Journal for ImmunoTherapy of Cancer

Tailoring CAR surface density and dynamics to improve CAR-T cell therapy

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To cite: Hinckley-Boned A, Barbero-Jiménez C, Tristán-Manzano M, *et al*. Tailoring CAR surface density and dynamics to improve CAR-T cell therapy. *Journal for ImmunoTherapy of Cancer* 2025;**13**:e010702. doi:10.1136/jitc-2024-010702

► Additional supplemental material is published online only. To view, please visit the journal online (https://doi.org/10.1136/jitc-2024-010702).

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Accepted 23 March 2025

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ABSTRACT

Chimeric antigen receptor (CAR)-T cell therapy has revolutionized the treatment landscape for relapsed and/or refractory B-cell neoplasms, garnering Food and Drug Administration/European Medicines Agency approval for six commercial products. Despite this success, challenges persist, including a relapse rate of 30–50% in hematologic tumors, limited clinical efficacy in solid tumors, and severe side effects. This review addresses the critical need for therapeutic enhancement by focusing on the often-overlooked strategy of modulating CAR protein density on the cell membrane. We delve into the key factors influencing CAR surface expression, such as CAR downmodulation following antigen encounter and antigen-related factors. The dynamics of CAR downmodulation remain underexplored; however, recent data point to its modification as a useful tool for improving functionality. Notably, transcriptional control of CAR expression and the incorporation of specific elements into the CAR design have emerged as interesting strategies to tailor CAR expression profiles. Therefore, controlling CAR dynamic density may represent an attractive strategy for achieving optimal therapeutic outcomes.

INTRODUCTION

Chimeric antigen receptor (CAR)-T cell therapy has demonstrated unprecedented success in treating relapsed and/or refractory B-cell neoplasms,[1] leading to the approval of seven commercial CAR-T-based medicinal products by the US Food and Drug Administration (FDA) and the European Medicines Agency (EMA). Despite its profound impact on immuno-oncology, CAR-T therapy still faces significant limitations in the treatment of hematologic tumors. Lack of persistence results in a relapse rate of approximately 30–50% in patients treated with CAR-T cells,[2] while hyperactivation of CAR-T cells triggers severe side effects (eg, cytokine release syndrome (CRS) and immune effector cell-associated neurotoxicity syndrome).[3] On the other hand, CAR-T cell therapies face substantial challenges against solid tumors, where responses remain limited due to antigen heterogeneity and immunosuppressive microenvironments.[4 5] However, recent clinical trials have shown promising but modest efficacy in different solid tumors[6] such as glioblastoma[7] or sarcoma.[8] In any case, challenges persist, highlighting the pressing need for advancements in terms of CAR-T cell efficacy and safety, and the relevance of a more in-depth understanding of the mechanism of action of the CAR molecule.

Various studies have highlighted the importance of regulating CAR expression levels on the surface of CAR-T cells to enhance therapeutic efficacy.[9–14] Nevertheless, the influence of CAR protein density on the membrane as a strategy to improve CAR-T cells has often been overlooked. In this context, the primary focus has been to investigate the mechanisms of action of various CAR configurations, cell types (T cells, natural killer (NK) cells, and macrophages), T-cell subpopulations, gene editing strategies, metabolic intervention, and combination therapy to address existing limitations, as revised elsewhere.[15 16] However, these investigations frequently neglect a crucial factor that can affect the efficacy and safety of CAR-T cells: the density and kinetics of CAR molecules on the cell surface.

CAR levels on the surface of T cells depend on two main factors: the transcriptional regulation of CAR transgene expression, and the dynamics of internalization, degradation, and recycling of CAR proteins on T cells (ie, CAR kinetics). The mechanisms governing the kinetics of CAR expression on the cell membrane remain largely unexplored despite the consensus that CAR membrane expression is dynamic.[12 17–19] The transcriptional

control of CAR expression is better understood since it depends on the promoter and RNA structure. The decision on how to express the CAR can have important consequences on T-cell signaling, persistence and proinflammatory cytokine release, impacting the antitumor efficacy and safety of the final product.[10 11 14 20] However, all approved CAR-T cell products, and the majority under evaluation in ongoing clinical trials, use autologous T cells transduced with ɣ-retroviral or lentiviral vectors expressing the CAR under strong constitutive promoters, such as the human elongation factor-1 alpha (EF1α), the murine stem cell virus (MSCV) long terminal repeat (LTR) or the myeloproliferative sarcoma virus MPSV enhancer (MND) promoters.

In this review, we outline the key factors that influence CAR density and expression kinetics in the T-cell membrane. We will explore the role of these factors in determining their efficacy and safety. Special emphasis will be placed on how the concentration of CAR molecules on the surface of T cells can impact CAR-T cell activity and how this can be modulated to attain optimal efficacy and safety. Highlighting the pivotal role of controlling CAR expression in CAR-T products, our aim is to raise concern about this overlooked matter to potentially develop CAR-T products that are not only safer but also more effective.

