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REVIEW

CAR-Ts: new perspectives in cancer therapy

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Chimeric antigen receptor (CAR)-T-cell therapy is a promising anticancer treatment that exploits the host's immune system to fight cancer. CAR-T cell therapy relies on immune cells being modified to express an artificial receptor targeting cancer-specific markers, and infused into the patients where they will recognize and eliminate the tumour. Although CAR-T cell therapy has produced encouraging outcomes in patients with haematologic malignancies, solid tumours remain challenging to treat, mainly due to the lack of cancer-specific molecular targets and the hostile, often immunosuppressive, tumour microenvironment. CAR-T cell therapy also depends on the quality of the injected product, which is closely connected to CAR design. Here, we explain the technology of CAR-Ts, focusing on the composition of CARs, their application, and limitations in cancer therapy, as well as on the current strategies to overcome the challenges encountered. We also address potential future targets to overcome the flaws of CAR-T cell technology in the treatment of cancer, emphasizing glycan antigens, the aberrant forms of which attain high tumour-specific expression, as promising targets for CAR-T cell therapy.

Keywords: cancer; CAR-T; cell therapy; chimeric antigen receptor; glycans; glycosylation; tumour therapy

Despite all efforts made to better understand the molecular and cellular basis of tumour progression and, consequently, to develop more effective treatments, cancer continues to have a major impact on the society worldwide, remaining one of the leading causes of death. In 2020, according to the World Health Organization (WHO) estimates, more than 19 million cases of cancer were diagnosed, and close to 10 million deaths were attributed to cancer [1]. The high incidence and mortality rates associated with cancer are mainly due to resistance of advanced disease to conventional cancer treatments, such as chemotherapy, radiotherapy and surgical resection.

Immunotherapy, which consists in the exploitation of the patient's own immune system to fight cancer, has recently emerged as the fourth pillar of cancer treatment, mainly due to the introduction of immune check point inhibitors. However, a more sophisticated

Abbreviations
AAL, acute lymphoid leukaemia; ACT, adoptive cell transfer; AML, acute myeloid leukaemia; CAR, chimeric antigen receptor; CSD, co-stimulatory domain; EBV, Epstein–Barr virus; EGFR, Epidermal Growth Factor Receptor; FDA, Food and Drug Administration; Le^y, Lewis y; MAb, monoclonal antibody; MHC, major histocompatibility complex; MM, multiple myeloma; NHL, non-Hodgkin lymphoma; NK, natural killer; scFv, single-chain variable fragment; Siglec, sialic acid-binding immunoglobulin-type lectin; SLe^x, sialyl Lewis x; STn, sialyl Tn; TCR, T cell receptor; TMD, transmembrane domain; TRUCK, T cell redirected for universal cytokine-mediated killing; VH, variable heavy chain; VL, variable light chain.

cell-based immunotherapy is now producing unprecedented results. This strategy consists in the modification of effector immune cells such as T or natural killer (NK) cells, thanks to the improvements in the field of biotechnology and immune cell manipulation over the past decades [2–4]. The principle of cell-based immunotherapy relies on the manipulation and subsequent infusion of immune cells into the patient. In most cases, such cells are isolated from the same patient (autologous), but can also come from a donor as primary material (allogeneic) or as a cell line (e.g. NK-92). Their tumour-targeting efficiency is increased by means of *ex vivo* genetic modification.

Within the repertoire of immunotherapeutic approaches against cancer, this review will focus on the adoptive cell transfer (ACT) method, which consists in the transfer of immune cells previously modified to express receptors that recognize specific markers presented by cancer cells so as to trigger a specific and effective anticancer immune response (Fig. 1) [5]. Due to their central role in cell-mediated immune response, T lymphocytes, also called T cells, are the cells of choice for genetic manipulations aiming at cancer treatment, such as the introduction of a therapeutic T-cell receptor (TCR) [6] or a chimeric antigen receptor (CAR) (Box 1). Both TCRs and CARs are transmembrane proteins that bind their cognate targets through their extracellular domain and bear signal transduction capacities that trigger the cytotoxic functions of host effector T cells [7–9]. Thus, these receptors have to be highly specific to distinguish malignant versus healthy cells and sensitive enough to activate cytotoxic signalling pathways upon engagement with a cell surface antigen displayed by a target cell. The main difference between engineered TCRs and CARs relates to their mode of recognition: CARs can only recognize surface antigens, whereas TCR binding relies on the presentation of a peptide on the major histocompatibility complex (MHC), which involves molecular compatibility between patient and donor. However,

