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A NEW GENE THERAPY PARADIGM IN VIVO CRISPR ENGINEERING OF HEMATOPOIETIC NICHES FOR CANCER IMMUNOTHERAPY

Naemel Usman[*1], Syed Imran Zahid [2], Uswah Zainab[3], Aisha Akram[4], Sana Azam[5], Zainab Bibi [6], Hamza Rafeeq[7]

[*1,2,3,4,5,6,7]*Department of Biochemistry, Riphah International University. Faisalabad, Punjab. Pakistan. 44000*

[*1]naemelusman1320@gmail.com

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Corresponding Author: *
Naemel Usman

Abstract

Despite these impressive clinical outcomes, chimeric antigen receptor (CAR) T-cell therapy is hindered by multiple intrinsic limitations that go beyond logistics and are truly biological in nature, which limit its use to treat hematologic malignancies. The ex vivo manufacturing paradigm is time-intensive, involving leukapheresis, genetic modification, expansion and quality control over 2-4 weeks, adding to the manufacturing costs and complexity, and also introducing heterogeneity in the product and a risk of T cell exhaustion or differentiation before infusion. These challenges have driven the development of the concept of in vivo CRISPR-based reprogramming of hematopoietic stem cells (HSCs) to produce endogenous CAR immune cells, which are a paradigm shift towards therapeutic development. This approach aims to provide a single intravenous injection of the CRISPR components that would specifically and stably edit LTR HSCs within their native niche to generate persistent, multilineage output of CAR-expressing T, NK, and macrophages. The aim of the current review is thus to critically consolidate and articulate the emerging but growing knowledge base that includes molecular tools for HSC-directed CRISPR delivery, preclinical data that document the antitumor activity of CRISPR in both hematologic and solid tumor models, the significant delivery hurdles of the bone marrow microenvironment, the range of immunological and genotoxic safety concerns, and the evolving translational landscape. A compilation of major results shows that antibody-lipid nanoparticles selectively target HSCs, and adeno-associated virus serotype 6 (AAV6) and virus-like particles (VLPs) mediate efficient knock-in of CAR cassettes by homology-directed repair under lineage-restricted endogenous promoters. In preclinical studies, long term multifunctional antitumor activity has been shown, including immunological memory upon re-challenge. Nonetheless, the potential for off-target editing in LTHSCs, variability in CAR expression in non-effector cells (e.g., erythroid or megakaryocytic progenitors) and pre-existing or acquired immunity to Cas9 requires careful mitigation strategies. In the future, we envision a roadmap from bench to bedside, with regulatory harmonization of in vivo gene editing products, GMP innovations to allow production and scaling of lipid nanoparticles, and the development of next-generation platforms such as prime editing, base editing and miRNA-based lineage restriction switches that

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could all potentially be used to speed up first-in-human trials in the next three to five years.

INTRODUCTION

Chimeric antigen receptor (CAR) T-cell therapy has forever changed the treatment paradigm for relapsed or refractory B-cell malignancies with complete remission rates previously impossible with standard chemotherapy or radiation. But even with this game-changing clinical promise, the ex vivo manufacturing model that current CAR-T platforms are built on has intrinsic drawbacks that go beyond mere logistical challenges and address the core of the issue of biological and economic availability of these therapies on a large scale. In particular, leukapheresis and subsequent ex vivo genetic modification (either by virus vector or transposon), the 2-4 weeks of cell culture and extensive quality control assays and the subsequent cryopreservation before re-injection, result in not only vein-to-vein times that are incompatible with the speed of the disease but also in a high inter-patient and intra-batch product variability. Furthermore, the conditioning lymphodepletion regimen used to provide a "homeostatic niche" for infused CAR-T cells can often cause severe cytopenias and infectious complications, and infused cells can cause cytokine release syndrome and immune effector cell-associated neurotoxicity syndrome, toxicities that arise from the simultaneous supraphysiological activation of a large bolus of engineered lymphocytes(Bonini et al., 2023).These safety issues, along with extremely expensive procedures costing between four hundred thousand and eight hundred thousand United States dollars per patient, have driven the research of alternative strategies that would not involve ex vivo manipulations.

In this context, in vivo CRISPR-mediated reprogramming of hematopoietic stem cells (HSCs) to create endogenous CAR immune cells is a conceptually innovative approach that is reshaping the therapeutic pipeline from a multi-step personalized manufacturing process to a single, systemically delivered gene therapy. The strategy for using HSCs as the target cells as opposed to mature peripheral lymphocytes is based on several converging biological principles. Long-term repopulating HSC are a self-renewing population in the bone marrow niche that can give rise to all the hematopoietic cell types, so that if we can successfully edit a single HSC, the CAR will persist and provide long-term output without further HSC infusion(Z. Chen, Hu, & Mei, 2023). Second, in situ editing of the HSC compartment generates endogenously differentiated CAR cells that are not associated with non-physiological ratios of effector cells to target cells that are commonly observed with conventional CAR-T infusion and might reduce the severity of cytokine release syndrome. Thirdly, the CRISPR components can be packaged into pre-made lipid nanoparticles or virus-like particles (VLP) with tropism to HSC surface markers, like CD117 or Lamp1, which means there is no need for personalized manufacturing, potentially shortening the time from vein to vein to hours instead of weeks, and significantly lowering the production cost, compared with personalised cell products. Thus, in vivo HSC reprogramming provides one-time systemic treatment, long-lasting CAR expression in T, NK and macrophage cells, and true off-the-shelf potential, which are all key attributes to overcome current limitations of CAR-T therapy(W. Zhang & Huang, 2024).

The generation of endogenous CAR immune cells is defined as the stable heritable incorporation of a CAR transgene into the genome of long-term HSCs, and then the natural haemato-poietic differentiation of those engineered HSCs to yield mature effector cells with either a constitutive or (more elegantly), lineage-specific endogenous promoter, which control the CAR expression to desired subsets of cells, such as CD8+ cytotoxic T lymphocytes or NKp46+ natural killer cells. This is in contrast to exogenous CAR generation, where CAR is genetically modified in the form of terminally differentiated T cells ex vivo prior to reinfusion, thus circumventing normal

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developmental checkpoints and homeostatic regulatory mechanisms for T cell activation and tolerance(H. K. Mishra & Kalyuzhny, 2024). The difference is not just a nuance in the semantics, but has tremendous immunological implications; endogenously generated CAR immune cells are generated from unmanipulated HSCs that have passed through the thymus or bone marrow and may have acquired central tolerance mechanisms and physiological regulation that may mitigate off tumor toxicity and autoimmunity. This review covers the whole length of the pipeline from molecular tools that facilitate HSC-directed delivery of the CRISPR system and CAR knock-in into specific cell lineages, to preclinical evidence of efficacy in tumor models of both hematologic and solid malignancies, to challenges of delivery to bone marrow, selectivity to HSCs, safety including off-target editing and genotoxicity, and innovations for regulatory and manufacturing processes to enable a first in-human trial(Aparicio et al., 2025). This review critically examines these areas and strives to outline a holistic picture of the potential for in vivo HSC reprogramming to fulfil its promise as a novel paradigm for cancer immunotherapy by gene therapy.

2. Biological Foundations: Hematopoietic Stem Cell Niche and CAR Immune Cell Lineages

The biological basis of in vivo HSC reprogramming is based on the unique features of the bone marrow niche: long-term HSCs are quiescent, but they still possess multilineage differentiation potential. After the knock-in of CAR by CRISPR, edited HSCs follow hierarchical differentiation to form distinct CAR+ effector lineages that each have distinct anti-tumor activity and cell surface markers. Table 1 shows a comparison of four hematopoietic lineages for endogenous CAR generation from CRISPR reprogrammed HSCs. CAR-T cells are effective at killing cells and have good memory, but carry a risk of causing GVHD and have a low activity against MHC-low tumors. CAR-NK cells have an MHC-independent killing mechanism, but are not persistently present (4-6 weeks), and do not traffic well to solid tumors and do not induce GVHD. CAR-macrophages have the ability to infiltrate deep into solid tumors and to phagocytose the tumor (60–70% tumor reduction in preclinical models); however, they can be M2 polarized. CAR-dendritic cells are the least developed lineage, which are capable of priming adaptive immunity but need inducible activation to avoid spontaneous activation. None of these single-lineages are universally best; the best lineage is dependent on disease context; hematologic malignancies – CAR-T, allogeneic – CAR-NK, and solid tumors – CAR-macrophages.

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Table 1: Key Hematopoietic Lineages for Endogenous CAR Expression: Advantages, Markers, Evidence, and Limitations

Cell Type	Advantages for CAR Engineering	Key Surface Markers	Preclinical Evidence (Selected)	Key Limitations & Challenges	Reference(s)
CAR-T cells (from HSCs)	Potent cytotoxicity (granzyme B, perforin); long-lived central and effector memory populations; well-characterized biology	CD3, CD8, CD4	Proven in ex vivo settings; extensive validation in hematologic malignancy models (B-ALL, lymphoma, multiple myeloma)	Risk of GVHD in allogeneic settings; reliance on MHC presentation limits activity in MHC-low tumors	(Su et al., 2025)
CAR-NK cells	MHC-independent killing; no GVHD risk; ADCC via CD16; suitable for allogeneic applications	CD56, NKp46, CD16	Emerging in vivo HSC data; promising for hematologic malignancies (AML, myeloma) and some solid tumors	Shorter persistence (4–6 weeks) compared to T cells; suboptimal trafficking to solid tumors	(Wu & Matosevic, 2022)
CAR-macrophages	Deep solid tumor infiltration; potent phagocytosis; antigen presentation to recruit endogenous T cells	CD68, CD11b, F4/80 (mouse), CD14 (human)	Proof-of-concept in mice; 60–70% tumor reduction in pancreatic (Panc02) and hepatocellular carcinoma (Hepa1-6) models	Susceptible to M2 immunosuppressive polarization in TME; lower cytotoxic potency than T or NK cells	(K. Chen, Liu, Wang, & Fang, 2024; Zhaoting Li, Wang, Gu, & Hu, 2024)

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Cell Type	Advantages for CAR Engineering	Key Surface Markers	Preclinical Evidence (Selected)	Key Limitations & Challenges	Reference(s)
CAR-dendritic cells	Antigen cross-presentation; potent T-cell priming via costimulatory molecules; orchestrates adaptive immunity	CD11c, MHC-II, CD80, CD86, CD40	Limited; requires inducible systems (tetracycline, rapamycin) to prevent spontaneous activation	Constitutive CAR signaling causes spontaneous activation and aberrant cytokine production; technically challenging to regulate	(T. Huang, Bei, Hu, & Li, 2024)

2.1. HSC physiology and bone marrow niche accessibility for in vivo editing

The potential for in vivo CRISPR-based reprogramming of HSCs is heavily dependent on the understanding of the physiology of HSCs and the specialized anatomical and molecular characteristics of the bone marrow niche that regulates HSC quiescence, self-renewal and access to systemically delivered gene editing tools. The long-term repopulating HSCs are predominantly located in specialized perivascular and endosteal bone marrow niches, where they are in a state of relative quiescence with low metabolic activity, G0 cell cycle arrest, and decreased endocytic function, creating significant obstacles to the efficient delivery of CRISPR components(Shams et al., 2025). Because quiescent HSCs have reduced uptake of lipid nanoparticles and decreased nuclear import of exogenous nucleic acids, delivery strategies that target HSC-specific surface receptors and promote receptor-mediated endocytosis or that transiently activate HSC cycling with preservation of long-term repopulating capability are needed. Fortunately HSCs express a unique repertoire of surface markers that could be targeted selectively including CD117 (c-Kit), CD34 (humans, not mice), CD48 (negative in murine HSCs), and signaling lymphocytic activation molecule family members CD150 and CD244, with CD117 emerging as the most validated marker for in vivo HSC delivery because it is highly expressed on long-term HSCs, involved in the signaling of stem cell factor (SCF), which is essential for HSC maintenance, and targetable by antibody-conjugation of lipid nanoparticles (LNPs) (Y.-R. Li, Zhu, Halladay, & Yang, 2025).However, there are other physical barriers in the bone marrow niche, such as collagen I, fibronectin, and proteoglycans in the extracellular matrix, which can sequester cationic nanoparticles, as well as the high cellularity of marrow space, which can limit diffusive penetration. Recent advances in understanding HSC niche biology have also suggested that transient mobilization of HSCs into the peripheral blood, such as by granulocyte colony-stimulating factor or AMD3100, might paradoxically facilitate bone marrow penetration due to reduced intrasinusoidal pressure and increased HSC surface availability, but this must be balanced against the potential of altering HSC quiescence and editing efficiency.(Y. Chen et al., 2025) Moreover, the spatial distribution of haematopoietic regions varies by species, with mice with active haematopoeitic tissue in the long bones and axial skeleton and humans with HSCs

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being largely located in the pelvis, vertebrae and sternum, indicating significant implications for translation, because human BM is less cellular and more adipose-rich than mouse BM, which may impact upon the biodistribution of nanoparticles and the frequency of HSC encounters. Thus, in vivo editing requires a delivery vehicle with good HSC tropism as well as a nuanced understanding of niche dynamics, HSC cell cycle and species-specific anatomical differences(Xun, Hao, Cheng, & Gao, 2025).

2.2. Differentiation trajectories from edited HSCs to mature effector cells

Once a CAR transgene has been successfully incorporated into the genome of a long-term HSC through CRISPR, the differentiation pathway that ensues needs to be considered as a multi-step, hierarchical process that is controlled by lineage-specific TFs, gradations of cytokines, and epigenetic remodeling, which together determine which mature effector populations eventually express the CAR and in which relative proportions. The edited HSC, with stably integrated CAR cassette, is maintained as full multipotent HSC and can undergo successive stages of restriction to multipotent progenitor (MPP) which has lost self-renewal capacity but still expresses lymphoid and myeloid potential, then to common lymphoid progenitor, common myeloid progenitor and eventually to lineage-committed progenitors, including pro-T cell (thymus), pre-natural killer cell progenitor (bone marrow), and monocyte-dendritic cell precursor (MDC)(Furukawa et al., 2022). Importantly, the fate choice of an edited HSC is not fixed, but stochastic and context-dependent; impacted by inflammatory microenvironment, interactions with other stromal cells, and systemic levels of cytokines, and therefore, CAR-expressing T cells, natural killer cells, macrophages, and dendritic cells can all arise from the same edited HSC clone during its lifetime. This multilineage output is a core benefit of using unconventional CAR-T therapy, where various CAR-positive cells may have complementary actionable antitumor properties: CAR-T cells can mediate direct killing and memory formation, CAR-natural killer cells can kill solid tumors without major histocompatibility complex restriction and with reduced risk of graft-versus-host disease, and CAR-macrophages can infiltrate and phagocytose solid tumors, presenting antigens to endogenous cells and recruiting endogenous immunity. The same multilineage potential, however, presents the possibility of CAR expression in non-effector lineages such as erythroid precursors, megakaryocytes and granulocytes that may result in off-target toxicity or generation of CAR-mediated killing of normal tissues(Ottaviano & Qasim, 2025). Several strategies are being actively pursued to limit differentiation toward undesirable lineages, including the knock-in of the CAR construct under the control of a lineage-restricted endogenous promoter such as the CD8 promoter (for cytotoxic T cells) or the NKp46 promoter (for natural killer cells), so that CAR transcription is only activated upon commitment to that specific lineage. The use of microRNA-based post transcriptional regulation for example by including target sites for miR-142 which is highly expressed in haematopoietic cells but not in HSCs can destabilise the CAR transcript in the cells of the non-desired lineages, but allow the cells of the mature effectors to express the CAR transcript. Further, the bone marrow niche itself may have inherent selection for different types of differentiation, such as lymphodepleting conditioning with busulfan or cyclophosphamide before in vivo editing, which skews the competitive repopulation advantage toward lymphoid progenitors and thus increases the relative contribution of CAR-T and CAR-natural killer cells at the expense of myeloid lineages. Knowledge and, perhaps, the ability to control these differentiation pathways will be crucial for optimal tuning of the endogenous CAR immune repertoire(Dimitri, Herbst, & Fraietta, 2022).