FACTORS INFLUENCING CAR SURFACE EXPRESSION PROFILE

Internalization, recycling and degradation processes

While CAR expression kinetic is not fully explored and there's limited data available, T-Cell Receptor (TCR) expression kinetic is well understood. Since CAR and TCR share structural features that are involved in the internalization process, we decided to use the knowledge of TCR kinetics to compare and depict what is known and which theoretical gaps could be investigated regarding CAR internalization, degradation and recycling (table 1). The presence of TCR and CAR in the membrane has been proven not to be static. The TCR follows different kinetics depending on whether the TCR is engaged or not, and its surface expression depends on the newly synthesized TCRs and processes such as internalization, recycling, or degradation (figure 1).[21 22] On strong antigen recognition, the surface TCR is downmodulated, the engaged TCRs are ubiquitinated by two of the Casitas B-lineage lymphoma family of ubiquitin ligases, c-Cbl and Cbl-b,[23]

Table 1 Detailed comparison of internalization, kinetics, fate, and regulation of CAR and TCR in T cells

Parameter	CAR	TCR
Internalization – absence of target antigen	Clathrin-independent. Intracellular domain dictates CAR distribution in T cells.[37] Tumor microenvironment could affect CAR internalization.[30]	Constitutive internalization in resting T cells via clathrin-dependent endocytosis.[26 31 32]
Kinetics of antigen-induced downmodulation	Quickly downmodulated on encountering tumor antigen.[12 18 28 29] Influenced by antigen density. Can be influenced by CAR affinity.[17] Trogocytosis.[19]	Internalized by unknown clathrin-independent pathway.[24–26] In the immunological synapse, activated TCRs are ejected from the cell membrane by ectocytosis.[38] Bystander internalization of TCRs adjacent to engaged TCRs.[34] Strong correlation between downmodulation and avidity.[49 50]
Fate of internalized protein complexes	Rapid ubiquitination of intracellular domains.[29] Degraded in lysosomes.[12 29 30] Highly influenced by the intracellular domains.[37]	Ubiquitinated by c-Cbl and Cbl-b.[23] Lysosomal degradation or recycled to the immunological synapse through flotillins.[27] The balance between recycling and lysosomal degradation depends on activation strength.[34]
Regulation of expression	Strong promoters: required in some contexts but generally lead to early CAR-T cell exhaustion and stronger CRS.[11 12 28 65] Physiological/weak promoters: improved memory-like phenotype, lower exhaustion and reduced CRS[9] Genome editing: improved memory-like phenotype, lower exhaustion and reduced CRS.[12 69 70]	Expression downregulation on antigen encounter.[10 12]

CAR, chimeric antigen receptor; TCR, T Cell Receptor.

