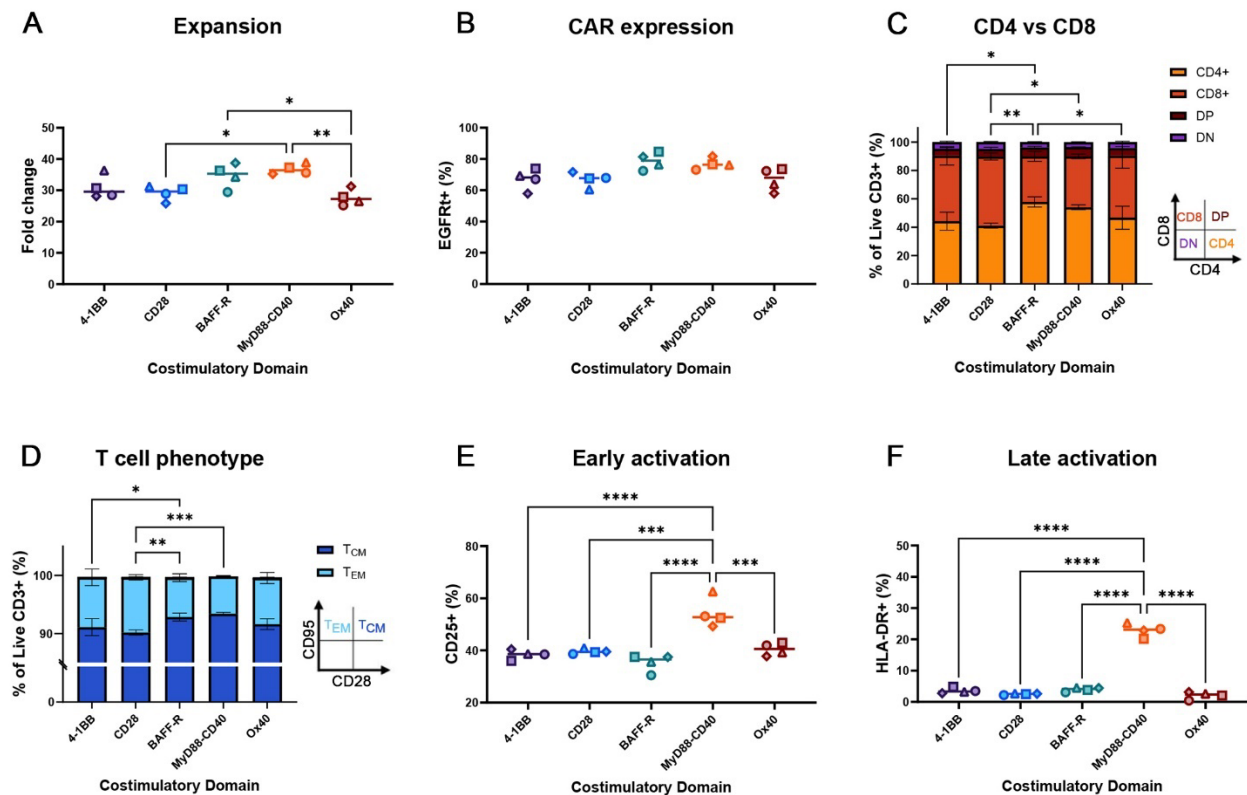


Figure 2. Phenotypic characterization of individual CAR-T variants. (A) Following arrayed *ex vivo* manufacturing, CAR-T cell variants V1-V20 were harvested and counted. Fold change represents change in total cell counts over 7 days of expansion in G-REX flasks. (B-F) At harvest, each CAR-T variant was assessed via flow cytometry for (B) CAR expression via truncated EGFR (EGFRt); (C) CD4/CD8 T cell composition including CD4⁺ single positive (CD4⁺ CD8⁻), CD8⁺ single positive (CD4⁻ CD8⁺), double positive (DP, CD4⁺ CD8⁺), and double negative (DN, CD4⁻ CD8⁻); (D) T cell subsets, T_{CM} (Central memory, CD28⁺ CD95⁺), T_{EM}

(Effector memory, CD28⁺ CD95⁺); (E) early T cell activation marker CD25 in EGFRt⁺ subsets; and (F) late T cell activation marker HLA-DR in EGFRt⁺ subsets. Each datapoint represents the average from three biological replicates ($n = 3$ pigtail macaques), except for V17 which represents the average from two biological replicates ($n = 2$ pigtail macaques). Statistical significance was determined using one-way ANOVA with Tukey's correction for multiple comparisons. $*P < 0.05$, $**P < 0.005$, $***P < 0.0005$, $****P < 0.00005$. In panels A, B, E, and F, shapes and color codes correspond to Table 1.

CAR-T variants display distinct functional profiles ex vivo

We next extended our *ex vivo* experiments to explore the impact of CAR architecture on CAR-T cell function, including proliferative, cytokine, and cytolytic responses. To measure CAR-specific proliferation, we quantified the dilution of CellTrace Violet (CTV) dye (**Figure 3A**). We observed robust division of CAR⁺ cells when stimulated with a cell line overexpressing CD20 antigen (K562-CD20), with minimal bystander proliferation in the CAR⁻ fraction (**Figure 3B**, $P < 0.00005$). Only MyD88-CD40 CAR-T cells exhibited significant tonic signaling, as indicated by antigen-independent proliferation of CAR⁺ cells (**Figure 3C**, $P < 0.00005$). These cells also displayed phenotypic features of tonic signaling,⁸⁷ including elevated CD25 and HLA-DR, as well as reduced expression of exhaustion markers PD-1 and TIGIT (**Figure 3D**). Following K562-CD20 stimulation, all CAR variants exhibited antigen-specific cytokine secretion (**Supplemental Figure 1A-G**), with distinct patterns across costimulatory domain groups. Most notably, CD20-stimulated MyD88-CD40 CAR-T cells produced minimal IL-6 (**Figure 3E**), significantly reduced IL-10 (**Figure 3F**, $P < 0.005$), and increased IL-13 (**Figure 3G**, $P < 0.005$), a cytokine less characterized in CAR-T cell biology but previously linked to MyD88-CD40 signaling.⁷⁶ Assays measuring the specific lysis of an adherent CD20-overexpressing cell line (LLCMK2-CD20) over time (**Figure 3H**), confirmed antigen-specific cytotoxicity across all CAR variants, with minimal non-specific killing of the parental cell line (LLCMK2-wt) (**Figure 3I**, $P < 0.00005$). Interestingly, MyD88-CD40 CAR-T cells also displayed modest-to-significantly enhanced antigen-dependent cytotoxicity (**Supplemental Figure 1H**). Finally, we applied principal component analysis (PCA) to integrate data from functional assays (**Figure 3B-I**). PCA further highlighted the distinctiveness of MyD88-CD40 CAR-T cells, which clustered separately from other variants (**Figure 3J**). Collectively, these findings underscore the influence of costimulatory domains on CAR-T function, with MyD88-CD40 CARs distinguished by tonic signaling, unique cytokine profiles, and potent cytolytic activity.

Chronic antigen stimulation highlights phenotypic and functional differences among CAR-T variants

To investigate the influence of CAR design on *in vivo* persistence,^{69,70} we next compared the long-term function of CAR-T variants in an *ex vivo* chronic antigen stimulation assay. CAR-T cells were repeatedly exposed to K562-CD20 cells to evaluate CAR-T durability amid exhaustive conditions (**Figure 4A**).^{88–91} All variants demonstrated robust expansion, marked by increased EGFRt⁺ expression across successive stimulations (**Figure 4B**). Consistent with previous reports,^{75,76} only MyD88-CD40 CARs retained lower PD-1 (**Figure 4C**, $P < 0.0005$) and TIGIT (**Figure 4D**, $P < 0.005$) after chronic stimulation, suggesting this domain confers superior resistance to exhaustion. MyD88-CD40 CAR-T cells also produced less IL-10 (**Figure 4E**, $P < 0.005$) and substantially more IL-13 (**Figure 4F**, $P < 0.00005$). Interestingly, 4-1BB CAR-T cells failed to retain antigen-specific cytotoxicity after chronic stimulation (**Figure 4G**). PCA of chronic stimulation data further highlighted the distinct clustering of MyD88-CD40 and 4-1BB CARs (**Figure 4H**), underscoring the role of costimulatory domains in shaping CAR-T exhaustion, cytokine secretion, and cytotoxicity during prolonged antigen exposure.

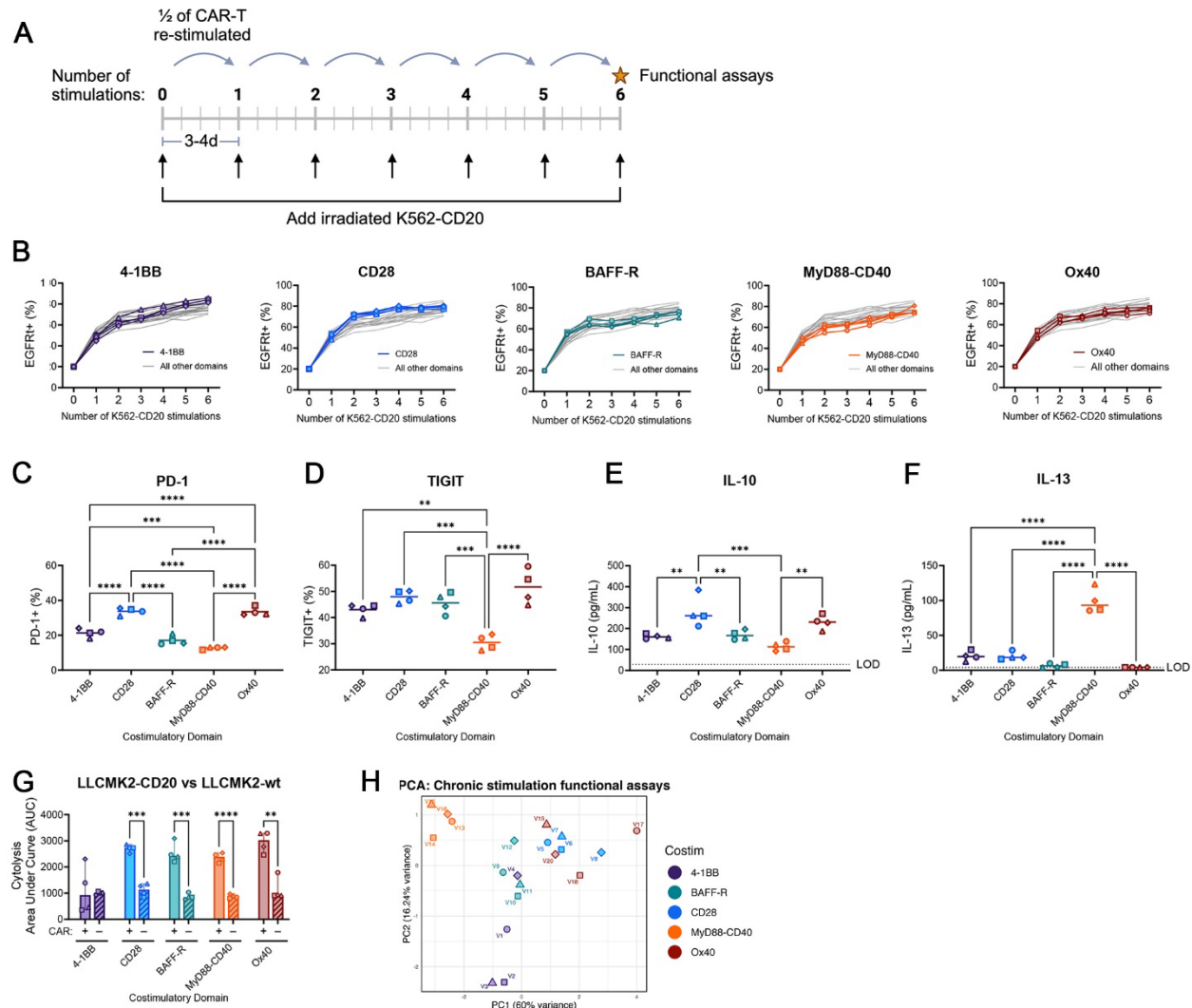


Figure 4. Chronic antigen stimulation ex vivo reveals functional divergence between CAR-T cell variants. (A) *Ex vivo*-manufactured CAR-T cells were quantified and normalized to 20% CAR⁺ by addition of untransduced T cells, then repeatedly stimulated with irradiated K562-CD20 every 3–4 days. Over six rounds of stimulation, cells were harvested for immunophenotyping and half were restimulated. After the final round of stimulation, cells were assessed for cytotoxicity and cytokine secretion. (B) CAR expression levels; each panel highlights one of 5 costimulatory domain groups. (C–D) Expression of exhaustion markers (C) PD-1 and (D) TIGIT by flow cytometry. (E–F) Secretion of (E) IL-10 and (F) IL-13 following chronic stimulation. (G) Cytolysis AUC for LLCMK2-CD20 and LLCMK2-wt target cells after chronic stimulation. (H) Data from (C–G) was compiled, Z-normalized and analyzed via PCA. Each datapoint in (B–F) and LLCMK2-CD20 cytotoxicity in (G) represents the average of three biological replicates ($n = 3$ pigtail macaques), except for V17 ($n = 2$). LLCMK2-wt cytotoxicity in (G) represents two biological replicates. Statistical significance was determined using one-way ANOVA with Tukey’s correction for (C–F) and multiple

unpaired T-tests with Holm-Šídák correction for (G). * $P < 0.05$, ** $P < 0.005$, *** $P < 0.0005$, **** $P < 0.00005$.

Pooled chronic stimulation ex vivo reveals donor-specific proliferation of CAR-T variants

Prior to *in vivo* experiments where CAR variants were pooled and infused into single recipient NHPs, we developed a competitive *ex vivo* stimulation assay in which pooled CAR-T variants were serially stimulated with K562-CD20 as in **Figure 4A**. Since flow cytometry cannot distinguish unique CAR variants, we turned to CAR quantitation at the DNA level using digital droplet PCR (ddPCR). Our variant-specific ddPCR assay was designed to enable precise quantification of individual CAR variants within pooled samples from *ex vivo* and *in vivo* experiments. Primer-probe sets were designed for individual CAR variants and validated to ensure variant-specific amplification (**Supplemental Figure 2A**), and assay sensitivity to single-copy thresholds (**Supplemental Figure 2B**). Donor-specific expansion patterns emerged, with 4-1BB or CD28 CARs preferentially expanding (**Figure 5**). Notably, V7 (CD8 hinge, CD28 transmembrane, CD28 costimulatory domain), showed significant enrichment in two donors, whereas BAFF-R or MyD88-CD40 CARs exhibited no expansion across all donors. These data suggest that CD28 and 4-1BB costimulatory domains promote efficient proliferation in competitive *ex vivo* assays. We next performed a corresponding competitive *in vivo* study using the same biological donors to determine whether these *ex vivo* findings were recapitulated *in vivo*.

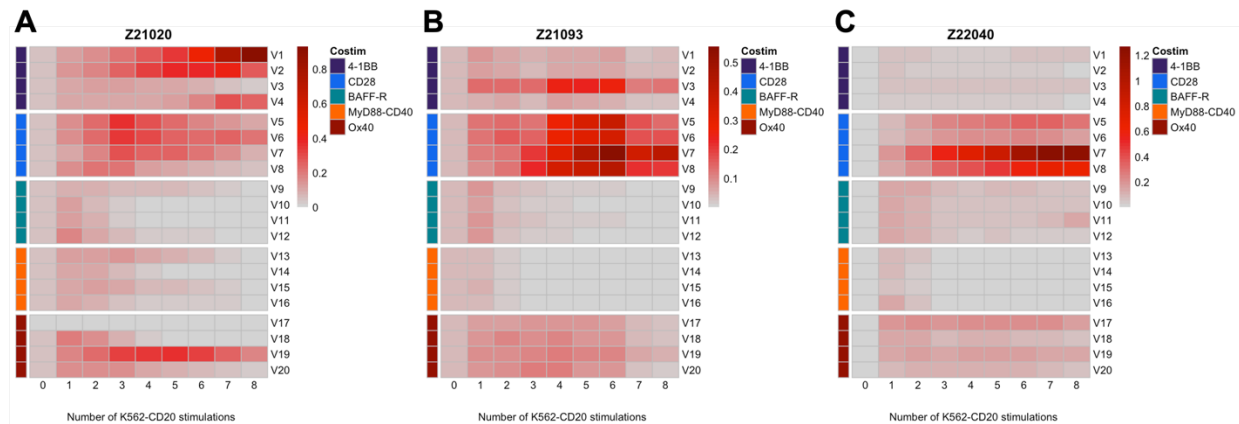


Figure 5. Pooled *ex vivo* chronic stimulation assay reveals donor-specific expansion of CAR-T cell variants. (A-C) Equal numbers of CAR⁺ cells from all CAR-T variants were pooled, subjected to chronic antigen stimulation, and analyzed for relative CAR variant abundance using ddPCR. Heatmaps depict normalized vector copy number (VCN), calculated by normalizing the measured VCN at each timepoint to the CAR Variant VCN abundance ratio in the initial pooled sample (see Methods), across three biological donors,

as indicated above. Donors correspond to 3 pigtail macaques that were subsequently studied in *in vivo* experiments.

Pooled CAR-T cells expand in vivo, induce B-cell aplasia and display distinct inflammatory profiles

Following extensive *ex vivo* characterization, we next evaluated our CAR array in a cohort of three pigtail macaques. We pooled CAR-T variants in equal proportions (**Supplemental Figure 3**) and infused into autologous NHPs preconditioned with a low-dose cyclophosphamide regimen.¹⁷ Each animal received a dose of 9×10^6 CAR⁺ cells/kg (**Figure 6A and Supplemental Table 1**). To align with our *ex vivo* data and methods in prior studies from our group and others,^{16,42} we provided supplemental antigen in the form of irradiated K562-CD20 cells that were administered at 3 and 5 weeks post-CAR infusion. CAR-T proliferation, trafficking, and persistence were evaluated in longitudinal blood and tissues for up to 102 days post-CAR infusion (**Figure 6B**).

Total CAR-T cells expanded significantly after infusion, constituting 30-96% of peripheral CD3⁺ T cells, with peak levels observed between 7-11 days post-infusion (**Figure 6C-E**). CAR-T expansion corresponded with rapid B-cell depletion in peripheral blood (**Figure 6C-E**) and substantial B-cell follicle loss in lymph nodes and spleen, as confirmed by immunohistochemistry (**Figures 6F-G**). At day 25, we detected a modest secondary CAR-T expansion following a single infusion of K562-CD20 in Z21020 (**Figure 6C**). Z22040 exhibited extreme CAR-T expansion within the first week (92-96% of CD3⁺ T cells) and developed immune effector cell-associated neurotoxicity syndrome (ICANS) despite intensive supportive care (**Supplemental Table 1**), requiring an end of study at day 9. Importantly, integration site analysis of infusion products and PBMCs from peak expansion revealed highly polyclonal expansion of CAR-T cells in all NHPs, with 7036—17,501 unique clones expanding and no concerning enrichment of clones with integrations near oncogenes (**Supplemental Table 3**). For Z21020 and Z21093, B-cells recovered between 30- and 40-days post-infusion, although levels remained low through study completion (**Figure 6G**). Z21020 and Z21093 developed gut infections approximately 80 days post-infusion that did not respond to antibiotic treatment. Based on clinical signs and low levels of CAR⁺ cells at these timepoints, these animals ended study at days 92 and 102. Collectively, these data indicate increased CAR-T activity in NHPs receiving the pooled V1-V20 CAR array, relative to a previously-published cohort that received a single infusion of V1, a 4-1BB based CAR.¹⁷ Intriguingly, inflammatory biomarkers in serum were distinct between these cohorts. Unlike the historical V1 cohort, which exhibited a significant IL-6 spike after CAR-T infusion, the pooled CAR array cohort showed no IL-6 elevation (**Figure**

6H) but had markedly higher ferritin levels (**Figure 6I**). C-Reactive protein (CRP) levels were comparable (**Figure 6J**). Multiplex plasma cytokine analyses during peak CAR expansion confirmed reduced IL-6 levels, as well as decreased IFN γ and TNF α and elevated IL-2 in the CAR array cohort (**Supplemental Figure 4A**). We also performed lumbar punctures to collect cerebrospinal fluid (CSF) and observed distinct cytokine trends, with CAR array NHPs exhibiting trends toward increased IL-6 and IL-18 compared to the historical cohort (**Supplemental Figure 4B**). Collectively, the pooled CAR-T array demonstrated robust CAR-T expansion, durable B-cell depletion, and distinct, compartment-specific inflammatory dynamics, including muted IL-6 responses, elevated ferritin, and altered cytokine profiles.