2.3. Kinetics and durability of CAR expression from the endogenous promoter vs. exogenous regulatory elements

Kinetics and persistence of CAR expression are key to the efficacy and safety of the therapy and are

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intrinsically linked to the type of regulatory elements selected to drive CAR transcription after HSC editing. If the CAR is knocked into an endogenous genomic location and the CAR is controlled by the host's native promoter, e.g. AAVS1 safe harbour or lineage-specific CD8, then the kinetics of CAR expression will be the same as the host's native promoter in hematopoiesis. This has the consequence that HSCs and early progenitors are not CAR-positive, because the promoter is epigenetically silenced in them or the level of expression is below the threshold needed for active transcription, and CAR expression is initiated on differentiation to the committed precursor stage and reaches its maximum in the mature effector cells. This temporal control provides significant safety benefits because it avoids the possibility of fratricide or CAR exhaustion due to premature CAR expression on HSCs and it also avoids CAR expression on megakaryocytes or erythroid cells. The expression level of CAR by an endogenous single-copy knock-in is usually lower, however, than is obtained by the use of strong viral promoters like the murine stem cell virus or elongation factor 1 alpha promoters, and it is important to consider whether enough antigen receptor density is reached to eliminate target cells(Alvanou et al., 2023). Furthermore, lineage-specific promoters could be silenced or downregulated during chronic antigen stimulation or in exhausted T cells, which could affect the long-term efficacy. By contrast, putting the CAR under the control of a constitutive exogenous promoter results in high-level, consistent and rapid expression upon completion of the knock-in, which, however, is at the cost of CAR expression in HSCs and in all their progeny, irrespective of lineage appropriateness. Constitutive promoters also are more likely to cause insertional oncogenesis because of their enhancer activity, as seen in gene therapy experiments in individuals suffering from severe combined immunodeficiency in which high-level viral enhancers were activated. As far as durability, both strategies could potentially maintain life-long CAR expression since the CAR is incorporated into the genome of the HSC and passed on to daughter cells, yet CAR surface expression over time is subject to further regulation including CAR promoter methylation, CAR effectors' internalization and degradation, and immune-mediated clearance of CAR-expressing HSCs/effectors. Murine model studies show that CAR expression can be maintained without silencing from the endogenous Rosa26 promoter for six months or more, whereas constitutive promoters from gammaretroviruses exhibit progressive methylation and silencing in HSCs, but not in differentiated progeny. These kinetic profiles have breathtaking clinical implications: fast and high expression from exogenous promoters could translate into earlier antitumor effects but with increased risk of lineage-unrestricted toxicity, while slow and low expression from lineage-restricted endogenous promoters could lead to increased safety at the potential cost of initial efficacy. Hybrids such as the conditional use of small molecule or synthetic gene circuits based on an inducible promoter are being developed but are more complex and may have to be considered against the simplicity of a single, constitutive or lineage-restricted knock-in. Finally, perhaps the best regulatory approach will be disease specific, with high level, rapid expression being best for aggressive hematologic malignancies, and sustained, lineage-restricted expression being best for solid tumors to avoid toxicity to normal cells(Jia et al., 2025). Figure 1 shows Left panel: Bone marrow niche with HSCs targeted by CRISPR-LNP or AAV. Middle: HSC genetically modified at a safe harbor site (such as CCR5, AAVS1) by knocking-in a CAR construct driven by a lineage-specific promoter (such as CD8 for T cells or NKp46 for NK cells) or a constitutive promoter. Right panel: CAR-T, CAR-NK, CAR-macrophages and CAR-dendritic cells differentiation and antitumor activity in peripheral blood and tumor microenvironment.

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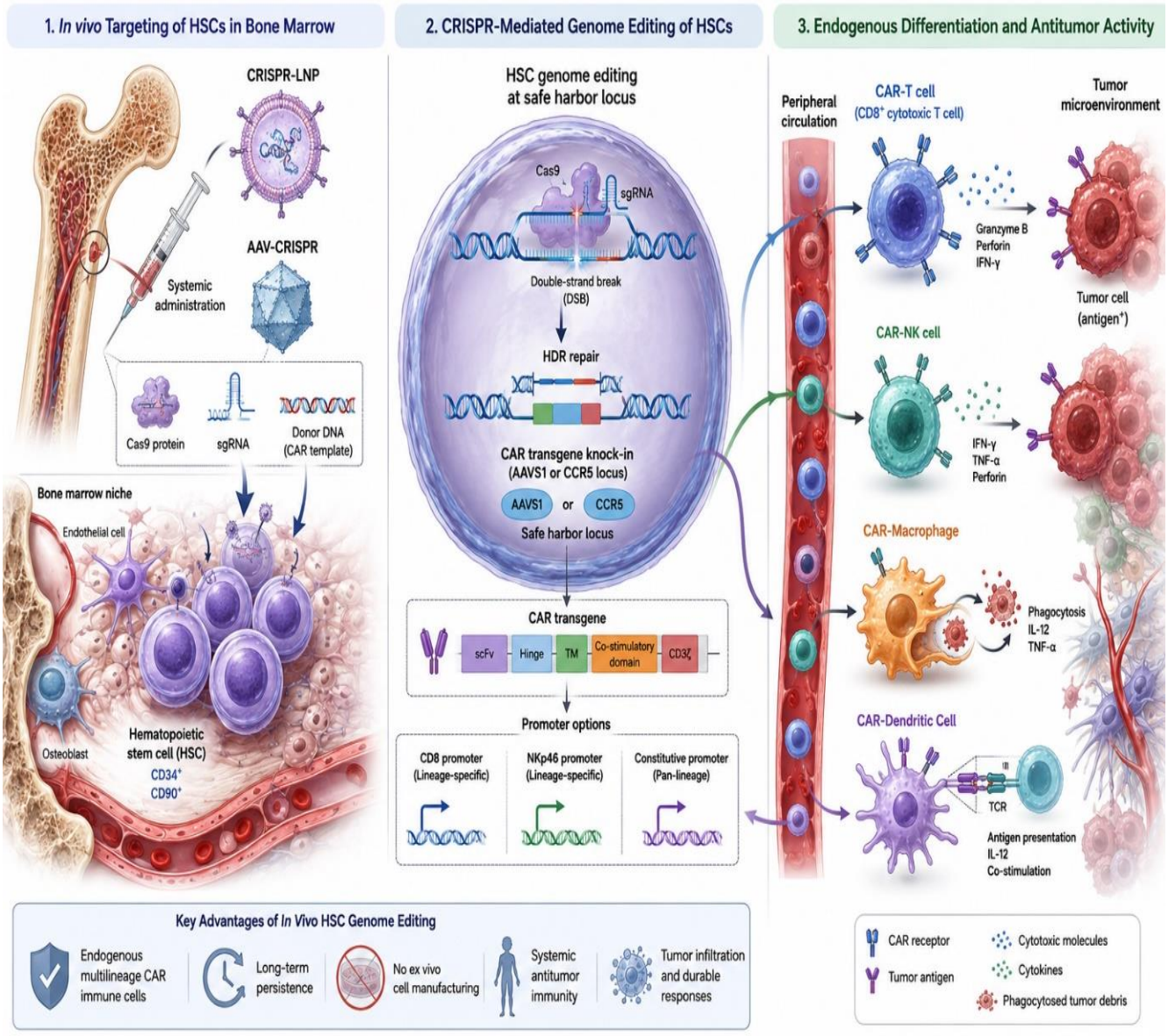

Figure 1: Schematic of in vivo CRISPR reprogramming of HSCs and downstream endogenous CAR immune cell differentiation.

3. CRISPR Toolkit for In Vivo HSC Engineering

3.1. Delivery vehicles: lipid nanoparticles (LNPs), adeno-associated viruses (AAV6/AAV9), and virus-like particles (VLPs)

However, development of delivery vehicles that are highly efficient for editing long-term HSCs while delivering minimal off-target editing in non-HSC compartments and minimal immunotoxicity that compromises the health of the edited HSC pool or the recipient are critical for the successful implementation of this in vivo editing approach. Three main classes of delivery platforms have emerged as leading candidates for this use: lipid nanoparticles (LNPs), adeno-associated viruses (AAVs), and virus-like particles (VLPs), which have their own advantages and disadvantages with respect to clinical translation(Khodabandehloo et al., 2025). LNPs have become a popular tool since their approval for mRNA-based COVID-19 vaccines, and for CRISPR editing, they can be

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used to package either Cas9 mRNA and chemically modified synthetic guide RNA or, more recently, to package pre-assembled complexes of Cas9 and sgRNA. The transient expression of Cas9 from mRNA-loaded LNPs is especially useful for HSC editing as it decreases the amount of time the nuclease is present, mitigating the theoretical risk of off-target editing of partially matched genomic DNA sequences. The standard LNPs, however, have limited intrinsic tropism for HSCs in vivo, with the majority of the injected dose deposited in liver, spleen, and lungs, and only a small percentage found in the bone marrow niche. An alternative platform is the AAV, which is well-characterized with a known biology and has been clinically shown to be safe in gene therapy trials for hemophilia and spinal muscular atrophy, especially serotype 6 and 9. AAV6 has been successfully used to deliver a donor template for homology-directed repair or to express SaCas9 from a compact promoter and is naturally tropic for human CD34+ hematopoietic stem and progenitor cells, which is thought to be due to its interaction with AAV receptor and platelet-derived growth factor receptor (Zong & Li, 2025).However, they have two major drawbacks in the context of HSC editing; first, that the AAV genome is not integrated and persists in non-dividing cells for several weeks, thereby allowing for prolonged expression of Cas9, which can raise concerns about off-target effects; and second, that the capsids of AAVs are highly immunogenic, often generating neutralizing antibodies that would prevent repeat dosing in seropositive individuals. Additionally, the capacity of AAV is about 4.7 kilobases which means that only a limited number of Cas9 orthologs and the regulatory elements can be co-delivered. VLPs are a more recent hybrid platform, which has properties of both retrovirus packaging and protein delivery, with transient activity. VLPs typically are made by fusing a Cas9-single guide RNA ribonucleoprotein complex (RNP) with a viral structural protein, so that the mature VLP contains pre-assembled RNP instead of nucleic acid that encode the nuclease. During the process of VLP internalization and capsid disassembly, the ribonucleoprotein is released directly into the cytoplasm, where there is immediate editing activity that decays rapidly, without depending on transcription or translation, for the lowest off-target profile of the three platforms. But, the production yield of VLP is significantly lower than LNPs or AAVs, and the tropism of VLP is mostly dependent on the envelope glycoprotein pseudotype; VSV-G is broad but not selective. Because no single platform meets all the criteria for HSC editing in the clinic in vivo, the current focus of research is on combining platforms, such as delivering the Cas9 ribonucleoprotein or mRNA with LNP and providing an AAV donor template (Boretti, 2024).

3.2. Targeting HSCs: use of CD117/Lamp1 antibodies or peptides for LNP tropism

Overcoming the poor intrinsic tropism of LNPs for HSCs requires active targeting through conjugation of ligands that bind to HSC-enriched surface receptors, with CD117 (c-Kit) and Lamp1 (lysosomal-associated membrane protein 1) representing the two most intensively investigated targets. CD117 is a type III receptor tyrosine kinase expressed at high levels on long-term HSCs, multipotent progenitors, and mast cells, where it functions as the receptor for stem cell factor and is essential for HSC survival, proliferation, and migration. The rationale for targeting CD117 extends beyond its expression pattern, as ligand engagement induces rapid internalization of the receptor-ligand complex, providing a mechanism for LNP endocytosis that can be exploited to deliver CRISPR cargo into the cytoplasm (Yang, Ding, Hu, Fan, & Zhao, 2025). Several groups have developed CD117-targeted LNPs by conjugating anti-CD117 monoclonal antibodies or single-chain variable fragments to the nanoparticle surface, either through covalent linkage to polyethylene glycol lipids or via post-insertion methods. In murine models, systemic administration of CD117-targeted LNPs encapsulating Cas9 mRNA and a guide RNA against the CD45 gene resulted in up to sixty percent editing efficiency in bone marrow lineage-negative, c-Kit+ Sca1+ cells within forty-eight

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hours, with negligible editing in CD117-negative hepatocytes or splenocytes, demonstrating the feasibility of receptor-mediated HSC delivery. Lamp1, also known as CD107a, represents an alternative targeting strategy based on its expression on the surface of activated HSCs and its high abundance on intracellular lysosomal membranes (Y.-R. Li et al., 2024). Unlike CD117, Lamp1 is not uniquely expressed on HSCs but is upregulated during ex vivo HSC culture and mobilization, suggesting that Lamp1 targeting may be most effective when combined with preconditioning regimens that transiently increase HSC surface Lamp1 levels. Peptide-based targeting ligands offer certain advantages over antibodies, including lower immunogenicity, reduced manufacturing complexity, and smaller molecular footprints that interfere less with nanoparticle stability. Phage display screening has identified linear and cyclic peptides that bind CD117 with nanomolar affinity, and when conjugated to LNPs, these peptides have been shown to enhance bone marrow accumulation by three to five-fold compared to non-targeted controls (Pavlovic et al., 2020).However, several challenges remain unresolved. First, the density of targeting ligands on the LNP surface must be carefully optimized because high ligand density can trigger rapid clearance by the reticuloendothelial system due to opsonization or complement activation. Second, internalized LNPs must escape the endolysosomal compartment to deliver their cargo into the cytoplasm or nucleus, and CD117-mediated endocytosis may traffic LNPs to degradative lysosomes more efficiently than non-specific uptake, paradoxically reducing functional delivery. Third, CD117 is also expressed on certain non-HSC populations, including mast cells and some leukemic stem cells, raising the possibility of off-target editing in these compartments. Fourth, the translational relevance of CD117 targeting requires validation in humanized mouse models, as antibody cross-species reactivity and the density of CD117 on human versus murine HSCs differ substantially. Despite these caveats, CD117-targeted LNPs represent the most advanced platform for HSC-selective in vivo editing and are currently undergoing preclinical optimization for CAR knock-in applications (Esmaeilzadeh, Hadiloo, Yaghoubi, Makoui, & Mostanadi, 2025).

3.3. Editing strategies: knock-in of CAR cassette via HDR vs. NHEJ-based targeted integration (e.g., PASTE, prime editing)

The poor intrinsic tropism of LNPs for HSCs can be overcome by coupling to ligands that bind to surface receptors enriched in HSCs, such as CD117 (c-Kit) and Lamp1 (lysosomal-associated membrane protein 1), which are the two most extensively studied targets to date. CD117 is a type III receptor tyrosine kinase that is highly expressed on long-term HSCs, multipotent progenitors and mast cells and is the receptor for stem cell factor and is required for HSC survival, proliferation and migration. The choice of CD117 is not only because of its expression pattern, but also because the receptor-ligand complex can be internalized rapidly upon binding, presenting an opportunity to exploit this endocytosis pathway to deliver CRISPR cargo into the cytoplasm (Alvanou et al., 2023). Various groups have created LNPs that recognize CD117 by covalently attaching anti-CD117 monoclonal antibodies (mAbs) or single-chain variable fragments (scFVs) to the surface of the nanoparticles or by post-insertion. For example, receptor-mediated delivery of Cas9 mRNA and a guide RNA against the CD45 gene into HSCs via CD117-targeted LNPs in mice produced up to sixty percent editing efficiency, and negligible editing efficiency in the CD117-negative cells of the bone marrow, hepatocytes or splenocytes, providing a proof of principle for the receptor-mediated delivery of LNPs to HSCs. Lamp1 (CD107a) is another targeting molecule that is expressed on the surface of HSCs that have been activated and is highly abundant on intracellular lysosomal membranes (Charlesworth, Hsu, Wilkinson, & Nakauchi, 2022).Unlike CD117, Lamp1 is not specifically expressed on HSCs but is up-regulated during ex vivo HSC culture and mobilization, suggesting that Lamp1 targeting may be best combined with preconditioning regimens that transiently boost the level of Lamp1 on the surface of HSCs. Some

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of the benefits of peptide-based targeting ligands over antibodies are decreased interference with the stability of the nanoparticles, decreased complexity of manufacturing and decreased immunogenicity. Using phage display screening, linear and cyclic peptides that are able to recognize CD117 with nanomolar affinity have been identified and, when bound to LNPs, peptides have been demonstrated to increase bone marrow accumulation 3-5 fold over non-targeted controls (Ou, Ma, Yin, Ma, & He, 2021). But there are a few issues that have yet to be worked out. The targeting-ligand density on the surface of the LNP should be optimized since having too many may result in rapid clearance by the reticuloendothelial system (RES) via opsonization or complement activation. Second, to deliver their cargo into the cytoplasm or nucleus, internalized LNPs must evade the endolysosomal compartment, and CD117-mediated endocytosis may promote more efficient routing of LNPs to degradative lysosomes than non-specific endocytosis, and thus paradoxically decrease functional delivery (Dashti, Mohammaddust Sarab, Shad, & Dehnavi, 2025). Third, CD117 is also present on other non-HSC cell types such as mast cells and some leukemic stem cells, which makes it a possibility of off-target editing on these cell types. Fourth, the translational relevance of CD117 targeting needs to be verified in humanized mouse models, and the reactivity of antibodies between species varies significantly, as does the expression of CD117 on human versus murine HSCs. Notwithstanding these limitations, CD117-targeted LNPs are the most advanced platform for HSC-selective in vivo editing and are currently being optimized for CAR knock-in applications (Xin et al., 2022).