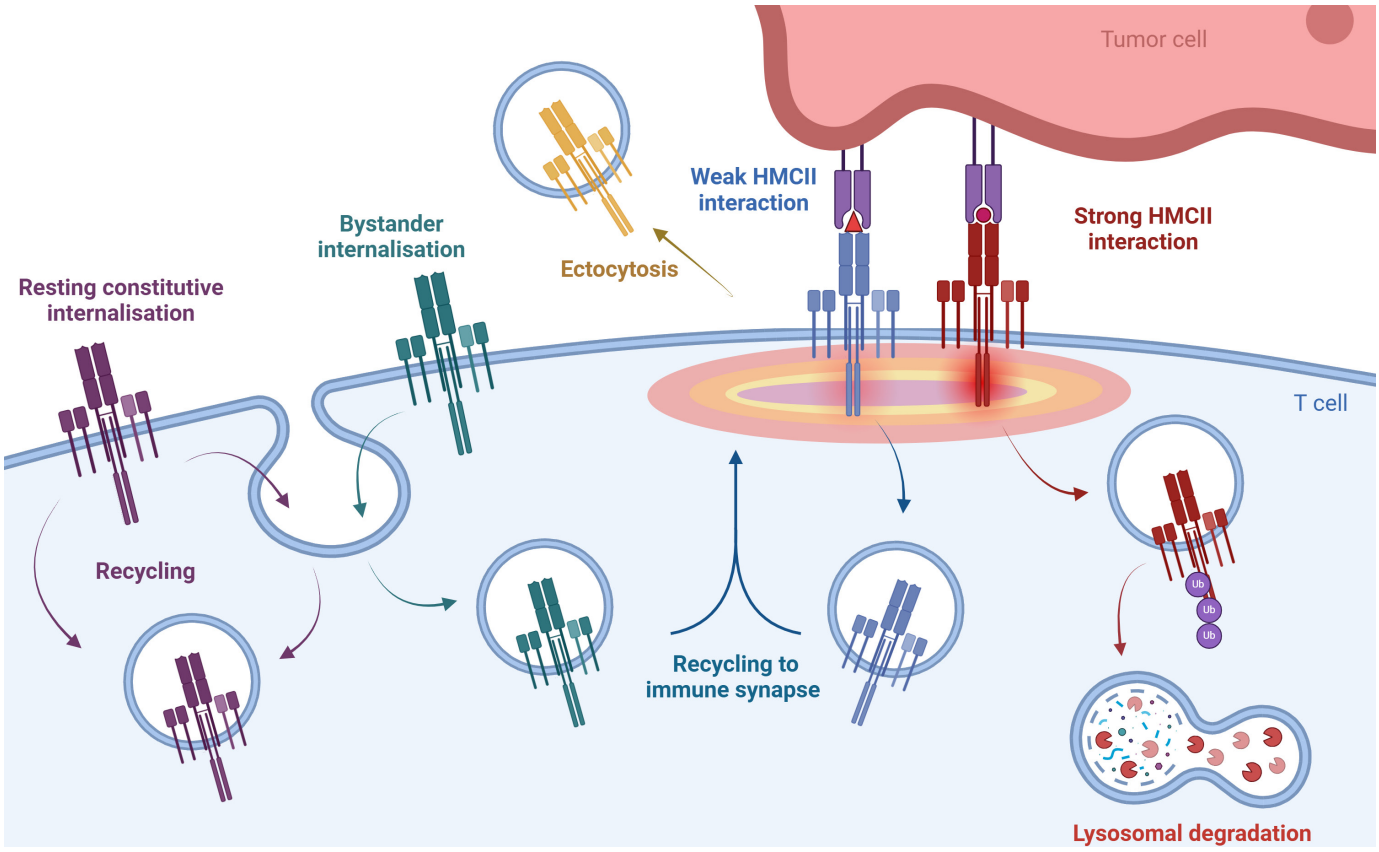

Figure 1 Kinetics of the TCR complex on the T-cell membrane during resting and activation states. Only the internalization and degradation routes after strong interaction with major histocompability complex (MHC) class II molecules (shown in red) have been reported for chimeric antigen receptor molecules.[12 29 30]

internalized by a yet unknown clathrin-independent pathway, and undergo lysosomal degradation.[24–26] Activated TCRs can also be recycled to the immunological synapse through flotillins after clathrin-independent uptake.[27] Similarly, when CAR encounters the tumor antigen, it is quickly downmodulated.[12 18 28 29] This occurs, at least in part, by the rapid ubiquitination of intracellular domains.[29] The CAR is also internalized and it can be degraded in lysosomes.[12 29 30]

In resting T cells, the TCR undergoes constitutive internalization.[26 31 32] This internalization occurs through clathrin-dependent endocytosis, which directs the TCR to the endosomal recycling pathways instead of lysosomal degradation.[33 34] This same clathrin-dependent route enables the bystander internalization of TCRs adjacent to engaged TCRs in activated T cells.[34] This bystander internalization allows these TCRs to be re-routed to the immunological synapse to maintain TCR signaling.[35 36] Essentially, there seems to be a balance between recycling and lysosomal degradation that is dependent on the strength of TCR activation.[34] Mutating the clathrin adaptor AP2 internalization motifs in all TCR:CD3 complex chains, except CD3ζ, still resulted in 40% TCR internalization.[33] Thus, AP2 motifs in the CD3ζ chain of CARs may enable clathrin-dependent internalization and recycling, similar to TCR. However, CAR clathrin-dependent internalization and recycling have not been described in CAR-T cells, and the question remains of whether CAR activation strength influences the commitment to a recycling or degradation route, or whether non-CD3ζ TCR-related structures play a more decisive role in the recycling/degradation balance. A recent report by Wang *et al*[37] describe the ionic interactions between the intracellular domains of CARs and the endomembrane system as important contributors to CAR surface distribution.

In the TCR immunological synapse, activated TCRs are ejected from the cell membrane by ectocytosis instead of being internalized by endocytosis, which terminates their signaling.[38] However, this recent discovery has not yet been reported in CAR synapses. When considering immunological synapse formation, TCR and CAR molecules exhibit dissimilarities. In contrast to TCR, CAR molecules form a disorganized synapse[39 40] forming clusters without recruiting TCR molecules to the CAR synapse.[38 41] In brief, even if TCR and CAR kinetics present resemblances they also show differences in their internalization and synapse formation.

Recently, non-antigen-related downmodulation through internalization and lysosomal degradation has been reported. Li *et al* observed that acute myeloid leukemia (AML) cells induced much higher CAR downmodulation than acute lymphoblastic leukemia (ALL) cells when assessing the same CARs and antigens at

low effector-target ratios.[30] They discovered that Gal-1 produced by AML cells was able to induce CAR downmodulation, which was blocked by internalization and lysosomal activity inhibitors. Although the study has some limitations, it suggests that the tumor microenvironment could affect CAR internalization and degradation, which would then imply that not all CAR downmodulation is antigen-specific.

The importance of this downmodulation on activation and its experimental suppression in CAR-T cells has been reported in several studies. The blockage of ubiquitination by the mutation of intracellular lysine residues enhanced the recycling of internalized CAR, which resulted in more effective long-term tumor killing activity. In this model, internalization occurs, but the recovery rate is faster and higher. The increased effectiveness was much higher when the costimulatory domain 4-1BB was used rather than CD28. Although counterintuitive, CAR-T cells presented less surface CAR when intracellular lysines were mutated. Additionally, preventing CAR degradation increased 4-1BB signaling by endosomal CAR molecules, which enhanced mitochondrial oxidative function and promoted differentiation towards memory T cells. Notably, only 4-1BB signaled from the endosome, while the CD3ζ chain did not.[27] Although the authors do not believe the improvement was due to reduced tonic signaling, it could be a combined effect with the endosomal signaling of 4-1BB, as lower surface CAR levels would reduce tonic signaling. Consistent with these findings, mutating the intracellular lysines of an anti-CD33 CAR containing 4-1BB demonstrated benefits in vitro but not in vivo.[30] However, the study had limitations, and the in vivo experiments lacked statistical significance. Additionally, although initial CAR levels were lower in the mutated CAR-T cells, the AML model used a different substance that also caused downmodulation. This may have independently reduced CAR surface levels, potentially preventing the observation of effective cytotoxic activity in vivo.