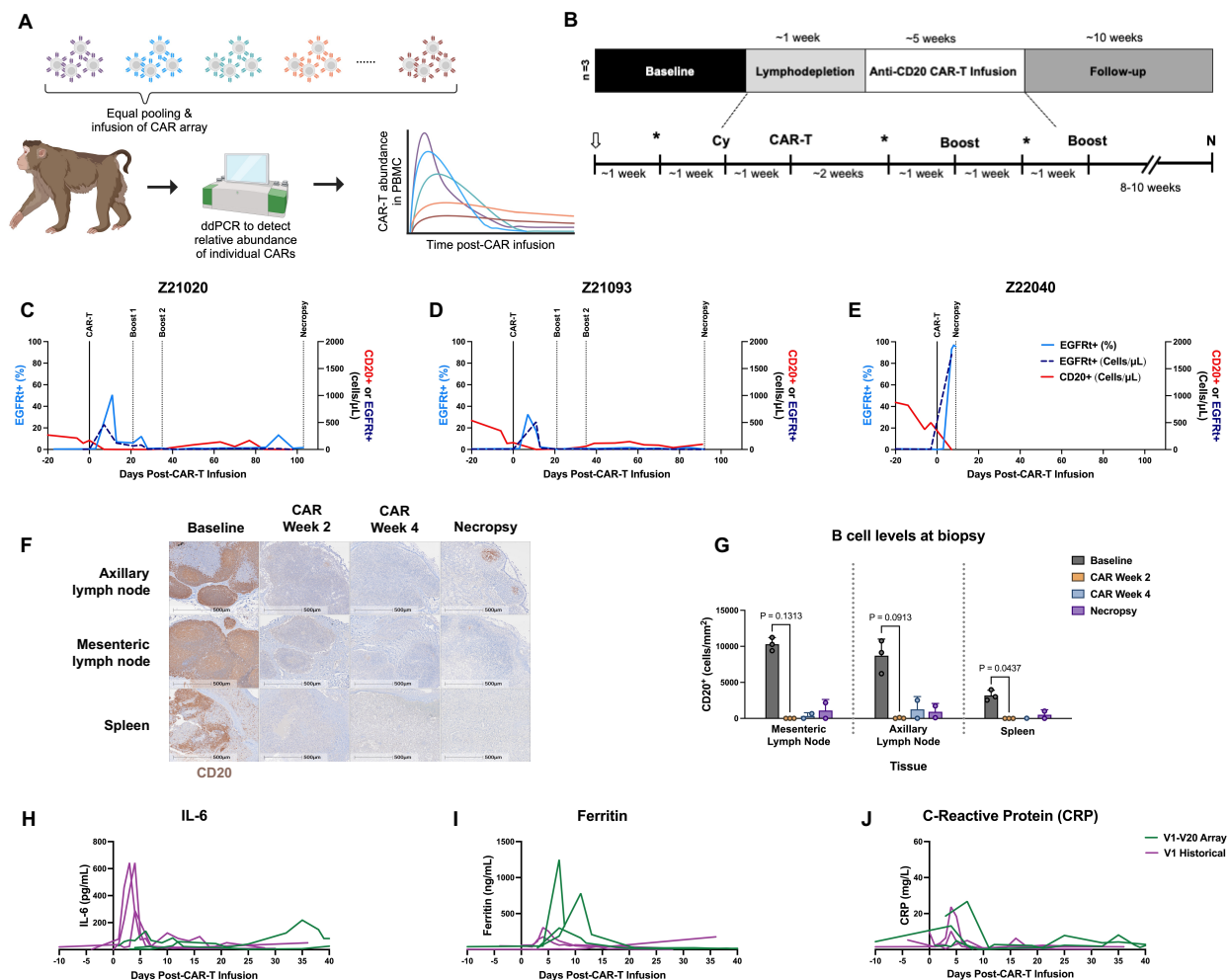


Figure 6. Infusion of the pooled CAR-T cell array drives robust expansion, durable B-cell depletion, and distinct inflammatory dynamics. (A) CAR-T variants were manufactured ex vivo, pooled in equal proportions, and infused into conditioned autologous NHPs. CAR variant abundance was tracked longitudinally using ddPCR. (B) Animals underwent leukapheresis (arrow) followed by cyclophosphamide (Cy) conditioning. Pooled CAR-T products were infused (CAR-T), and two K562-CD20 antigen

boosts (Boost) were administered at weeks 3 and 5 post-infusion. Tissue samples (*) were collected at -2, 2, and 4 weeks relative to CAR-T cell infusion. Necropsy (N) occurred 9-102 days after CAR infusion. (C-E) Longitudinal analysis of EGFRt⁺ (%) (light blue), EGFRt⁺ counts (cells/ μ L) (dark blue) and B-cell counts (cells/ μ L) (red) in PBMCs, for animal ID (C) Z21020, (D) Z21093, and (E) Z22040. (F) Representative immunohistochemistry (IHC) images from Z21020 showing CD20⁺ B-cells (brown) in lymphoid tissues at indicated timepoints. (G) Quantification of CD20⁺ B-cells from IHC across timepoints, with each data point representing an individual animal. (H-J) Longitudinal quantification of serum (H) IL-6, (I) ferritin, and (J) C-Reactive protein levels in the pooled CAR array cohort (V1-V20 CAR array) compared to a comparable historical single-CAR study that infused V1 (V1 Historical). Statistical significance for (G) was determined using Kruskal-Wallis tests with Dunn's correction for multiple comparison. * $P < 0.05$, ** $P < 0.005$, *** $P < 0.0005$, **** $P < 0.00005$.

MyD88-CD40 CARs preferentially expand, traffic, and persist in vivo

We surmised that the increased CAR activity, toxicity, and divergent inflammatory profiles in CAR array animals relative to our historical V1 cohort were driven by distinct CAR variant(s) within the array. Consistent with this hypothesis, longitudinal variant-specific ddPCR revealed significant enrichment of MyD88-CD40 CAR-T cells (V13-V16) in PBMCs across all timepoints and NHPs (**Figure 7A-C**, $P < 0.005$). Likewise, MyD88-CD40 CAR-T cells were strikingly enriched in lymph nodes, spleen, bone marrow, BAL, and CSF samples collected within 2 weeks of CAR-T infusion (**Figure 7D**, $P < 0.005$). Among these, V14 (CD28 hinge/transmembrane domain) was most abundant during the primary expansion phase (**Figure 7E**) and clustered distinctly from other MyD88-CD40 variants via PCA (**Figure 7F**). 4-1BB CARs showed some early expansion and modest tissue trafficking, but did not persist beyond two weeks. Neither CD28 nor BAFF-R CARs showed appreciable expansion in peripheral blood or tissues.

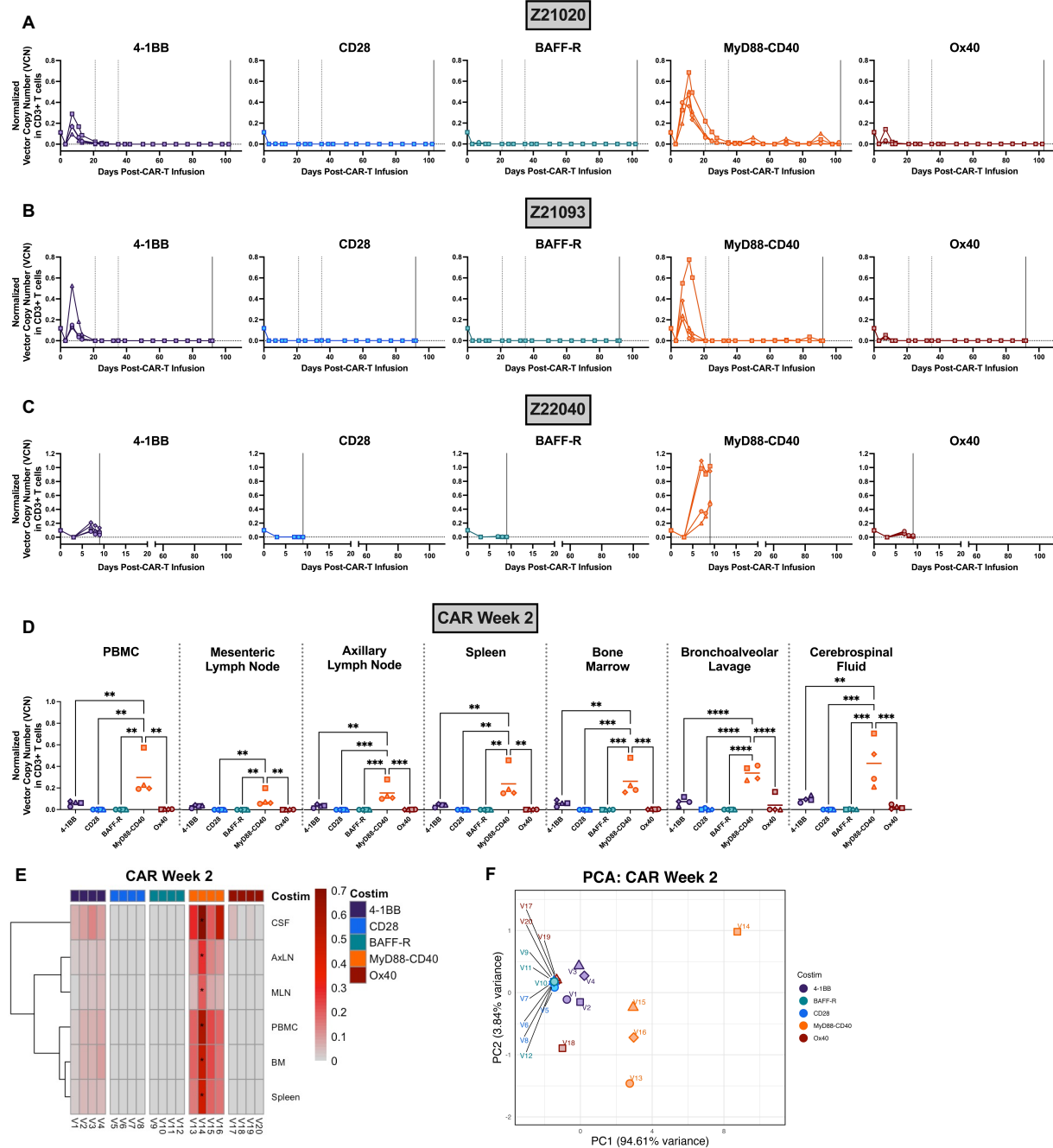


Figure 7. MyD88-CD40 CAR-T cells exhibit superior proliferation, durability, and tissue trafficking *in vivo*. Genomic DNA was extracted from blood and tissue samples to quantify the relative abundance of each CAR variant by ddPCR. (A-C) Normalized vector copy number (VCN) of CAR variants, grouped by distinct costimulatory domains, for animal ID (A) Z21020, (B) Z21093, and (C) Z22040 PBMCs. Dashed lines denote the timing of K562-CD20 antigen boosts, while solid lines mark the day of necropsy. (D) Normalized VCN from tissues collected between 9- and 14-days post-infusion, with each datapoint representing the average across three NHPs ($n = 3$ pigtail macaques), except for V17 ($n = 2$). (E)

Normalized VCN from tissues collected between 9- and 14-days post-infusion, as in (D), displayed as a heatmap for statistical analysis by one-sample Z test with Benjamini-Hochberg correction to identify specific significantly enriched variants. Stars in the V14 column indicate significant abundance of this variant. (F) Data from (D) was compiled, Z-normalized and analyzed via PCA. Statistical significance for (D) was determined using one-way ANOVA with Tukey's correction for multiple comparisons. $*P < 0.05$, $**P < 0.005$, $***P < 0.0005$, $****P < 0.00005$.

MyD88-CD40 CARs persisted in blood and tissues until necropsy, with marked enrichment in bronchoalveolar lavage (BAL) and lung tissue samples (**Figure 8A-B**). At necropsy, V13, V14, and V15 showed significant enrichment across various tissues (**Figure 8C**), and PCA revealed these variants clustered distinctly from each other and the rest of the CAR array (**Figure 8D**). Our NHP study demonstrates that MyD88-CD40 domains drive superior CAR-T expansion, persistence, and tissue trafficking *in vivo*, significantly outperforming conventional 4-1BB and CD28 CAR-T cells.

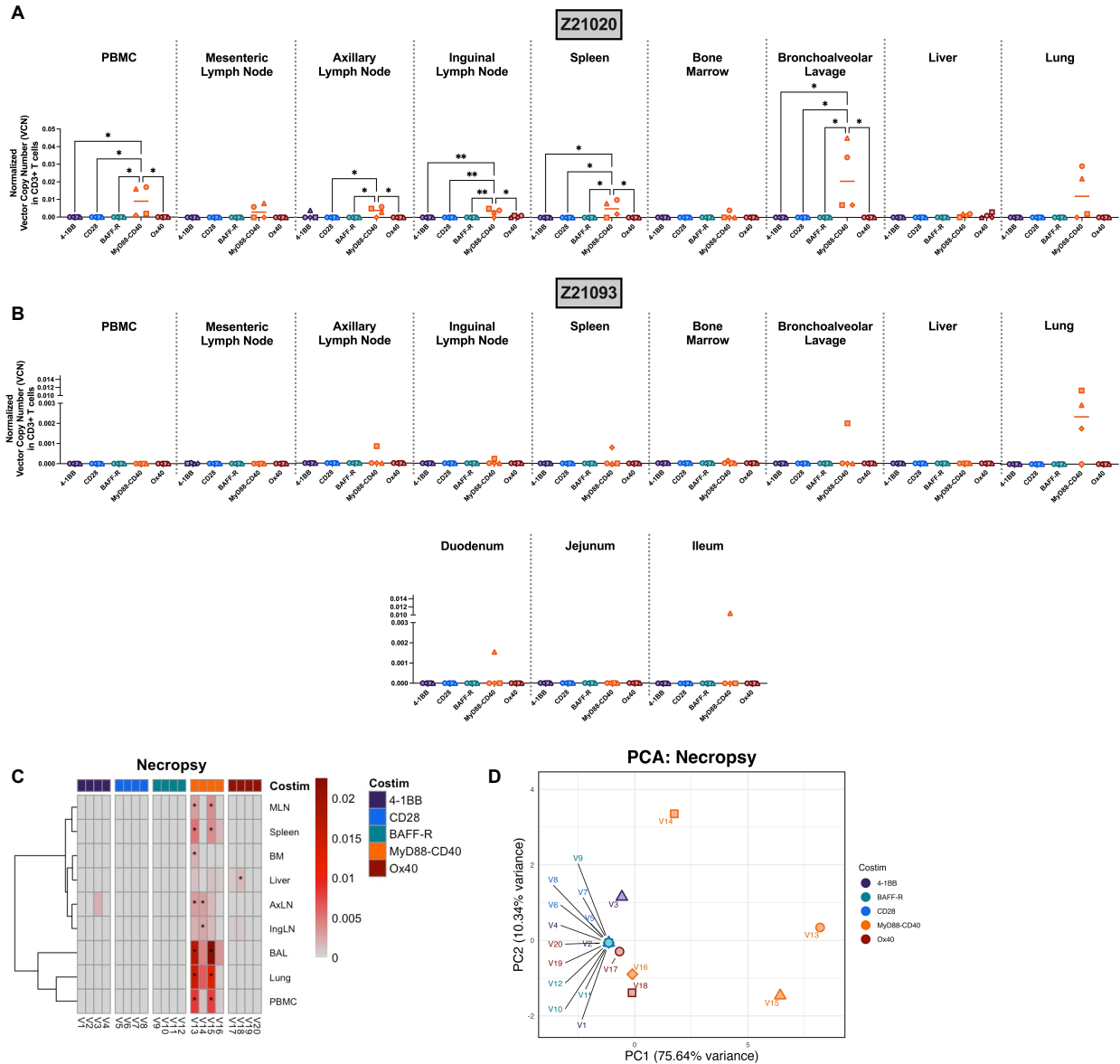


Figure 8. Necropsy analysis reveals durable persistence of MyD88-CD40 CAR-T cells. (A-B) Normalized VCN from tissues collected at necropsy for (A) Z21020 and (B) Z21093. (C) Data from (A-B), averaged and displayed as a heatmap for statistical analysis by one-sample Z test with Benjamini-Hochberg correction to identify significantly enriched variants, indicated with stars. (D) Compiled data from (C) were Z-normalized and analyzed via PCA. Statistical significance in (A) was determined using one-way ANOVA with Tukey's correction for multiple comparisons. * $P < 0.05$, ** $P < 0.005$, *** $P < 0.0005$, **** $P < 0.00005$.

Discussion

While CAR-T cell therapy has redefined the treatment landscape for numerous B-cell malignancies, achieving long-term efficacy remains a challenge, in part due to suboptimal

CAR-T cell persistence *in vivo*. Addressing this limitation will be paramount for unlocking the full potential of CAR-T therapies, not only in the context of B-cell malignancies but also for expanding their application to solid tumors, infectious diseases, and autoimmunity.^{17,92} We developed a novel *in vivo* CAR competition assay in NHPs to simultaneously evaluate the behavior of multiple CAR designs in an immunocompetent background. We identified specific CAR architectures, notably the MyD88-CD40 costimulatory domain, that drive enhanced CAR-T cell proliferation, persistence, and tissue trafficking.

In primary cell-based assays, MyD88-CD40 CAR-T cells exhibited four key features: (1) enhanced activation, (2) tonic signaling, (3) distinct cytokine secretion, and (4) resistance to exhaustion following chronic antigen stimulation. These characteristics likely supported the robust *in vivo* proliferation of MyD88-CD40 CARs. MyD88-CD40 V14, which featured a CD28 hinge and transmembrane domain, demonstrated the strongest proliferation *in vivo*, consistent with prior reports that the CD28 transmembrane domain enhances CAR activity and antigen sensitivity.⁸⁰ This feature may have supported the sustained persistence of MyD88-CD40 CAR-T cells even as B-cells declined. Importantly, other groups have similarly identified MyD88-CD40 as a superior costimulatory domain in pooled screening approaches, though predominantly in liquid tumor models, suggesting the efficacy of this domain may vary with tumor type and the associated microenvironment.⁹³ This group also linked MyD88-CD40 to a hyperactivation and tonic signaling phenotype, reinforcing the reproducibility of our findings.

The impact of tonic signaling on CAR-T cell efficacy remains controversial. Many groups have linked tonic signaling to scFv stability and characterized it as a detrimental feature associated with excessive activation, terminal differentiation, and exhaustion.^{94,95} Others have noted that modest tonic signaling may improve CAR-T persistence and anti-tumor function.^{96,97} In our study, we maintained a consistent anti-CD20 scFv across all CAR designs, allowing us to attribute the tonic signaling primarily to intracellular domains. Notably, the tonic signaling we observed in MyD88-CD40 CARs did not lead to exhaustion; instead, these CARs displayed a robust resistance to exhaustion, a unique feature of this domain. This lack of exhaustion, combined with tonic signaling, likely synergized to enhance MyD88-CD40 CAR-T activity and durability *in vivo*.

The unique cytokine profile of MyD88-CD40 CAR-T cells offers key insights into their mechanisms of action and implications for next-generation CAR-T therapies. IL-6 is a hallmark of CAR-T expansion and CRS observed in patients and in prior NHP studies with 4-1BB CARs.^{16,17,98} The minimal IL-6 elevation we observed in MyD88-CD40 CAR-T cells both *ex vivo* and *in vivo* suggests that the characteristic IL-6 spike is primarily driven by

conventional costimulatory domains like 4-1BB and CD28. As novel CAR architectures advance to the clinic, our findings underscore the importance of considering domain-specific toxicities. For example, our data indicate that IL-6 assays would be insufficient to diagnose CRS in patients receiving MyD88-CD40 CAR-T cells. As in prior studies, MyD88-CD40 CAR-T cells also exhibited significantly reduced IL-10 secretion *ex vivo*⁷⁶. Given IL-10's potent anti-inflammatory properties and critical role in regulating immunological homeostasis,⁹⁹ its diminished secretion may have facilitated the robust expansion that we observed *in vivo*. These results align with emerging interest in combining CAR-T cells with IL-10 blockade, which has been shown to reinvigorate CAR-T activity in preclinical models.^{100,101} Rather than IL-6, MyD88-CD40 CAR-T cells secreted significantly increased IL-13 *ex vivo*, a cytokine associated with Th2-mediated immune responses and allergic inflammation, and previously linked to MyD88-CD40 signaling.^{76,102} This inflammatory signaling may have supported the hyperactivation and tonic signaling observed in these CARs, suggesting that IL-13 secretion could be applied in armored CAR-T therapies to enhance activation and persistence.

Our pooled *ex vivo* stimulation assays, which were intended to simulate the pooled *in vivo* competition assay, revealed superior expansion of CD28 and 4-1BB CARs, and almost immediate depletion of MyD88-CD40 CARs. Critically, these results were not replicated *in vivo*, where we saw striking expansion of MyD88-CD40 CAR-T cells and near-complete absence of CD28 CAR-T cells. This discrepancy may potentially be attributed to paracrine signaling factors present in the *ex vivo* setting that supported the expansion of CD28 and 4-1BB CAR designs. Our findings caution against overreliance on artificial *ex vivo* pooled screening approaches and emphasize the importance of evaluating CAR-T cell function in physiologically relevant preclinical models. We also demonstrated the value of the NHP model in identifying acute toxicities, including those associated with MyD88-CD40 domains. In our study, one NHP experienced uncontrolled CAR-T expansion, suggesting that hyperactivation, tonic signaling, and dampened regulatory mechanisms may also contribute to safety concerns. Similar toxicities have been observed clinically; in an early trial evaluating PSCA-specific CAR-T cells incorporating an inducible MyD88-CD40 costimulation switch for prostate cancer, dose-limiting toxicities led to trial discontinuation.⁷⁷ While biological activity was observed, these findings are consistent with prior data indicating that MyD88-CD40 CARs may be more effective in liquid tumors than in solid tumors.⁹³ Our results underscore the importance of immunocompetent NHP models in recapitulating key CAR-mediated toxicities observed in the clinic, which are rarely captured by murine models. Future NHP studies should focus not only on the continued development of novel CAR architectures but also on identifying prognostic

markers of toxicity and devising strategies to mitigate the hyperactivity observed in MyD88-CD40-containing CARs, such as cis-acting safety switches.¹⁰³

In summary, our study highlights the power of the NHP model as a platform for pooled screening of CAR designs, enabling the identification of superior architectures such as MyD88-CD40. The exceptional proliferation, persistence, and trafficking conferred by this domain may address fundamental challenges in CAR-T therapies, including limited persistence. The unique features associated with MyD88-CD40 include enhanced activation, tonic signaling, distinct cytokine secretion, and resistance to exhaustion. These key attributes that drive MyD88-CD40 potency provide valuable insights that can be leveraged in the design of next-generation therapies.

Methods

CAR-T cell design and manufacturing

The CAR array was designed to span all combinations of hinge (CD8, CD28), transmembrane (CD8, CD28), and costimulatory (4-1BB, CD28, BAFF-R, MyD88-CD40, Ox40) domains, with a common anti-CD20 scFv and CD3 ζ activation domain. The CAR array was codon-optimized for expression in *Macaca nemestrina* (pigtail macaques) and synthesized individually as gBlocks (Integrated DNA Technologies). We included a co-expressed marker (truncated epidermal growth factor receptor, EGFRt) downstream of the CAR, separated by a T2A sequence, as a surrogate for detecting CAR-transduced T cells. The CAR array was cloned into a self-inactivating lentivirus backbone (vector plasmids kindly provided by Dr. Michael Jensen) and sequence verified via next-generation sequencing (Plasmidsaurus). Lentiviral vectors were generated using a third-generation packaging system pseudotyped with Vesicular Stomatitis Virus G-protein (Fred Hutch Preclinical Vector Core), and titered via qPCR in HT1080 cells.¹⁰⁴ CAR-T cells were manufactured *ex vivo* as described.^{17,42} Briefly, CD4⁺ and CD8⁺ T cells were isolated from peripheral blood mononuclear cells (PBMCs) using bead-based selection (STEMCell Technologies) and cultured in X-VIVO-15 (Thermo Fisher Scientific) supplemented with 10% FBS, 1% penicillin/streptomycin, 1% L-glutamine, 55 μ M 2-mercaptoethanol, and 5 ng/mL recombinant IL-7 and IL-15 (Peprotech). T cells were activated with artificial antigen-presenting cells (aAPCs; kind gift from Dr. James Riley¹⁰⁵) at a 2:1 ratio. Three days later, T cells were transduced with lentiviral vectors at a multiplicity of infection from 1–10 with 5

μL of LentiBlast Transduction Reagent (OZ Bioscience), followed by 7-day expansion in G-REX24 flasks (Wilson Wolf).