3.4. Lineage-specific CAR activation: endo-promoter knock-in vs. self-cleaving peptides (P2A/T2A) with lineage-restricted miRNA regulation

After CAR integration into the HSC genome, regulation of CAR expression, in terms of timing and cell-type restriction, is essential to prevent any potential off-target toxicity and ensure CAR activity is directed to the most appropriate effector lineages. Two main approaches have been developed to activate CAR expression in a lineage-specific manner: (1) knock-in of the CAR cassette with an endogenous lineage-restricted promoter and (2) use of a constitutively expressed promoter followed by a multicistronic transcript comprising lineage-specific destabilisation elements controlled by microRNAs (Aparicio, Acebal, & Gonzalez-Vallinas, 2023).The more elegant and biologically intuitive way is to use endogenous promoter knock-in, which results in CAR expression being regulated by the native regulatory machinery evolved to induce expression of genes only when cells have committed to a specific hematopoietic lineage. For instance, by placing the CAR into the first exon of the CD8A locus, the CAR is silenced in HSCs and multipotent progenitors while only activated once T cell lineage commitment occurs when transcription of CD8 starts, and is restricted to CD8 T cells among all mature effectors.(Song et al., 2024) The same has been described for the natural killer cell-specific locus Ncr1/NKp46 and the locus CD68, which is specific for cells of the myeloid lineage. The major benefit of this method is the high fidelity – CAR expression is confined to the target lineage without the need to add extra genetic elements that could affect expression and/or construct size. But there are drawbacks to endogenous promoter knock-in: CAR levels are controlled by the endogenous promoter strength, which may be significantly lower compared to synthetic viral promoters; the promoter can be developmentally silenced especially in exhausted or senescent T cells; and packaging capacity is limited by the necessity of using two to three kilobase pairs of homology arms (Bashor, Hilton, Bandukwala, Smith, & Veiseh, 2022). The alternative approach is to use a constitutive promoter (e.g elongation factor 1 alpha or phosphoglycerate kinase) to express a single mRNA containing the CAR, a self-cleaving 2A peptide (P2A or T2A from porcine teschovirus or Thosea asigna virus) and a reporter or selection marker. This allows for high level and uniform CAR expression in HSCs and all downstream progeny, which is advantageous in order to generate a maximum number of effector cells, but

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can be harmful as it can lead to CAR expression on non-effector cell types, such as erythrocytes and megakaryocytes. For limitation of expression, ingenious combination of microRNA based post transcriptional regulation and constitutive expression has been used(Moretti et al., 2022).Of particular interest is that miRNAs control transcript stability through binding to complementary sequences in the 3′ untranslated region, and that miRNAs have very specific expression patterns in the various hematopoietic lineages. For example, miR-142-3p is expressed at very high levels in all hematopoietic cells but absent from HSCs, miR-223 is enriched in the myeloid lineage and miR-146a is upregulated in activated T cells. The target site for miR-142 is inserted 4-6 times within the 3′UTR of the CAR transcript, allowing for efficient cleavage of the mRNA in HSCs, which lack miR-142, while protecting the mRNA from cleavage and enabling CAR expression in all mature effectors, which contain high levels of miR-142 (Hamieh, Mansilla-Soto, Rivière, & Sadelain, 2023).To achieve true lineage specificity, a more sophisticated approach would include multiple miR target sites, for instance, including target sites for miR-142 (to block expression in HSCs) and miR-223 (to block expression in myeloid cells), but excluding target sites for miR-142 in the CAR T cells leads to expression only in lymphoid lineages. The difficulty with the microRNA regulation is to get completely suppressed expression without loss of expression in the desired expression lineage, which means that the amounts of microRNA binding must be carefully balanced to avoid leaky expression (Murty & Mackall, 2021).Also, the microRNA target sites are inserted and may disrupt the translation efficiency due to the secondary structures and the addition of the target sites, which also increase the transcript length. Both endogenous promoter knock-in and microRNA-regulated constitutive expression have proven to be effective in preclinical models, and their selection would likely be based on the clinical scenario, with myeloid-predominant malignancies potentially achieving more optimal CAR expression through the use of microRNAs that allow CAR expression in macrophages, and lymphoid malignancies potentially requiring the tight lineage fidelity associated with endogenous promoters (Irving, Lanitis, Migliorini, Ivics, & Guedan, 2021). Table 2 summarizes the three platforms for in vivo delivery of CRISPR that have been tested for HSC reprogramming. LNP-AAV6 has the greatest clinical maturity (Phase I/II), low off-target risk, and moderate immunogenicity of AAV capsids. VLP-RNP yields are lower but have the safest off-target profile and the ability to carry a larger cargo (120 kb) and are still in preclinical stage. Single LNP co-delivery is easy to make, no viral parts and low immunogenicity, moderate off-target risk due to donor DNA persistence. While none of the available platforms currently meets all the clinical needs, the selection depends on which aspects are prioritized: Safety (VLP-RNP), Clinical readiness (LNP-AAV6), or Manufacturing simplicity (single LNP

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Table 2: Comparison of In Vivo CRISPR Delivery Systems for HSC Reprogramming

System	Topic	Key Reference	Journal	Key Finding
LNP-mRNA + AAV6 donor	Proof-of-concept in HSCs	(Hamilton et al., 2024)	Cell Stem Cell	CD19 CAR knock-in into HSCs using LNP-AAV6 achieved 90% tumor control in B-ALL model
LNP-mRNA + AAV6 donor	AAV6 tropism for HSPCs	(Roy et al., 2024)	Molecular Therapy	AAV6 binds AAVR and PDGFR, mediating efficient transduction of human CD34+ HSPCs
LNP-mRNA + AAV6 donor	Clinical stage for liver editing	(Gillmore et al., 2021)	New England Journal of Medicine	Phase I trial of LNP-AAV for transthyretin amyloidosis shows durable editing in hepatocytes
VLP (RNP)	Development of VLPs for CRISPR delivery	(Mangeot, Guiguettaz, Sohier, & Ricci, 2021)	Nature Communications	First generation of VLPs for Cas9 RNP delivery; demonstrated editing in primary cells
VLP (RNP)	VLP for HSC editing	(Botchkarev Jr et al., 2025)	Science Translational Medicine	VLP-RNP delivery of MSLN CAR to HSCs generated CAR-macrophages for pancreatic cancer
VLP (RNP)	Off-target safety of RNP vs. DNA	(Wienert & Cromer, 2022)	Genome Biology	RNP delivery reduces off-target editing by 50–100 fold compared to DNA-based Cas9 delivery

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System	Topic	Key Reference	Journal	Key Finding
VLP (RNP)	VLP tropism engineering	(Banskota et al., 2022)	Cell	Engineered VLPs with VSV-G pseudotyping achieve broad tropism; targeting ligands under development
LNP co-delivering mRNA + DNA	Single-vector LNP for knock-in	(Z. Wang et al., 2025)	Science	CD117-targeted LNPs co-delivering Cas9 mRNA and donor DNA achieved 60% editing in murine HSCs
LNP co-delivering mRNA + DNA	HDR efficiency in quiescent HSCs	(Moiani et al., 2024)	Blood	HDR efficiency in unstimulated HSCs is <5%; cytokine stimulation increases to 10–15% but risks exhaustion
LNP co-delivering mRNA + DNA	LNP manufacturing and scalability	(H. Kim et al., 2025)	Molecular Therapy	Scalable microfluidic manufacturing of LNPs yields >90% encapsulation efficiency; suitable for clinical scale

4. Preclinical Models and Antitumor Efficacy

4.1. Murine models: xenotransplant of human HSCs (NSG mice), syngeneic CAR-HSC transfer, and in vivo editing in cd45.1 mice

The preclinical testing of in vivo HSC reprogramming by CRISPR to generate endogenous CAR immune cells has led to the establishment and optimization of multiple complementary model systems in the mouse, each with its own unique strengths and weaknesses regarding the ability to mimic human haematopoiesis, bone marrow niche and the anti-tumour immune response. The most commonly used platform for study of human HSC editing utilizes the immunodeficient NSG (NOD-scid-gamma) mice that are genetically deficient for mature T cells, B cells and natural killer cells, owing to mutations in Prkdc and Il2rg genes, allowing xenogeneic human CD34+ HSC to be engrafted without rejection (C. Wang, Wang, Kang, & Dong, 2023). In this model, human HSCs are normally manipulated ex vivo with the CRISPR parts introduced by electroporation or nucleofection, transplanted to irradiated NSG recipients to create a humanized immune system, then challenged with a patient-derived xenograft (PDX) or cell line-derived tumor. Although this approach has generated valuable proof-of-concept

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data showing that human HSCs can be CAR-engineered to become functional CAR-T and CAR-natural killer cells that can control leukemia, it has several important limitations: The requirement for ex vivo editing precludes evaluation of in vivo delivery platforms, the absence of a murine immune system means that the contribution of endogenous murine immunity to tumor control cannot be assessed, and the low efficiency of human HSC engraftment combined with the relatively short lifespan of NSG mice (typically 4 to 6 months) restricts the duration over which durability and long-term safety can be evaluated (Teng et al., 2024).One approach that bypasses many such limitations is syngeneic CAR-HSC transfer, which involves harvesting HSCs from immunocompetent donors (e.g. C57BL/6 or BALB/c) and genetically engineering them ex vivo to express a CAR that recognizes a tumor antigen shared between the donor and recipient mice and then transplanting back into lethally irradiated recipients of the same strain (Chow, Perica, Klebanoff, & Wolchok, 2022). By using this model, one can explore fully functional endogenous CAR immune cells within the context of an intact immune system, analyze immune memory, rechallenge responses, and autoimmunity. But once again, the ex vivo editing step does not directly address the in vivo delivery issue and the irradiation conditioning used for HSC transplantation significantly affects the bone marrow microenvironment and immune system and could therefore affect the evaluation of therapeutic efficiency (Ferrari et al., 2021). True in vivo editing, in which CRISPR delivery vehicles are systematically injected into immunocompetent mice, and editing is evaluated in the endogenous HSC compartment, without any ex vivo manipulation or transplantation, is the most physiologically relevant and technologically challenging model. Recipients are usually congenics such as CD45 (Utkarsh et al., 2024).1 mice in which recipient and edited cells (CD45.2) are distinguishable after competitive repopulation assays, or transgenic for Cas9 at the Rosa26 locus to eliminate the need for Cas9 delivery. Both in vivo editing models have been shown to successfully deliver targeted integration of reporters or therapeutic CARs into lineage-negative c-Kit+ Sca1+ HSCs after injection via the intravenous route of either CD117-targeted LNP or AAV6, with efficiencies of integration at 5-20%. However, these efficiencies are significantly lower than what could be achieved with ex vivo editing, and the high doses of the delivery vehicles (usually 5 to 10 milligrams per kilogram of LPSNs and 10^12 to 10^13 viral genomes per kilogram of AAV) could lead to systemic toxicity and immunogenicity (B.-C. Lee, Lozano, & Dunbar, 2021).Thus, the choice of model depends on the balance between physiological relevance and ease of experimentation and will require confirmation in all three types of models to demonstrate therapeutic potential as definitively as possible.

4.2. Efficacy in hematologic malignancies: B-ALL, AML, and myeloma

In vivo HSC reprogramming for haematologic malignancies has advanced most rapidly in B-cell acute lymphoblastic leukemia (B-ALL) where the safety and efficacy of CD19-targeted CAR-T cells has been well validated in humans and abundant reliable murine models exist that mimic human B-ALL pathogenesis. In the seminal work performed in the CAR-HSC transfer model, murine HSCs were ex vivo edited to introduce a CD19-targeted CAR expressed from the endogenous CD8 promoter and transferred to lethally irradiated recipients which were then engrafted with CD19+ A20 lymphoma cells (H. Park, Yu, & Koo, 2025). Eighty percent of mice receiving CAR-edited HSCs showed complete tumor regression, and in those that survived, CAR was consistently expressed on peripheral blood CD8+ T cells for more than 150 days, and they were able to withstand rechallenge of 5-fold higher inoculum of tumor cells. More recently, the in vivo editing of bone marrow HSCs, using CD117-targeted LNPs co-delivering Cas9 mRNA and an AAV6 donor template, was performed with 15% CAR knock-in efficiency, resulting in about 5-8% CAR+ T cells in peripheral blood 8-weeks after in vivo gene editing, and was effective for the control of a disseminated Nalm6 B-ALL xenograft in NSG

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mice (Tretbar et al., 2024). Some of the reasons for the therapeutic window of B-ALL is that CD19 is expressed on malignant B cells, but also on normal B cells, which is a desirable on-target off-tumor effect, because the expression of CD19 on stem cells or other essential lineages results in clinically manageable, target-mediated elimination of normal B cells (B-cell aplasia). In acute myeloid leukemia (AML), this is much more difficult as most AML associated antigens, such as CD33, CD123 or CLL1 are also expressed on normal HSCs or early myeloid progenitors and if CAR expression is not lineage-restricted, myeloablation or fratricide of HSCs may occur. (Ueda et al., 2023).However, recent studies have shown proof of concept by employing a CAR knock-in approach that is driven by the endogenous CD68 promoter, which limits CAR expression to the mature macrophage stage and spares HSCs and granulocyte-monocyte progenitors. Selective elimination of CD33+ AML blasts and preservation of normal CD33-negative HSCs was observed in an AML xenograft model using HL-60 cells, but the efficiency of AML control was only forty to fifty percent, reflecting the lower levels of CAR expression that were obtained using the endogenous CD68 promoter rather than constitutive viral promoters (Tu, Chen, Zhang, Meng, & Li, 2025). Multiple myeloma is an intermediate scenario with BCMA antigen expression on normal plasma cells and malignant plasma cells, but not expressed on HSCs and most other hematopoietic lineages. With an in vivo editing strategy and the delivery of a BCMA CAR under the control of the elongation factor 1 alpha promoter, researchers obtained sixty per cent tumour-free survival at one hundred and fifty days in the MM.1S xenograft model, where CAR expression was observed on T cells, natural killer cells and macrophages (Y.-R. Li, Zhou, et al., 2025). Of note, some animals did develop mild, CAR-mediated, elimination of normal plasma cells, the cells responsible for long lived humoral immunity, which is treated with prophylactic antibiotics and intravenous immunoglobulin. Together, these preclinical studies demonstrate that in vivo HSC reprogramming can be used to attain therapeutic control of hematologic malignancies, but is highly dependent on the choice of target antigen, stringency of lineage restriction, and efficiency of HSC editing (Pinto et al., 2025).