Another strategy is to delete the member Casitas B-cell lymphoma-B (CBL-B) from the E3 ubiquitin ligase families that promotes ubiquitination of the CD3ζ chain,[42 43] resulting in resulting in reduced expression of exhausting markers and increased tumor cell killing of a CAR against carcinoembryonic antigen (CEA) with CD28 as a costimulatory domain.[44] Even so, this improvement could also be related to other CBL-B functions, such as its role in TGF-β signaling[45] or its possible role in the inhibitory programmed death-1 (PD-1) signaling pathway.[46] There are no data on CAR levels to confirm that it might have less surface expression and more cytosolic expression.

In a different approach, Zhou *et al*[47] fused cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) cytoplasmic tails (CTTs), which contain internalization motifs that interact with clathrin adaptor AP-2 to the C-terminal region of a third generation CAR containing 4-1BB and CD28. This resulted in accelerated CAR endocytosis, degradation, and recycling, together with a reduction in trogocytosis, activation, and proinflammatory cytokine secretion. In vivo, CAR-T cells containing one or two CTTs showed improved survival, persistence, and an increased T central memory phenotype, which resulted in enhanced antitumor functionality. The authors showed lower levels of inhibitory receptors and lower tonic signaling in CTT-CARs compared with standard CARs. Interestingly, the addition of CTTs also decreased the surface expression of CAR and increased cytosolic expression.[47] We believe that the evidence from these articles suggests that lowering CAR surface expression and increasing CAR cytosolic expression reduces tonic signaling while maintaining 4-1BB signaling. An alternative explanation could be that the 4-1BB endodomain sequesters the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) inhibitor A20 to the cell membrane. This prevents A20 from performing its cytoplasmic activities, leading to hyperactivation of the NF-κB pathway, which, in turn, increases CAR-T cell death and necroptosis.[48] The authors suggest that the internalization of the CAR would imply that A20 could carry out its function from the cytoplasm, improving the antitumor ability of the CAR-T cell. To confirm the mechanism by which the internalization in 41BB CARs improves cell efficacy, it would be interesting to analyze in depth both the effect of CAR downmodulation on CAR-derived tonic signaling and the possibility of A20 recovering its activity by the CAR internalization.

These studies indicate that modifying CAR downmodulation could be a strategy to control CAR surface expression, potentially enhancing the effectiveness of CAR-T therapy. However, it is crucial to conduct extensive in vitro and in vivo analysis of T-cell fitness in long-term cytotoxic assays to thoroughly assess the advantages and disadvantages of altering the downmodulation kinetics.

Antigen-related factors

As has been found for the TCR,[49 50] antigen recognition is a pivotal factor influencing CAR downmodulation. When presenting CAR-T cells to the same tumor models lacking antigen expression, CARs are not equally downmodulated.[18 29] Walker *et al*[18] demonstrated that CAR T-cell function is limited by both CAR receptor density and target antigen density, and they showed that CAR downmodulation occurs on antigen encounter and persists on repeated exposure to antigen-positive tumors. In the same direction, Greenman *et al*[28] found that CAR downmodulation occurs rapidly following the encounter with target cells and is dependent on antigen density. The authors suggest that the proportion of downmodulated CARs is determined solely by antigen density, whereas the overall quantity of downmodulated CARs is influenced by both antigen density and the initial receptor count. Interestingly they did not find a clear effect of CAR affinity on receptor downmodulation. Conversely, Caruso *et al*[17] showed that CAR affinity for its antigen does play a pivotal role in CAR downmodulation. In this work, CARs were generated based on cetuximab and nimotuzumab,

which bind highly overlapping epitopes of the epidermal growth factor receptor (EGFR) protein with different on-rate binding kinetics. The authors showed that cetuximab-CAR, which has a higher affinity, exhibited significant downregulation from the T-cell surface after interaction with EGFR, while nimotuzumab-CAR, which has a lower affinity, did not show appreciable downregulation. This downregulation of cetuximab-CAR was observed to be a function of both affinity and antigen density, impacting the T-cell's ability to respond to repeated challenges with EGFR.

The potency of CAR-T cells is related to antigen expression density on the tumor and insufficient reactivity against cells with low antigen density has emerged as a pivotal determinant of CAR-T cell therapy resistance.[14 51–53] However, unlike canonical αβTCRs, there is consensus on the need for CARs to surpass a defined signaling threshold to exert an antitumor response.[54–57]

As described throughout this manuscript, the spatiotemporal dynamics of CAR distribution on the cell membrane profoundly affect the physiological behavior and antitumor capacity of CAR-T cells. Indeed, Caruso *et al* also noticed that during a second antitumor response, the internalization of the high-affinity CAR hindered an effective response, a phenomenon not observed with the lower-affinity CAR, where internalization was not detected.[17] Collectively, these studies suggest that the downmodulation of this receptor is a conserved process among different CAR constructs, driven by receptor internalization. This phenomenon can significantly affect signaling, and consequently, the potency of CAR-T cells.