CellTrace Violet Proliferation Assay

CAR-T cell proliferation was assessed immediately after manufacturing using CellTrace Violet dye (CTV, Thermo Fisher Scientific). CAR-T cells were CTV-labeled according to manufacturer instructions, and 5×10^5 labeled CAR-T cells were co-cultured with 1×10^5 irradiated CD20-expressing K562 cells (K562-CD20, kindly provided by Dr. Brian Till¹⁰⁶) or wild-type K562 cells (K562-wt; ATCC) (effector-to-target ratio 5:1) for three days at 37°C. Proliferation was quantified by flow cytometry based on CTV dilution in CAR⁺ and CAR⁻ subsets.

Real-time cell analysis of cytotoxicity

CAR-T cell cytotoxicity was assessed using the xCELLigence (Agilent Technologies) real-time cell analysis (RTCA) platform and an adherent target cell line. CAR-T cells were co-cultured with CD20-expressing LLCMK2 (LLCMK2-CD20)¹⁷ or wild-type LLCMK2 (LLCMK2-wt; ATCC) target cells at varying effector-to-target ratios, and target cell death was quantified as percent cytolysis (%). Cytotoxicity was measured immediately after manufacturing and following six rounds of stimulation with irradiated K562-CD20 cells.

Flow cytometry and chronic antigen stimulation assay

All antibodies and clones for flow cytometry are listed in **Supplemental Table 2**. All flow panels included a live/dead stain to exclude dead cells. The long-term function of CAR-T cell variants was evaluated through serial stimulation with irradiated K562-CD20 cells. To standardize initial conditions, CAR-T variants were analyzed for CAR expression via flow cytometry and diluted to 20% CAR⁺ cells by adding untransduced autologous T cells. 5×10^5 CAR-T cells were co-cultured with 1×10^5 irradiated K562-CD20 or K562-wt cells (effector-to-target ratio of 5:1). Every 3-4 days, CAR-T cells were harvested, with half being restimulated and half stained with immunophenotyping panels and analyzed by flow cytometry. The same experiment was performed with equal numbers of pooled CAR⁺ cells from each CAR-T variant, with half being restimulated and half snap-frozen for analysis via ddPCR.

Cytokine measurements

NHP plasma samples and CAR-T cell supernatants collected 24 hours after co-culture with K562-CD20 or K562-wt cells were assayed using the LEGENDplex™ 10-plex NHP Th

Cytokine Panel (BioLegend). Cerebrospinal fluid (CSF) cytokines were measured using the ProcartaPlex™ 14-plex NHP Th Panel (Invitrogen), following the manufacturer's instructions.

***In vivo* NHP CAR-T cell studies**

This study was approved by the Institutional Animal Care and Use Committees of the Fred Hutchinson Cancer Center/University of Washington (protocol no. 3235-04). Pigtail macaques were housed and cared for under conditions that meet NIH standards as stated in the *Guide for the Care and Use of Laboratory Animals* (National Research Council, National Academy Press, Washington, DC, 1996), ILAR recommendations, and AAALAC accreditation standards, as described previously.⁴² Animals received cyclophosphamide (40 mg/kg) on days -7 and -6 prior to CAR-T infusion. To ensure equal pooling of CAR variants in the infusion product, CAR-T variants were analyzed for CAR expression by flow cytometry, and equal numbers of CAR⁺ variants were pooled to achieve a final dose of 9×10^6 CAR⁺ cells/kg. Irradiated K562-CD20 cells were infused at 2.5×10^7 cells/kg on days 21 and 35 following CAR-T infusion for antigen restimulation, as previously described.⁴² Blood was collected longitudinally until necropsy. Sampling in other compartments including spleen, lymph nodes, CSF, bone marrow and bronchoalveolar lavage (BAL) was performed at -2, 2- and 4-weeks following CAR-T infusion. Tissues were processed and dissociated for downstream analyses using established protocols.¹⁷

CRS management and serum cytokine assays

Animals received tocilizumab (8 mg/kg per dose), anakinra (1 mg/kg per dose), and dexamethasone (0.5 - 2 mg/kg per dose) under clinician direction to prevent and treat CRS (**Supplemental Table 1**). Z22040 experienced ongoing symptoms of immune effector cell-associated neurotoxicity syndrome (ICANS) and was given Keppra (15 - 60 mg/kg), furosemide (2 mg/kg), and diazepam (0.5 mg/kg) to prevent and treat seizure activity. Blood samples were analyzed by the University of Washington Clinical Laboratory for C-reactive protein (CRP), ferritin and IL-6.

Immunohistochemistry

Immunohistochemistry was performed as previously described.¹⁷ Briefly, 5- μ m tissue sections mounted on glass slides were deparaffinized in xylene and rehydrated through a series of graded ethanol to distilled water solutions. Heat-induced epitope retrieval (HIER) was performed with the antigen retrieval citrate buffer (pH 6.0) in a NxGen decloaking chamber (Biocare Medical) at 110°C for 15 min, cooled for 20 min, and then rinsed twice in ddH₂O and 1x TBS with 0.05% Tween 20 (TBS-T). Slides were incubated with blocking buffer (TBS-T with 0.25% casein) for 30 min at room temperature, rinsed with TBS-T, and incubated at room temperature for 1 hour with goat anti-CD20 (Invitrogen; cat. no. PA1-9024; at

1:800), which was diluted in blocking buffer. Endogenous peroxidases were blocked with 1.5% H₂O₂ in TBS-T for 5 min. Slides were then incubated with goat Polink-1 HRP (Origene). Slides were developed using Impact DAB (3,3'-diaminobenzidine; Vector Laboratories), washed in ddH₂O, counterstained with hematoxylin (Biocare Medical), mounted in Permount (Fisher Scientific), and scanned at 20x magnification. Quantitative image analysis was performed using HALO software (v.3.6.4134; Indica Labs) on lymph node or splenic sections from each animal. For CD20 quantification, the Multiplex IHC module v3.4.9 was used to detect CD20⁺ cells; quantification is presented as a proportion of total tissue (CD20⁺ cells/mm²). Manual curation was performed to confirm accurate annotations.

Droplet digital PCR assay

Droplet digital PCR (ddPCR) was used to quantify CAR variant abundance. Unique primer/probe sets were designed and validated for specific detection. Genomic DNA (gDNA) was extracted (QIAGEN; AllPrep DNA), diluted to ~100 ng/μL, and divided across six ddPCR assays, with 1-10 μL of gDNA per reaction. Each multiplex assay targeted a subset of CAR variants (2- to 6-plex). To normalize these data, 1:10 diluted gDNA was used to detect a housekeeping gene (macaque RNase-P p30 subunit, MRPP30)¹⁰⁷ and T cell receptor D (TRD), a gene that is excised during T cell receptor gene rearrangement and is therefore only present in non-T cells.¹⁰⁸ The ddPCR reactions were performed using the Droplet Digital PCR System (Bio-Rad) with ddPCR Multiplex Supermix reagents. Negative controls (untransduced T cells, H₂O) were routinely included to ensure the specificity of amplification. Samples with >3 ddPCR-positive droplets were considered positive.

ddPCR vector copy number (VCN) calculation

For all ddPCR reactions, 1–10 μL of gDNA was used as input, and raw ddPCR counts are reported as counts per μL of gDNA sampled. The total T cells in each sample were estimated by calculating the total cell genomes (based on MRPP30 counts, representing all cells) and subtracting TRD counts (representing non-T cells).¹⁰⁸ This difference was then divided by 2 to account for the two copies of each gene per genome:

$$\text{Total T cells} \left(\frac{\text{genomes}}{\mu\text{L}} \right) = \frac{\text{MRPP30} \left(\frac{\text{counts}}{\mu\text{L}} \right) - \text{TRD} \left(\frac{\text{counts}}{\mu\text{L}} \right)}{\left(\frac{2 \text{ copies}}{\text{genome}} \right)}$$

The vector copy number (VCN) for individual CAR variants in both unmixed and pooled

samples was calculated as the number of CAR variant counts per total T cell genomes in each sample:

$$\text{CAR variant VCN} \left(\frac{\text{copies}}{\text{T cell genome}} \right) = \frac{\text{CAR variant} \left(\frac{\text{counts}}{\mu\text{L}} \right)}{\text{Total T cells} \left(\frac{\text{genomes}}{\mu\text{L}} \right)}$$

Normalization of ddPCR data from pooled assays

In our *ex vivo* and *in vivo* experiments involving pooled CAR variants, we applied normalization to account for minor discrepancies in the initial composition of CAR variants in the pool. These discrepancies could arise from imprecise flow cytometry quantification of CAR marking prior to pooling, pipetting errors during pooling, or differences in vector copy number (VCN) across CAR variant transductions. To address this, we normalized longitudinal ddPCR counts by the relative composition of CAR variants in the initial pooled sample. For example, if a particular CAR variant was found to be over-represented by a factor of 2 in the initial pool via ddPCR, we divided subsequent ddPCR counts for that

variant by 2 to correct for this initial bias. The normalization process involved the following steps:

1. We calculated the relative abundance of each CAR variant (“CAR variant VCN abundance ratio”) in the initial pooled sample:

$$\text{CAR variant VCN abundance ratio} = \frac{\text{CAR variant VCN} \left(\frac{\text{copies}}{\text{T cell genome}} \right)}{\text{Average of all CAR variant VCNs in pooled sample} \left(\frac{\text{copies}}{\text{T cell genome}} \right)}$$

2. Next, raw CAR variant ddPCR counts from subsequent *ex vivo* or *in vivo* timepoints were normalized by the CAR variant VCN abundance ratio in the initial pooled sample:

$$\text{Normalized CAR variant} \left(\frac{\text{counts}}{\mu\text{L}} \right) = \frac{\text{CAR variant} \left(\frac{\text{counts}}{\mu\text{L}} \right)}{\text{CAR variant VCN abundance ratio in initial pooled sample}}$$

3. We then calculated the normalized CAR variant VCN in total T cells for each CAR variant by dividing the normalized CAR variant counts to the measured total T cell counts from the same sample:

$$\text{Normalized CAR variant VCN} \left(\frac{\text{copies}}{\text{T cell genome}} \right) = \frac{\text{Normalized CAR variant} \left(\frac{\text{counts}}{\mu\text{L}} \right)}{\text{Total T cells} \left(\frac{\text{genomes}}{\mu\text{L}} \right)}$$

Quantifying pooled CAR ddPCR sensitivity

To evaluate ddPCR sensitivity, we simulated conditions of low CAR abundance. We manufactured a subset of 14 CAR variants using our standard CAR-T manufacturing, resulting in CAR-T cell variants ranging from 15–64% CAR expression, as measured by flow cytometry. Genomic DNA (gDNA) was extracted from each variant and pooled equally by mass (1:1 ratio). The pooled gDNA was then diluted with wildtype (CAR⁻) gDNA (wt gDNA) at six levels: 0%, 10%, 50%, 90%, 96%, and 99% wt gDNA, creating a dilution series with varying CAR variant content. To confirm the ability of ddPCR to reliably detect each of the 14 variants across the dilution series, we applied variant-specific ddPCR assays to quantify variant abundance. To account for differences in transduction efficiency and vector copy number (VCN) among the variants, we also measured individual CAR variant VCNs from

gDNA isolated from unmixed CAR-T variants. We plotted observed ddPCR counts against expected counts, where the expected ddPCR counts were calculated as follows:

1. The vector copy number (VCN) for unmixed CAR variants was calculated by dividing the CAR variant counts by the MRPP30 counts, then adjusting for the two copies of MRPP30 per genome:

$$\text{CAR variant VCN} \left(\frac{\text{copies}}{\text{genome}} \right) = \left(\frac{\text{CAR variant} \left(\frac{\text{counts}}{\mu\text{L}} \right)}{\text{MRPP30} \left(\frac{\text{counts}}{\mu\text{L}} \right)} \right) \cdot \left(2 \text{ MRPP30} \left(\frac{\text{copies}}{\text{genome}} \right) \right)$$

2. We quantified the number of genomes per μL in the six dilution samples by measuring MRPP30 counts using ddPCR and dividing by 2 to account for the two copies of MRPP30 per genome:

$$\left(\frac{\text{Genomes}}{\mu\text{L}} \right) \text{ in dilution series} = \frac{\text{MRPP30} \left(\frac{\text{counts}}{\mu\text{L}} \right)}{2 \text{ MRPP30} \left(\frac{\text{copies}}{\text{genome}} \right)}$$

3. We then calculated the expected CAR variant copies per μL by multiplying the CAR variant VCN by $\left(\frac{1}{14} \right)$, to account for the 14 CAR variants pooled, and by the number of genomes per μL :

$$\text{Expected CAR variant} \left(\frac{\text{copies}}{\mu\text{L}} \right) = \text{CAR variant VCN} \left(\frac{\text{copies}}{\text{genome}} \right) \cdot \left(\frac{1}{14} \right) \cdot \left(\frac{\text{genomes}}{\mu\text{L}} \right)$$

4. Next, we adjusted the expected CAR variant copies per μL to account for the applied dilution factor by multiplying the expected copies per μL by the fraction of wt gDNA vs CAR⁺ gDNA in the sample:

$$\text{Adjusted CAR variant} \left(\frac{\text{copies}}{\mu\text{L}} \right) = \text{CAR variant} \left(\frac{\text{copies}}{\mu\text{L}} \right) \cdot (1 - \text{fraction wt gDNA})$$

5. To match the observed sample measurements, we multiplied the expected variant copies per μL by 10 to account for the 10 μL of gDNA assayed. The expected variant copies per 10 μL were then plotted against the observed ddPCR counts per 10 μL of gDNA from the dilution series.

Supplemental Figures

Supplemental Table 1: Overview of nonhuman primates infused with pooled CAR-T cell products.

Animal ID (sex)	Weight* (kg)	Dosage (CAR ⁺ cells/kg)	Peak CAR expansion**	Days to B cell recovery [†]	Clinical observations during peak expansion	Supportive medications during peak expansion	Duration of study (days)
Z21020 (M)	4.7	9 x 10 ⁶	50.6%	39 days	Fever, inappetence	-	102
Z21093 (M)	4.1	9 x 10 ⁶	32.5%	32 days	Fever, inappetence, diarrhea, dehydration, itching	Benadryl, tocilizumab, anakinra, dexamethasone	92
Z22040 (M)	3.7	9 x 10 ⁶	96.8%	-	Fever, inappetence, tremors, altered consciousness, seizure activity	Benadryl, tocilizumab, anakinra, dexamethasone, diazepam, furosemide, Keppra	9

* Weight on day of infusion

** Peak CAR expansion *in vivo* as a percentage of total CD45⁺ CD3⁺ T cells in PBMCs by flow cytometry

[†] Days to B cell recovery defined as the number of days until CD20⁺ cell counts exceed 20 cells per μ L.

Supplemental Table 2: List of antibodies used

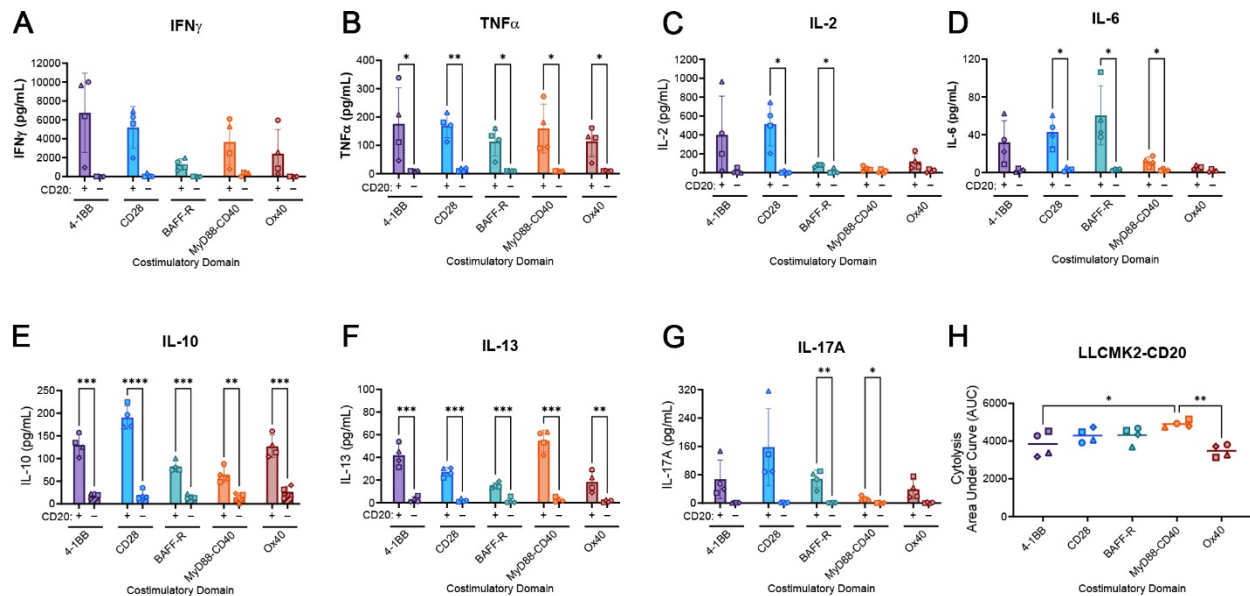
Target	Clone	Supplier	Assay
CD3	SP34-2	BD	Flow cytometry
CD45	D058-1283	BD	Flow cytometry
CD4	L200	BioLegend	Flow cytometry
CD8	SK1	BD	Flow cytometry
PD-1	Z12.2H7	eBioscience	Flow cytometry
TIGIT	MBSA43	eBioscience	Flow cytometry
CD25	BC96	BioLegend	Flow cytometry
HLA-DR	G46-6	BD	Flow cytometry
Ki67	DX2	BD	Flow cytometry
EGFRt	Hu1	Biotechne	Flow cytometry
CD22	S-HCL-1	BD	Flow cytometry
CD28	CD28.2	BD	Flow cytometry
CD95	DX2	Invitrogen	Flow cytometry
CD20	Polyclonal (cat. no. PA1-9024)	Invitrogen	IHC

Supplemental Table 3. Integration site analysis of pooled CAR-T cell products and PBMCs.

Animal ID	Timepoint	Number of unique IS*	IS in transcription units (%)	IS near oncogenes (%)
Z21020	d0	21833	56.92	5.10
Z21020	d11	9660	58.96	5.69
Z21093	d0	17322	56.86	5.28
Z21093	d11	7036	58.16	5.97
Z22040	d0	26382	57.02	5.54
Z22040	d9	17501	57.21	5.92

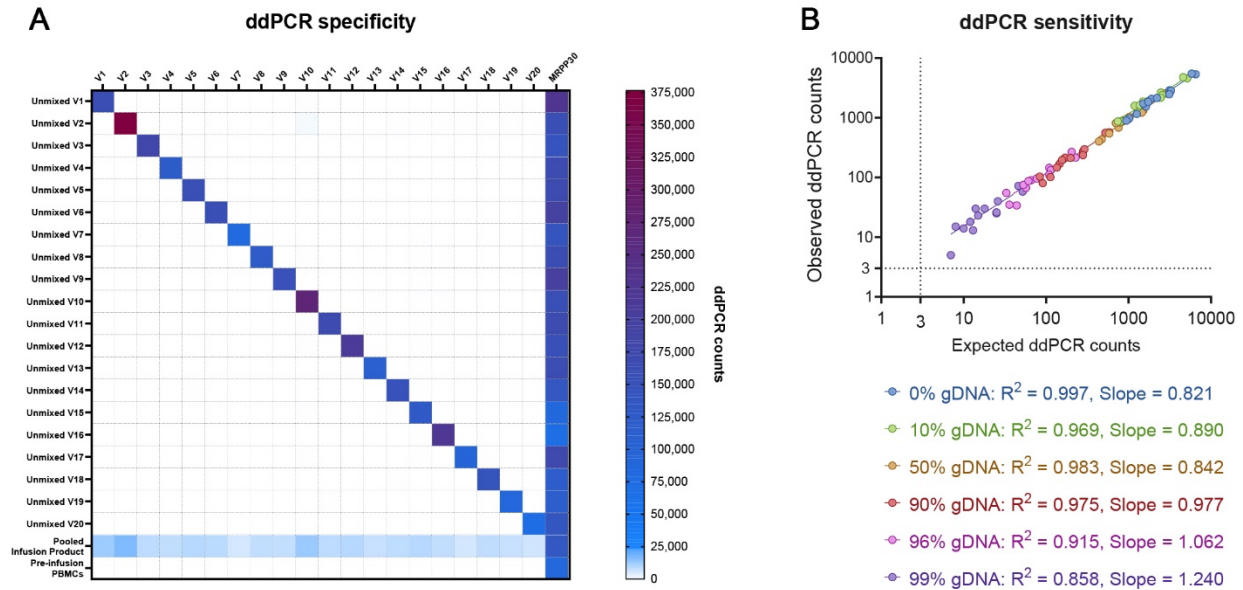
The day 0 (d0) timepoint represents the infused CAR-T cell product. PBMC samples were collected at peak CAR-T cell expansion post-infusion for each animal, either 9 or 11 days post- infusion, as indicated.

*IS, integration site.