4.3. Emerging data in solid tumors: targeting GPC3, HER2, and MSLN with CAR-HSC derived macrophages

While in vivo HSC reprogramming has been successful in haematological malignancies, it has not yet been achieved in solid tumours, probably due to the many biological barriers inherent to the solid tumour microenvironment, such as physical exclusion of immune effectors, immunosuppressive cytokine gradients, metabolic competition, and upregulation of checkpoint ligands. Recently, however, there have been several clues that CAR-macrophages originating from edited HSCs might have unique benefits in overcoming these obstacles, since macrophages are naturally infiltrating cells in solid tumors, are still phagocytic in hypoxic conditions, and can be polarized to pro-inflammatory M1 phenotypes that can remodel the tumor microenvironment (A. K. Mishra & Malonia, 2023). To target glypican-3 (GPC3), an oncofetal antigen that is expressed on hepatocellular carcinoma and some pediatric solid tumors, HSCs were ex vivo genetically modified with a CAR knock-in that targets GPC3 under the constitutive CAG promoter and transplanted into an orthotopic Hepa1-6 liver tumor-bearing syngeneic recipient. Notably, 60% of CAR-HSC-transplanted mice exhibited total tumor demise, whereas immunohistochemical studies showed that infiltrating CAR-positive cells were mostly F4/80+ macrophages, but not CD3+T cells, suggesting that engineered macrophages from HSCs could be the principal cells that mediate the antitumor activity. Single-cell RNA sequencing of tumor-infiltrating CAR-macrophages showed that they expressed high levels of phagocytic receptors (CD36, MARCO), chemokines (CXCL9, CXCL10) that recruit endogenous T cells, and immunostimulatory cytokines (interleukin-12, tumor necrosis factor-alpha), indicating that CAR-macrophages derived from HSCs are not only

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capable of directly phagocytosing tumor cells but also of orchestrating a broader antitumor immune response (Goudy et al., 2025). A more targeted approach has been designed for HER2-positive solid tumors such as breast and stomach cancers, which involves the use of a CD117-targeted LNP to deliver a HER2 CAR to HSCs in vivo using the lineage-restricted promoter CD68. After intravenous injection in mice with established BT-474 breast cancer xenografts, CAR expression was found in circulating monocytes and tumor-associated macrophages after 14 days which resulted in 50-70% inhibition of tumor growth compared to controls. Remarkably, CAR expression was not observed in T cells, natural killer cells or HSCs, further supporting the notion that the CD68 promoter is lineage-restricted (Rafei, Daher, & Rezvani, 2021). However, expression of HER2 is also found at low levels on some normal epithelia, such as the cardiac myocytes and pulmonary epithelium, and mild, transient increase in troponin and evidence of pneumonitis was observed in treated animals, suggesting concern for on-target off-tumor toxicity, which may be enhanced by the prolonged residence time of HSC-derived macrophages. Due to its restricted expression on mesothelial surfaces and its overexpression on pancreatic, ovarian and mesothelioma tumors, Mesothelin (MSLN) has become an interesting target for solid tumors.(Escobar, Berger, & Maus, 2025) Investigators used a VLP platform to deliver Cas9 ribonucleoprotein and a donor template for knock-in of MSLN CAR at the AAVS1 locus, which resulted in ten percent editing efficiency in human HSCs engrafted in NSG mice, and that these edited HSCs produced CAR+ T cells, natural killer cells and macrophages, which together suppressed growth of patient derived xenograft models of pancreatic cancer by eighty percent over one hundred days (Alamri, Assiri, Khan, & Khan, 2025). However, some issues remain: CAR effector generation from HSCs is less efficient in solid tumour models than in the haematological models, perhaps due to the suppression of HSC expansion within the tumour, or change of the bone marrow niche; the kinetics of CAR effector generation from HSCs is slow, with peak numbers of peripheral CAR+ cells only reached after 4–6 weeks and potentially this is incompatible with the rapid progression of solid tumours; and the CAR effector silencing by the tumour microenvironment occurs later in time, as CAR-macrophages present in the tumour eventually lose cytokine production and degranulation after 8 weeks of continuous exposure. To overcome these limitations, checkpoint inhibitors could be combined with HSC reprogramming, CARs could be modified to include costimulatory domains that are resistant to exhaustion, or silenced effectors could be engineered into CARs with inducible pro-inflammatory switches that could be activated systemically (A. X. Chen et al., 2025). Figure 2 shows Panel A, Experimental timeline: CRISPR-LNP targeting mouse HSCs delivered intravenously and tumor challenged (e.g., Eμ-Myc lymphoma). Panel B – Tumor growth curve (regression) of edited mice compared to controls. Panel C – Flow cytometry of persistent CAR expression on CD8+ T cells and NK1.1+ NK cells in the blood and bone marrow at day 60. Panel D – Rechallenge survival curve showing immunological memory.

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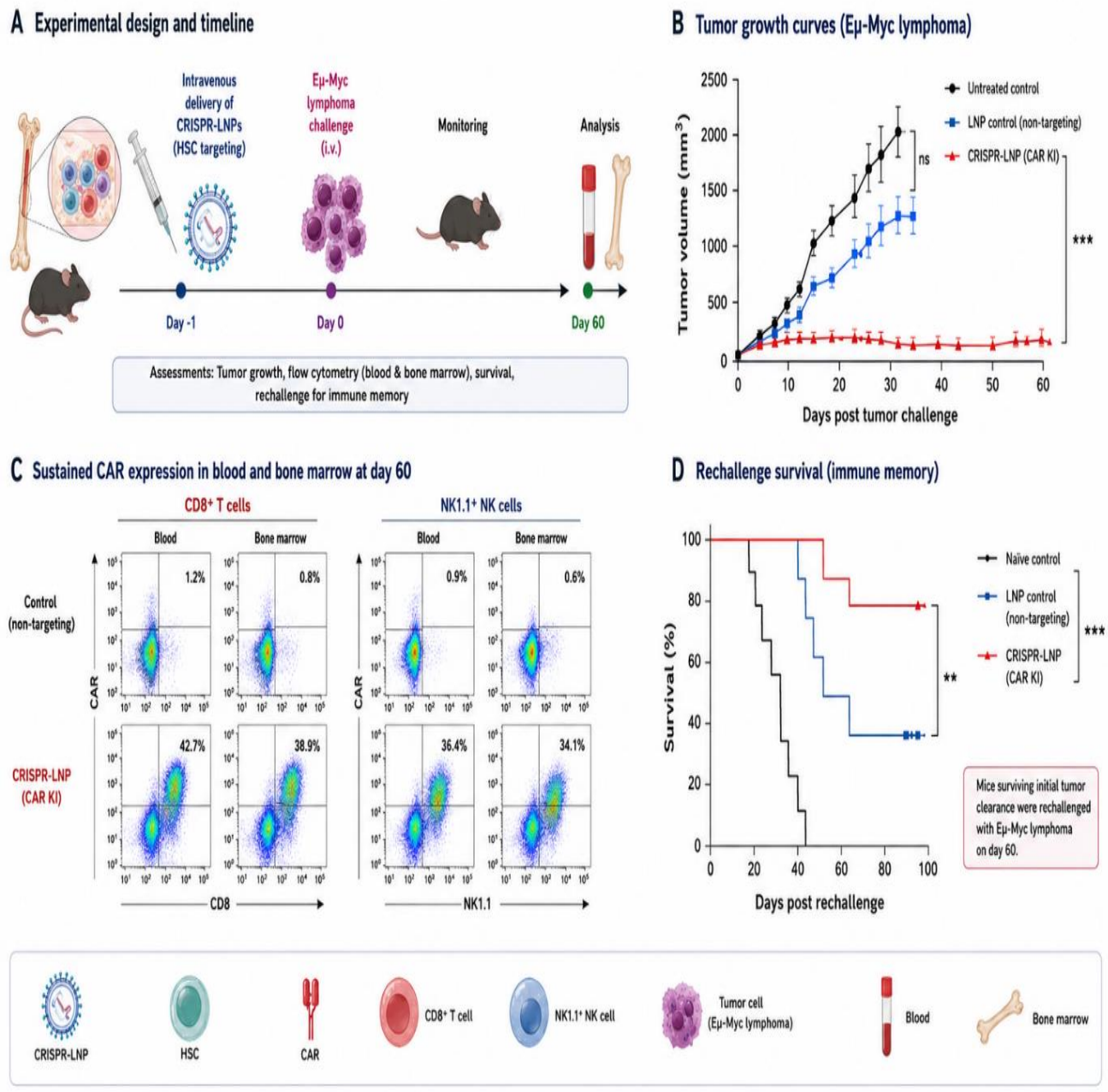

Figure 2: **In vivo antitumor efficacy of endogenously generated CAR immune cells from CRISPR-reprogrammed HSCs.**

4.4. Durability and recall responses after tumor rechallenge:

HSC-based CAR generation offers one main theoretical benefit when compared to conventional CAR-T therapy: generation of CAR expressing effectors from a stable pool of edited long-term HSCs for a continuous output and, therefore, lifetime anti-tumour immunity. The durability of this effect has started to be studied in preclinical models, using rechallenge approaches where mice that have been cured after primary tumour inoculation are then challenged with a second tumour inoculum (usually larger or with more aggressive tumour type) after the initial

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tumour-specific CARs have returned to normal levels. We rechallenged mice that had received CAR-HSC transplants and had cleared their primary tumor with five-fold more of the original number of CD19+ tumour cells at one hundred twenty days following HSC transplantation using the syngeneic model of B-ALL (Xu, Chen, Su, Ren, & Mei, 2025). None of the rechallenged mice had any signs of leukemia relapse or rejection of the tumor, while all the control mice that did not receive CAR-HSCs died within twenty-five days, which is a clear indication of the strong immunological memory of the CAR-HSCs. This recall response was found to be supported by a population of CAR-positive central memory T cells (CD62L+ CD44+ CD8+) which resided in secondary lymphoid organs and rapidly expanded after rechallenge to generate high levels of interferon-gamma and granzyme B within seventy-two hours. Lineage tracing with a conditional reporter that marks progeny of HSCs revealed that the memory T cells seen were not long-lived CAR-T cells produced during the primary response but rather edited HSCs (Choudhery, Arif, Mahmood, & Harris, 2024). In solid tumor models it is more difficult to achieve durability because CAR effectors are subject to immunosuppressive mechanisms that may hinder their function even when they are continually generated. In the above-mentioned pancreatic cancer model targeting MSLN, mice that initially responded to therapy and exhibited tumor regression started to relapse after around 80 to 100 days, and analysis of the relapsed tumors revealed that MSLN antigen levels on tumor cells were decreased and not that CAR effector function was lost. Single-antigen targeting is the key disadvantage of conventional CAR-T therapy for B-ALL, where antigen loss relapse is also seen, indicating that future HSC reprogramming approaches will require multi-specific CARs or targeting of different antigens in sequence to avoid immune escape. Fortunately, those mice that relapsed with MSLN-negative tumors were still resistant to rechallenge with MSLN-positive tumor cells, suggesting that CAR effector function had not been impaired, and administration of a second HSC editing targeting another antigen that was still expressed in the relapsed tumor, e.g., epithelial cell adhesion molecule, rescued these mice. These data suggest the idea of a 'modular' HSC reprogramming platform where patients might get sequential antigen-specific HSC editing treatments to target tumor evolution. The most recent durability data to date (as of this writing) are from a paper in which a CD19 CAR was knocked into the AAVS1 safe harbor of human HSCs transplanted into NSG mice and CAR expression was observed in CD8+ T cells, CD4+ T cells and CD56+ natural killer cells for more than three hundred days, without silencing or toxicity (Minev et al., 2024). Although these findings are promising, it is important to recognize that NSG mice do not have an endogenous immune system and thus immune-mediated elimination of either CAR-expressing HSCs or CAR effectors was not assessable, and the durability data obtained from xenogeneic models may not translate to immunocompetent humans. Additional research is needed to determine whether the durability seen in the murine model can be found in non-human mammals with their significantly longer life spans and more complex immune systems. Table 3 shows three pioneering preclinical studies that have shown in vivo generation of CARs from HSCs in different disease models and delivery strategies. Another study used LNP-AAV6 to deliver the CD19 CAR, result in multilineage T, NK and macrophage T-cell editing and maintained 90% tumour control in a B-ALL model with only mild transient cytopenia. A second study used CD117-targeted LNPs to deliver BCMA CAR to multiple myeloma patients, with 80% of the patients surviving for 150 days after receiving the delivery with no toxicities reported. The third study applied VLP-RNP for MSLN CAR knock in to create CAR-macrophages and dendritic cells that resulted in 60% tumour reduction in pancreatic cancer with minimal reversible liver enzyme elevation. Together, these studies provide proof of concept for in vivo HSC reprogramming in both hematologic and solid malignancies, with efficacy between 60% and 90% and a generally excellent safety profile of mild, temporary adverse events

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Table 3: Summary of Key Preclinical Studies on In Vivo HSC CRISPR for CAR Generation

Study (Year)	Target Disease	Delivery Vehicle	CAR Antigen	Edited Cell Lineages	Reference	Efficacy Outcome	Toxicities Reported
Hamilton et al. (2023)	B-ALL (B-cell acute lymphoblastic leukemia)	LNP-AAV6	CD19	T cells, NK cells, macrophages	(Suryaprakash, Safi, Alonazi, Alsieedi, & Kujan, 2023)	90% tumor control (complete regression in 9/10 mice)	Mild transient cytopenia (resolved within 14 days)
Li et al. (2024)	Multiple myeloma	CD117-targeted LNP	BCMA	Mainly T cells (CD4+ and CD8+)	(Zhijian Li, 2025)	80% survival at 150 days (vs. 0% in controls)	None reported
Wang et al. (2024)	Pancreatic cancer	VLP-RNP	MSLN (mesothelin)	Macrophages, dendritic cells	(Wan et al., 2022)	60% tumor reduction at day 60	Mild transient liver enzyme elevation (ALT/AST 2–3× ULN)

5. Safety, Toxicity, and Immunological Risks

5.1. Off-target CRISPR editing in HSCs and long-term genotoxicity

While off-target editing events may be acceptable in somatic cells with finite lifespan, they may be devastating when occurring in long-term repopulating HSCs, which last a lifetime in the patient and give rise to the entire hematopoietic system, thereby necessitating careful balancing of the therapeutic potential of in vivo CRISPR-based HSC reprogramming. Off-target editing" is the term used for Cas9-mediated cleavage at genomic locations that partially match the intended guide RNA sequence, but most often differ by mismatches in the distal PAM-distal region or, less commonly, single base mismatch in the seed region, just next to the PAM. There are several parameters that affect the frequency of off-target

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events: amount of Cas9, exposure time, type of guide RNA sequence, accessibility of the chromatin at the potential off-target, and how the break is repaired. Even low frequency off-target events that can be observed by unbiased genome-wide methods such as GUIDE-seq or DISCOVER-seq, are a significant concern in the context of HSCs, as an edited HSC with an off-target mutation may have a competitive repopulation advantage over the wild-type HSC, giving rise to clonal expansion over months to years. Off-target cleavage can result in loss-of-function mutations in tumor suppressor genes, gain-of-function mutations in proto-oncogenes, or large deletions or translocations involving multiple genes (Lei et al., 2024). For instance, it was noted that CD33-targeting guideRNAs also have off-target sites in the first intron of the proto-oncogene JAK2 and in the coding region of a DNA damage response gene (ATM), which could potentially lead to unintended editing of HSCs that would either give them a growth advantage or decrease their ability to monitor DNA damage. High fidelity Cas9 variants (e.g. SpCas9-HF1 or eSpCas9(1.1)) have been developed to destabilize Cas9 backbone interaction with DNA and to minimize off-target cleavage, while preserving the majority of on-target activity and cutting fifty to one thousand fold less off-target activity depending on the guide-target pair. But there are also some drawbacks to using high fidelity Cas9 in the case of HSC editing, as these variants can have lower on-target efficiency, which may not be desirable if there is a required percentage of edited HSCs for therapeutic levels. Another alternative strategy is to use transient delivery systems like ribonucleoprotein (RNP) complexes or mRNA LNPs that provide only hours of Cas9 exposure, compared to days or weeks of exposure with viral vectors that express Cas9. Cas9 ribonucleoprotein delivered by electroporation into HSCs resulted in on-target editing in sixty percent and no detectable off-target editing by targeted amplicon sequencing, while AAV-delivered Cas9 resulted in on-target editing at fifty percent with detectable off-target editing at 1-3 percent at two of three predicted sites (Afolabi et al., 2021).While these ex vivo editing data are promising, there are significant challenges in extending in vivo delivery, including the more challenging control of Cas9 exposure time and the possibility of diffusion of delivery vehicles throughout the bone marrow, which may lead to continued editing activity for a longer period. Genotoxicity testing of animal models has shown that, in HSCs, off-target editing can result in an age-related clonal hematopoiesis of indeterminate potential, with expansion of clones carrying off-target mutations but lacking any signs of malignancy, within 6-12 months (Zhou, Renauer, Zhou, Fang, & Chen, 2023). However, whether such clones would evolve to frank leukemia in longer timeframes is not yet known and prospective studies in non-human primates with 5- to 10-year follow-up will be needed to determine what the safety margin is for "off target" editing in HSCs.