The impact of the interplay between CAR and antigen densities on CAR signaling has not been fully explored, with the underlying mechanism yet to be formally elucidated. CAR signaling appears to be strongly influenced by both CAR and antigen densities.[18 28] High-density CAR-T cells can benefit from downmodulation, which limits hyperactivation and exhaustion resulting from chronic antigenic stimulation. Conversely, low-density CAR-T cells may be effective for treating tumors with high antigen density, provided that the activation threshold for the T-cell response is effectively reached while minimizing cross-targeting to antigen-low positive healthy tissues. This approach can potentially achieve tumor elimination while significantly reducing the release of proinflammatory cytokines, thereby enhancing the safety profile of the therapy.[58] In this direction, Andreu-Saumell[14] showed that CAR-T cells containing high CAR levels can overcome programmed death-ligand 1 (PD-L1)-mediated inhibition observed in low-affinity CARs, and this result is highly dependent on antigen density. The authors also demonstrated that PD-1 knockout (KO) provided an advantage to low-affinity CAR-T cells when human epidermal growth factor Receptor 2 (HER2) levels were high but not to high-affinity CAR-T cells. However, in co-culture with HER2-low cells, PD-1 KO conferred an advantage to high-affinity CAR-T cells.[14] These reports indicate the importance of antigen and CAR densities on CAR-T cell activity and introduce CAR affinity as an important parameter.

Another antigen density-dependent phenomenon leading to CAR depletion from the T-cell membrane is trogocytosis. It has been well documented that CAR-T cells can accept targeted antigens from tumor cells, promoting tumor escape via trogocytosis and reducing CAR surface expression.[19] However, it was recently demonstrated that CAR molecules can also be transferred to tumor cells, not only to dampen CAR-T cell killing but also to contribute to antigen-masking.[59] The extent of these phenomena is highly influenced by antigen density and CAR affinity.[59–61] In fact, trogocytosis itself also affects the antigen and CAR density, lowering antigen levels in the tumor cell and the CAR levels in the T cell. Interestingly, the addition of CTT tails to the CAR, which generates an increase in CAR kinetic speed, has been linked to a reduction in trogocytosis.[47] This highlights the importance of deciphering whether trogocytosis can be influenced not only by antigen levels but also by CAR density.

TRANSCRIPTIONAL CONTROL OF CAR EXPRESSION

Previous research has identified critical factors influencing CAR-T cell response, including extrinsic factors such as the lymphodepletion regimen, blood lactate dehydrogenase levels, and monocyte chemoattractant protein-1. Additionally, intrinsic factors such as T-cell activation prior to viral transduction, the CD4/CD8 ratio, and the composition of different T-cell subpopulations in the final infusion product also play a key role.[53] Beyond these factors, the transcriptional control of CAR expression is another crucial determinant of CAR-T cell functionality. One of the main parameters directly influencing CAR expression is the chosen strategy for T-cell engineering. In this regard, the CAR transgene can be integrated into the chromosome of T cells semi-randomly[62] (by classical viral and non-viral integrative vectors) or into a selected locus[63] (by genome editing) (figure 2). Moreover, transient CAR expression can also be achieved via electroporation of CAR messenger RNA (mRNA) into T cells.[64]

For viral gene transfer, modulating CAR density is possible by altering viral titers aimed at achieving different vector copy numbers or the type of viral vector used for genetic modification.[65] For both viral and non-viral strategies, this can also be accomplished by using different promoters[20] or by arranging transgene configurations.[11] All CAR-T cell products approved by the FDA and the EMA are generated using γ-retroviral or lentiviral vectors with strong promoters (MSCV-LTR, MND or EF1α-based).[66 67] However, high and sustained CAR concentrations on the surface of T cells and/or high antigen avidity may lead to spontaneous ligand-independent CAR aggregation. This can result in tonic signaling, potentially accelerating T-cell exhaustion and off-target toxicities, thereby causing side effects and relapses.[11 12 28 65] However, as mentioned previously, these negative effects are highly