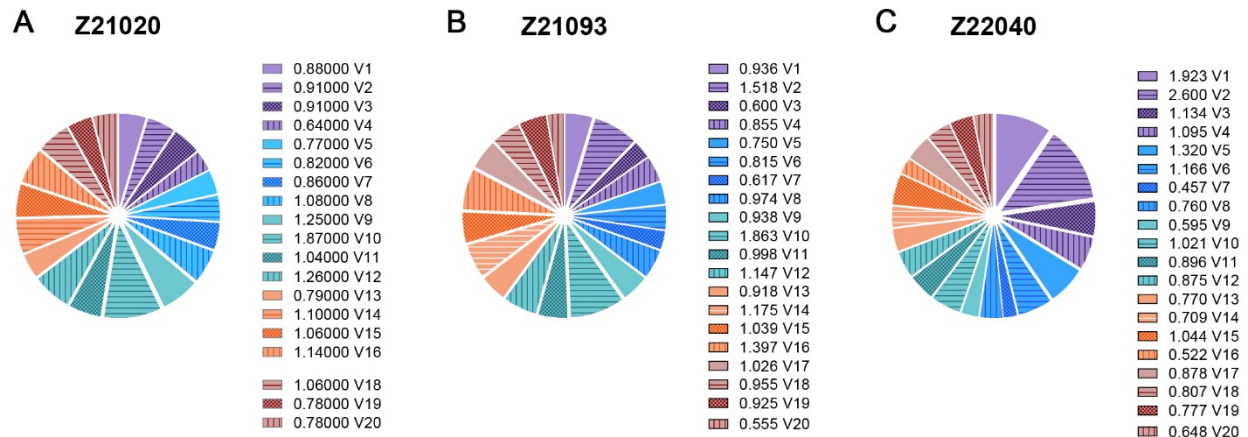


Supplemental Figure 1. Antigen-specific cytokine secretion and cytotoxicity profiles across CAR-T cell variants. (A-G) CAR-T variants were co-cultured with irradiated K562-CD20 or K562-wt cells. Supernatants were collected after 24 hours and analyzed for (A) IFN γ , (B) TNF α , (C) IL-2, (D) IL-6, (E) IL-10, (F) IL-13, or (G) IL-17A cytokine secretion. Antigen status of the target cells (CD20⁺ or CD20⁻) is indicated on the x-axis. (H) Area-under-the-curve (AUC) quantification of CAR variants' cytotoxicity of co-cultured LLCMK2-CD20 target cells, as outlined by methods depicted in Figure 3H-I. To compare cytokine secretion between CD20⁺ and CD20⁻ conditions, data in (D-F) were reanalyzed from Figure 3E-G using unpaired t-tests with Holm-Šídák correction. To assess antigen-specific cytotoxicity

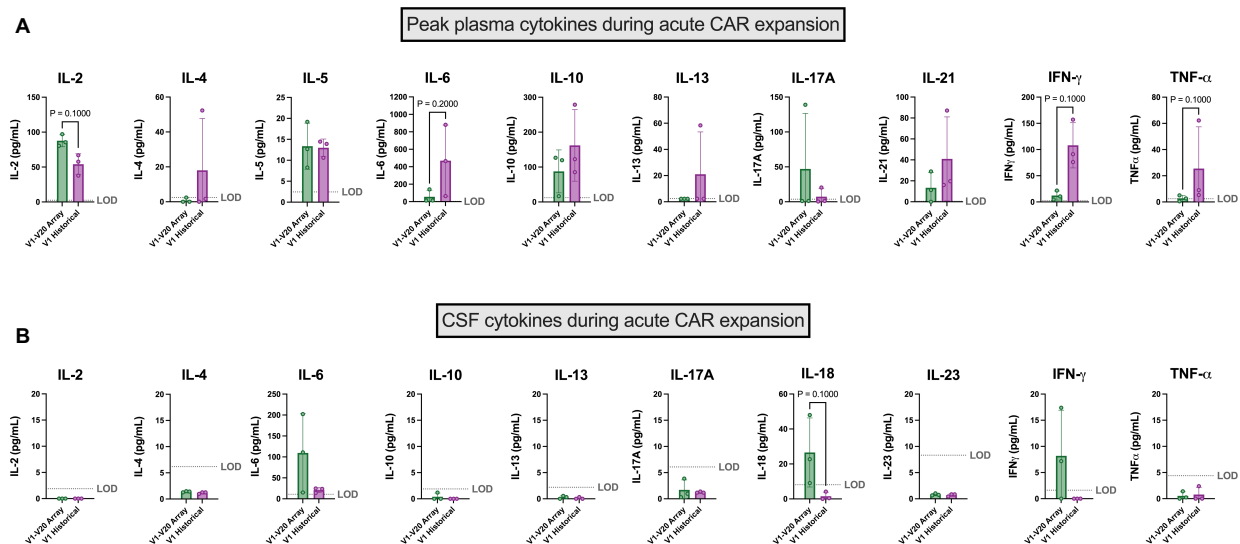
across costimulatory groups, LLCMK2-CD20 cytotoxicity data in (H) were reanalyzed from Figure 3I using one-way ANOVA with Tukey's correction. Each data point in (A-G) represents the mean of three biological replicates ($n = 3$ pigtail macaques), except for V17 ($n = 2$). (H) includes two replicates. Statistical significance: $*P < 0.05$, $**P < 0.005$, $***P < 0.0005$, $****P < 0.00005$.



Supplemental Figure 2. Validation of ddPCR specificity and sensitivity. (A) Variant-specific ddPCR was used to analyze samples indicated on the y-axis, including genomic DNA (gDNA) from individually manufactured CAR-T variants (unmixed V1– unmixed V20), pooled CAR-T cell infusion products, and pre-infusion (CAR⁻) PBMCs. Heatmap displays the average ddPCR counts across three biological replicates ($n = 3$ pigtail macaques), except for V17 ($n = 2$); white boxes indicate zero cross-variant background for each CAR-specific primer probe set (x-axis). The far-right column in the matrix shows a genomic control/housekeeping locus (macaque gene RPP30, MRPP30), used to quantify total genomes for each y-axis sample. (B) To evaluate ddPCR sensitivity, we simulated conditions of low CAR abundance. gDNA from 14 CAR-T variants was mixed in equal proportions by mass and serially diluted with wild-type gDNA (0%, 10%, 50%, 90%, 96%, 99%). Expected ddPCR counts, computed based on the measured VCN of the individual CAR-T variants and the applied dilution factors (see Methods), were compared with observed counts. The assay reliably resolved expected 1:1 input ratios across all dilution levels, maintaining strong correlations between expected and observed counts ($0.858 < R^2 < 0.997$; $0.821 < \text{slope} < 1.240$). Individual CAR variants were consistently detected up to 99% wild-type gDNA spike-in levels, above the assay's positivity threshold of ≥ 3 ddPCR counts.



Supplemental Figure 3. Composition of pooled infusion products by variant. The relative abundance of each CAR variant in the pooled infusion product was quantified by ddPCR-based vector copy number (VCN) assay (see Supplemental Methods). Shown are data from animal IDs (A) Z21020, (B) Z21093, and (C) Z22040. Pie charts indicate each CAR variant with distinct colors/patterns. To assess the representation of each variant within the initial pooled sample, the VCN of each variant was normalized to the average VCN across all variants. The resulting CAR Variant VCN abundance ratio is shown for each CAR variant to the right of the pie chart. If all variants are precisely pooled, each would contribute equally with a relative ratio of 1. Z21020 did not receive V17 due to technical difficulties in manufacturing.



Supplemental Figure 4. Cytokine profiles in plasma and cerebrospinal fluid (CSF) during acute CAR-T cell expansion. (A) Peak cytokine levels in plasma during the acute phase of CAR-T cell expansion, defined as days 3 to 14 post-infusion. For each animal, the highest concentration observed during this window is plotted, in order to account for inter-animal variability in cytokine kinetics and slight differences in sample collection timepoints across

animals and cohorts. (B) Cytokine levels in CSF samples obtained 9-14 days after CAR-T infusion. Cytokine concentrations for the pooled V1-V20 CAR array (green) were compared to a comparable historical single-CAR cohort that received V1 (purple). Each data point represents one animal (n = 3 pigtail macaques per cohort). Cohorts were matched for species, Cytoxan preconditioning, CAR-T manufacturing workflow, and total CAR-T cell dose (9×10^6 CAR⁺ cells per kg). All samples were assayed concurrently under identical conditions. Statistical comparisons were performed using two-tailed Mann-Whitney tests.

Chapter 3: Antigen-boosted CD4CAR-T cells fail to expand or control viremia in multiple nonhuman primate models of HIV

This chapter is adapted from the following manuscript:

Maynard, Lucy H., et al. "Antigen-boosted CD4CAR-T cells fail to expand or control viremia in multiple nonhuman primate models of HIV." (manuscript in preparation)

Abstract

Chimeric antigen receptor T (CAR-T) cell therapy has demonstrated curative potential in B cell malignancies, yet translating this success to chronic infections like human immunodeficiency virus (HIV) remains a major challenge. In people living with HIV (PLWH) on suppressive antiretroviral therapy (ART), low antigen levels limit CAR-T cell expansion and persistence. Our prior work reported in Rust et al., 2020 suggested that HIV-targeted CD4CAR-T cells could overcome this barrier through exogenous antigen supplementation, leading to robust *in vivo* expansion. Here, we sought to rigorously reproduce and extend those findings through a comprehensive series of preclinical studies in nonhuman primates (NHPs). We tested a broad array of strategies to enhance CD4CAR-T cell efficacy, including CRISPR-Cas9-mediated gene editing of immune checkpoint and HIV-associated genes, single and pooled competitive infusions of engineered CAR-T cells, distinct CAR constructs incorporating either CD28 or 4-1BB costimulatory domains, and exogenous antigen boosting. These approaches were evaluated across multiple NHP models of HIV, including both simian immunodeficiency virus (SIV)- and simian-human immunodeficiency virus (SHIV)-infected, ART-suppressed NHPs. Although CD4CAR-T cell products exhibited

antigen-specific proliferation and cytotoxicity *ex vivo*, they consistently failed to expand, persist, or control viremia *in vivo*. Given these findings, we reexamined our original findings in Rust et al. and uncovered major data inconsistencies that undermine the validity of the reported CD4CAR-T cell expansion. Together, these studies reveal fundamental limitations of CD4CAR-T cell therapy for HIV cure strategies and highlight the critical need for rigorous validation and reproducibility in preclinical immunotherapy research.

Introduction

Adoptive transfer of chimeric antigen receptor (CAR)-T cells has revolutionized the treatment of hematologic malignancies and has emerged as a promising strategy for addressing chronic viral infections such as HIV. However, HIV presents unique barriers to successful CAR-T cell therapy that differ substantially from the cancer setting. In people living with HIV (PLWH) on durable antiretroviral therapy (ART), circulating antigen levels are extremely low, limiting CAR-T cell expansion and persistence.⁴¹ In addition, HIV persists in difficult-to-access tissue reservoirs such as gastrointestinal-associated lymphoid tissue (GALT) and the central nervous system (CNS),¹⁰⁹ posing additional hurdles for effective CAR-T cell trafficking and clearance. The high mutability of HIV also presents a challenge for targeting viral epitopes.¹¹⁰ Our group and others have developed HIV-targeted CAR-T cells, termed CD4CAR-T cells, that incorporate the extracellular domain of CD4 as the targeting moiety, leveraging the conserved nature of the CD4 binding site to minimize susceptibility to viral escape mutations.^{42,111,112} Preclinical studies in humanized mouse models of HIV-1 infection have demonstrated that CD4CAR-T cells are able to expand in response to antigen, provide partial protection of CD4⁺ T cells from infection, and reduce viremia after ART cessation.^{111,112}

Previously, in Rust et al., 2020, we reported that exogenous antigen supplementation using K562 cells engineered to overexpress the HIV envelope glycoprotein (Env) could overcome the low antigen environment enabled by ART and enable robust CD4CAR-T cell proliferation in simian-human immunodeficiency virus (SHIV)-infected, ART-suppressed nonhuman primates (NHPs). This strategy was conceptually inspired by analogous efforts in oncology, such as the use of lipid nanoparticle (LNP)-delivered Claudin-6 antigen to boost anti-Claudin-6 CAR-T cell responses.¹¹³ In this study, all four CAR-treated ART-suppressed NHPs showed expansion of CD4CAR-T cells up to 40% in peripheral CD3⁺ T cells, with two animals exhibiting transient control of viral replication following ART withdrawal. In the two animals without initial viral suppression, subsequent administration of a rhesusized anti-PD-1 antibody (nivolumab) induced CAR-T cell re-expansion and temporary reduction in viral load. To our knowledge, this was the first demonstration of meaningful expansion of

Figure 1. Overview of Phase 1A (SHIV) NHP study. (A) Phase 1A (SHIV) study design overview. Rhesus macaques were infected with SHIV-1157ipd3N4, treated with ART, and administered CD4CAR-T cells with or without K562-Env boosting, followed by ART withdrawal. Stars (*) indicate tissue collection timepoints. (B) Manufacturing schema for CD4CAR-T cells, including separate isolation and activation of CD4⁺ and CD8⁺ T cells, lentiviral transduction with CD4CAR vectors, CRISPR-mediated CCR5 editing, and G-REX expansion.

CD4CAR-T cells fail to expand or control viremia in SHIV-infected, ART-suppressed NHPs

CD4CAR-T cells were manufactured using established protocols,^{17,42} including separate isolation of CD4⁺ and CD8⁺ T cells, activation with artificial antigen-presenting cells (aAPCs), lentiviral transduction, CRISPR-ribonucleoprotein (RNP)-mediated CCR5 editing, and expansion in G-REX flasks (**Figure 1B**). On average, 2.35×10^8 total cells per kg, including 9.144×10^7 CAR⁺ cells per kg, were infused into each animal (**Figure 2A**). Transduction efficiencies ranged from 32.7% to 47.15% (average 40.6%), and CCR5 editing efficiency in the Δ CCR5 CAR-T group ranged from 76.11% to 96.24% (average 87.39%) (**Figure 2B**). Following CAR-T infusion, K562-Env boosting, and ART withdrawal, we did not detect CAR-T cell expansion in post-infusion PBMCs (**Figure 2C**), conflicting with the previous Rust et al. report. Consistent with this lack of CAR signal in peripheral blood, CAR-T cells were undetectable in tissues collected longitudinally throughout the study, including samples from pre-infusion, 2-, 4-, and 6-weeks post-infusion, and at necropsy (**Supplemental Figure 1**). To further investigate potential factors contributing to differences between studies, we profiled plasma from the Rust et al. cohort and our Phase 1A (SHIV) cohort at matched timepoints. We hypothesized that differences in the peripheral immune environment could explain the observed discrepancies in CAR-T cell expansion. Across 37 analytes measured, we detected no significant differences in cytokines, chemokines or growth factors (**Supplemental Figures 2-4**). Additionally, we assayed plasma for anti-Env antibodies, hypothesizing that differences in immunologic responses to the K562-Env antigen boost in Phase 1A (SHIV) animals may have contributed to the lack of *in vivo* CAR-T cell expansion we observed in this cohort. However, while all boosted animals across Rust et al and the Phase 1A study showed increases in plasma anti-Env antibodies following K562-Env boost (**Supplemental Figure 5A**), we did not detect any significant differences in the level of anti-Env antibodies at any time-matched timepoints between Phase 1A (SHIV) and Rust et al. plasma samples (**Supplemental Figure 5B**).

Following ART interruption, viral rebound occurred in a subset of animals across all groups, while others remained suppressed through necropsy (**Figure 2E**). However, the lack of

CAR-T cell expansion in these animals suggests that viral control was not CAR-mediated but instead reflected the high rate of spontaneous control observed in the SHIV model, which is a known limitation of this system.¹¹⁴ Consistent with this, many animals in our cohort exhibited declining viremia or spontaneous viral control prior to ART initiation (**Figure 2D**). We also evaluated an additional cohort of ART-suppressed, SHIV-infected NHPs, referred to as Phase 1B (SHIV). Animals either received no CAR-T cells (No CAR; n=3), serving as a control for assessing viral rebound after ART withdrawal in the absence of CD4CAR-T therapy and K562-Env boosting, or received CCR5-edited CD4CAR-T cells manufactured using a revised protocol and K562-Env regimen (Δ CCR5 CAR-T + Boost (MOD); n=2) (**Supplemental Table 1**). In this modified arm, we reduce soluble IL-15 supplementation from 5 ng/mL to 0.5 ng/mL during CAR-T manufacturing, and active T cells with a modified aAPC-based approach: aAPCs were cultured in soluble IL-15 and engineered to overexpress IL-15 receptor alpha (IL-15R α), providing superagonist IL-15 signaling during T cell activation.¹¹⁵ CAR-T cells were manufactured from PBMCs collected both before and after SHIV infection, based on the hypotheses that (1) including SHIV-specific T cells might enhance CAR-T potency, and (2) using post-infection PBMCs better models real-world scenarios where pre-infection samples are unavailable. These manufacturing changes are summarized in **Supplemental Table 3**. Finally, we adjusted the treatment timeline: K562-Env boosting and ART withdrawal were both performed 7 days after CAR-T infusion. Despite these protocol changes, Δ CCR5 CAR-T + Boost (MOD) animals showed no CAR-T cell expansion and no measurable impact on viral load compared to No CAR controls (data not shown). Taken together, these findings suggest that CD4CAR-T cell expansion in SHIV-infected NHP models may be limited.

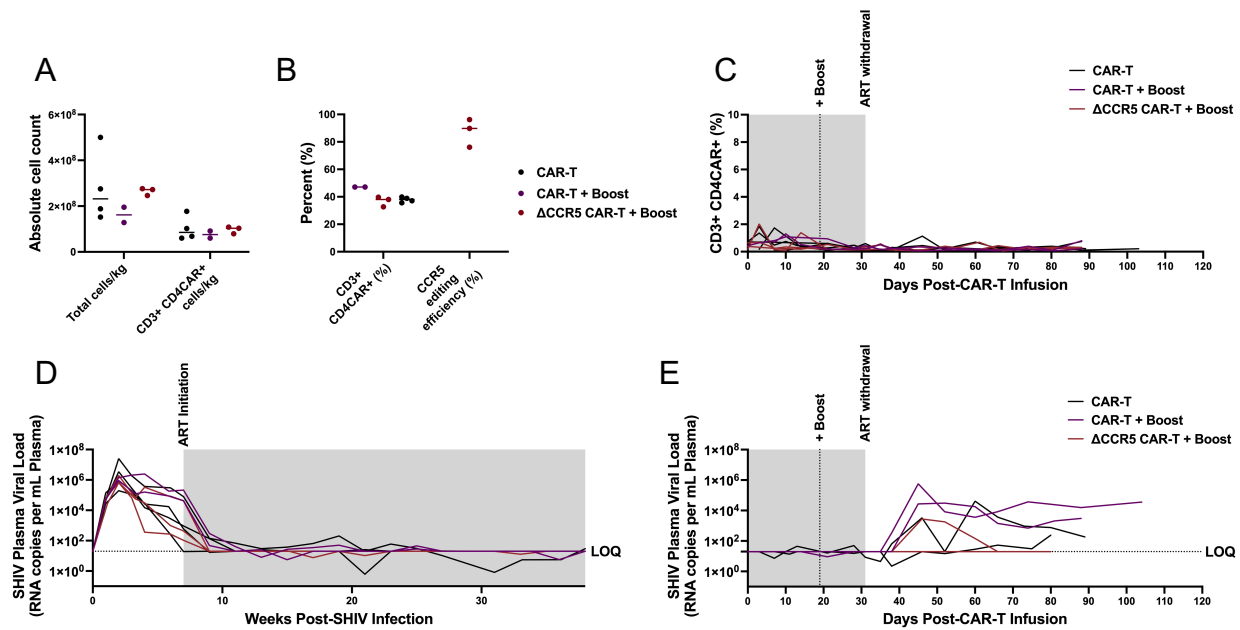


Figure 2. CD4CAR-T cells fail to expand or control viremia in SHIV-infected, ART-suppressed NHPs in Phase 1A (SHIV). (A) Absolute number of infused T cells and CD4CAR⁺ T cells per kg body weight across treatment groups. (B) CD4CAR transduction efficiency and CCR5 editing efficiency in infusion products. (C) Frequency of CD4CAR⁺ T cells in PBMCs following infusion, K562-Env boosting, and ART withdrawal. (D) Longitudinal plasma viral load measurements following at SHIV infection and ART initiation. (E) Plasma viral loads following CAR-T infusion and ART withdrawal. Each data point represents an individual NHP. LOQ, limit of quantification.

Design and implementation of Phase 2 (SIV) CD4CAR-T cell studies

To address limitations observed in our Phase 1A and Phase 1B (SHIV) cohorts, where low antigenicity and/or weak/delayed viral rebound may have impaired CAR-T cell activation and expansion, we transitioned to a simian immunodeficiency virus (SIV)-infected NHP model for Phase 2 (referred to herein as Phase 2 (SIV)). Compared to SHIV, SIV infection induces more consistent viral rebound following ART withdrawal and has a lower rate of spontaneous viral control, providing a more robust platform for evaluating CAR-T cell function *in vivo*.^{116,117} Building on findings from Rust et al., which demonstrated that CAR-T cells could be reinvigorated through immune checkpoint blockade via administration of an anti-PD-1 antibody, Phase 2 was designed to assess whether targeted disruption of additional immune checkpoint and HIV-associated genes could further enhance CAR-T cell expansion and persistence. Eight genes were selected for knockout: six immune checkpoint regulators (PD-1,¹¹⁸ TIGIT,¹¹⁹ CTLA-4,¹²⁰ DKGα,¹²¹ LAG-3,¹²² and TIM-3¹²³), one gene involved in HIV pathogenesis that was also investigated in the Phase 1 experiment

(CCR5)¹²⁴, and one gene linked to T cell effector (T_{EFF}) function, identified in *in vivo* CRISPR screens (FLI-1)¹²⁵. We evaluated three cohorts. The first received CCR5-knockout CD4CAR-T cells combined with K562-Env boosting (Δ CCR5 CAR-T, n=3). The second received a pooled infusion product containing single-plex gene-edited CD4CAR-T cells targeting one of the eight genes listed above, combined with K562-Env antigen boosting (CRISPR Array CAR-T, n=3). The third received no CAR-T cell infusion (No CAR, n=3) (**Figure 3A**). For the CRISPR Array group, single-plex gene-edited CAR-T cells were manufactured individually in an arrayed format, following the same standard workflow as for Δ CCR5 CAR-T cells, pooled in equal proportions, and infused per our established competitive repopulation study design (Chapter 2). Longitudinal PBMC samples were collected pre- and post-infusion, and editing at each locus was quantified over time to determine whether particular knockouts were enriched or depleted relative to baseline. This approach provided a surrogate measure of the fitness advantage or disadvantage conferred by each gene edit (**Figure 3B**).