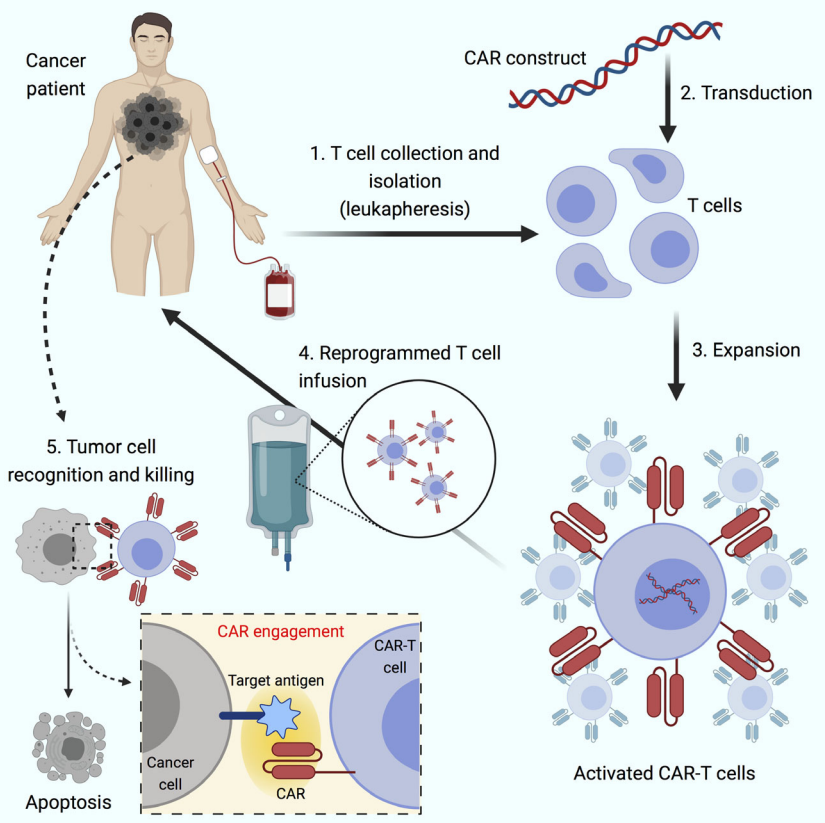

Fig. 1. Principle of adoptive cell therpy (ACT). Peripheral blood lymphocytes are collected and isolated from the patient, a procedure known as leukapheresis (1). The T cells are subsequently transduced with an expression vector, typically of viral aetiology, such as gamma retroviruses or lentiviruses, to introduce the chimeric antigen receptor (CAR) of interest (2). After a period of *in vitro* expansion and activation of the engineered cells (3), the resulting CAR-T cells are infused into the patient (4). This product is expected to recognize the target antigens expressed at the surface of cancer cells and initiate a cascade of events that will trigger tumour cell killing (5).

TCR-specific peptides can be derived from any cellular protein, which greatly increases the spectrum of recognition [10].

The potential of CAR-Ts as an effective cancer therapy has been robustly demonstrated for liquid tumours. For instance, high remission rates in acute lymphoid leukaemia (ALL) [11] and non-Hodgkin lymphoma (NHL) patients [12] were reported when the B-cell marker CD19 was targeted by a specific CAR [13]. Nevertheless, regardless of the groundbreaking and encouraging clinical performance of the anti-CD19 CAR in the treatment of haematological malignancies, it has been challenging to develop such an efficient and safe genetically modified T-cell immunotherapy for solid tumours and other types of liquid tumours, such as acute myeloid leukaemia (AML) and multiple myeloma (MM). The main difficulties encountered are the lack of cancer-specific cell surface markers and the immunosuppressive nature of the tumour microenvironment [7,14]. With this in mind, and despite all the progress towards the understanding of tumour immunology and of the factors determining the safety and efficacy of these therapies, the discovery of cancer-specific antigen cell surface targets is imperative for the generation of promising new immune cell therapies against cancer.