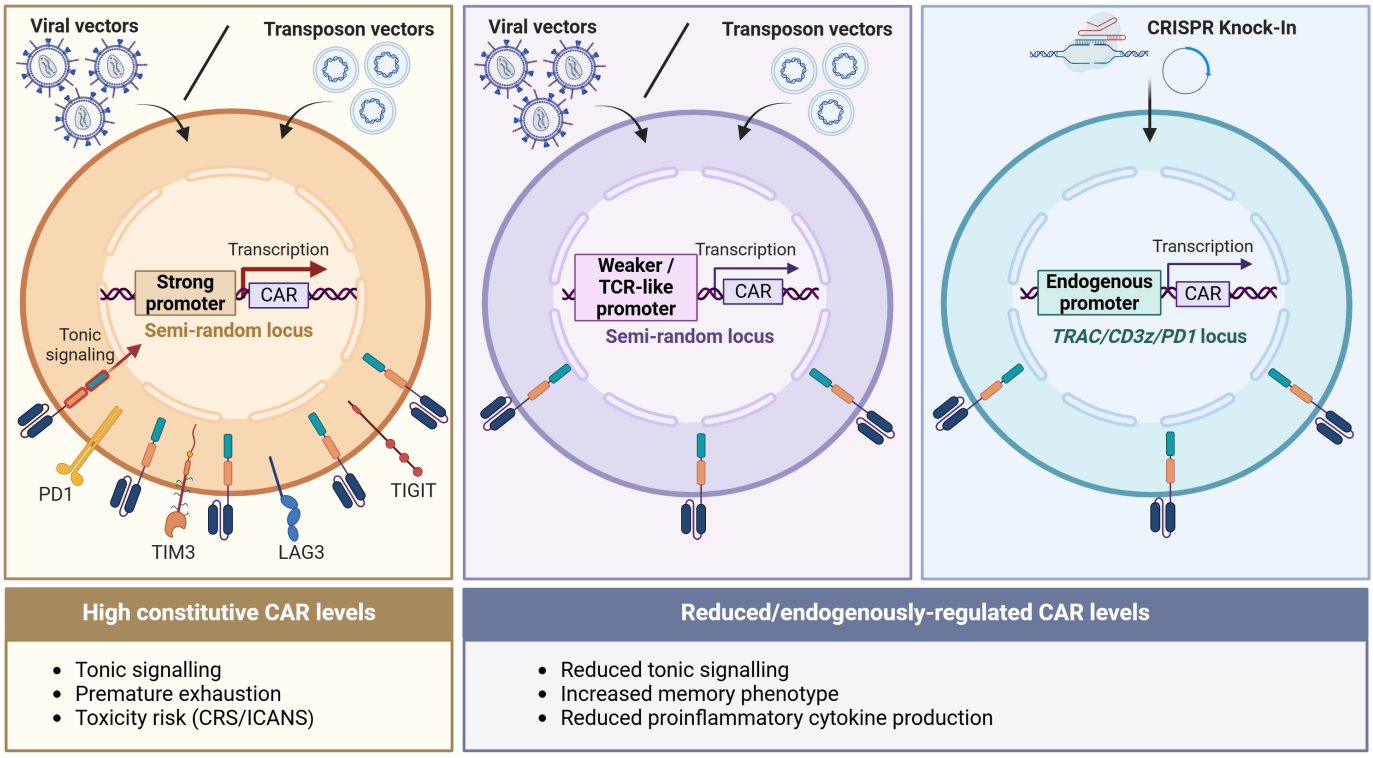

Figure 2 Different transcriptional control methods used to drive CAR expression and their impact in the final CAR-T cell products. CAR, chimeric antigen receptor, CRS, cytokine release syndrome; ICANS, immune effector cell-associated neurotoxicity syndrome; PD-1, Programmed Death-1; TIM3, T-cell Immunoglobulin and Mucin-domain containing-3; TIGIT, T cell immunoreceptor with Ig and ITIM domains; TCR, T cell receptor; LAG3, lymphocyte activation gene-3.

dependent not only on the CAR density but also on the CAR design.[14] Recently, Rodriguez-Marquez *et al* found that increased levels of sorted CAR high T cells within the infusion product were associated with decreased responses in different hematological malignancies.[9] This study highlights the link between CAR density and CAR-T cell activity and its impact on clinical outcomes.

The possibility of expressing the CAR through weaker promoters has been explored with opposing results.[10 18 20 68] Ho *et al* investigated the safety and efficacy of anti-CD19-CAR-T cells driven by the synthetic promoter MND (weaker than the EF1α promoter), which contains a myeloproliferative sarcoma virus enhancer.[20] They observed higher viral titers and a reduction in CAR expression driven by MND when compared with the EF1α promoter, but CAR-T cells retained a similar killing ability in vitro and in vivo, with reduced proinflammatory cytokine production. These results suggest that lower levels of CARs on the cell surface may also reduce CRS without compromising CAR-T cell cytotoxicity. The feasibility of this MND promoter is endorsed by Abecma, the first CAR-T product approved against B-cell maturation antigen (BCMA) for the treatment of multiple myeloma. In a different study, Guedan *et al* generated anti-mesothelin CAR-T cells carrying a 4-1BB costimulatory domain and observed a reduction in CAR expression levels when the EF1a promoter was replaced with pGK300, a truncated promoter from the phosphoglycerate kinase gene. Although pGK300-BBz T cells showed benefits in tonic signaling and exhaustion markers, when administered to mice bearing xenograft pancreatic tumors, they were unable to induce any antitumor effect compared with mice treated with untransduced cells. Thus, low CAR surface expression does not always guarantee improved antitumor responses, indicating the complexity of the CAR-antigen interaction, the heterogeneity based on the tumor model used when reaching a threshold that triggers the cytotoxic activity of T cells, as well as the difference in efficacy based on the components that make up the different domains of the CAR and their interaction.