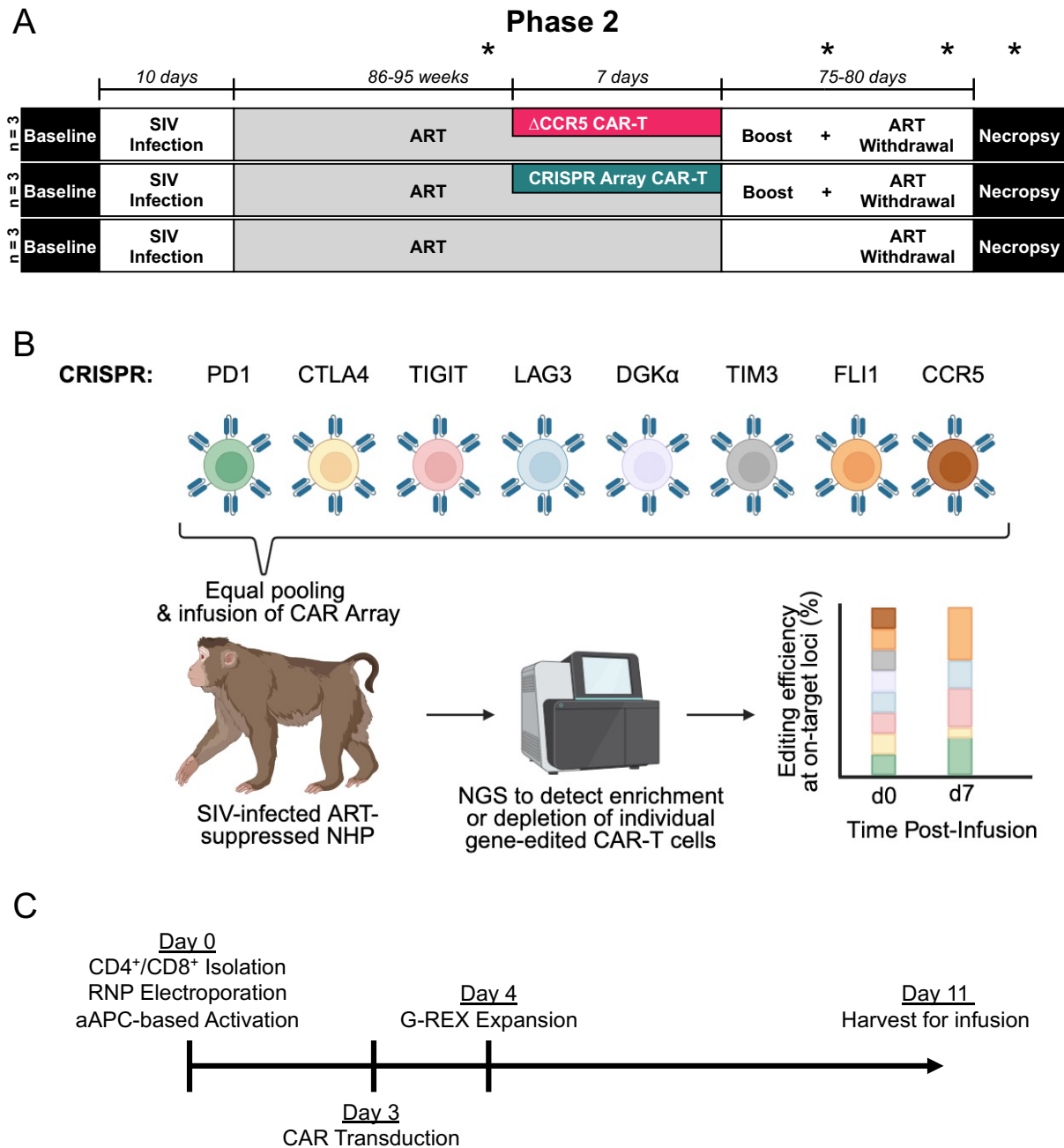


Figure 3. Overview of Phase 2 (SIV) study design and pooled CRISPR CAR-T screening approach. (A) Schematic of the Phase 2 (SIV) *in vivo* study. Rhesus macaques were infected with SIVmac239M, treated with ART, and infused with CD4CAR-T cells, followed by K562-Env boosting and ART withdrawal. (B) Design of the pooled CRISPR Array screen. CD4CAR-T cells gene-edited at distinct immunoregulatory or HIV susceptibility genes were manufactured *ex vivo*, pooled and infused into single SIV-infected, ART-suppressed NHPs. On-target editing was quantified by Next Generation Sequencing (NGS) at longitudinal timepoints to detect enrichment or depletion of specific edits, indicating potential *in vivo*

selective advantages or disadvantages. (C) Summary of CAR-T cell manufacturing, including CD4⁺ and CD8⁺ T cell isolation, CRISPR-mediated gene editing, activation with aAPCs, lentiviral transduction with CD4CAR, and expansion in G-REX flasks.

In addition to changes in the HIV model and study design, several refinements were introduced into the CAR-T cell manufacturing process and dosing scheme (**Figure 3C and Supplemental Table 3**). As in Phase 1B (SHIV), CAR-T cells were activated with aAPCs engineered to provide superagonist IL-15 signaling, but soluble IL-15 was removed entirely from the T cell media. CAR-T cells were manufactured from PBMCs collected both before and after SIV infection. Here we refer to SIV⁻ and SIV⁺ fractions as input T-cells that were collected before SIV infection (SIV⁻) or after SIV infection and ART suppression (SIV⁺); notably, the SIV⁺ fraction is not composed entirely of infected cells, but rather may contain a subset of SIV-infected and SIV-specific T cells. Residual ART in SIV⁺ cells should minimize recrudescent virus replication during CAR manufacturing, but also limits the potency of our lentiviral vectors.¹²⁶ To mitigate the risk of chromosomal loss associated with CRISPR electroporation following T cell activation,¹²⁷ the workflow was adjusted such that RNP electroporation was performed on Day 0, prior to aAPC stimulation. Finally, based on prior reports suggesting enhanced persistence and expansion with mixed costimulatory domains,¹¹² CAR-T cells were co-transduced with dual lentiviral vectors encoding CD4CAR constructs bearing either a 4-1BB or a CD28 costimulatory domain. Collectively, these manufacturing modifications were intended to optimize CAR-T cell fitness, improve *in vivo* persistence, and better simulate clinically relevant therapeutic manufacturing processes.

Manufacturing and characterization of gene-edited CD4CAR-T cells

We manufactured CD4CAR-T cells using our revised protocol (**Supplemental Table 3**) and infused either pooled or single gene-edited CAR-T cell products at an average dose of 1.45×10^8 total cells per kg, including 3.315×10^7 CAR⁺ cells per kg (**Figure 4A**). In the CRISPR Array cohort, pooled infusions were designed to balance each of the eight distinct single-plex gene-edited products across SIV⁻ and SIV⁺ CAR-T cell fractions (**Figure 4B**). CAR expression among gene-edited products ranged from 6.6% to 45% (**Figure 4C-D**), with higher transduction efficiency in SIV⁻ fractions (average 24.8%) compared to SIV⁺ fractions (average 14.4%), likely due to ART-mediated inhibition of lentiviral transduction. Amplicon-based Next Generation Sequencing (NGS) showed robust on-target editing across all eight loci (**Figure 4E-F**), with an average editing efficiency of 91.2% in SIV⁻ CAR-T cells and 75.8% in SIV⁺ CAR-T cells. We hypothesize that the lower editing rates observed in SIV⁺ CAR-T cells may reflect impaired cellular fitness due to SIV infection.

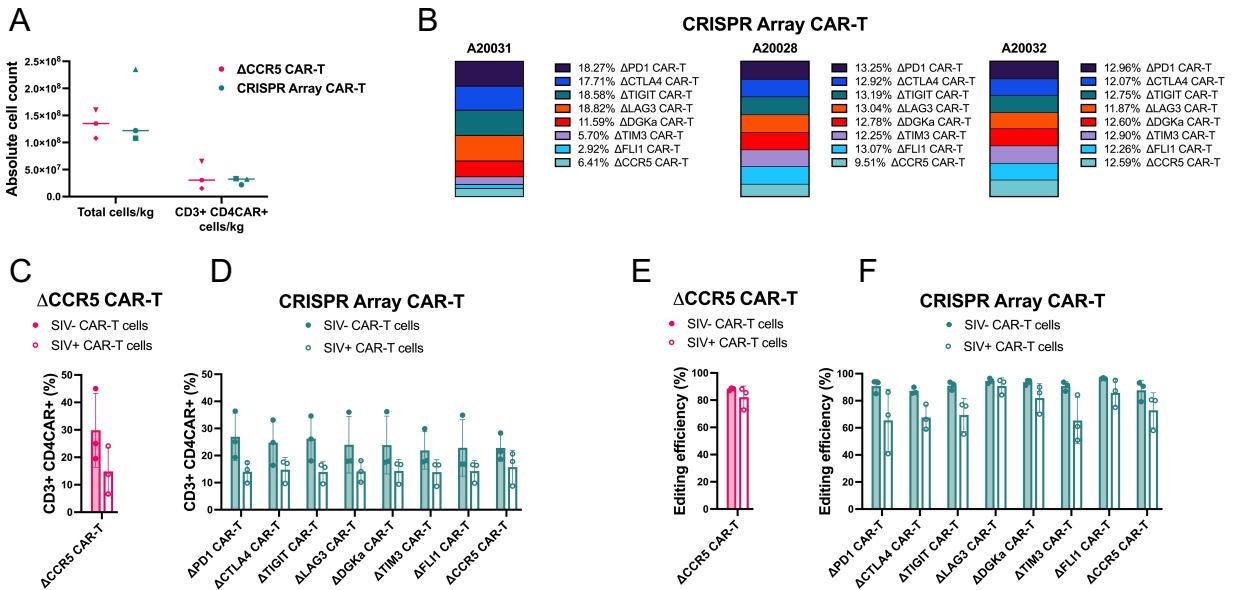


Figure 4. Composition and characterization of Phase 2 (SIV) CD4CAR-T cell products.

(A) Total CAR-T cell dose and CD3⁺CD4CAR⁺ cell dose infused into animals in the ΔCCR5 and CRISPR Array CAR-T cell cohorts. (B) Relative composition of the CRISPR Array CAR-T cell products for each indicated animal, based on the frequency of CD3⁺CD4CAR⁺ cells corresponding to each individual edit, as measured by flow cytometry. (C–D) CAR transduction efficiency in (C) ΔCCR5 CAR-T cell products and (D) individual gene-edited products within the CRISPR Array CAR-T cell cohort, shown separately for cell products manufactured from T-cells collected before (SIV[−]) or after (SIV⁺) SIV infection and ART suppression. (E–F) On-target editing efficiency measured by NGS in (E) ΔCCR5 CAR-T cells and (F) individual gene-edited CAR-T cells within the CRISPR Array cohort, separated by SIV status. In (C–F), open and filled circles represent SIV[−] and SIV⁺ CAR-T cell fractions, respectively.

Gene-edited CD4CAR-T cells demonstrate antigen-specific cytotoxicity and proliferation ex vivo

We rigorously evaluated the function of gene-edited CAR-T cells *ex vivo* prior to infusion into SIV-infected, ART-suppressed NHPs. To assess cytotoxicity, we co-cultured gene-edited CAR-T cells with either antigen-expressing (LLCMK2-Env) or antigen-negative (LLCMK2-GFP) target cells and measured target cell killing using the xCELLigence Real-Time Cell Analysis (RTCA) platform. In both the ΔCCR5 and CRISPR Array CAR-T cell groups, gene-edited CAR-T cells exhibited robust antigen-specific cytotoxicity against antigen-positive targets, with minimal non-specific killing of antigen-negative cells (**Figure 5A–D**). We further assessed proliferative capacity using a CellTrace Violet-based proliferation assay. Upon coculture with antigen-positive K562-Env target cells, all gene-

edited CAR-T cells demonstrated strong antigen-specific proliferation, indicated by dilution of CellTrace Violet within the CAR⁺ T cell fraction (**Figure 5E–H**). No evidence of non-specific or tonic CAR signaling was observed in the absence of antigen stimulation.

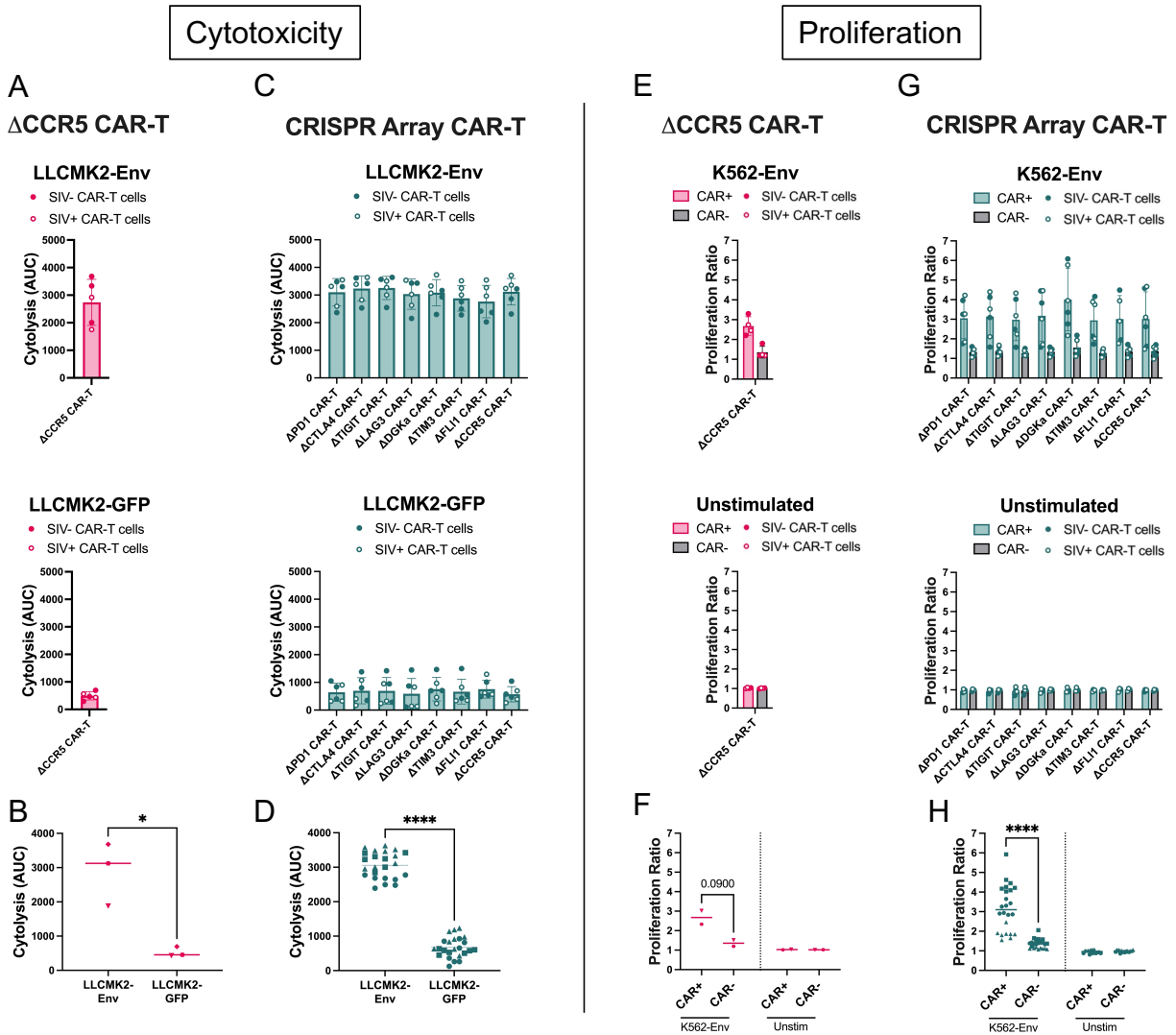


Figure 5. Ex vivo antigen-specific cytotoxicity and proliferation of gene-edited CD4CAR-T cell products from Phase 2 (SIV). (A–D) Cytotoxicity was measured using real-time impedance-based cytotoxicity assays with Env-positive (LLCMK2-Env) or control (LLCMK2-GFP) target cells. Killing activity is reported as area under the curve (AUC) over 48 hours. (A) Cytolytic activity of Δ CCR5 CAR-T cells, shown for SIV⁻ and SIV⁺ fractions. (B) Summary AUC values for Δ CCR5 CAR-T cells, averaged across paired SIV⁻ and SIV⁺ fractions for each NHP. (C) Cytolytic activity of individual gene-edited CAR-T cell products from the CRISPR Array cohort. (D) Summary AUC values for CRISPR Array CAR-T cells, averaged across SIV⁻ and SIV⁺ fractions for each edited product (8 edits per NHP; n = 3 NHPs). (E–H) CAR-T cells were labeled with CellTrace Violet and co-cultured with Env-

expressing (K562-Env) or control (wild-type K562) cells. Proliferation was calculated as the ratio of CellTrace Violet (MFI) between stimulated and control conditions (See Methods). (E) Proliferation of Δ CCR5 CAR-T cells, shown separately for CAR⁺ and CAR⁻ subsets across SIV⁻ and SIV⁺ fractions. (F) Summary Proliferation Ratios for Δ CCR5 CAR-T cells, averaged across SIV⁻ and SIV⁺ fractions for each NHP. (G) Proliferation of individual gene-edited CAR-T cell products from the CRISPR Array cohort, shown for CAR⁺ and CAR⁻ subsets across SIV⁻ and SIV⁺ fractions. (H) Summary Proliferation Ratios for CRISPR Array CAR-T cells, averaged across SIV⁻ and SIV⁺ fractions for each edited product (8 edits per NHP; n = 3 NHPs). In (A), (C), (E), and (G), open and filled circles represent SIV⁻ and SIV⁺ CAR-T cell fractions, respectively. Statistical comparisons in (B), (D), (F), and (H) were performed using paired two-tailed t-tests. *P < 0.05; **P < 0.005; ***P < 0.0005; ****P < 0.00005.

Gene-edited CD4CAR-T cells fail to expand or control viremia in SIV-infected ART-suppressed NHPs

We observed robust plasma viremia following SIVmac239M infection of NHPs, and initiation of ART effectively suppressed viral replication (**Figure 6A**). However, after CAR-T cell infusion, followed by K562-Env boosting and ART withdrawal, no significant CAR-T cell expansion was detected by flow cytometry (**Figure 6B**). Given the low CAR-T cell abundance observed and the potential for technical artifacts with flow cytometry, such as subjective gating and high background signal, we quantified CAR-T cells at the DNA level using highly sensitive digital droplet PCR (ddPCR) assays. Analysis of infusion products confirmed CAR-specific amplification and high vector copy numbers (VCNs), ranging from 0.70 to 1.63 (**Figure 6C**). In post-infusion PBMC samples, ddPCR detected very low levels of CAR DNA, with peak expansion occurring at day 7 (**Figure 6D**). The average vector copy number (VCN) in CD3⁺ T cells was 0.019 in the Δ CCR5 cohort and 0.005 in the CRISPR Array cohort, corresponding to approximately 0.5–1.9% CAR marking in peripheral CD3⁺ T cells across the two groups. Viral rebound occurred between 10- and 20-days following ART withdrawal in all animals (**Figure 6E**). We further assessed editing frequencies across the eight target loci to determine whether any specific gene-edited T cell populations preferentially expanded *in vivo*. In the Δ CCR5 CAR-T cell cohort, peak editing levels at day 7 ranged from 1.2% to 4.14% of total PBMCs, declining to 1.16–2.44% by day 28 (**Figure 6F**). In the CRISPR Array cohort, editing was generally lower, with most edits detected at frequencies below 1% at all timepoints assessed (**Figure 6G–I**). Together, these data indicate that gene-edited CD4CAR-T cells failed to expand, persist, or exert measurable antiviral activity in SIV-infected, ART-suppressed NHPs.

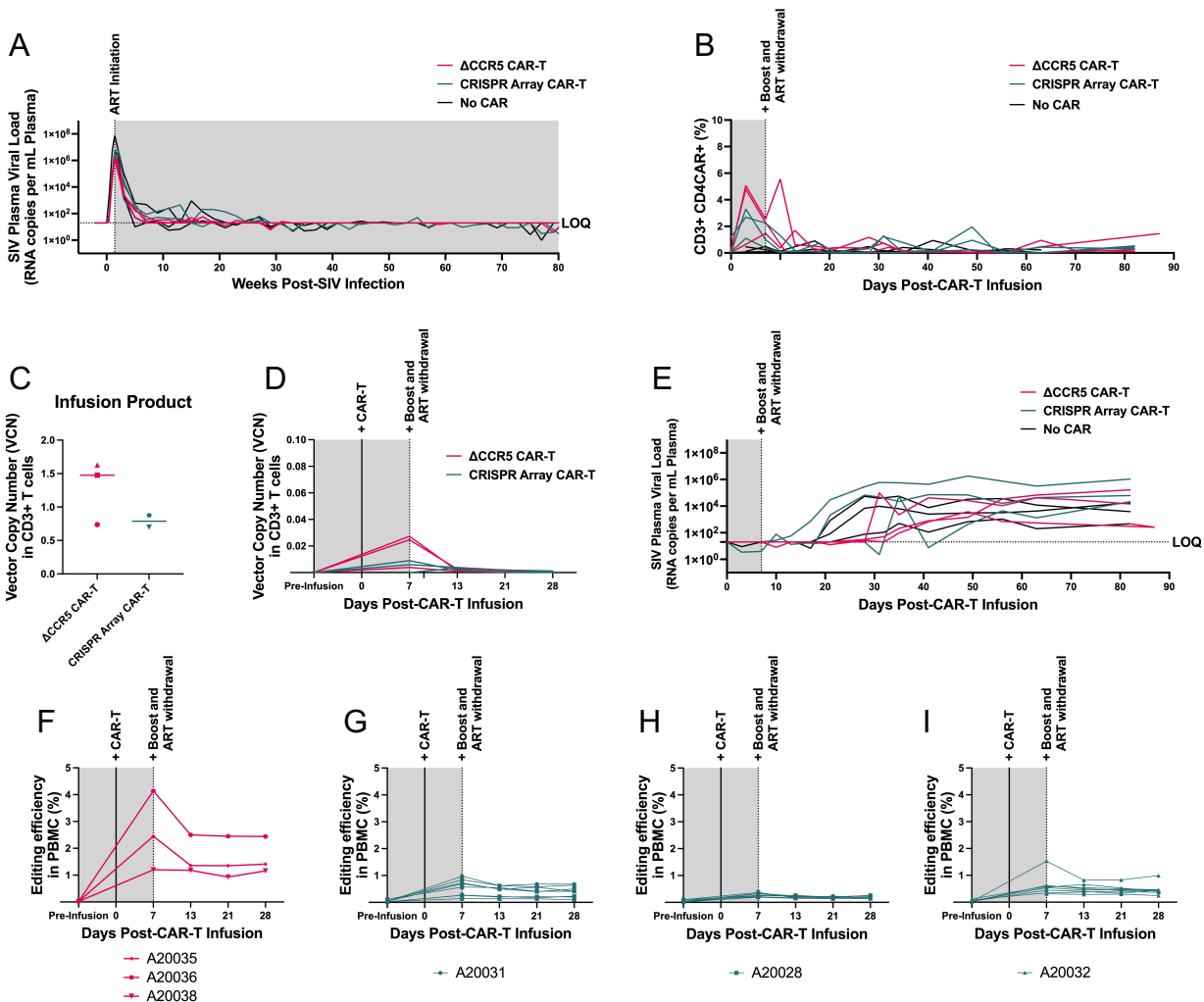


Figure 6. Gene-edited CD4CAR-T cells fail to expand or control viremia in SIV-infected ART-suppressed NHPs. (A) Longitudinal plasma viral load measurements following SIVmac239M infection and ART treatment. (B) Frequency of CAR-T cells in PBMCs following infusion, K562-Env boosting, and ART withdrawal. (C) Vector copy number (VCN) in CD3⁺ T cells from Δ CCR5 and CRISPR Array CAR-T cell infusion products. (D) VCN in CD3⁺ T cells from PBMCs collected longitudinally before and after CAR-T cell infusion. (E) Plasma viral load measurements following CAR-T infusion and ART withdrawal. (F) Editing efficiency at the CCR5 locus in PBMCs from animals treated with Δ CCR5 CAR-T cells. (G–I) Editing efficiency across CRISPR Array targets (PD-1, TIGIT, CTLA-4, LAG-3, DKG α , TIM-3, FLI-1, and CCR5) in PBMCs from animals treated with CRISPR Array CAR-T cells: (G) A20031, (H) A20028, and (I) A20032. Each datapoint in (A–F) represents an individual NHP; each datapoint in (G–I) represents gene-editing measurements across the eight edited loci for the specified NHP.