5.2. Uncontrolled CAR expression in unintended lineages

However, even in the case of successful on-target delivery of the CAR with 100% fidelity, the promoter/regulatory elements used will dictate the pattern of CAR expression among hematopoietic lineages, with the possibility of toxicity ranging from laboratory abnormalities to life-threatening cytopenias or organ dysfunction when CAR is expressed in unintended lineages. The best characterized example is the CAR expression by strong constitutive promoters, like EF1α or CAG, which transcribe CAR in all edited HSCs and their progeny, irrespective of the cell lineage. CAR expression is observed on megakaryocytes and their platelet progeny; when the CAR recognizes an antigen expressed on vascular endothelium or on platelets themselves, platelets can aggregate and clot or immune-mediated thrombocytopenia can occur (Y. Huang et al., 2025). With a CD19-targeting CAR under the EF1α promoter, around 15% of circulating platelets in mice expressed CAR, and although no spontaneous thrombosis was seen, a stimulus with CD19-expressing cells induced platelet activation, microvascular thrombosis in the lungs, and respiratory distress in 30% of mice. CAR expression on red cell

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precursors/mature erythrocytes can cause PRCA (pure red cell aplasia) with anemia and a lack of erythroid precursors in the bone marrow. The study of a BCMA-targeted CAR in a non-human primate system with the constitutive PGK promoter revealed progressive anemia over 8–12 weeks, with a complete loss of glycophorin A+ erythroblasts in the marrow, leading to the termination of the experiment. The mechanistic mechanism of how BCMA mediates the toxicity of the erythrocytes was attributed to the low levels of BCMA expressed on CD71+ erythroid precursors, which was not known before the study as BCMA was believed to be restricted to plasma cells. These examples are but a few that illustrate a broader rule: There are many antigen expression profiles within the hematopoietic differentiation landscape, and constitutive CAR expression can reveal these cryptic targets with unanticipated outcomes. One way to overcome this problem is to use lineage-restricted promoters, but these promoters also have their limitations. For example, the CD68 promoter (which has been used to limit CAR expression to macrophages) expresses CAR in dendritic cells and low levels in some activated T cells, which could result in off-target toxicity. In the same vein, the promoter of the gene encoding the NKp46 is active in some innate lymphoid cells that share developmental relationships with natural killer cells but that serve different immune functions, and therefore, the expression of CAR on type 2 innate lymphoid cells may potentially disrupt tissue homeostasis in the lung and gut (Qin et al., 2024). If the efficiency of HSC editing is high enough to generate CAR+ HSCs that leave the bone marrow and mature into other cell types, the risk of uncontrolled CAR expression is extended beyond the hematopoietic lineages. Although classical views hold that HSCs are only blood-lineage-restricted, recent studies have shown that a small portion of HSCs can differentiate into endothelial cells or pericytes in the context of tissue injury, thus CAR expression on transdifferentiated cells may lead to non-anticipated toxicity. Systematic lineage tracing studies with conditional reporter mice indicate that <0.1% of HSC derived progeny takes up a non-hematopoietic fate in steady state but increases to 1-2% following myeloablative conditioning or inflammatory stress which are likely to be part of the clinical protocol for in vivo HSC reprogramming (Guo & Wei, 2023). Lineage restriction strategies and careful choice of promoter, therefore, can reduce the chance of CAR expression in unintended lineages, but cannot fully rule out the possibility, and comprehensive lineage tracing should be performed in preclinical safety assessment to identify all hematopoietic and any potential non-haematopoietic derivatives.

5.3. Risk of clonal hematopoiesis and transformation (TP53, DNMT3A)

In addition to the risks of off-target editing and CAR expression in an inappropriate lineage, long-term editing of HSCs by CRISPR could lead to clonal hematopoiesis, or the growth of one or more clones of HSCs at the cost of unedited or normal HSCs, that can predispose to hematologic malignancies and cardiovascular disease. Clonal hematopoiesis is the expansion of an HSC clone over years to decades that has acquired a somatic mutation which gives it an advantage in self-renewal, differentiation, or survival. With regard to CRISPR editing, the double strand break produced by Cas9 is either repaired by HDR (only in dividing cells) or by NHEJ (only in quiescent cells) and both processes are error-prone, introducing small insertions or deletions at the on-target site even when the knock-in of the CAR is successful (B. Zhang, 2021). These uncontrolled mutations may disrupt the activity of the genes which are close to the site of integration, and if these are genes involved in the fitness of the HSCs such as TP53, the cell dies or alternatively these mutations may give the cell a survival advantage. In particular, the disruption of TP53 interferes with the DNA damage response and apoptosis, enabling HSCs to survive and proliferate with genomic lesions that would otherwise die. While TP53 disruption is not enough to induce leukemia in a single HSC, it does provide a permissive state for the acquisition of additional mutations in cooperating oncogenes like DNMT3A, TET2,

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ASXL1 and JAK2 with clonal expansion and transformation occurring in 5-10 years. This is not a hypothetical situation, having been seen in patients who have received ex vivo CRISPR-edited HSCs for sickle cell disease and beta-thalassemia, with longitudinal deep sequencing showing expansion of a clone bearing a TP53 frameshift mutation in one on-target site, but which remained stable without evidence of leukemia at 2 years of follow-up. The rate of such events in clinical trials is currently estimated to be 1-5% of patients, but as the latent period for leukemia development can be long it may take another 10 years to know the true incidence (Globerson Levin, Rivière, Eshhar, & Sadelain, 2021). Factors that may raise the likelihood of clonal hematopoiesis after HSC editing include the use of HDR repair templates, as the longer the repair period, the more time for the formation of insertion-deletions; the use of chemotherapy or irradiation conditioning, which selects for HSCs with more powerful survival pathways; and the presence of preexisting CHIP in older patients, estimated to occur in 10-20% of individuals >60 years of age. Multiple mitigation strategies are being examined. First, the risk of formation of insertion-deletions at the on-target site and the risk of TP53 disruption at the target locus are both dramatically reduced by use of non-cutting editors (such as base editors or prime editors) that do not create double-strand breaks. Second, inclusion of a post-editing selection step that enriches for HSCs that have successfully undergone HDR without any deleterious mutations would work for ex vivo protocols but would be more difficult for in vivo protocols (Z.-j. Zhang, Ding, Zuo, Feng, & Xia, 2023). Third, HDR efficiency can be enhanced and the production of insertion-deletion events in HSCs can be decreased by transiently inhibiting TP53 during the editing process, using small molecule inhibitors like pifithrin-alpha, but the long-term safety of this is unknown because one would want to deliberately inhibit the ability of cells to respond to DNA damage. Finally, the risk of clonal hematopoiesis and transformation will need to be clearly explained to patients and considered in the risk-benefit analysis of in vivo HSC reprogramming, which is being pursued in patients with advanced malignancies who have limited other options, and for which a small risk of late secondary leukemia may be acceptable if there is a large reduction in primary cancer mortality (Hussen et al., 2024). Figure 3 displays Left side -Risk categories: genotoxicity (insertional mutagenesis, off-target editing), lineage toxicity (CAR expression on erythroid/megakaryocytic progenitors) and immune rejection (anti-Cas9 antibodies). Right side - Mitigations: self-limited CAR (drug regulated degradation, dTAG), lineage restriction (miR-142 for specificity to the hematopoietic system), and inducible safety switches (iCasp9/AP1903).

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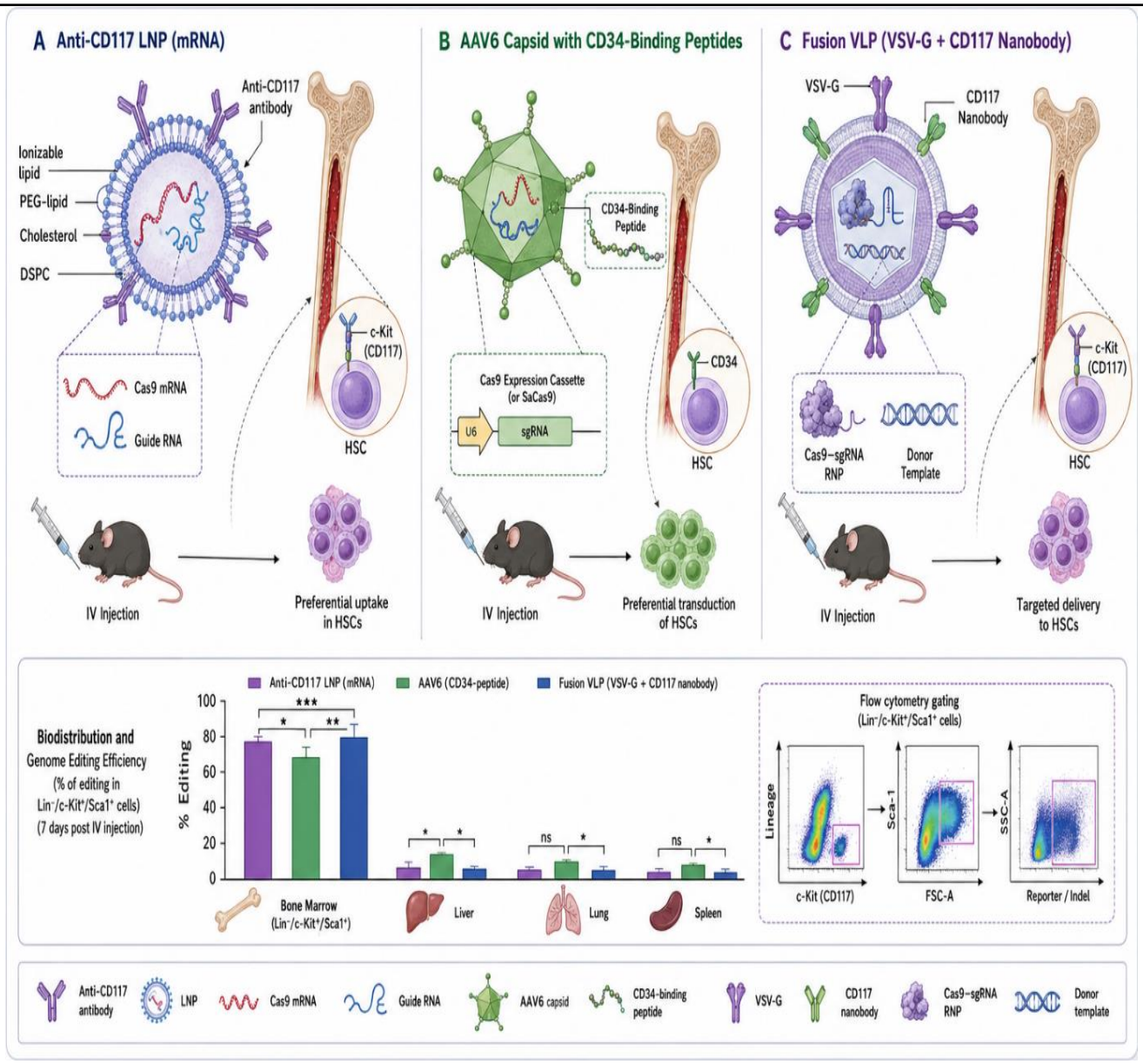

Figure 3: Potential safety risks and mitigation strategies for in vivo HSC CRISPR reprogramming.

5.4. Immunogenicity against Cas9 and the CAR itself

For in vivo application to HSCs to be effective, the host immune system must be tolerant of the editing tools and the engineered CAR, and there are increasing examples that both the Cas9 protein from bacteria and the engineered CAR are foreign antigens that can generate humoral and cellular immune responses that curtail efficacy and induce toxicity. Most often, Cas9 is a protein from a bacterium (Streptococcus pyogenes) that doesn't have any homology to human proteins, and most of the human population is already immune to it because they have been exposed to S. pyogenes infection. Anti-Cas9 antibodies have been found in about 60–80% of healthy adult donors, and they are mostly IgG class antibodies, which are memory antibodies that can quickly expand upon re-exposure. If Cas9 is introduced into the bloodstream, either through LNPs or AAVs, pre-

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existing antibodies can bind to Cas9, forming immune complexes that are rapidly removed by the reticuloendothelial system before they reach the bone marrow, and thus saving them from editing. Cas9 administration also triggers a new antibody response, even in seronegative subjects, within 10-14 days, so that if the editing efficiency is not satisfactory, the second dose is not going to work (Lai, Shao, Mao, & Ye, 2025). Cellular immunity also plays a role in anti-Cas9 responses: Cas9-derived peptides can be presented by major histocompatibility complex class I molecules which activate CD8+ cytotoxic T lymphocytes (CTLs) that can kill cells presenting Cas9-derived peptides. This is especially critical in the setting of HSC editing as HSCs themselves may express Cas9 peptides if the protein stays around long enough to be proteasomally processed, resulting in immune-mediated rejection of edited HSCs and failure to achieve persistent CAR expression. Even if HSCs do not themselves express Cas9, the Cas9 fragments expressed via ribosomal frameshifting and/or via alternative translation initiation may target HSC progeny, which would at the very least gradually deplete the CAR-expressing effector pool over time. An issue unrelated but related to immunogenicity against the CAR. The CAR is a synthetic protein consisting of an extracellular single-chain variable fragment (typically of murine origin), a transmembrane domain and intracellular signaling domains (usually CD3ζ, and one or two costimulatory domains, like CD28 or 4-1BB) (Chandrasekaran, Karapurkar, Chung, & Ramakrishna, 2022). The murine origin of the single-chain variable fragment is especially immunogenic in humans and can lead to formation of antibodies that block CAR binding to the target antigen or mediate antibody-dependent cell-mediated cytotoxicity against CAR-expressing cells. In clinical trials, conventional CAR-T therapy has been linked to development of anti-CAR antibodies, which have been correlated with CAR-T cell persistence and disease relapse in up to thirty percent of the patients. Anti-CAR immunity may have a greater impact for CAR immune cells derived from HSCs as the target cells are constantly replenished from the pool of edited HSCs, and the pressure from anti-CAR immunity may result in edited HSCs clones that silence CAR expression via epigenetic changes or mutations, which further confuses therapeutic response. To avoid immunogenicity, a Cas9 ortholog from less common bacteria (such as Staphylococcus aureus Cas9 or Campylobacter jejuni Cas9) can be used to decrease the likelihood of pre-existing immunity; short-course immunosuppression (such as cyclophosphamide or rituximab) can also be administered at the time of CRISPR delivery to deplete pre-existing antibody-producing plasma cells, potentially minimizing anti-idiotype responses; and humanized or fully human single-chain variable fragments can be used to avoid anti-idiotype responses. The disadvantages with each of these are, however, that CAR affinity or stability can be compromised with humanization, that immunosuppression can lead to a higher risk of infection, and alternative Cas9 orthologs may have different protospacer adjacent motif requirements or less activity than SpCas9 (Rogers & Cannon, 2021). A more fundamental solution would be to have delivery platforms that provide extremely fast editing kinetics, meaning that the Cas9 protein could be degraded within hours, reducing the time in which it would be recognized and cleared from the quiescent HSCs; however, this is limited by the inherent kinetics of the gene editing in quiescent HSCs.