Glycoconjugates, a complex and abundant group of carbohydrate-based biomolecules displayed by all living organisms, constitute major and promising molecular targets in cancer therapy. The biosynthesis of glycan chains and their attachment to target molecules, known as glycosylation, constitutes a tightly regulated cellular process. Glycans can be attached to different types of macromolecules, such as proteins and lipids, giving rise to the glycoconjugates that densely decorate every cellular surface [15]. In cancer context, genetic and epigenetic mechanisms can lead to major alterations in the cellular glycosylation profile, or glycome, originating the so-called cancer-associated carbohydrate antigens. Common modifications include the expression of short-truncated *O*-glycans and the increased expression of sialylated and fucosylated terminal glycan structures that are frequently associated with tumour development and progression [16]. The aberrant tumour cell surface glycan repertoire (glycome) constitutes a promising source of molecular candidates for the development of novel CAR formulations, as illustrated by the development of several highly specific glycan-directed monoclonal antibodies (mAbs). However, the pre-clinical and clinical application of glycan-directed CAR-T cells remains largely unexplored.

The present review will thoroughly discuss the CAR structure, design and application in cancer therapy. We will also discuss underexplored yet promising molecular targets, with particular emphasis on glycan-based antigens, which might help overcoming the technical and clinical shortcomings of CAR technology in the cancer treatment.

CAR basic concepts

A CAR construct was first described 30 years ago when Kuwana and colleagues associated the antigen recognition domain of an antibody to the constant regions of a TCR while exploring the signalling machinery required for T-cell activation [17]. Since then, although the structure and composition of a CAR's cytoplasmic domain has evolved, the overall configuration has remained largely the same.

Briefly, CARs are synthetic receptors with pre-specified properties resulting from the combination of different functional subdomains. These receptors are typically composed of four regions: an extracellular antigen-binding domain, an extracellular hinge region, a transmembrane domain (TMD), and an intracellular signalling module (Fig. 2). Due to their design, CARs provide the ability to detect pre-defined antigens with a high degree of specificity and trigger the effector functions found in natural immune cells [7–9].

Each of the regions that make up a CAR construct confers different capabilities to the receptor, influencing not only its expression and stability, but also the avidity of ligand–receptor interactions and, consequently, the downstream cell signalling and effector cell's response [9]. Below, we explore the composition of these receptors and illustrate how subtle alterations in their composition deeply impact the function and clinical efficacy of the CAR-T cells.

Extracellular antigen-binding domain of CARs

In a classical design, the ectodomain of a CAR is the region that dictates its specificity. Most of CAR's ectodomain compositions tested nowadays have been designed from mAbs' single-chain variable fragments (scFvs), although peptides, nanobodies and ectodomains of specific proteins have also been exploited [18]. scFvs are ideal targeting tools in the sense that they are small, single-chained and are supposed to conserve the affinity and the specificity of the antibody they were built from (Fig. 2). These variable regions consist of two chains – heavy (VH) and light (VL) – connected via a flexible linker sequence [8,9,19].