No CD4CAR-T expansion detected in reevaluation of prior data from Rust et al.

Given the lack of CAR-T cell expansion observed in both Phase 1A (SHIV), Phase 1B (SHIV) and Phase 2 (SIV) studies across a total of 17 CD4CAR-T cell-treated NHPs, we re-evaluated our initial findings from Rust et al., 2020. Post-infusion PBMC samples from Rust et al. corresponding to timepoints of peak CD4CAR-T cell expansion were analyzed via single cell RNA sequencing (scRNA-seq),¹²⁸ but bioinformatic analysis failed to detect CD4CAR⁺ cells in any samples analyzed (data not shown). We additionally analyzed post-infusion PBMC samples with our established ddPCR assay and found no evidence of CAR-T cell expansion, particularly at 30 to 40 days post-infusion, which corresponds to the time frame of peak expansion previously reported (**Figure 7A**). Instead, ddPCR measurements closely mirrored those from our Phase 2 (SIV) cohorts. As a positive control, we applied a comparable ddPCR workflow to post-infusion PBMCs from a separate study (Bui et al., 2024) in which NHPs received CD20-targeted CAR-T cells.¹⁷ These samples showed robust CAR-T cell expansion and corroborated previously reported flow cytometry data, with VCNs reaching a peak of up to 1.976 at 7 to 10 days post-infusion (**Figure 7B**).

Prompted by these findings, we reanalyzed the original flow cytometry data that had formed the basis for the reported 15-40% CAR-T cell expansion in Rust et al. Notably, the original gating strategy was established without the use of appropriate negative controls (e.g., pre-infusion PBMC samples or fluorescence-minus-one [FMO] controls) to distinguish CAR⁺ from CAR⁻ events. To address this limitation, we re-stained available post-infusion PBMC samples from the reported expansion phase in Rust et al., including both negative and positive controls to establish well-defined flow cytometry gates. When gated on pre-infusion PBMCs, a true biological negative control (**Supplemental Figure 7A**), these samples showed no appreciable CD4CAR signal above background (**Supplemental Figure 7C**). When gated on a representative FMO control (**Supplemental Figure 8A**), these samples showed CD4CAR marking between 0.56—3.3% (**Supplemental Figure 8C**), though pre-infusion PBMCs also showed high background of 2.16% (**Supplemental Figure 8D**), and *ex vivo* manufactured CAR-T cells showed clear overlap of the applied CD4CAR gate into the CD4CAR-negative population (**Supplemental Figure 8B**). A summary of these findings, including experimental distinctions on the two negative control samples (i.e., pre-infusion PBMCs and FMO sample) used to establish gates in Supplemental Figure 7 and 8 are provided in **Supplemental Table 6**. These two analysis approaches demonstrate the oftentimes tenuous nature of using flow cytometry to quantify low-abundance events and discriminate signal from noise. As such, more quantitative and sensitive methods, including ddPCR, should be used to corroborate flow cytometry findings from CAR-T cell studies. While we were unable to re-stain all original PBMC timepoints by flow cytometry

due to limited sample availability as many PBMC samples were diverted toward scRNA-seq, our extensive ddPCR analysis, together with repeated flow cytometry data, collectively indicate that minimal CAR-T cell expansion occurred in Rust et al. and suggest that the previously reported expansion was likely attributable to a technical artifact in flow cytometry analysis.

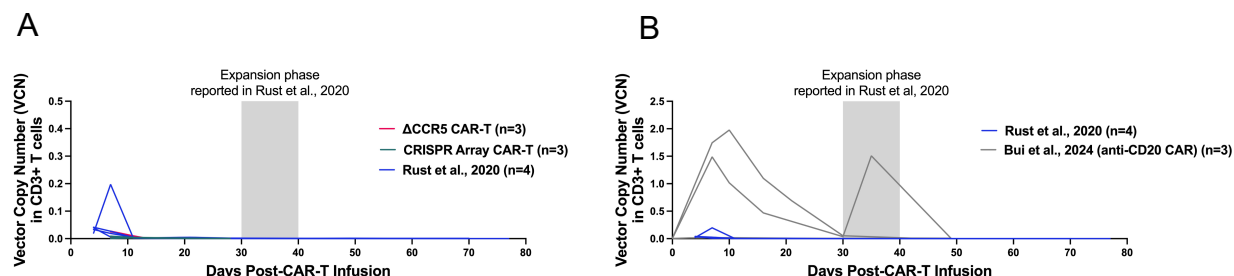


Figure 7. Quantification of CAR-T cells in post-infusion PBMCs by ddPCR across NHP studies. (A) VCN in CD3⁺ T cells from post-infusion PBMCs from Rust et al., 2020 and Phase 2 (SIV) animals treated with Δ CCR5 or CRISPR Array CD4CAR-T cells. (B) VCN in CD3⁺ T cells from post-infusion PBMCs from Rust et al., 2020 (as in A), and Bui et al., 2024, which used a CD20-targeted CAR-T cell. Gray shaded regions indicate the reported expansion phase in Rust et al.

Discussion

HIV cure strategies face major challenges due to the persistence of long-lived viral reservoirs and the lack of therapeutic approaches that are both safe and effective. While a few cases of sustained HIV remission have been achieved through hematopoietic stem cell transplantation (HSCT) with CCR5-null donor cells, effectively reconstituting the immune system with HIV-resistant cells, these transplants were performed primarily to treat underlying hematologic malignancies, with HIV cure as a secondary outcome.^{129,130} The intensive preconditioning regimens required for HSCT carry significant toxicity and mortality risks, rendering this approach unsuitable for otherwise healthy PLWH.¹³¹ In addition, HIV persists in latent reservoirs within tissues such as the gut and central nervous system, which are difficult to access with conventional therapies. CAR-T cell therapy for HIV cure represents a promising alternative approach, with the potential to deliver small numbers of highly potent effector cells that can persist *in vivo*, traffic to reservoir sites, and selectively eliminate infected cells.

However, one of the major barriers to successful CAR-T cell therapy for HIV is the low antigen environment in ART-suppressed individuals. CAR-T cell activation requires a threshold level of antigen at the surface of target cells to trigger proliferation, cytokine

production and cytotoxicity, estimated around 5,000 to 60,000 molecules per target cell.^{132,133} Understanding the quantitative antigen threshold required to trigger CD4CAR-T cell effector function will be critical for determining whether these cells can effectively respond in the context of acute, chronic, or suppressed viral replication. To address this, we are developing assays to evaluate how CD4CAR-T cell activity varies in response to different levels of antigen, with the goal of defining their antigen sensitivity. In PLWH on ART, active viral replication is effectively repressed, and although precise quantification of HIV antigen levels remains challenging, it is likely that antigen availability falls below the threshold required to drive robust CAR-T cell activation.⁴¹ To address this, we previously developed an *in vivo* antigen-boosting strategy that reportedly induced robust CD4CAR-T cell expansion in NHPs. In the present study, we sought to systematically replicate and extend those findings through a series of rigorous NHP experiments. Despite efforts to improve CAR-T design, optimize manufacturing, and enhance trafficking and HIV resistance via gene editing, we observed no evidence of CAR-T cell expansion *in vivo*. Retrospective re-analysis of archived samples from the original study reported in Rust et al. likewise failed to detect CAR-T expansion. Collectively, our current studies and retrospective analysis do not support evidence of CD4CAR-T cell expansion in NHP models of HIV infection.

These results also underscore the importance of technical rigor and reproducibility in preclinical HIV cure research. NHP studies are uniquely valuable due to their ability to capture human-like immunology and reservoir dynamics, yet they are inherently resource-intensive and often underpowered. As such, careful validation, standardized methodologies, and transparent reporting are essential to ensure that promising preclinical findings reflect true biological potential and can reliably guide the development of clinical strategies, particularly as CD4-based CAR-T cell therapies enter the clinic.¹³⁴

Beyond the challenge of antigen scarcity, the biological properties of the HIV envelope glycoprotein (Env) may further limit its suitability as a CAR-T cell target. Env expression on infected cells is often sparse and short-lived, with rapid internalization and shedding reducing its stability as a target.^{135–138} Structurally, Env is heavily glycosylated and conformationally flexible, shifting between closed and open states that may alternately expose or shield key epitopes.^{139–141} These features may collectively impair the ability of CAR-T cells to consistently recognize and eliminate Env-expressing cells, even when using binding domains directed at conserved regions.

Despite these limitations, some Env-targeting approaches, particularly those involving broadly neutralizing antibodies (bNAbs), have recently reported promising clinical results.

bNAbs can engage both infected cells and free virions, which may provide broader antiviral coverage.^{142–144} CD4-based CAR-T cells, in contrast, may be functionally limited by binding to circulating Env-containing virions, which could act as decoys and reduce their ability to engage infected cells. Recent trials, including the RIO study of dual bNAbs,¹⁴⁵ the FRESH study combining bNAbs with a TLR7 agonist,¹⁴⁶ and a Phase 2 study of long-acting lenacapavir with bNAbs,¹⁴⁷ have shown partial or sustained virologic control in subsets of participants. These promising recent reports highlight the potential of antibody-based and combinatorial immunotherapies to overcome the structural and biological barriers that may limit the effectiveness of CD4CAR-T cell therapies, and suggest a path forward for achieving durable HIV remission.

Methods

CD4CAR-T cell design

The CD4CAR transgene was adapted directly from our previous studies, including a long EF1 α promoter driving CD4CAR, which incorporates domains 1 to 4 of the CD4 ectodomain and a CD8 hinge and transmembrane domain.^{42,111} For Phase 1A and 1B (SHIV) studies, the CD4CAR incorporated a 4-1BB costimulatory domain, while in Phase 2 (SIV) studies we co-transduced cells with dual CD4CAR constructs that incorporated either a 4-1BB or a CD28 costimulatory domain. Both CD4CAR constructs were codon-optimized for expression in *Macaca mulatta* (rhesus macaques) and cloned into lentiviral vectors derived from the nonpathogenic simian immunodeficiency virus isolate SIVmac1A11, which is specifically designed to efficiently transduce rhesus macaque cells.¹⁴⁸ Lentiviral vectors were produced and titered via qPCR in HT-1080 cells, as previously described.¹⁰⁴

CD4CAR-T cell manufacturing

CAR-T cells were manufactured *ex vivo* as described.^{17,42} Briefly, CD4⁺ and CD8⁺ T cells were isolated from PBMCs via bead-based selection (STEMCell Technologies) and cultured in T cell media composed of X-VIVO-15 (Thermo Fisher Scientific) supplemented with 10% FBS, 1% penicillin/streptomycin, 1% L-glutamine, 55 μ M 2-mercaptoethanol, and 5 ng/mL recombinant IL-7 (Peprotech). In Phase 1A (SHIV), cultures were additionally supplemented with 5 ng/mL IL-15 (Peprotech). T cells were activated with artificial antigen-presenting cells (aAPCs; kindly provided by Dr. James Riley¹⁰⁵) at a 2:1 T cell:aAPC ratio. In Phase 1A (SHIV), aAPC 3.1 cells expressing CD3 scFv, CD64, and CD86 were used; in Phase 1B (SHIV) and Phase 2 (SIV), aAPC 5.1 cells co-expressing CD3 scFv, CD64, CD86, IL-7, 4-1BBL, and IL-15R α were used (**Supplemental Table 3**). CD4⁺ and CD8⁺ T cells were electroporated separately with single-plex CRISPR-Cas9 ribonucleoprotein (RNP) complexes targeting NHP loci. In Phase 1A and 1B, CCR5 was targeted; in Phase 2, one or

more of the following were targeted: PD-1, TIGIT, CTLA-4, LAG-3, DGK α , TIM-3, CCR5, or FLI-1. Guide RNA sequences are provided in **Supplemental Table 5**. Each RNP consisted of 180 pmol TrueCut Cas9 Protein v2 (Thermo Fisher Scientific) and 900 pmol synthetic gRNA (Synthego), incubated for 10 minutes at room temperature prior to use. Electroporation was performed on the Lonza 4D platform using the P3 Primary Cell 4D Kit L and program CY100. T cells were electroporated on Day 3 (post-activation) in Phase 1A and 1B (SHIV), and on Day 0 (pre-activation) in Phase 2 (SIV). Three days after activation, T cells were transduced with lentiviral vectors encoding the CD4CAR transgene at a multiplicity of infection (MOI) of 1–10 in media supplemented with protamine sulfate. The following day, CD4⁺ and CD8⁺ T cells were combined at a 1:1 ratio and expanded in G-REX flasks (Wilson Wolf) for 7–8 days.

CellTrace Violet proliferation assay

CAR-T cell proliferation was assessed immediately after manufacturing using CellTrace Violet dye (CTV, Thermo Fisher Scientific). CAR-T cells were CTV-labeled according to manufacturer instructions, and 5×10^5 labeled CAR-T cells were co-cultured with 1×10^5 irradiated Env-expressing K562 cells (K562-Env, generated as previously described¹¹¹) or wild-type K562 cells (K562-wt; ATCC) (effector-to-target ratio 5:1) for three days at 37°C. Proliferation was quantified by flow cytometry based on CTV dye dilution in both CAR⁺ and CAR⁻ subsets. A Proliferation Ratio was calculated by dividing the mean fluorescence intensity (MFI) of CTV in CAR-T cells stimulated with K562-wt by the MFI in those stimulated with K562-Env, with separate values computed for CAR⁺ and CAR⁻ populations. Higher Proliferation Ratios reflect increased antigen-driven proliferation. Unstimulated CTV-labelled CAR-T cells were included as a control.

Real-time cell analysis of cytotoxicity

Cytotoxic activity was evaluated using the xCELLigence Real-Time Cell Analysis (RTCA) platform (Agilent Technologies). Adherent LLCMK2 target cells (ATCC) were electroporated with mRNA encoding SIVmac239 Env (TriLink Technologies), plated onto RTCA E-plates, and allowed to adhere for 20–24 hours. CAR-T cells were added at effector-to-target (E:T) ratios of 10:1, 5:1, and 2.5:1 in duplicate wells. Mock-transduced CAR-T cells were included as controls at matched E:T ratios. Target cell killing was monitored via impedance-based measurements over 48 hours post-effector addition, and cytolytic activity was quantified by calculating the area under the curve (AUC) of the cytolysis trace. The amino acid sequence for SIVmac239 Env is:

MGCLGNQLLIAILLLSVYGIYCTLYVTVFYGVPAWRNATIPFLCATKNRDTWGTTQCLPDNGDYSEV
ALNVTESFDAWNNTVTEQAIEDVWQLFETSIPKPCVKLSPLCITMRCNKSETDRWGLTKSITTTASTTS
TTASAKVDMVNETSSCIAQDNCTGLEQEQMISCKFNMTGLKRDKKKEYNETWYSADLVCEQGNN

TGNESRCYMNHCNTSVIQESCDKHYYWDAIRFRYCAPPGYALLRCNDTNYSGFMPKCSKVVVSSC
TRMMETQTSTWFGFNGTRAENRTYIYWHGRDNRTIISLNKYYNLTKCRRPGNKTVLPVTIMSGLVF
HSQPINDRPKQAWCWFGGKWKDAIKEVKQTIVKHPRYTGTNNTDKINLTAPGGGDPEVTFMWTN
CRGEFLYCKMNWFLNWVEDRNTANQKPKEQHKRNYVPCHIRQIINTWHKVGKNVYLPPREGDLT
CNSTVTSLIANIDWIDGNQTNITMSAEVAELYRLELGDYKLVEITPIGLAPTDVKRYTTGGTSRNKRGV
FVLGFLGFLATAGSAMGAASLTLTAAQSRSTLLAGIVQQQQQLLDVVKRQQELLRLTVWGTKNLQTRVT
AIEKYLKDQAQLNAWGCAFRQVCHTTVPWPNASLTPKWNNETWQEWERKVDFFLEENITALLEEA
QIQQEKNMYELQKLNSWDVFGNWFDLASWIKYIQYGVYIVGVILLRIVYIVQMLAKLRQGYRPVF
SSPPSYFQQTHIQQDPALPTREGKERDGGEGGGNSSWPWQIEYIHFLIRQLIRLLTWLFSNCRTLL
SRVYQILQPILQRLSATLQRIREVLRTELTYLQYGWSYFHEAVQAVWRSATETLAGAWGDLWETLRR
GGRWILAIPRRIRQGLELTLL*

Flow cytometry

All antibodies and clones used for flow cytometry are listed in **Supplementary Table 4**. Each staining panel included a viability dye to exclude dead cells. For detection of low-frequency populations (e.g., CD4CAR-T cells in PBMCs) or markers with less distinct positive–negative separation (e.g., PD-1, HLA-DR), appropriate controls, such as fluorescence minus one (FMO) controls and/or true biological negatives (e.g., pre-infusion PBMCs), were routinely included to guide gating and ensure accurate identification.

In vivo NHP CAR-T cell studies

Rhesus macaques were housed in accordance with NIH standards as outlined in the *Guide for the Care and Use of Laboratory Animals* (National Research Council, 1996), ILAR recommendations, and AAALAC accreditation guidelines, as previously described.⁴² All animal procedures were approved by the Institutional Animal Care and Use Committees (IACUC) of the Fred Hutchinson Cancer Research Center/University of Washington (protocol no. 3235–04).

In Phase 1A and 1B (SHIV), animals were infected with SHIV-1157ipd3N4, and antiretroviral therapy (ART) was initiated 7 weeks post-infection. ART consisted of preformulated tenofovir disoproxil fumarate (TDF, 5.1 mg/kg), emtricitabine (FTC, 40 mg/kg), and dolutegravir (DTG, 2.5 mg/kg) generously provided by Gilead Sciences. After 37–45 weeks of viral suppression, animals received CD4CAR-T cells, followed 19 days later by an infusion of irradiated K562-Env cells (2.5×10^7 cells/kg), as previously described.^{42,111} Twelve days post-K562-Env infusion, animals underwent analytical treatment interruption (ATI) for the remainder of study. In Phase 2 (SIV), animals were infected with SIVmac239M and began the same ART regimen 10 days post-infection. Following 86–95 weeks of ART suppression

in SIV animals, each received CD4CAR-T cells, followed 7 days later by K562-Env infusion (2.5×10^7 cells/kg) and subsequent ATI.

Peripheral blood was collected throughout both studies via venipuncture for plasma viral load quantification and PBMC immunophenotyping. Tissue samples, including colon (lower GI), duodenum/jejunum (upper GI), spleen, axillary lymph nodes, and bronchoalveolar lavage (BAL), were collected at specified timepoints. In Phase 1, tissues were sampled pre-infusion and at 2-, 4-, and 6-weeks post-CAR-T cell infusion; in Phase 2, samples were collected pre-infusion and at 2- and 6-weeks post-infusion. Tissue processing followed previously established protocols.^{17,42}

Next Generation Sequencing

Genomic DNA was extracted from CD4CAR-T cell products and Ficoll-separated PBMCs using the QIAamp DNA Blood Mini Kit (Qiagen) according to the manufacturer's instructions. Indel frequencies at gene-edited loci were quantified using a two-step PCR amplification protocol with Platinum Taq DNA Polymerase High Fidelity (Thermo Fisher Scientific). Target-specific primers (**Supplementary Table 5**) were designed to amplify a 200–300 bp region spanning the CRISPR cleavage site for each edited locus. Libraries were purified using AMPure SPRI beads (Beckman Coulter), quantified with a Qubit Fluorometer (Thermo Fisher Scientific), and pooled for sequencing using a barcoded, 2×150 bp paired-end MiSeq assay (Illumina). Sequencing data were analyzed using a custom pipeline built with CRISPResso2,¹⁴⁹ with a minimum average Phred quality score of 30 ($-q \ 30$) and a quantification window of 15 base pairs on either side of the predicted cleavage site ($-w \ 15$).

Luminex assays

Cytokine, chemokine, and growth factor levels were quantified from Ficoll-separated plasma collected in Phase 1A (SHIV) and Rust et al., 2020 using the ProcartaPlex™ Non-Human Primate Cytokine/Chemokine/Growth Factor 37-Plex Panel (Thermo Fisher Scientific). Assays were performed in technical duplicate according to the manufacturer's instructions.

Anti-Env ELISA assays

Plasma samples from Phase 1A (SHIV) and archived samples from Rust et al., 2020 were analyzed for HIV Env-specific antibody responses via ELISA. 96-well plates were coated overnight at 4°C with recombinant HIV-1 gp120 (strain SF162, Clade B), then blocked and dried according to standard protocols.¹⁵⁰ Heat-inactivated plasma samples were serially diluted in PBS with 0.05% Tween-20 and 0.5% BSA, and incubated for 2 hours at 37°C. Antibody binding was detected using a peroxidase-conjugated rabbit anti-human IgG,

followed by SureBlue substrate development and TMB stop solution. Absorbance at 450 nm was recorded, and antibody titers were calculated as the inverse plasma dilution corresponding to half-maximal binding (EC_{50}), determined by nonlinear regression.