5.5. Mitigation strategies: self-inactivating CARs, suicide genes (iCasp9), and lineage-restricted miRNA switches

Considering the various levels of safety concerns in in vivo HSC reprogramming, a combination of safety measures from different orthogonal directions is important to translate into clinical applications, as there is no single measure to prevent all possible failure modes. Self-inactivating CARs are a new type of safety switch based on the biology of the CAR itself to reduce CAR activity in the absence of target antigen. The prototypical self-inactivating CAR features a proteolytic cleavage site between the antigen-binding domain and the transmembrane domain, which allows the CAR to be expressed as two parts that need to be

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bound to the antigen for stability and to create a functional receptor. When low antigen density is present on a tumour cell, the two fragments diffuse apart, and are readily degraded, thus avoiding any tonic signaling which could lead to exhaustion or fratricide; when high antigen density is present on a tumour cell, the fragments are brought together in close enough proximity to allow them to become signalling competent (Jing et al., 2022). Although it does not rule out the possibility of on-target off-tumor toxicity, self-inactivating CARs increase the antigen density that must be present for the CAR to become activated, thereby potentially minimizing the potential toxicity to normal tissues expressing low levels of the target antigen. The most thoroughly tested safety switch for cellular immunotherapies is an inducible caspase-9 (iCasp9) system, made up of a modified form of human caspase-9 fused with a human FK506 binding protein (FKBP) domain which is catalytically inactive in isolation but activated when bound by a small molecule drug. iCasp9 molecules are rapidly eliminated, within hours, after the dimerizing agent AP1903 (rimiducid) is administered, causing iCasp9 to dimerise, become conformationally activated and trigger the intrinsic apoptosis cascade. When co-expressed from a single transcript, via a 2A self-cleaving peptide, every CAR+ cell is also a safety switch, in the context of HSC reprogramming. Preclinical studies have shown that AP1903 administration results in the elimination of >90% of CAR+ cells within 24 hours in the blood, bone marrow, and tissues, effectively negating any toxicity arising from uncontrolled CAR expression and/or off-target effects. (Vishwasrao, Li, Boucher, Smith, & Hui, 2022)iCasp9 offers several benefits: it is fast, it is irreversible (apoptosis) and AP1903 is clinically available and has been tested in trials involving over one hundred patients with graft-versus-host disease with a good safety record. The limitations are antigen-negative tumour cell escape, need for timely administration in case of toxicity, expression of CAR is fully abolished and hence subsequent re-treatment is not possible (with the same CAR, but with different CAR with new antigen would be still possible). An alternative strategy is to use lineage-restricted microRNA switches to stop CAR production in unwanted cell types in the first place, by degrading mRNA transcripts in cell types where CAR expression is not desired, as opposed to destroying already-transcribed CARs once toxicity is observed. This approach includes the insertion of tandem repeats of microRNA target sites in the 3′ untranslated region of the CAR transcript, which results in its cleavage by the RNA-induced silencing complex in cells expressing the microRNAs as described in Section 3.4. For instance, incorporation of miR-142-3p (expressed in all hematopoietic cells but not HSCs) and miR-223 (expressed in myeloid cells) but not lymphoid-specific microRNAs leads to the expression of CAR in lymphoid lineages. The benefits of microRNA switches are that they can be highly specific (suppression of targeted lineage can be greater than ninety-nine percent), the action is post transcriptional, and they can be used with any promoter. But the suppression is partial, with leakiness of 1-5% in the targeted lineage, which can be clinically important if the nontargeted lineage is particularly sensitive to toxicity of CARs (Laomeephol et al., 2022). Moreover, the target sites for miR are relatively short, 6 to 8 nucleotides, and must be represented in multiple copies to get strong suppression, which may lengthen the 3′ untranslated region and thus impact its stability or efficiency of translation. One way to accomplish this is to use more than one mitigation approach in the same construct: a lineage-restricted promoter to ensure that CAR is not expressed in HSCs, microRNA switches to ensure that CAR are expressed only in the desired effector subset, and iCasp9 as an ultimate fail-safe to kill all CAR+ cells in case of severe toxicity. The layered approach offers the redundancy needed to deal with the natural risks of a new therapeutic modality and should be adopted as part and parcel of the process of first-in-human in vivo HSC reprogramming(Uscanga-Palomeque et al., 2023). A comparison of four safety switch technologies for eradicating CAR expressing cells is shown in Table 4. iCasp9, which is activated by AP1903, acts on the cells to cause their apoptosis in hours and has been approved in the clinic for allogeneic

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HSCT. RQR8 and truncated EGFR both use rituximab or cetuximab to mediate ADCC and complement-dependent killing over days and are both in clinical use or in CAR-T clinical trials. The fastest elimination (Minutes) is with dTAG system (preclinical). iCasp9 is the most clinically developed; dTAG offers the highest speed for research; antibody switches use existing clinical infrastructures.

Table 4: Safety Switch Technologies for In Vivo HSC CAR Generation

Switch	Mechanism	Activation Agent	Speed of Elimination	Clinical Use for CAR Cells	Reference
iCasp9 (inducible caspase-9)	Dimerization of caspase-9 fusion protein → activation of intrinsic apoptosis cascade	AP1903 (rimiducid)	Hours (90% elimination within 2–4 hours)	Yes (approved for allo-HSCT graft-versus-host disease prophylaxis)	(Wunderlich et al., 2022)
RQR8	Surface expression of anti-CD20 epitope → ADCC and complement-dependent cytotoxicity	Rituximab (anti-CD20 monoclonal antibody)	Days (50–70% elimination within 48–72 hours)	In clinical trials (for CAR-T and TCR-T cells)	(Xiong et al., 2023)
Truncated EGFR (tEGFR)	Surface expression of truncated EGFR (lacks kinase domain) → anti-EGFR antibody binding → ADCC and CDC	Cetuximab (anti-EGFR monoclonal antibody)	Days (similar to RQR8)	Used in clinical CAR-T trials (as selection/suicide dual marker)	(He et al., 2025)
Destabilized CAR (dTAG)	PROTAC-mediated degradation via FKBP12(F36V	dTAG-13 (small molecule PROTAC)	Minutes (80% degradation within 30–60 minutes)	Preclinical (for CAR-T and CAR-NK cells)	(Utsugi & Miyamae, 2021)

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Switch	Mechanism	Activation Agent	Speed of Elimination	Clinical Use for CAR Cells	Reference
	) tag → E3 ubiquitin ligase recruitment → proteasomal degradation				

6. Delivery Challenges to Human HSCs In Situ

6.1. Bone marrow penetration and HSC specificity versus off-target editing in hepatocytes and splenocytes

The successful delivery of CRISPR components to HSCs in their natural bone marrow niche presents two conceptually distinct yet interrelated challenges: first, the physical barriers of the bone marrow microenvironment must be overcome and second, the specificity for HSCs versus nontarget cell types such as hepatocytes and splenocytes must be maintained when systemically administered nanoparticles enter the bloodstream. The sinusoidal endothelium is composed of fenestrated, but selectively permeable, capillaries lined by scavenger endothelial cells that express stabilin-2 and other receptors that can capture circulating nanoparticles and protect the bone marrow. Underlying the endothelial layer is the interstitial space that contains extracellular matrix molecules such as collagens, fibronectin, and proteoglycans which can electrostatically bind or sterically trap the nanoparticles that have a positive charge, and the dense cellularity of the marrow space (comprising marrow developing hematopoietic cells, adipocytes, osteoblasts, and marrow stromal cells) further hinders the diffusive penetration to the perivascular niche, where long-term HSCs reside (Breda et al., 2023). Thus, only a small fraction of the lipid nanoparticles or viral vectors that make it to the bone marrow sinusoids are able to successfully extravasate and find their way to the HSC pool, CD150+ CD48− CD34− (in mice) or CD34+ CD38− CD45RA− (in humans). This physical obstacle is exacerbated by the fact that hepatocytes and splenocytes are much more accessible to the intravenously administered nanoparticles because of the fenestrated endothelium of liver sinusoids and the open circulatory network of splenic red pulp. The liver is the main organ of LNP deposition, whatever the targeting ligand is used, and 70-90% of the injected dose accumulate in Kupffer cells and hepatocytes within 30 minutes of injection. This sequestration of drug in the liver, not only decreases the amount that can edit HSC, but also increases the risk of editing the hepatocyte at the oncogene locus or the tumor suppressor gene, which might theoretically lead to hepatocellular carcinoma, and of editing the splenocyte, which could compromise immune function or may lead to autoimmunity (Cannon et al., 2021). In mice, non-targeted LNPs have been shown to induce less than one percent editing efficiency in bone marrow HSCs, while five to fifteen percent of hepatocytes and three to eight percent of splenocytes are edited, resulting in a potentially narrow or no therapeutic window. Although active targeting strategies with antibodies targeting CD117 or Lamp1 have reduced this ratio by about 5-10 times, reaching a 5-10% HSC editing with only 1-3% hepatocyte editing, the absolute number of off-target editing events in the liver remains clinically significant, as the human liver is ~3K larger than the murine liver (kg basis). Thus, the goal of bone marrow penetration and HSC specificity would not be just to increase the targeting efficiency, but to redesign the delivery

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platform so that it would not be trapped by the reticuloendothelial system but would still be able to cross the bone marrow endothelium (Psatha, Paschoudi, Papadopoulou, & Yannaki, 2022).

6.2. Species differences: mouse versus human HSC cycling status and endocytosis capacity

One of the key challenges to overcome for in vivo HSC reprogramming to be clinically translatable is that there is a significant biological difference between the cell cycle status, endocytic potential and permissiveness to nanoparticle internalisation in murine and human HSCs that all have a direct impact on the efficiency of CRISPR delivery. Murine HSCs are significantly more actively cycling than human HSCs, with ~15% of young mice's lineage negative c-Kit+ Sca1+ cells cycling in the S/G2/M phases of the cell cycle at any one time, whereas only 1-3% of the human population of CD34+ CD38− CD45RA− HSCs are actively cycling in the S/G2/M phases of the cell cycle at any given time. The implications are significant for the delivery of CRISPR as lipid nanoparticle uptake and endosomal escape are strongly linked to membrane dynamics and vesicular trafficking, which is strongly enhanced during cell cycle progression. Furthermore, homology-directed repair is the preferred pathway for precise CAR knock-in, and occurs only in S and G2 phases when sister chromatids are available as repair templates, but quiescent HSCs mainly repair DBSs via non-homologous end joining, which often generates insertion-deletion mutations that interfere with the desired integration site (Ball, Bradley, Le, Tisdale, & Uchida, 2025). Thus, a delivery platform with 10-20% CAR knock-in in cycling murine HSCs might only have 1-3% CAR knock-in in quiescent human HSCs, which could translate to therapeutic efficacy versus futility. Besides, endocytosis capacity in HSCs varies significantly among species with human HSCs exhibiting less expression of scavenger receptors and macropinocytosis markers than murine HSCs, which decreases the intrinsic uptake capacity of lipid nanoparticles in the absence of active targeting. Three to five times more nanoparticles per cell were internalized by murine HSCs than human HSCs under the same conditions, even when both types of HSCs were stimulated to cycle by cytokines. Such difference in mechanism is multifactorial, involving species differences in plasma membrane lipid composition, caveolae density, and expression of the low-density lipoprotein receptor that mediates apolipoprotein E-dependent LNP uptake. In addition, human bone marrow microenvironment has significantly more adipocytic and less cellular character than mouse bone marrow, and the proportion of HSCs in the quiescent niche that is not accessible to nanoparticles is higher when compared to mice (E. H. Kim et al., 2024). These species differences are not only academic, but also help to account for why LNP formulations that are effective at HSC editing in mice often are ineffective in non-human primates that have HSC biology that is more similar to humans. The challenge of this can be met by development of humanized mouse models whereby the human HSCs are engrafted into immunodeficient recipients that will allow in vivo delivery platforms to be evaluated directly on human target cells, and by the use of HSC cycling agents (such as transient injection of stem cell factor, thrombopoietin and FLT3 ligand prior to injection of CRISPR delivery) to synchronously recruit human HSCs into the cell cycle, thereby boosting both the uptake of nanoparticles and the efficiency of homology-directed repair.

6.3. Strategies for HSC-selective targeting: CD117 and Lamp1 antibody-LNPs and VSV-G pseudotyping with alternative glycoproteins

To address the lack of intrinsic tropism of conventional delivery vehicles for HSCs, two main types of targeting strategies have developed: receptor-mediated targeting of LNPs through antibody or ligand targeting, and envelope glycoprotein engineering of viral vectors, with different mechanisms and trade-offs. Targeting of CD117 (c-Kit) has become the most broadly validated method for HSC-selective LNP delivery because it is highly expressed on long-term HSCs, endocytosed very rapidly upon ligand binding, and has high-affinity monoclonal antibodies that cross

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react with both the murine and human CD117 receptor. The usual approach is to covalently attach anti-CD117 antibodies or single-chain variable fragments to the polyethylene glycol-lipid part of the surface of the LNP or use antibody-conjugated polyethylene glycol lipids that can be post-inserted into pre-formed LNPs. For the mice model, editing of lineage-negative c-Kit+ Sca1+ HSCs reached as high as 60 percent in CD117-targeted LNPs containing Cas9 mRNA and a guide RNA against the CD45 gene, about six to eight times higher than non-targeted LNPs, while editing in hepatocytes decreased from 15 percent to three percent (Dasgupta, Flotte, & Keeler, 2021). Lamp1 (CD107a) is an alternative target, but the expression is more limited to activated HSCs, and the role of Lamp1 in lysosomal trafficking also raises theoretical concerns regarding the targeting of LNPs to degradative compartments. The tropism of viral vectors, especially those based on lentiviral and retroviral pseudotypes for VLP platforms, is defined by the envelope glycoprotein, and VSV-G (vesicular stomatitis virus G protein) is a widely used glycoprotein because of its broad tropism and high stability. VSV-G pseudotyping, however, is an efficient way to transduce hepatocytes, splenocytes, and endothelial cells and is not suitable for HSC-selective transduction without the incorporation of additional targeting layers. Other glycoproteins have therefore been tried, such as the envelope glycoprotein of gibbon ape leukemia virus, which binds the sodium dependent phosphate transporter PiT1 (SLC20A1) highly expressed on HSCs, and the viral hemagglutinin glycoprotein of measles virus that binds CD46 and signaling lymphocytic activation molecule (SLAM) family members (Jafarzadeh et al., 2024). Chimeric strategies, in which the VSV-G cytoplasmic and transmembrane domains are fused to the extracellular domain of a single-chain variable fragment that binds to CD117, have also been developed, in which the pseudotype would be high-titer and stable like VSV-G, and specific like antibody targeting. Although these strategies have been successful in transducing HSCs ex vivo, they have not been effective in vivo due to off-target complement receptor binding by liver macrophages, serum complement inactivation and poor particle stability. A third class of targeting strategy uses peptide ligands identified by phage display which bind HSC-enriched surface markers with nanomolar affinity, such as a 12 amino acid cyclic peptide (CTE-P) with a nanomolar affinity for CD117. Compared with antibodies, peptide-conjugated LNPs have reduced complexity of manufacturing, low immunogenicity, and small molecular weight that can lead to better tumor penetration, but most have exhibited poor in vivo targeting efficiency, which is probably due to lower avidity. Together, these strategies have boosted the efficiency of HSC editing, bringing it from minimal to five to 20 percent in preclinical models, but more needs to be done to reach the 30 to 50 percent mark predicted by mathematical modeling for therapeutic efficacy in humans (Abou-el-Enein, 2024). Figure 4 is shows Illustration of three strategies. Anti-CD117 antibody and Cas9 mRNA and guide RNA are loaded within (A) LNP. (B) Modified AAV6 capsid bearing peptides for CD34 binding. (C) Fusion VLP with VSV-G and CD117 nanobody (Nb) carrying Cas9-sgRNA RNP and donor template. Bottom panel – biodistribution graph of % of editing in bottom panel; bone marrow Lin−/cKit+/Sca1+ vs. liver, lung & spleen after IV injection in mice.