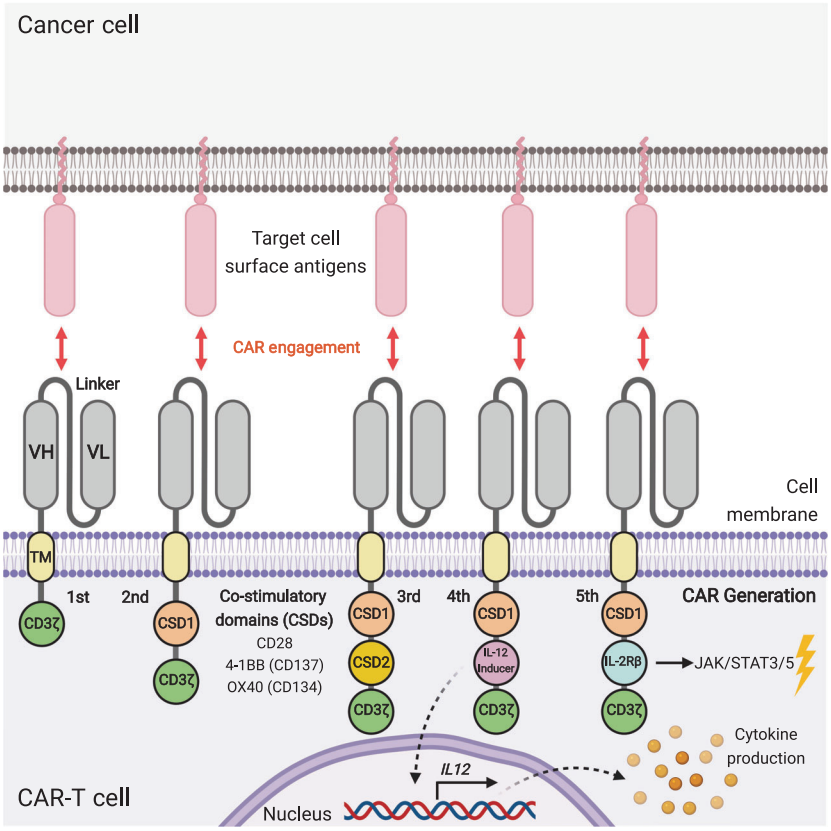

Fig. 2. Schematic diagram of the five different generations of CAR-T cells. A CAR construct is typically composed of four regions – an extracellular antigen-binding domain, commonly a single-chain variable fragment (scFv) fragment composed of variable light (VL) and heavy (VH) chains connected via a flexible linker sequence, an extracellular hinge region, a transmembrane domain (TMD) that anchors the CAR to the cell membrane and an intracellular signalling module. CARs can be classified according to the complexity of their endodomain, being grouped into five different generations. First-generation CARs present a single activation signalling motif, typically derived from a CD3-ζ chain. Second- and third-generation CARs include one or two co-stimulatory domains (CSDs), respectively, such as CD28, 4-1BB (CD137) or OX40 (CD134). The fourth-generation CARs, based on second-generation CARs, contain a constitutive or inducible expression cassette, leading to the transcription of a protein, such as IL-12. Fifth-generation CARs, also based on second-generation CARs, present intracellular domains of cytokine receptors, such as the IL-2 receptor β-chain (IL-2Rβ) chain fragment, with a STAT3/5 binding motif.

The orientation (VL or VH first) and the size of the linker have also been shown to impact the CAR potency [20]. Importantly, not all scFvs retain their ability to bind their cognate epitopes as seen in the original antibody, which can be explained by an ineffective cell surface expression, loss of binding specificity or even due to protein aggregation via Fv fragments [9,21].

The extracellular antigen-binding domain of these engineered receptors has already been vastly studied and, thus, several studies have reported its contribution to overall CAR performance. In 2011, Chmielewski and colleagues showed that the amount of antigen and the CAR's target binding affinity are decisive elements for the successful activation of CAR-T cells, and further demonstrated that the cytolytic efficacy was not considerably affected by the addition of co-stimulatory modules to the receptor's intracytoplasmic domain [22]. Later, Liu et al. have developed different CAR constructs with scFvs with variable affinities against ErbB2, revealing that a CAR's scFv depicting greater affinity does not necessarily reflect improved therapeutic efficacy, and could even be more harmful to the patients compared to a CAR with a binding domain bearing reduced target affinity [23]. These two examples reflect the enormous importance of an appropriate choice of the antigen-binding domain when performing rational CAR design.

Hinge region of CARs

In addition to the antigen-binding region that defines a CAR's specificity, these receptors also display in

their extracellular domain the so-called hinge or spacer region. The hinge region of a CAR is often adapted from spacer regions found in natural receptors, such as IgG, CD28 and CD8α [24]. As the name implies, the spacer region represents a connecting sequence between the ecto- and TMDs, and impacts a CAR's function by defining important structural properties, such as receptor's length, flexibility and even membrane stability [8,9]. Of note, the hinge length can be connected to the position of the epitope on the target protein, as suggested for CD22 [25]. Consequently, the design of this region should be wisely considered when developing a CAR.