Another strategy for controlling CAR density involves using transcriptional elements to drive a more physiological expression pattern.[10 12 20] CAR expression can also be regulated by targeting a specific endogenous gene and inserting it via homologous recombination into its locus, controlled by its promoter. In 2017, Eyquem *et al* pioneered the generation of universal T-cell receptor alpha constant (*TRAC*)-CAR-T cells by inserting an anti-CD19-28z CAR complementary DNA into the *TRAC* locus using CRISPR/Cas9 technology. This approach made CAR expression dependent on the TCR endogenous promoter, driving a decrease in CAR expression after encountering the antigen. This physiological expression of CAR molecules resulted in reduced signaling in the absence of stimulation and a more memory-like and lower expression of exhaustion markers (PD-1, lymphocyte activation gene-3 (LAG3) and T-cell Immunoglobulin and Mucin-domain containing-3 (TIM3)) of CAR-T cell

products. Consequently, this led to increased antitumor efficacy and decreased toxicity compared with high and uncontrolled levels of CAR generated by gamma retroviral transduction.[12] More recently, Kath *et al* used CRISPR-Cas9 editing to integrate truncated CD3ζ-deficient CARs in-frame into the *CD3ζ* locus,[69] thus causing TCR ablation and bringing the CAR under the transcriptional regulation of the *CD3ζ* gene. Compared with *TRAC*-edited CAR-T cells, CD3ζ-CAR-T cells exhibited comparable effectiveness in tumor killing in vitro but displayed reduced susceptibility to activation-induced cell death and cell differentiation. This was likely attributable to the lower CAR expression levels of this CAR fusion gene in these cells. In the same direction, Mansilla-Soto *et al*[70] went a step further introducing hybrid TCR-CAR receptors into the *TRAC* locus, which were expressed following the TCR expression kinetic. They form a complex that uses both the TCR alpha and beta chains fused with the immunoglobulin heavy and light chains, achieving human leukocyte antigen (HLA)-independent recognition and HLA-dependent signaling.

Additionally, another physiological control system was developed, targeting the CAR construct to the *PDCD1* locus, which generates a feedback loop that links CAR expression to T-cell activation. This system, which produces temporary CAR expression, could aid in the disappearance of CAR-T cells after tumor control, while breaking the immunosuppressive axis interaction PD-1/PD-L1.[71] This strategy improved the cytotoxic capacity both in vitro and in vivo.

Combining the previous approaches, Dharani *et al* generated universal dual inducible CAR-T cells by inserting an anti-fibroblast activation protein (FAP) CAR at the *TRAC* locus (for physiological expression) and an anti-mesothelin CAR at the *PDCD1* locus (for inducible expression).[72] When exposed to FAP, the two CARs exhibited nearly opposite kinetics due to the different expression pattern of TCR and PD-1 molecules on T-cell activation. Anti-FAP CAR interaction with its cognate ligand led to its internalization, while T-cell activation initiated the expression of anti-ML CAR through the *PDCD1* locus. Within 3–4 days of FAP antigen exposure, anti-ML CAR expression was downregulated, and the anti-FAP CAR expression was restored. Still, it would be interesting to study the kinetics when both antigens are presented to better understand the kinetics of the anti-ML CAR when its antigen is also present

To ensure controlled and physiological expression of the CAR using lentiviral vectors, our laboratory designed a chimeric promoter based on the Wiskott-Aldrich promoter (WAS) to express ARI-0001 CAR in T cells.[10 73–79] Previous studies demonstrated that WAS-promoter-based lentiviral vectors exhibit a moderate, physiological and hematopoietic-specific expression pattern.[68–74] In fact, CAR-T cells driven by the WAS chimeric promoter showed a TCR-like expression pattern of the CAR after T cell stimulation. Interestingly, WAS-promoter-driven CAR-T cells maintained a less differentiated phenotype, less tonic signaling, milder secretion of proinflammatory cytokines, and higher specific lysis rates than EF-1 alpha-driven CAR-T cells.[10]

Further consideration for promoter choice is possible silencing in vivo, which would lead to receptor extinction. Multiple epigenetic processes can cause an instantaneous or gradual decrease in gene expression after the differentiation or proliferation of cells transduced with integrating viral vectors, potentially leading to poor clinical responses.[80 81]

CAR DESIGN FOR OPTIMAL EXPRESSION

Even though one of the main ways to control CAR density is through its expression, changing CAR design can also modify CAR density and kinetics. As mentioned above, changing the intracellular lysines of the CAR increases recycling and improves CAR-T cell treatment.[29] In addition, modifications to the positively charged patches on the CAR single-chain variable fragment (scFv) have been demonstrated to modulate the formation of CAR clusters, varying CAR density on the membrane, and causing tonic signaling.[82]

The addition of different elements to the CAR structure has also been shown to control CAR surface expression. Weber *et al* added a destabilizing domain to the CAR, allowing it to be expressed on the surface only when a drug was administered.[83] The temporary pause in CAR surface expression helped to reset the exhausted state of anti-GD2 CAR-T cells through epigenetic changes. In another study, the addition of CTLA-4 tails to the CAR accelerated the CAR kinetics, increasing internalization and recycling, which translated to a better overall T phenotype and antitumor activity.[47] CAR density in the membrane has also been optimized to target tumor cells with low antigen levels by fusing intrinsically disordered regions (IDRs) to the CAR structure, which leads to the formation of biomolecular condensates. The fusion of IDRs to CAR proteins against CD19, CD22 and HER2 increased membrane-proximal signaling of the CARs, which resulted in higher cytotoxicity against low antigen-expressing cancers in vitro and in vivo. Interestingly, no higher tonic signaling was observed.[84]

Taken together, these studies suggest that modifying or incorporating elements within the CHead1AR structure design may be another interesting strategy for controlling optimal surface CAR levels.