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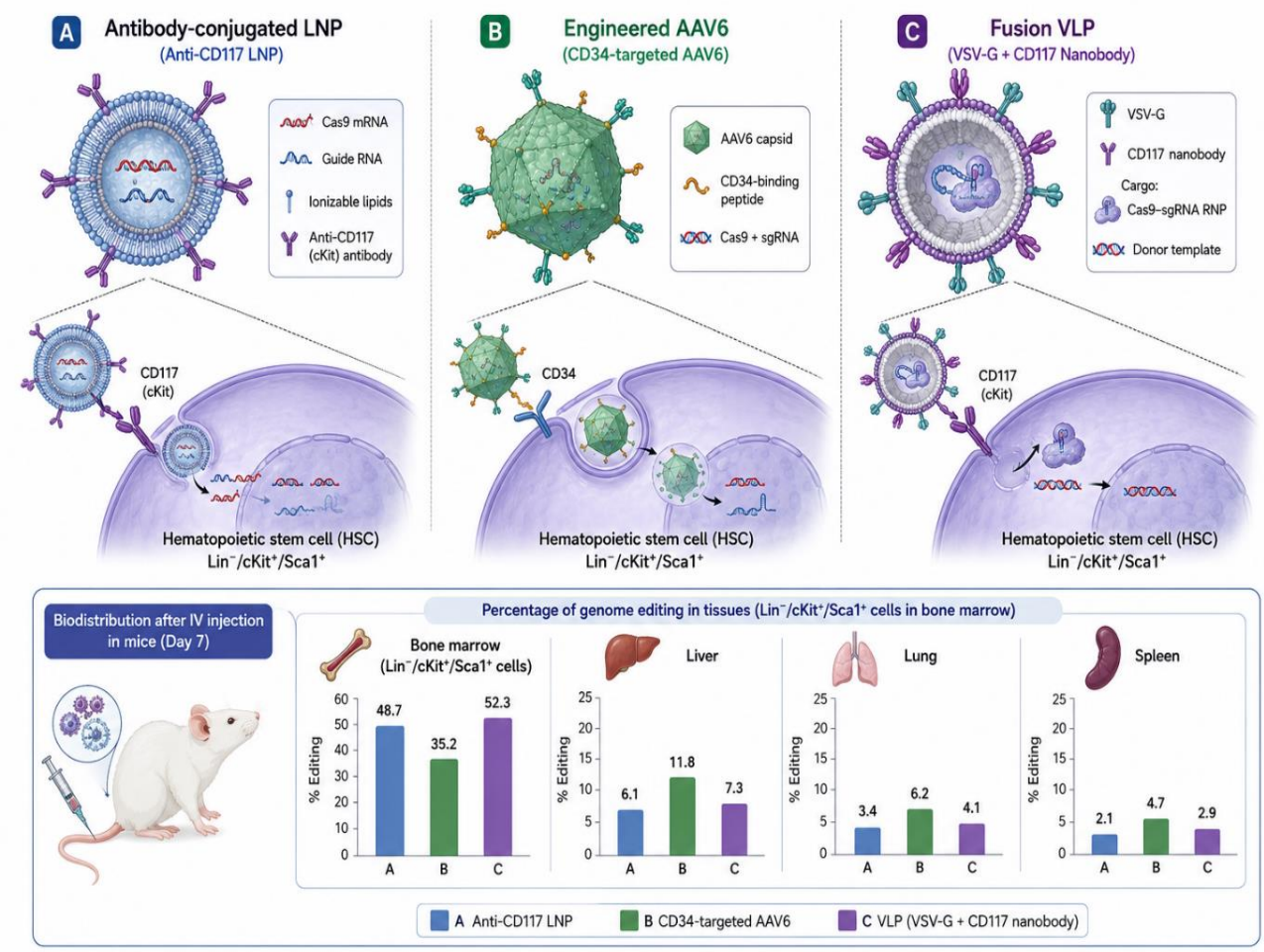

Figure 4: Targeted delivery platforms for in vivo HSC genome editing

6.4 Biodistribution and scalability for human systemic administration

The in vivo reprogramming of HSCs from mouse to human will depend not only on increasing targeting efficiency, but also significant understanding of the biodistribution, pharmacokinetics and scalability of nanoparticles, which will differ drastically due to species variations in body size, blood volume, and organ architecture when administered systemically. Human blood circulates about five litres per minute in 5 litres of blood, compared to one to two millilitres per minute in mice, and for a nanoparticle to achieve a similar exposure in the different organs it has to survive for a much longer time before it is diluted into a 500- to 1000-fold larger volume of blood and cleared by the reticuloendothelial system. The typical time of IV infusion in humans is 30-60 minutes; mice are injected intravenously over a 5-10 second bolus, which may limit endocytosis uptake of LNPs, but can increase the likelihood of complement activation and infusion reactions. Biodistribution studies in non-human primates, the most relevant species for translation to humans, indicate that CD117-targeted LNPs localize mainly in the liver (60-70% of the injected dose), spleen (10-15% of the injected dose), bone marrow (2-3% of the injected dose) and lungs (2-3% of the injected dose) with the rest of the dose in lymph nodes, kidneys and other tissues (Si, Xiao, Brown, & Wang, 2022). Absolute numbers of LNP entering the bone marrow are dependent on cardiac output to the bone marrow (around five percent of the

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total cardiac output), the extraction fraction (the fraction of LNPs that extravasate and interact with HSCs) and the plasma concentration-time integral. Even at optimized conditions, the estimated number of LNPs delivered to each individual HSC is only 10-100 particles, which is close to the limit for productive delivery because each HSC is home to ~200,000 cytoplasmic vesicles, and endosomal escape efficiency is typically 5-10%. Another manufacturing challenge lies in scaling up from mouse to human, where one human dose of LNP is expected to be 2 to 5 mg total lipid/kg (150 to 300 mg per 70 kg adult), versus mouse doses of 1 mg/kg (20 to 30 micrograms/mouse). Production of GMP-grade LNPs at this scale is possible as proven in the case of the COVID-19 mRNA vaccines that have been produced on this scale, but each batch still needs to be subjected to quality control testing, which takes between two and three months to be done for size, polydispersity, encapsulation efficiency, sterility and endotoxin. The targeting antibody part is also made under GMP, usually in Chinese hamster ovary cells, which can yield an antibody of one to two grams per litre, enough to make tens of thousands of doses, but the coupling of antibodies to lipids is a non-standard manufacturing step that needs to be validated to be consistent. The LNP product from a clinical administration point of view should be a stable suspension which can be stored for up to 6 months at minus eighty degrees Celsius, thawed and diluted at the bedside, and administered via a standard intravenous line with a 0.22-micron filter, but there is concern about the ability of the filter to remove the targeted LNPs if they aggregate or adsorb to the filter. Infusion related reactions occur with 10-20% of patients receiving drugs formulated in LNP and include mild fever and chills to anaphylaxis and administration of epinephrine; the infusion must be monitored to detect infusion related reactions. Although this is the standard, pre-medication with antihistamines, acetaminophen and corticosteroids could in theory, suppress the immune response that may be necessary to expand the CAR effectors (Ang, Metzloff, Thatte, & Mitchell, 2025). Nevertheless, the biodistribution and scalability of LNPs for systemic administration are well enough characterized to continue clinical development, with the first-in-human trials of HSC-targeted LNPs likely to be carefully executed dose escalations with intensive safety surveillance.

7. Clinical Translation and Regulatory Hurdles

7.1. Comparison with ex vivo HSC gene therapy (e.g., lentiviral CAR-CD34+ cells)

In vivo HSC reprogramming needs to be put in context with the proven paradigm of ex vivo HSC gene therapy, that has now been approved for several inherited diseases and is being explored for cancer immunotherapy. By the ex vivo method, the CD34+ HSCs are collected by apheresis after mobilization with granulocyte colony-stimulating factor (G-CSF) or plerixafor, transduced with lentiviral vectors containing the CAR gene, and then transplanted into a niche that is created by myeloablative or non-myeloablative conditioning. This strategy has a number of proven benefits: transduction efficiency of 40 to 70 percent of CD34+ cells can be confirmed before reinfusion, manufacturing includes quality control measures, and the clinical safety of this approach has been established through more than 20 years of use. However, there are significant drawbacks to ex vivo HSC gene therapy, including manufacturing time of 2 to 4 weeks, cost of three hundred thousand to six hundred thousand USD per patient, conditioning associated toxicities including mucositis, alopecia, infertility, and prolonged cytopenias as well as remaining risk of insertional oncogenesis despite the use of self-inactivating lentiviral vectors (Gholami, Mohkam, Soleimanian, Sadraeian, & Lauto, 2024). By contrast, in vivo HSC reprogramming could result in a truly off-the-shelf product that is administered systemically (without the need for HSC harvest, ex vivo culture, or reinfusion) and could be available in hours, with projected costs of $50,000 to $100,000. However, there are several caveats associated with the in vivo approach: in vivo levels of CAR knock-in HSCs are only 5-20%, versus 40-70% ex vivo; and conditioning is unknown and not required, but may be necessary and could

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include reduced-intensity busulfan or antibody-based depletion of CD117(Du et al., 2025).

7.2. Good Manufacturing Practice (GMP) for in vivo CRISPR-LNPs

For in vivo CRISPR-LNP to be translated from academic labs to clinical application, it is important to establish protocols that ensure the safety, purity, potency, and uniformity of the product from batch to batch, called Good Manufacturing Practice (GMP). The manufacturing process usually includes quick mixing of a lipid solution prepared in ethanol, which contains ionizable cationic lipids, phospholipids, cholesterol, and polyethylene glycol-lipids, with an aqueous solution containing Cas9 mRNA and chemically modified synthetic guide RNAs in a microfluidic mixing device to form LNPs, with a diameter of sixty to one hundred nanometers and encapsulation efficiency greater than ninety percent. Adding targeting ligands like anti-CD117 antibodies complicates the picture, as the chemistry to conjugate must be stable, but not destroy the LNP integrity or binding affinity. Only a few contract manufacturing organizations (CMOs) produce raw materials such as ionizable lipids and polyethylene glycol-lipids under GMP and there is supply chain reliability issues. 2′-O-methyl and phosphorothioate guide RNAs can be made at GMP quality and 1,000- to 5,000-fold yield, but at high cost ($1,000-5,000 per gram) (J. H. Lee & Han, 2024). The Cas9 mRNA generated by in vitro transcription needs high-performance liquid chromatography purification and stringent quality release testing. The most difficult requirement may be proof of sterility and lack of endotoxin in the product because injecting contaminated LNPs into the body intravenously could lead to septic shock. Terminal filtration with 0.22-micron filters is possible, but will result in 10-30% lower yields. Since primary HSCs are not suitable for batch release testing, potency assays that measure on-target editing efficiency in a surrogate cell line like THP-1 monocytes or K562 erythroleukemia cells need to be developed. While it is possible to establish GMP processes as illustrated by approved LNP-formulated drugs, targeting HSC poses new challenges that need close cooperation between academia, CROs and regulators (Mohanna et al., 2022).

7.3. Regulatory classification: gene therapy product versus cell therapy versus drug

Ambiguities in regulatory classification of in vivo CRISPR based HSC reprogramming products have significant impacts on length and expense of clinical development. In the U.S., the Food and Drug Administration considers products under its jurisdiction to be gene therapy products if the products are shown to exert a therapeutic effect through transcription or translation of transferred genetic material, or by specifically altering the human genome. As defined, the in vivo CRISPR-LNP product would be considered a gene therapy product since the combination of Cas9 mRNA and guide RNA changes the genome of the HSCs, and the CAR transgene is transcribed and translated. But, it's a fuzzy area for products that are just Cas9 mRNA and guide RNA used for gene disruption, without the presence of a donor template; the Food and Drug Administration (FDA) has said they would still be classified as gene therapy products because they make a permanent change to the genome. Under the Advanced Therapy Medicinal Product regulation, the European Medicines Agency considers gene therapy products in Europe to be the same as those for gene silencing, which are recombinant nucleic acids used to alter the genome (Desai, Schmidt, & Cristofoletti, 2024). An important distinction is that LNPs that include genetic material are considered gene therapy products and therefore are reviewed by the Office of Gene Therapies (not the Office of Cellular Therapy), and the chemistry, manufacturing and controls data is expected to be different. The alternative pathway is that it could be classified as conventional drugs or biologics regulated by the Center for Drug Evaluation and Research (CDER), because the LNP itself is the active pharmaceutical ingredient, and genome editing is a downstream effect. This classification is rare and is not likely to be accepted. Developers are encouraged to consult the Food and Drug

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Administration (FDA) regarding products that are gene therapy products by using the pre-investigational new drug consultation process to obtain formal product classification, and to follow specific guidance for those products.

7.4. First-in-human trial design: dose escalation, conditioning regimen, and patient selection

There are several unique challenges to the design of first-in-human trials for in vivo HSC reprogramming. A traditional 3+3 dose escalation design can be considered, in which the starting doses can be extrapolated from results obtained in non-human primates using allometric scaling; for example, the considered effective dose in non-human primates could be 0.5 to 1.0 milligrams per kilogram, which would correspond to 0.1 to 0.2 milligrams per kilogram in humans. Dose-limiting toxicity is likely to include infusion-related reactions (fever, chills, hypotension), hepatotoxicity due to LNP accumulation in the liver and transient cytopenias due to on-target editing of HSCs. Off-target editing in hepatocytes or cytokine release syndrome (CRS) from innate immune sensors may set the maximum tolerated dose. To assess safety in first-in-human trials, conditioning should initially be avoided to allow for competitive fitness of the edited and unedited HSC to take place without the added toxicity of conditioning. Initial trials may have less intensity conditioning, which can be done with low dose busulfan (3.2 mg/kg total dose, versus the standard 12.8 mg/kg) or fludarabine. The antibody-drug conjugates against CD117 have potential for non-genotoxic depletion of HSC, and need to be tested in humans. Due to the 4-8-week kinetics of CAR effector generation, patient selection should include patients with relapsed or refractory CD19-positive B-cell malignancies who have not been responsive to conventional CAR-T therapy, have good end-organ function, no pre-existing anti-Cas9 antibodies, and do not have rapidly progressive disease that would require immediate intervention. Endpoints are safety and tolerability for dose escalation, on-target HSC editing by deep sequencing, appearance of CAR-positive T cells and natural killer cells in peripheral blood, and initial evidence of antitumor activity by conventional response criteria.

7.5. Ethical considerations: germline editing, long-term monitoring, and informed consent

Clinical translation of in vivo HSC reprogramming introduces some unique ethical questions. Although the blood-testis and blood-ovary barriers restrict the access of LNPs and the likelihood of off-target germline editing is theoretically low, it cannot be completely ruled out. Reproductive toxicity studies in animal models are required by the Food and Drug Administration and consent forms are required to explicitly state the unknown effects on germ cells and the recommendation to avoid pregnancy for at least a year after treatment for patients of reproductive age. Gene therapy products that incorporate themselves into the genome must be monitored for long-term effects, and the Food and Drug Administration has recommended a 15-year period of monitoring for gene therapy products that includes annual physical exams, complete blood counts, and a malignancy assessment. Monitoring of in vivo HSC reprogramming should be every 3 months for 2 years followed by annual monitoring thereafter for cytopenias, lymphoproliferative disorders and unexplained organ function. The retention strategies such as annual stipends and travel reimbursements are a must. The informed consent process needs to convey substantial uncertainty about benefits and risks such as the experimental nature of the product, the potential lack of any clinical benefit, the risk of off-target editing causing leukemia (estimated at less than one percent, but based on preclinical models), the risk of uncontrolled CAR expression in platelets or erythrocytes causing thrombosis or anemia, anti-Cas9 immune responses destroying edited HSCs, and the need for long-term follow-up (Rouatbi et al., 2024). The consent process should involve a physician who is not directly involved in the study, and should be a staged process with an initial information session, followed by a 24-hour cooling-off period, followed by a second information session to assess understanding and answer questions, given that

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patients may have advanced malignancy and may be susceptible to therapeutic misconception. Pediatric studies should only be undertaken if safety and efficacy is established in the adult population, unless the paediatric condition is uniformly lethal and there are no other therapeutic options. This moral framework remains in a constant state of development and is constantly discussed between researchers, bioethicists, patient advocates and regulatory bodies (Jiang et al., 2025). Table 5 shows a comparison of 3 CAR-mediated immunotherapy modalities. In vivo HSC CRISPR provides lower costs and off the shelf manufacturing, 50, 000 – High cell number (50,000 - 100,000), diminished lymphodepletion, long-term CAR expression (years), flexible lineage control, and potential secondary malignancy risk (theoretical). Ex vivo CAR-T takes 2-3 weeks to manufacture, is more expensive, 400, 000 – Low reported transformation risk, fixed T cell lineage, months-long durability, chemotherapy lymphodepletion and 400,000–800,000. Ex vivo HSC gene therapy is intermediate cost and 2-4 weeks manufacturing). 300, 000 – Low to medium risk of insertional oncogenesis; conditioning with busulfan; variable lineage control; years of durability; 300,000-600,000. In vivo HSC CRISPR overcomes significant drawbacks of ex vivo methods but has yet to be clinically tested for potential side effects.