A previous study has evaluated different scFvs targeting distinctive antigens, with and without spacer regions [26]. This work showed that, depending on the target epitope, the presence or absence of the connecting sequence could or could not be, respectively, beneficial for the binding efficiency, by determining the epitope's distance to the CAR-T cell's membrane [26]. A recent study with an anti-CD37 CAR has further demonstrated how the size of the hinge can impact not only the target recognition, but also the CAR activation. Here, the authors showed that a certain length (and composition) of the hinge could improve both these issues [27]. Unfortunately, no universal model of hinge region has been proposed and each new CAR should ideally be tested with a panel of distinct spacer candidates.

Transmembrane domain of CARs

The TMD of a CAR construct, usually adapted from common immune cell receptors such as CD3-ζ, CD4, CD8 or CD28, is responsible for anchoring the CAR to the cell membrane, and connecting the hinge region to the CAR's cytoplasmic domain [8].

Recently, a study aiming to understand the impact of this domain in the activity of the CAR-T cell developed CAR constructs with different TMD regions. This work demonstrated that the TMD has a significant impact on the CAR surface expression levels and stability [20]. An additional study has generated anti-CD19 CAR-T cells with distinct TMD to further comprehend their role in CAR-T cell activation. The results showed that CARs containing a CD28-derived TMD have the ability to establish heterodimers, as opposed to CD8-derived TMD, which lack such capacity. These results support that the TMD of a CAR is crucial for receptor dimerization and consequent modulation of the response of host effector T cells [28].

Cytoplasmic region of CARs

The cytoplasmic domain of a CAR, or the signal transduction region, is responsible for the triggering of T-cell activation, and further steers the direction and regulates the intensity of the effector T cell's cytotoxic response. In general, upon antigen binding to the extracellular domain, T-cell activation occurs via the phosphorylation of immunoreceptor tyrosine-based activation motifs (ITAMs) present in the cytoplasmic CD3-ζ domain, which is the activation domain most commonly found in CARs [29,30].

The composition of the cytoplasmic domain can be manipulated to enhance a CAR's specificity, potency and safety, giving rise to five different generations of CARs (Fig. 2). First-generation CARs were characterized by a single activation cytoplasmic domain. Second- and third-generation CARs contain one and two co-stimulatory modules, respectively. Fourth-generation CARs bear additional immunomodulatory properties, by harbouring a cytoplasmic module that sustains the release of transgenic cytokines. Fifth-generation CARs exhibit improved anticancer cytotoxic properties, due to the addition of signalling subdomains of cytokine receptors that activate cytokine-dependent signalling pathways [31].

First-generation CARs display the most minimalist composition and carry a single activation signalling motif, typically derived from a CD3-ζ chain [29,30]. Although this primary signal region is sufficient to activate T cells upon phosphorylation of ITAMs, and consequently trigger an effective response against cancer cells, it is unable to induce complete tumour cell eradication [32]. To enhance CAR-T cell efficacy towards cancer cells by modulating the persistence, proliferation and/or cytokine secretion capacities of engineered T cells, the second-generation of CARs emerged [9]. These receptors feature not only the CD3-ζ primary signal region common to first-generation CARs, but an additional co-stimulatory domain (CSD), typically derived from the CD28, 4-1BB (CD137) or OX40 (CD134) natural molecules [33,34]. Usually, CSDs are found near the cell membrane where they are able to establish interactions with their cognate signalling partners [9]. Currently, most of chimeric receptors approved for clinical use are second-generation CARs [35]. It is important to note that different CSDs lead to an increase in T-cell activity through the activation of different mechanisms [36], which should be carefully considered during CAR design.

In order to further increase the potency of CAR-T cell therapy, the third generation of CARs emerged

from the combination of different CSDs. This type of design takes advantage of the different signalling activities of distinct co-stimulatory molecules. However, it might also impact the expressing cells by allowing a robust tonic signal which will directly affect the clinical outcome [9].

The fourth- and fifth-generation of CARs are both based on the second-generation, but contain additional intracellular domains that confer enhanced antitumour properties to these receptors. The fourth-generation CARs, owing to the presence of cytokine secretion domains, are able to promote constitutive or inducible expression of desired inflammatory cytokines, such as the IL-12. T cells that are manipulated to express fourth-generation CARs are denominated T cells redirected for universal cytokine-mediated killing (TRUCKs) and have been explored for the treatment of several malignancies in pre-clinical models [37–39]. The CARs of fifth- and latest generation display a truncated cytoplasmic IL-2 receptor β-chain domain and a binding moiety for the transcription factor STAT3/5. These receptors are advantageous as they provide the three essential signals for a complete T-cell response – CAR engagement, co-stimulatory and cytokine-driven JAK-STAT3/5 signalling [40]. Although promising, this approach is still in its initial stages of development and further studies need to be performed to better understand its potential clinical applications.