Droplet digital PCR

Droplet digital PCR (ddPCR) was used to quantify CD4CAR vector copy number (VCN) in *ex vivo* CAR-T cell products and in post-infusion PBMC samples. A custom primer/probe set targeting the CD4 ectodomain of the CD4CAR transgene was designed and validated for specificity. Genomic DNA (gDNA) was extracted using the AllPrep DNA Kit (QIAGEN), diluted to ~100 ng/ μ L, and 1–10 μ L was used as input per ddPCR reaction. To normalize VCN relative to total T cells in each sample, 1:10 diluted gDNA was assessed in parallel for two reference targets: the macaque RNase P p30 subunit (MRPP30) as a housekeeping gene¹⁰⁷ and T-cell receptor delta (TRD), which is absent in mature T cells due to V(D)J recombination and thus used to distinguish non-T cell populations.¹⁰⁸ Reactions were prepared using the Bio-Rad ddPCR Multiplex Supermix and run on the Bio-Rad Droplet Digital PCR System. Negative controls (untransduced T cells, H₂O) were included in all runs to confirm specificity. Samples with >3 positive droplets were considered ddPCR-positive.

ddPCR vector copy number (VCN) calculation

For all ddPCR reactions, 1–10 μ L of gDNA was used as input, and raw ddPCR counts are reported as counts per μ L of gDNA sampled. The total T cells in each sample were estimated by calculating the total cell genomes (MRPP30 counts, representing all cells) and subtracting TRD counts (representing non-T cells). This difference was then divided by 2 to account for the two copies of each gene per genome:

$$\text{Total T cells} \left(\frac{\text{genomes}}{\mu\text{L}} \right) = \frac{\text{MRPP30} \left(\frac{\text{counts}}{\mu\text{L}} \right) - \text{TRD} \left(\frac{\text{counts}}{\mu\text{L}} \right)}{\left(\frac{2 \text{ copies}}{\text{genome}} \right)}$$

The vector copy number (VCN) was calculated as the number of CAR counts per total T cell genomes in each sample:

$$\text{CAR VCN} \left(\frac{\text{copies}}{\text{T cell genome}} \right) = \frac{\text{CAR variant} \left(\frac{\text{counts}}{\mu\text{L}} \right)}{\text{Total T cells} \left(\frac{\text{genomes}}{\mu\text{L}} \right)}$$

Supplemental Figures

Supplemental Table 1. Overview of NHPs in Phase 1A and 1B (SHIV).

Study ID	HIV Model	Cohort ID	Animal ID	CD4CAR Costim	CD4CAR Editing	K562-Env Boost	Sex	Age (Years) 1	Boost (Days) 2	ATI (Days) 3
Phase 1A (SHIV)	SHIV-1157ipd 3N4	CAR-T	A19232	4-1BB	-	-	F	4.05	-	31
			A20040	4-1BB	-	-	M	4.00	-	31
			A19228	4-1BB	-	-	M	4.42	-	31
			A19226	4-1BB	-	-	M	5.19	-	31
		CAR-T + Boost	A19233	4-1BB	-	+	M	3.94	19	31
			A19236	4-1BB	-	+	F	3.26	19	31
		ΔCCR5 CAR-T + Boost	A19229	4-1BB	CCR5	+	M	4.16	19	31
			A19227	4-1BB	CCR5	+	M	4.33	19	31
			A20029	4-1BB	CCR5	+	M	6.22	19	31
Phase 1B (SHIV)	SHIV-1157ipd 3N4	No CAR	A19231	-	-	-	M	4.09	-	-
			A19234	-	-	-	F	4.21	-	-
			A19235	-	-	-	M	3.33	-	-
		ΔCCR5 CAR-T + Boost (MOD) ⁴	A20034	4-1BB	CCR5	+	M	5.32	7	7
			A20037	4-1BB	CCR5	+	M	5.13	7	7

¹Age (Years): The age at infection.

²Boost (Days): Number of days between CAR-T infusion and K562-Env boost administration.

³ATI (Days): Analytical Treatment Interruption. Number of days between CAR-T infusion and ART interruption.

⁴ΔCCR5 CAR-T + Boost (MOD) refers to NHPs that received CD4CAR-T cell manufactured with a modified protocol, as outlined in **Supplemental Table 3**.

Supplemental Table 2. Overview of NHPs in Phase 2 (SIV).

HIV Model	Cohort ID	Animal ID	CD4CAR Costim	CD4CAR Editing	K562-Env Boost	Sex	Age (Years)	Boost (Days) 1	ATI (Days) 2
SIVmac-239M	No CAR	A20033	-	-	-	M	5.32	-	-
		A18032	-	-	-	M	5.47	-	-
		A20039	-	-	-	M	4.15	-	-
	ΔCCR5 CAR-T + Boost	A20038	4-1BB + CD28	CCR5	+	M	5.01	7	7
		A20036	4-1BB + CD28	CCR5	+	M	5.32	7	7
		A20035	4-1BB + CD28	CCR5	+	M	5.33	7	7
	CRISPR Array	A20031	4-1BB + CD28	CRISPR Array	+	M	5.38	7	7

	CAR-T + Boost	A20028	4-1BB + CD28	CRISPR Array	+	M	6.40	7	7
		A20032	4-1BB + CD28	CRISPR Array	+	M	5.39	7	7

¹Boost (Days): Number of days between CAR-T infusion and K562-Env boost administration.

²ATI (Days): Analytical Treatment Interruption. Number of days between CAR-T infusion and ART interruption.

Supplemental Table 3. Overview of CAR-T cell manufacturing differences in Phase 1A and 1B (SHIV) and Phase 2 (SIV).

Study	T cell media	Source of PBMCs	Timing of RNP EP	aAPC	CD4CAR Costim
Phase 1A (SHIV)	IL-7 + IL-15	SHIV-	Day 3 (after activation)	aAPC 3.1 (CD3 scFv + CD64 + CD86)	Single transduction with 4-1BB CD4CAR
Phase 1B (SHIV)	IL-7 + 0.1X IL-15	SHIV- and SHIV+	Day 3 (after activation)	aAPC 5.1 (CD3 scFv + CD64 + CD86 + IL7 + 4-1BBL + IL-15Rα)	Single transduction with 4-1BB CD4CAR
Phase 2 (SIV)	IL-7	SIV- and SIV+	Day 0 (before activation)	aAPC 5.1 (CD3 scFv + CD64 + CD86 + IL7 + 4-1BBL + IL-15Rα)	Co-transduction with 4-1BB CD4CAR + CD28 CD4CAR

RNP, ribonucleoprotein; EP, electroporation; aAPC, artificial antigen presenting cell.

Supplemental Table 4. List of antibodies used.

Target	Clone	Supplier	Assay
CCR5	3A9	BD	CAR-T cell & PBMC immunophenotyping
CCR7	3D12	BD	CAR-T cell & PBMC immunophenotyping
CD14	ME5	BioLegend	CAR-T cell & PBMC immunophenotyping
CD16	3G8	BioLegend	CAR-T cell & PBMC immunophenotyping
CD20	2H7	BioLegend	CAR-T cell & PBMC immunophenotyping
CD27	M-T271	BD	CAR-T cell & PBMC immunophenotyping
CD3	SP34-2	BD	CAR-T cell & PBMC immunophenotyping
CD4	SK3; OKT4	BD; BioLegend	CAR-T cell & PBMC immunophenotyping
CD4 (human, detects CD4CAR-T)	RPA-T4	BioLegend	CAR-T cell & PBMC immunophenotyping
CD45	D058-1283	BD	CAR-T cell & PBMC immunophenotyping
CD8	SK1	BD	CAR-T cell & PBMC immunophenotyping
CD28	CD28.2	BD	CAR-T cell & PBMC immunophenotyping
CD45	D058-1283; HI30	BD	CAR-T cell & PBMC immunophenotyping
CD69	FN50	BioLegend	CAR-T cell & PBMC immunophenotyping

CD95	DX2	Invitrogen	CAR-T cell & PBMC immunophenotyping
CD103	2G5	Beckman Coulter	CAR-T cell & PBMC immunophenotyping
CX3CR1	K0124E1	BioLegend	CAR-T cell & PBMC immunophenotyping
CXCR5	MU5BEEE	eBioscience	CAR-T cell & PBMC immunophenotyping
Granzyme b	BG11	BD	CAR-T cell & PBMC immunophenotyping
HLA-DR	G46-6	BD	CAR-T cell & PBMC immunophenotyping
Ki67	DX2; B56	BD	CAR-T cell & PBMC immunophenotyping
PD-1	Z12.2H7	eBioscience	CAR-T cell & PBMC immunophenotyping
Perforin	Pf344	MabTech	CAR-T cell & PBMC immunophenotyping
Tbet	4B10	eBioscience	CAR-T cell & PBMC immunophenotyping
TIGIT	MBSA43	eBioscience	CAR-T cell & PBMC immunophenotyping
HIV Env	VRC01	NIH HIV Reagent Program	Quantification of Env expression in K562-Env
PGT126	PGT126	NIH HIV Reagent Program	Quantification of Env expression in K562-Env
SIVmac293 Env	7D3	Kind gift from Dr. James Hoxie	Quantification of Env expression in LLC-MK2 cells electroporated with SIVmac293 Env mRNA
SIVmac293 Env	8C7	Kind gift from Dr. James Hoxie	Quantification of Env expression in LLC-MK2 cells electroporated with SIVmac293 Env mRNA

Supplemental Table 5. Sequences of guide RNAs and NGS sequencing primers.

Target	gRNA	NGS Forward Primer (5' to 3')	NGS Reverse Primer (5' to 3')
CCR5	ACAATGTGTCAACTCTTGAC	<u>TCGTCGGCAGCGTCAGATGTGTAT</u> <u>AAGAGACAGGCATGACTGACATCT</u> ACCTGC	<u>GTCTCGTGGGCTCGGAGATGTGTA</u> <u>TAAGAGACAGACCCCAAAGGTGA</u> CTGTCCT
CTLA-4	CUCGGCUCAACCUGGCUACC	<u>TCGTCGGCAGCGTCAGATGTGTAT</u> <u>AAGAGACAGATAAAGTCCTTGATTC</u> TGTGTGGGT	<u>GTCTCGTGGGCTCGGAGATGTGTA</u> <u>TAAGAGACAGGGGAGAAACACCT</u> CCTCCATC
DGKα	CUCUCAAGCUGAGUGGGUCC	<u>TCGTCGGCAGCGTCAGATGTGTAT</u> <u>AAGAGACAGCCTGTTCCCTTGG</u> CTAAGAT	<u>GTCTCGTGGGCTCGGAGATGTGTA</u> <u>TAAGAGACAGCAAGCATAACCCA</u> AGGCTGTC
FLI-1	CCUCUGCAGACCCUACACUG	<u>TCGTCGGCAGCGTCAGATGTGTAT</u> <u>AAGAGACAGATGGAGCCAGATGG</u> CTGTTC	<u>GTCTCGTGGGCTCGGAGATGTGTA</u> <u>TAAGAGACAGTGTCTGATCTCCATC</u> AAGCCG
LAG-3	AUGUGGGAGGCUCA GUUCCU	<u>TCGTCGGCAGCGTCAGATGTGTAT</u> <u>AAGAGACAGATCCTCTTCCTCCAC</u> CTCCC	<u>GTCTCGTGGGCTCGGAGATGTGTA</u> <u>TAAGAGACAGCCACCACAGAGCT</u> AAGGACC
PD-1	ACCCUGGCCCGGUCC UCUGGG	<u>TCGTCGGCAGCGTCAGATGTGTAT</u> <u>AAGAGACAGGCTCACATCTGCCT</u> CAGCTT	<u>GTCTCGTGGGCTCGGAGATGTGTA</u> <u>TAAGAGACAGGTATGGGGCAGAG</u> CTGGG

TIGIT	GACCUGGGUCACUU GUGCCA	<u>TCGTCGGCAGCGTCAGATGTGTAT</u> <u>AAGAGACAGT</u> GCTGACCCAGGAC TCACAT	<u>GTCTCGTGGGCTCGGAGATGTGTA</u> <u>TAAGAGACAGT</u> TCATGGTCAGCGA CTGGAG
TIM-3	AGAAGUGGAAUACAU AGCAG	<u>TCGTCGGCAGCGTCAGATGTGTAT</u> <u>AAGAGACAGCGCAGTGAATCATC</u> CTCCAAAC	<u>GTCTCGTGGGCTCGGAGATGTGTA</u> <u>TAAGAGACAGCCACGTTGCTACA</u> GTCAAACA

Underlined regions in the primer sequences indicate Illumina adapter overhangs.

Supplemental Table 6.

Study	Animal ID	HIV model	ART initiation (Days) ¹	Sample	Timepoint (Days post-infusion)	CD4CAR-T marking ² in fresh PBMCs (published ³) (%)	CD4CAR-T cell marking ² in cryo PBMCs (pre-infusion PBMC gating ⁴) (%)	CD4CAR-T cell marking ² in cryo PBMCs (FMO gating ⁵) (%)
Rust et al.	A15104	SHIV	91	Post-Infusion PBMC	31	8.46	0.67	2.47
Rust et al.	A15112	SHIV	91	Post-Infusion PBMC	31	15	0.61	3.30
Rust et al.	A15108	SHIV	91	Post-Infusion PBMC	28	2.45	0.25	0.56
Phase 2 (SIV)	A20031	SIV	10	Pre-infusion PBMC	-667	NA	0.66	2.16
Rust et al.	A15104, A15112, A15106 mix	SHIV	91	Post-infusion PBMC – FMO control	28-31	NA	0.05	0.3

¹The number of days after SHIV or SIV infection ART was initiated.

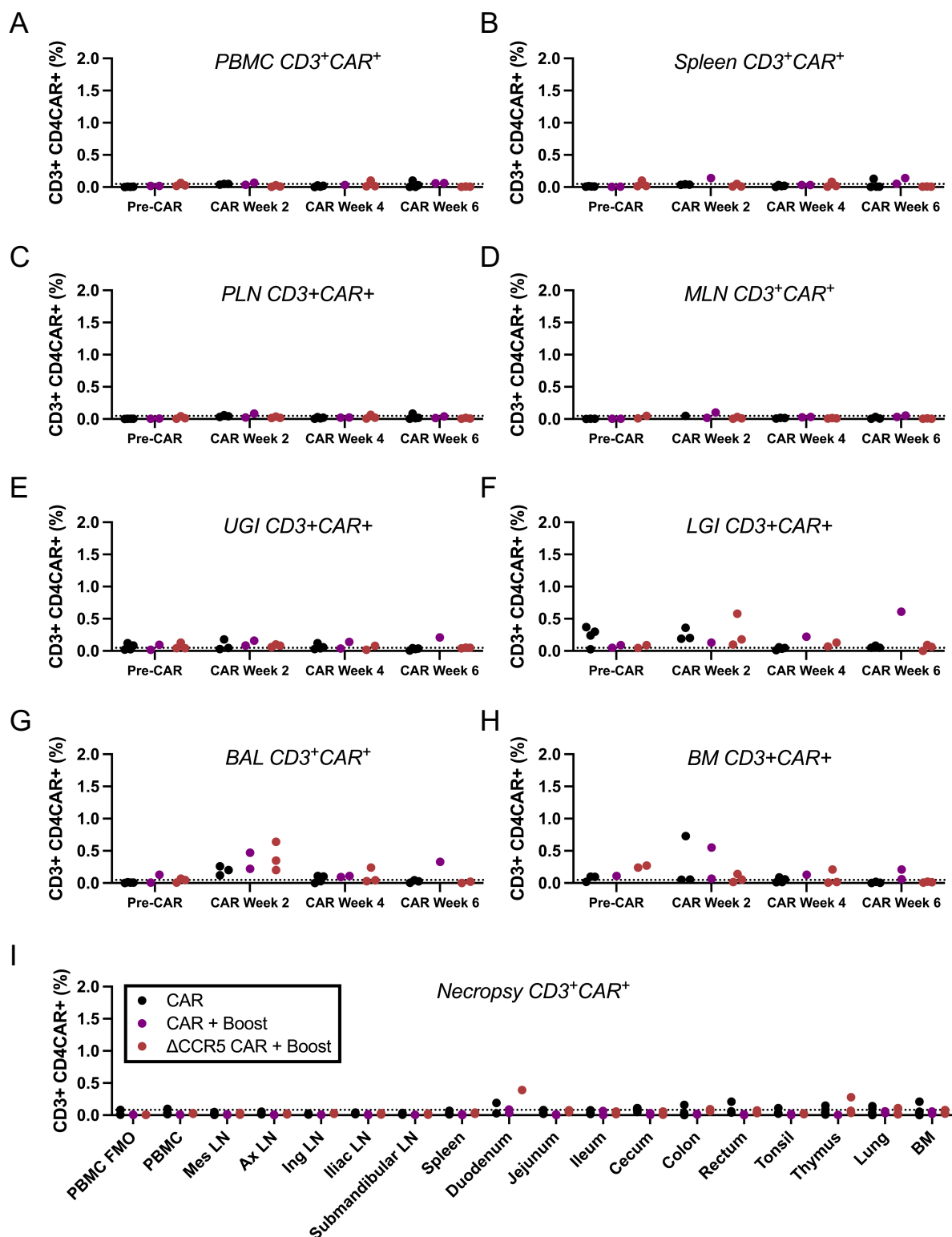
²CD4CAR marking in Live CD3⁺ CD45⁺ T cells.

³Published in Rust et al., 2020.

⁴CD4CAR-T cell marking, per flow cytometry gating with pre-infusion PBMC controls, as in **Supplemental Figure 7**

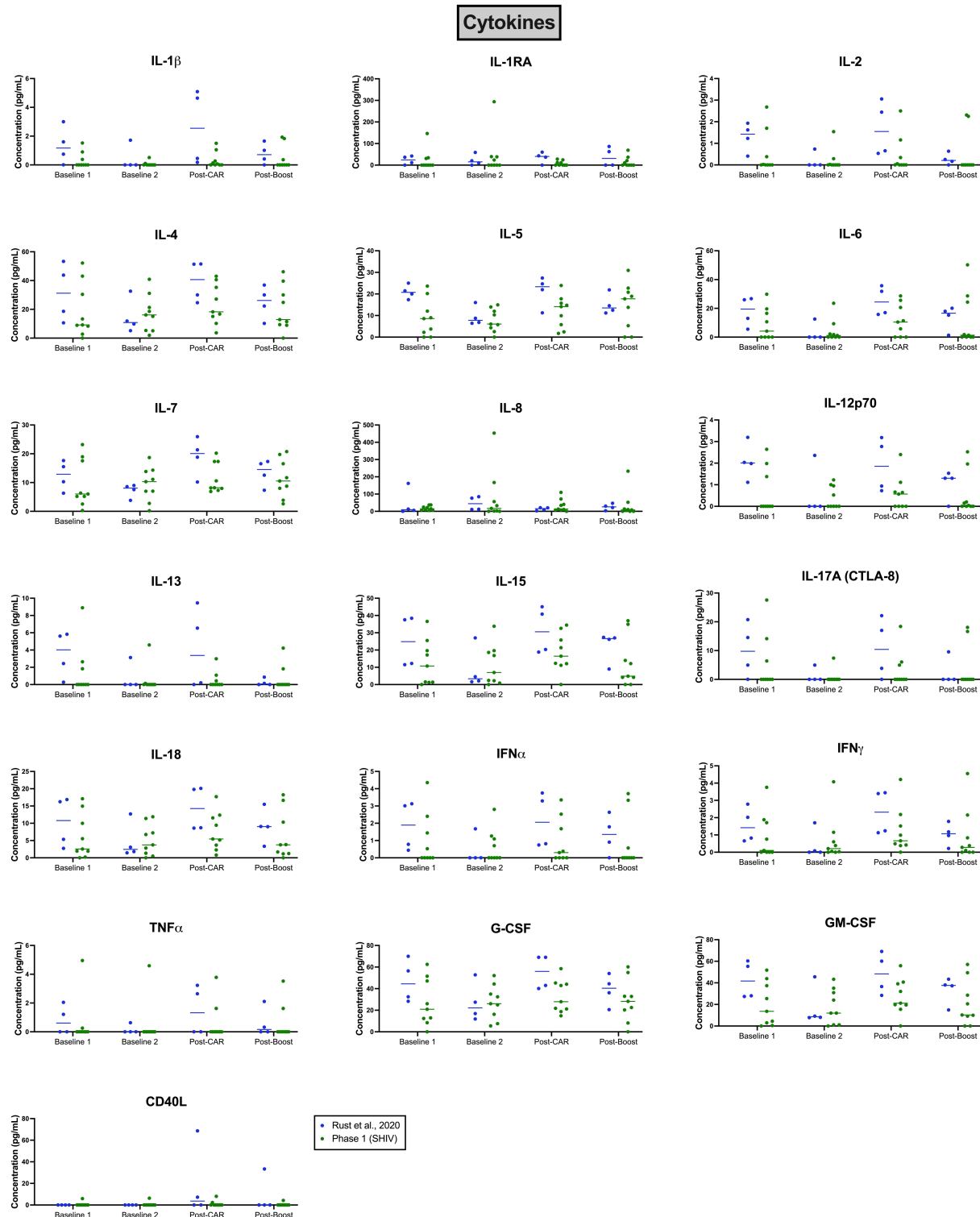
⁵CD4CAR-T cell marking, per flow cytometry gating with a FMO control, as in **Supplemental Figure 8**

Cryo, cryopreserved.



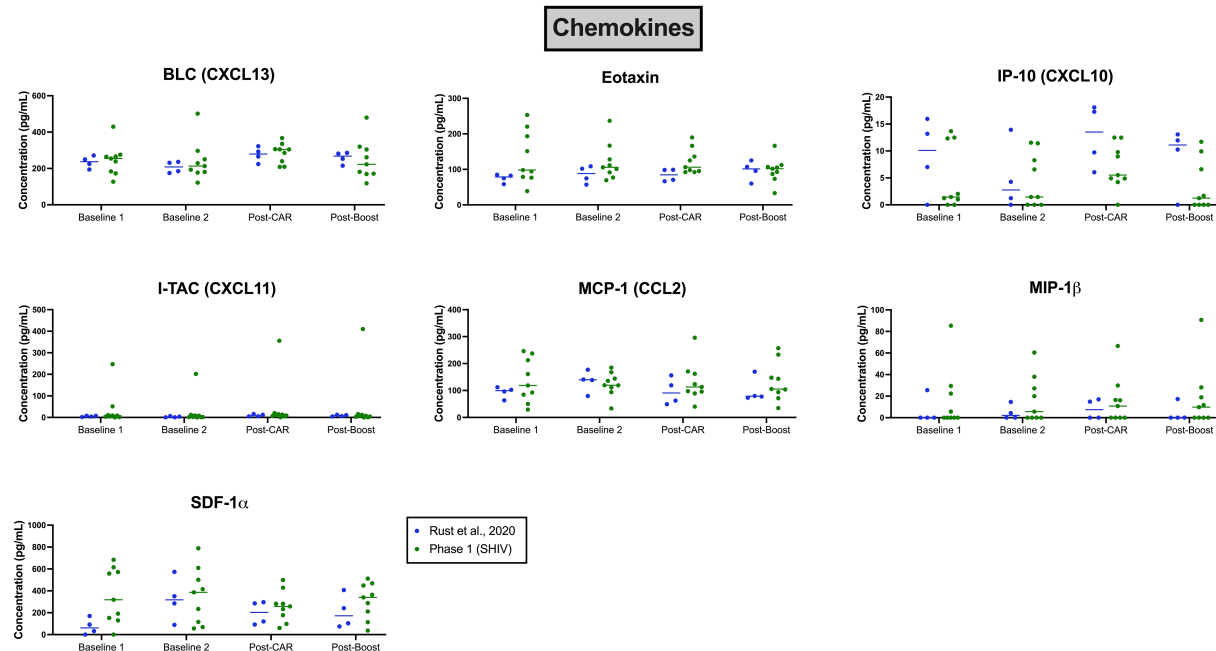
Supplemental Figure 1. CD4CAR T-cell biodistribution in SHIV-1157ipd3N4-infected, ART-suppressed rhesus macaques. Following isolation of single cell suspensions, flow cytometry was used to quantify CD3⁺CD4CAR⁺ T-cells from (A) PBMC, (B) Spleen, (C)

Peripheral Lymph Node (Axillary or Inguinal), (D) Mesenteric Lymph Node, (E) Upper Gastrointestinal Tract (Duodenum/Jejunum), (F) Lower Gastrointestinal Tract (Colon), (G) Bronchoalveolar Lavage (BAL), and (H) Bone Marrow at the indicated time points. (I) Broader tissue surveys were performed at necropsy (10 weeks post-CAR). Dotted lines represent thresholds established via PBMC fluorescence minus one [FMO] controls. Missing data points indicate sample was not collected.