METHODOLOGICAL APPROACHES AND TIMING FOR SURFACE CAR ANALYSIS

Determining the most appropriate method for detecting CAR molecules is essential for studying CAR expression levels on the cell surface. However, CAR detection methods can operate at the genomic, transcriptomic and proteomic levels and have been reviewed elsewhere.[85] There are translation-influencing elements and kinetic-related factors (such as CAR biodistribution and turnover

rate) which make detection at the proteomic level the most suitable choice to study CAR kinetics.

In most cases, to detect and quantify successfully transduced CAR-T cells, fluorescent proteins or truncated membrane receptors are incorporated into the vector sequence, typically downstream of the CAR construct, and both transgenes are expressed from a single promoter. Co-expression of various genes in one mRNA can be achieved by means of an internal ribosome entry site (IRES)[86] or 2A elements,[87] differing in the mechanisms involved in each approach. IRES are *cis*-acting RNA elements with the ability to recruit small ribosomal subunits and initiate translation in a 5' cap-independent manner.[86] Although detection of reporter gene expression often correlates with CAR protein levels, it must be considered that the mechanism of 2A-mediated "self-cleavage" was determined not to be due to proteolytic cleavage, but ribosome skipping.[88] Thus, only successful skipping and recommencement of translation results in two "cleaved" proteins. Moreover, the efficiency of ribosomal skipping for each 2A peptide varies between organisms, and it also depends on the 2A position and number of linked coding sequences.[89] Ho *et al*[20] quantified the absolute number of CAR molecules and found that these levels were inconsistent with truncated epidermal growth factor receptor (tEGFR) levels on the surface, with both transgenes separated by T2A. Another aspect to consider is the subcellular localization of the reporter protein. In the case of membrane receptors, turnover must also be considered, as each CAR protein and membrane receptor might differ. This suggests that CAR expression on the cell surface should be measured using CAR-specific detection methods, since the use of 2A elements may not always achieve equal amounts of co-expressed proteins.

Regarding the timing of CAR expression level measurements, we believe it would benefit the CAR-T therapy field to establish a consensus on when to measure the CAR molecules in the membrane. This would facilitate comparisons between studies and improve understanding of different CAR kinetics. It is important to ensure that enough time has passed to eliminate episomal expression of the CAR construct. Additionally, the time post-activation should be considered due to downmodulation kinetics

CONCLUDING REMARKS

In this review, we highlight the importance of modulating CAR protein density on the cell membrane to enhance the efficacy and safety of CAR-T cell therapy. Despite the remarkable success of CAR-T cell therapy in the treatment of relapsed and/or refractory B-cell neoplasms, challenges persist, such as relapse rates, limited efficacy in solid tumors, and severe side effects. Focusing on controlling CAR protein density as a strategy to improve CAR-T cell products, we investigated key factors influencing CAR surface expression and kinetics.

The dynamic nature of CAR expression on T cells is influenced by internalization, recycling, and degradation processes, as well as antigen-related factors such as antigen density and affinity. Emerging strategies to modulate CAR density, including innovative CAR designs and transcriptional control of its expression, hold promise for optimizing CAR-T cell therapy. Specific modifications within the CAR structure have been shown to alter CAR kinetics and should be considered to control optimal surface levels. The generation of CAR-T cells with modulated CAR expression has also been achieved using engineering tools such as promoter-driven lentiviral vectors (LVs) or CRISPR-mediated targeted insertion into specific loci. These tools have upgraded CAR-T cell performance and opened a world of possibilities in the design of future CAR-T cell therapies.

Overall, this review emphasizes the importance of fine-tuning CAR density and dynamics on the T-cell surface. Understanding these factors will pave the way for future research in this area and will enable the development of CAR-T products that are not only safer but also more effective.

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Acknowledgements All illustrations were created using Biorender.com.

Contributors AH-B, CB-J, MT-M, NM-P, PJ-L and FM wrote the manuscript. AH-B and CB-J were the review leaders; MH reviewed the article; PJ-L and FM managed the review.

Funding Instituto de Salud Carlos III (ISCIII) and the European Regional Development Fund (FEDER): Research grants, PI21/00298 and PI24/00888 (FM). Instituto de Salud Carlos III (ISCIII) – NextGenerationEU funds - actions of the Recovery and Resilience Mechanism: Red TerAv RD21/0017/0004 and TerAv+ RD24/0014/0005 (FM). Ministerio de Ciencia e innovación (MICIN). Plan de Recuperación, transformación y resilencia, Centro para el Desarrollo Tecnológico Industrial (CDTI) and European Union-Next Generation EU: Research grants 00123009/SNEO-20191072 (FM), PMPTA22/00060 (FM), DIN2018-010180 (PJ-L) and DIN2022-012828 (CB-J). Consejería de Salud y Familias (CSyF) -Junta de Andalucía - FEDER/European Cohesion Fund (FSE) for Andalucía: Grants: 2016000073332-TRA, CARTPI-0001-201, PECART-0031-2020 y PI-0236-2024 (FM). Ministerio de Ciencia e innovación (MICIN) – líneas estratégicas: Grant PLEC2021-008094 (FM).

Competing interests CB-J, MT-M and PJ-L are contractually associated with LentiStem Biotech S.L. All other authors declare they have no competing interests.

Patient consent for publication Not applicable.

Ethics approval Not applicable.

Provenance and peer review Commissioned; externally peer reviewed.

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