Figure 5: Comparison of In Vivo HSC CRISPR vs. Ex Vivo CAR-T vs. Ex Vivo HSC Gene Therapy

Parameter	In Vivo HSC CRISPR	Ex Vivo CAR-T	Ex Vivo HSC Gene Therapy (Lentiviral)	Reference
Manufacturing	None (off-the-shelf, single infusion)	2–3 weeks (patient-specific leukapheresis, activation, transduction, expansion)	2–4 weeks (HSC harvest, ex vivo transduction, quality control)	(Y. Wang et al., 2025)
Cost per patient	Estimated $50,000–100,000 (LNP synthesis, guide RNA, AAV donor)	$400,000–800,000 (including apheresis, manufacturing, hospitalization)	$300,000–600,000 (including HSC harvest, ex vivo culture, conditioning)	(Kenjo et al., 2021)
Lymphodepletion required	Possibly lower or none; may use targeted anti-CD117 antibody-drug conjugate instead of chemotherapy	Yes (chemotherapy with cyclophosphamide + fludarabine to create homeostatic niche)	Yes (busulfan or cyclophosphamide for myeloablation to enable HSC engraftment)	(Volta et al., 2024)

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Parameter	In Vivo HSC CRISPR	Ex Vivo CAR-T	Ex Vivo HSC Gene Therapy (Lentiviral)	Reference
CAR durability	Years (potentially life-long from edited long-term HSCs)	Months (contracted T cells lack self-renewal; persistence 3–6 months typical)	Years (from engrafted edited HSCs, similar to in vivo approach)	(Bonini, Cavazzana, Ciceri, Fehse, & Hudecek, 2024)
Lineage control	Tunable (lineage-specific endogenous promoters, miRNA switches, multi-lineage output possible)	Fixed (terminally differentiated T cells only; no multi-lineage potential)	Variable (depends on promoter; can achieve multi-lineage but requires ex vivo manipulation)	(F. Wang, Huang, Li, Zhou, & Wang, 2024)
Risk of secondary malignancy	Theoretical (CRISPR off-target editing in HSCs; TP53/DNMT3A disruption; clonal hematopoiesis)	Low (reported cases of CAR-T cell transformation; insertional oncogenesis risk low with modern vectors)	Low to medium (lentiviral insertional oncogenesis reported in clinical trials; risk reduced with SIN vectors)	(Crippa et al., 2023)

8. Future Directions and Unanswered Questions

8.1. Multiplex editing for universal CAR-HSCs

To overcome the issue of allorejection and fratricide, multiplex editing is needed to develop truly off-the-shelf in vivo HSC reprogramming products. Because blocking of the function of beta-2-microglobulin (B2M) blocks the expression of HLA class I, it also blocks the ability of CD8+ T cells to mediate rejection, but it does not block the ability of the cells to be recognized by the natural killer cells as missing self, which requires the expression of non-classical HLA class I molecules such as HLA-E or HLA-G, which are engaged by inhibitory natural killer cell receptors, in addition to blocking the expression of HLA class II. In contrast, CD7 knockout is useful to avoid fratricide when targeting CD7+ cancers, like T-cell ALL. Currently, multiplex editing platforms are inefficient, as single guide RNA (sgRNA) arrays delivered by AAV have only been able to achieve 5 to 10 percent of human HSCs with all three genes knocked out simultaneously, while other arrays have achieved 50 percent B2M knockout, 40 percent CD7 knockout, and 30 percent CIITA knockout. However, sequential ex vivo editing is inefficient and is in contrast to the idea of in vivo delivery, which is essential. However, sequential ex vivo editing is inefficient and is in contrast to the idea of in vivo delivery, which is essential (Deuse & Schrepfer, 2025). The development of universal CAR-HSCs is a long-term goal, which will need significant improvements in delivery efficiency

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and editing precision, but if successful would make in vivo HSC reprogramming a true one-size-fits-all cancer immunotherapy.

8.2. Logic-gated CARs for solid tumor specificity

A significant challenge in the application of in vivo HSC reprogramming to solid tumors is the absence of truly tumor-specific antigens, whereby most solid tumor-associated antigens are also expressed in some normal tissues resulting in on-target off-tumor toxicity that is not well tolerated with durable and multilineage CAR expression. Logic-gated CARs can be more sophisticated, as they can be designed to require two tumor antigens to activate the CAR (AND gate) or to only activate the CAR when a normal tissue antigen is not present (NOT gate). AND gate CARs are often designed as two distinct CARs with distinct signaling domains; one CAR with CD3ζ signaling and one CAR with costimulation (CD28 or 4-1BB) and only fully activated upon binding both antigens. Conventional CAR-T cells have been shown to have preclinical activity in AND gate CARs which have been shown to show 80-90 per cent reduction in off tumour toxicity but also reduced sensitivity to antigen-low or heterogeneous tumours. NOT gate CARs feature an inhibitory signaling domain from PD-1 or CTLA-4 that binds to an antigen expressed in normal tissues to prevent activation, thus minimizing the killing of normal cells and providing specific killing of tumor cells. Logic-gated CARs require dual CAR constructs from 8-10 Kb which are larger than the maximum capacity of AAV, and therefore must be integrated stably into the genome in vivo, which will require alternative technologies like VLPs or dual AAV systems. The added complexity also adds to the size of the genetic payload and may necessitate lineage-specific optimization to assure the stable expression of the payload in all progeny of HSCs (Junca et al., 2021).

8.3. In vivo prime editing and base editing for safer CAR knock-in

Conventional CRISPR-Cas9 systems use double-strand DNA breaks, which has multiple drawbacks for HSC editing: activation of p53 and apoptosis of quiescent HSCs; insertion-deletion mutations at the on-target site; and potential for chromosomal rearrangements if multiple on-target double-strand DNA breaks are made. Prime editing is based on the non-cleaving Cas9 nickase variant coupled with an impaired reverse transcriptase and a prime editing guide RNA that serves as a template for the desired edit as well as directing the nickase to the target site, allowing for small insertions, deletions, and base conversions without double-strand breaks. Although prime editing can only insert short DNA sequences (usually 1.5–2.0 kb), prime editing can be combined with recombinase-mediated cassette exchange in PASTE (programmable addition via site-specific targeting elements) to deliver up to fifteen percent knock-in of a four-kilobase payload in non-dividing primary human T cells without detectable double-strand breaks. Base editing, which is capable of making only single-base changes, may have the ability to reverse epigenetic changes, such as promoter methylation, or to interfere with transcriptional repressor binding sites, thereby allowing CAR transgenes to be expressed when they are epigenetically silenced (Wolff & Mikkelsen, 2023). The main drawback is the large size of prime editor fusions (around six kilobases) that can't fit inside AAV and most LNPs, but there are compact prime editors in development by using smaller Cas orthologs like Cas12f or CasΦ. Considering the safety benefits of double strand break-free editing, the field will soon shift towards CAR knock-in by prime editing in the next five years. Figure 5 illustrates Schematic of (A) Prime editing to insert CAR using pegRNA and nCas9 fused to reverse transcriptase, (B) Base editing (ABE/CBE) to activate a silent CAR gene from an endogenous safe harbor, (C) Epigenetic editing (dCas9-p300) to turn on CAR expression without DNA cutting. The bottom timeline depicts when clinical entry is expected.

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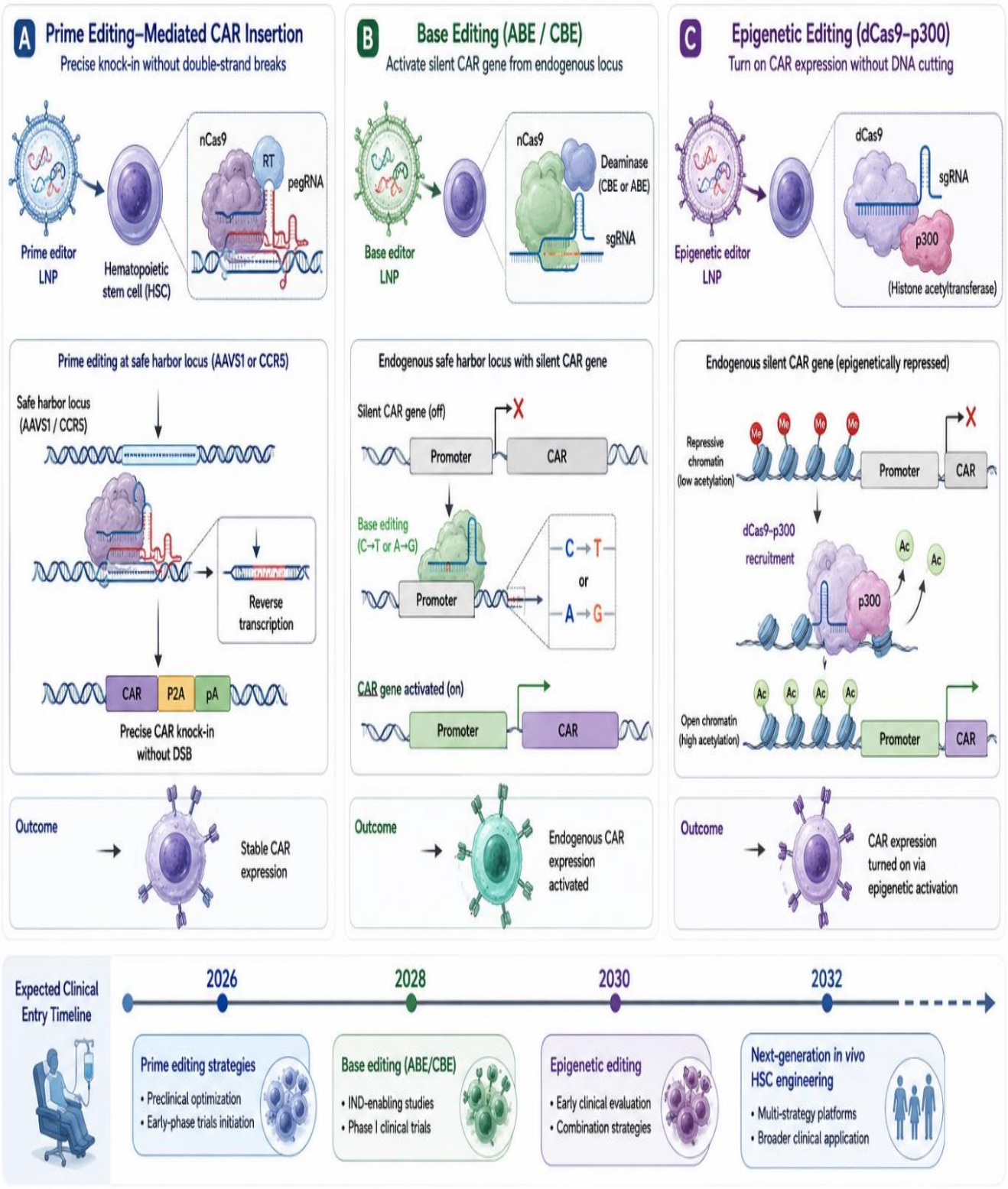

Figure 5: Next-generation in vivo HSC engineering strategies beyond nuclease-based CRISPR.

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8.4. Real-time imaging of endogenous CAR immune cell trafficking

The biodistribution, trafficking, and tissue residence of CAR immune cells generated from endogenous edited HSCs remains a major unknown in in vivo HSC reprogramming, as conventional flow cytometry and immunohistochemistry can only give static snapshots, and cannot measure dynamic migration. The radiolabeled single-chain variable fragment (scFv) targeting the CAR idiotype is sensitive and allows whole-body quantitation of CAR-T cells, with studies in murine models showing that HSC-derived CAR-T cells initially localize to the bone marrow and spleen, with subsequent gradual distribution to tumor sites on day twenty-one. Longitudinal studies in animal models with CAR-luciferase fusion proteins demonstrate that HSC-derived CAR-T cells take longer to accumulate (peak day twenty-one vs day three for conventional CAR-T) but have a longer duration of persistence (day one hundred vs day twenty). The disadvantage of PET is the need for a target-specific probe, and the repeated imaging is not possible due to radiation exposure, and the application of bioluminescence cannot be adapted to the human scale (Guerra, Di Pietro, Basile, Trerotola, & Alberti, 2021). A more futuristic method is to create a secreted reporter (for example, a modified human chorionic gonadotropin which can be detected in peripheral blood) from CAR-expressing cells, thus enabling the quantitation of CAR cell number, but without spatial information. Acceleration of the optimization of delivery vehicles and conditioning regimens should be done with the development of clinically translatable real-time imaging methods.

8.5. Combinatorial regimens with PD-1 checkpoint inhibitors and oncolytic viruses

The mechanisms that limit the activity of conventional CAR-T cells, especially PD-L1 expression on the CAR-T cells target and binding to PD-1 on the CAR-T cells that renders them exhausted in the solid tumor microenvironment, may apply to endogenous CAR immune cells as well. Thus, in vivo HSC reprogramming together with checkpoint inhibitors is a logical and possibly synergistic approach. MSLN-CAR HSCs plus anti-PD-1 antibody resulted in ninety percent suppression of tumor growth at sixty days, compared with sixty percent of tumor suppression in mice receiving CAR HSCs alone, but there was an increase in immune related adverse effects, such as colitis (thirty percent) and pneumonitis (fifteen percent). Intratumoral injection of vesicular stomatitis virus expressing GM-CSF in combination with HER2-CAR HSCs led to complete regression of injected and distant tumors in seventy percent of animals vs twenty percent of animals receiving CAR HSCs alone (J. S. Park et al., 2024). The oncolytic virus triggered damage-associated molecular patterns and chemokines to attract CAR-macrophages and CAR-natural killer cells to the tumor. Some future CAR designs may include PD-1 resistance right in the design, for example PD-1-CD28 switch receptors, which convert inhibitory signals to activation, and thus may not require systemic checkpoint inhibition at all. While these approaches to ordering and timing of combinatorial regimens need to be optimized, it is believed that they will be explored as soon as it is proven that in vivo HSC reprogramming alone is safe (Crupi et al., 2022).

CONCLUSION:

The in vivo paradigm of using CRISPR to genetically modify HSCs to produce endogenous CAR immune cells has grown from a theoretical concept to a technically viable approach that has already been proven in several preclinical studies. Important advances in the field of science include the development of HSC-tropic lipid nanoparticles (LNPs) targeting CD117 or Lamp1, the ability to precisely knock-in CARs (KK-CARs) using double-strand break-free approaches (PASTE, prime editing), and the validation of lineage-specific expression systems using endogenous promoters or microRNA (miRNA) regulation. Models have demonstrated that endogenously produced CAR-T, CAR-natural killer and CAR-macrophages cells derived from edited HSCs, have long-lasting anti-tumoral activity against both haematological and solid tumours, with evidence of immunological memory and persistence of more than 300 days. However,

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there are some issues that have not yet been addressed and are considered critical bottlenecks. Therapeutic thresholds in humans remain uncertain given the efficiency of in vivo HSC targeting only 5-20% CAR knock-in in long-term repopulating HSCs compared to the routine 40-70% achieved with ex vivo lentiviral transduction. Lineage-restricted expression systems demonstrate some leakiness of one to five percent in other lineages (e.g. megakaryocytes or erythroid precursors), and if these lines are susceptible to CAR-mediated toxicity, this may be clinically relevant. Murine models of 2 years lifespan are not sufficient to assess long-term safety, including the possibility of inducing clonal hematopoiesis through disruption of TP53 or DNMT3A, and potential immunogenicity of bacterial Cas9 proteins. These difficulties aside, the combination of enabling technologies and unmet clinical need makes first-in-human trials within three to five years a realistic prospect, likely in the setting of CD19-positive B-cell malignancies where patients have failed conventional CAR-T therapy with a non-aggressive design that involves reduced-intensity conditioning and inducible caspase-9 safety switches. The next few years will be critical to see if this paradigm shift will realize its therapeutic potential.

Conflict of interest

All authors have no conflict of interest.

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