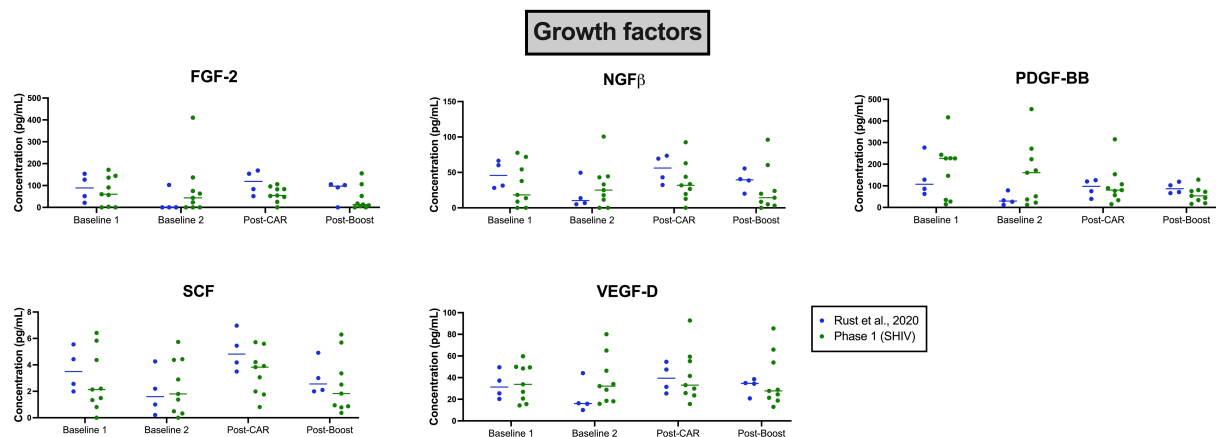


Supplemental Figure 2. Plasma cytokine concentrations in Rust et al., 2020 and Phase 1A (SHIV) cohorts. Plasma cytokine levels were quantified at matched timepoints: Baseline 1 (2 months prior to CAR-T infusion), Baseline 2 (1 month prior to CAR-T infusion), Post-CAR (7 days after CAR-T infusion), and Post-Boost (7 days after K562-Env boost)

administration). Statistical significance was assessed with multiple Mann-Whitney tests with Holm-Šídák correction for multiple comparison.

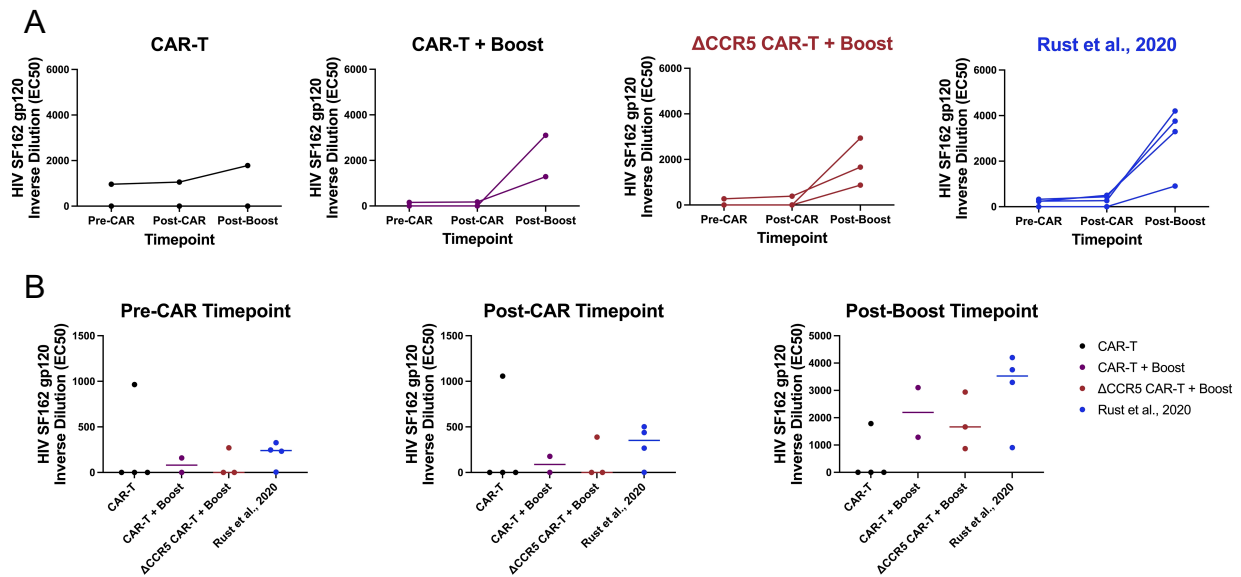


Supplemental Figure 3. Plasma chemokine concentrations in Rust et al., 2020 and Phase 1A (SHIV) cohorts. Plasma chemokine levels were quantified at matched timepoints: Baseline 1 (2 months prior to CAR-T infusion), Baseline 2 (1 month prior to CAR-T infusion), Post-CAR (7 days after CAR-T infusion), and Post-Boost (7 days after K562-Env boost administration). Statistical significance was assessed with multiple Mann-Whitney tests with Holm-Šídák correction for multiple comparison.



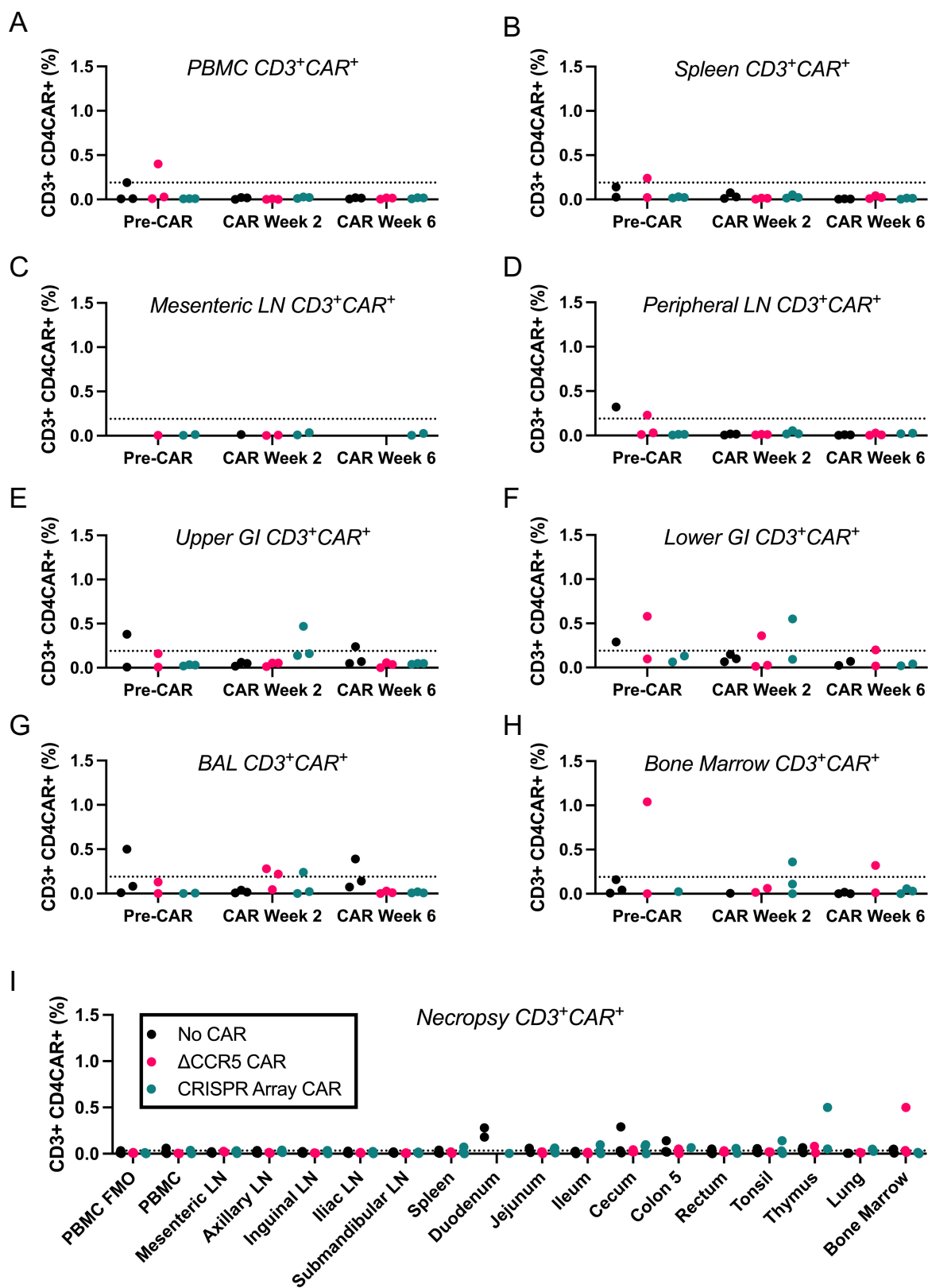
Supplementary Figure 4. Plasma growth factor concentrations in Rust et al., 2020 and Phase 1A (SHIV) cohorts. Plasma growth factor levels were quantified at matched

timepoints: Baseline 1 (2 months prior to CAR-T infusion), Baseline 2 (1 month prior to CAR-T infusion), Post-CAR (7 days after CAR-T infusion), and Post-Boost (7 days after K562-Env boost administration). Statistical significance was assessed with multiple Mann-Whitney tests with Holm-Šídák correction for multiple comparison.

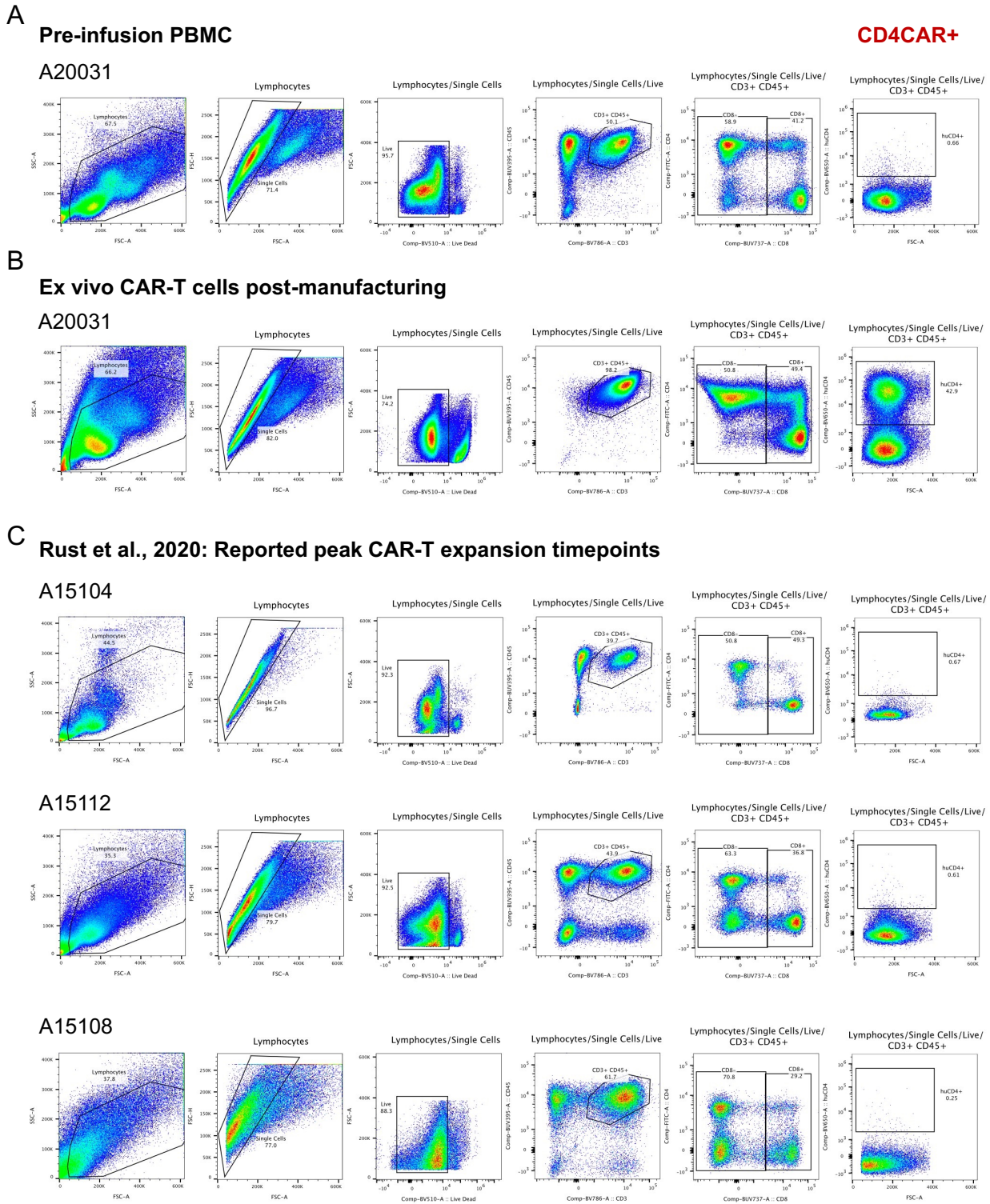


Supplemental Figure 5. Quantification of HIV anti-Env antibodies from NHP plasma.

(A) Quantification of anti-Env antibodies from SHIV-infected NHPs 1 month pre-CAR-T cell infusion (Pre-CAR), 7 days post-CAR-T cell infusion (Post-CAR), and 7 days after administration of K562-Env boost (Post-Boost) in Phase 1A (SHIV) and Rust et al., 2020 cohorts. (B) Quantification of anti-Env antibodies, as in A, compared across Phase 1A (SHIV) and Rust et al., 2020 cohorts at specified timepoints.



Supplemental Figure 6. CD4CAR T-cell biodistribution in SIVmac239M-infected, ART-suppressed rhesus macaques. Following isolation of single cell suspensions, flow cytometry was used to quantify CD3⁺ CD4CAR⁺ T-cells from (A) PBMC, (B) Spleen, (C) Mesenteric Lymph Node, (D) Peripheral Lymph Node (Axillary or Inguinal), (E) Upper Gastrointestinal Tract (Duodenum/Jejunum), (F) Lower Gastrointestinal Tract (Colon), (G) Bronchoalveolar Lavage (BAL), and (H) Bone Marrow at the indicated time points. (I) Broader tissue surveys were performed at necropsy (10 weeks post-CAR). Dotted lines represent thresholds established via PBMC fluorescence minus one (FMO) controls. Missing data points indicate sample was not collected.



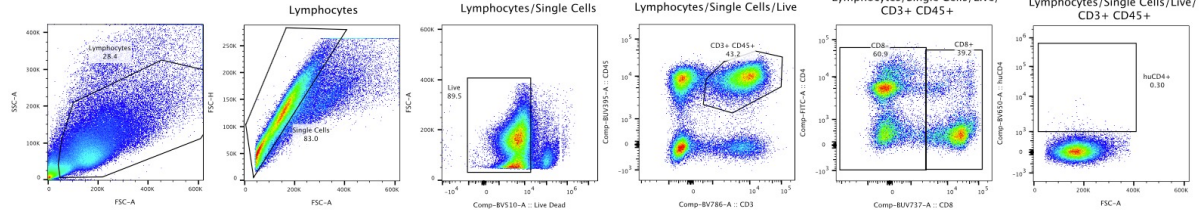
Supplemental Figure 7. CD4CAR-T cell staining from post-infusion PBMCs and ex vivo CAR-T cell products gated with pre-infusion PBMC controls. PBMCs and ex vivo CAR-T cell products were stained to assess CD4CAR expression via flow cytometry. (A) Pre-

infusion PBMCs served as a true biological negative control for establishing CD4CAR gating. (B) CD4CAR-T cell product was stained as a positive control. (C) Post-infusion PBMCs collected during the reported expansion phase from Rust et al., 2020 were stained and analyzed for CD4CAR⁺ cells. The flow cytometry gating hierarchy is shown above each plot and was applied consistently across all samples. Animal ID is indicated for each sample. Limited pre-infusion PBMC samples from Rust et al. were available. A20031 has several distinctions from Rust et al. samples: A20031 was a SIV-infected NHP, whereas Rust et al. samples were SHIV-infected NHPs. In A20031, ART was initiated at 10-days post-infection, whereas NHPs from Rust et al. were initiated on ART at 13-weeks post-infection. It is unclear whether these experimental differences would meaningfully affect background huCD4 staining or limit the suitability of A20031 pre-infusion PBMCs as a representative true biological negative control.

A

Fluorescence-minus-one (FMO) control

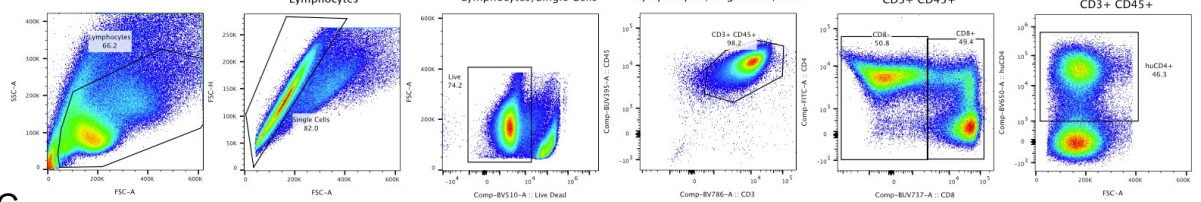
Mixed PBMCs from all samples



B

Ex vivo CAR-T cells post-manufacturing

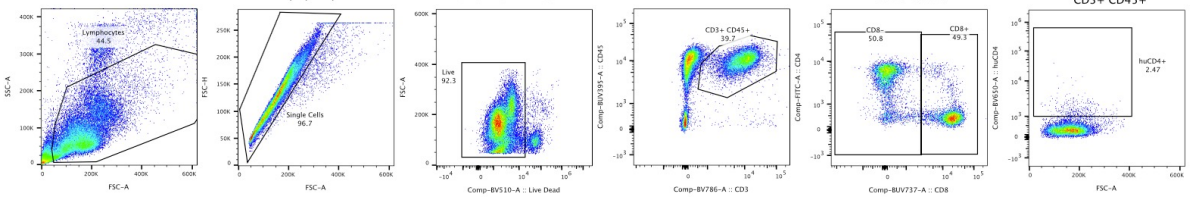
A20031



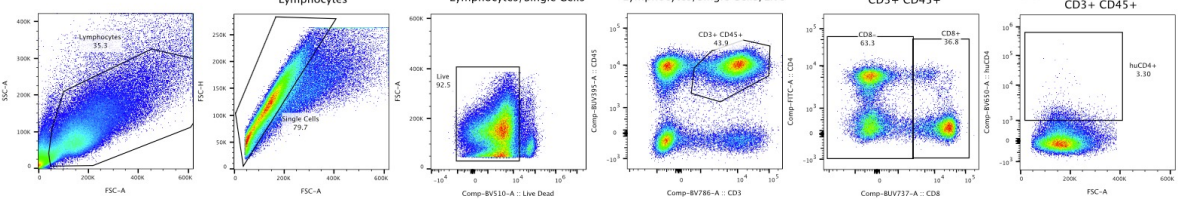
C

Rust et al, 2020: Reported peak CAR expansion timepoints

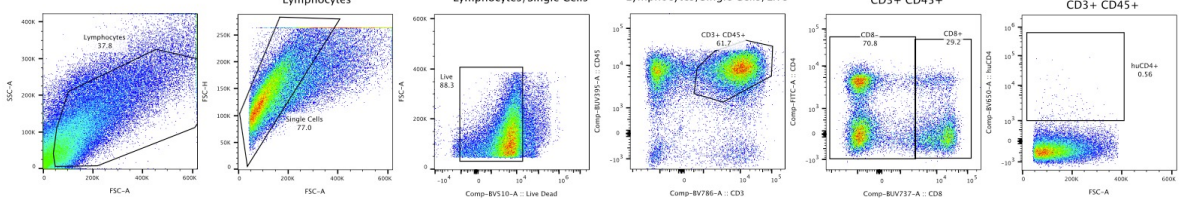
A15104



A15112



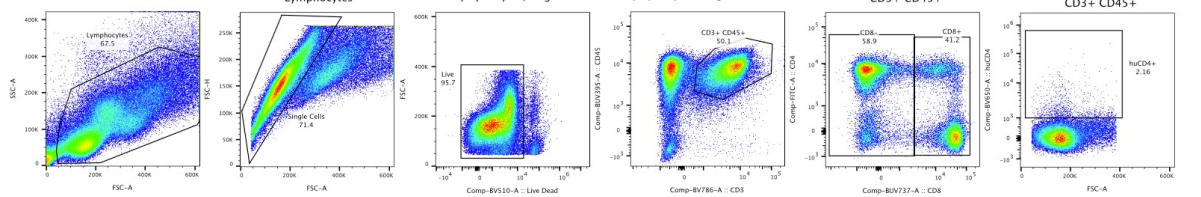
A15108



D

Pre-infusion PBMC

A20031



Supplemental Figure 8. CD4CAR-T cell staining from post-infusion PBMCs and ex vivo CAR-T cell products gated with FMO controls. Data, as in Supplemental Figure 7, is analyzed with flow cytometry gates established through fluorescence-minus-one (FMO) controls. (A) huCD4-BV650 FMO control, comprised of equally mixed PBMCs from (C). (B) CD4CAR-T cell product. (C) Post-infusion PBMCs collected during the reported expansion phase from Rust et al., 2020. The flow cytometry gating hierarchy is shown above each plot and was applied consistently across all samples. Animal ID is indicated for each sample.

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