Overall, the four composing regions of a CAR construct – the ectodomain, hinge region, TMD and cytoplasmic portion – are closely related and there is no standard configuration applicable to all cases, always requiring several pre-clinical tests in different models to finely tailor the receptor's specificity and efficacy.

A quick look at the CARs in the clinic

Given the versatility of CARs' structural composition, it is possible to redirect CAR-T cells to specifically target tumour-associated antigens and mount an effective antitumour immune response, by improving the ineffective host antitumour immune activity and overcoming the immunosuppressive tumour microenvironment.

In recent years, several clinical trials with CAR-T cells have been conducted, most of them being directed towards the treatment of haematological malignancies. The greatest success recorded in the history of CAR-Ts involves the CD19 antigen, a common marker of B cells [13]. Since CD19 expression is restricted to B cells, both normal and malignant, and their depletion can be effectively achieved by administering intravenous anti-CD19 immunoglobulins [41], this molecule represents a safe therapeutic target. Various clinical trials using anti-CD19 CARs were conducted and revealed impressive remission rates in patients with different types of haematological malignancies, including paediatric refractory or relapsed ALL [11] and NHL [12]. In 2017, following the demonstration of the antitumour efficacy and the survival and persistence rates of anti-CD19 CAR-T cells in pre-clinical *in vitro* and *in vivo* assays and several impressive results in phase I and II clinical trials, the U.S. Food and Drug Administration (FDA) approved Tisagenlecleucel (market name Kymriah®) as the first CAR-T cell therapy [42]. From then until early 2021, two other CAR-T drugs against CD19 – Axicabtagene Ciloleucel (market name Yescarta®) and Brexucabtagene Autoleucel (market name Tecartus™) – have been granted FDA approval [43,44]. Despite the encouraging results achieved with the CAR-T cell technology directed to the CD19 antigen, it is important to mention that CAR-based T cell formulations exhibit several drawbacks, including adverse and toxic clinical effects caused by an uncontrolled cytokine secretion occurring in excessive antitumour responses [45], as well as tumour relapse due to CAR-T cell exhaustion or the lack of antigen-presenting cancer cells [46].

Following the encouraging clinical performance of the CAR-T cell technology in the treatment of liquid malignancies, several efforts have been made to achieve similar success against solid tumours. Numerous clinical trials have interrogated the application of this immunotherapeutic approach on solid tumours, with the CAIX enzyme [47] and HER2 oncogenic receptor [48] being some examples of the selected molecular targets.

In an attempt to develop a CAR-T cell therapy against HER2, a member of the epidermal growth factor receptor (EGFR) family overexpressed in many human cancers [49–51], a CAR was developed based on the clinically used anti-HER2 mAb trastuzumab. After only a few hours, the treatment resulted in the patient's death probably due to the recognition of residual HER2 in the patient's lung tissues by the engineered T cells [48]. Similarly to what happened with the HER2-targeting CAR-T cells, a trial using T cells targeting CAIX, an enzyme overexpressed in kidney cancer, resulted in severe liver toxicity due to the low-level expression of CAIX in the normal biliary tree [47].

Despite extensive clinical evidence accounting for the efficacy of the CAR-T cell technology for cancer treatment, it is evident that serious issues remain. In the light of this, the continuous search for safer and more effective and personalized strategies is imperative.

Critical issues in CAR-T cell technology for cancer therapy

There is a series of challenges with regard to the CAR-T cell treatment efficiency, such as the persistence, survival and function of the T cells, as well as issues related to inherent tumour heterogeneity, which compromises the therapeutic efficacy of such T cell-based formulations.

The ACT strategy is a multistep process that involves the collection of the immune cells and their expansion and activation *in vitro* for long periods before and after genetic engineering (Fig. 1) [19]. Consequently, the ability of CAR-T cells to proliferate and persist after their infusion into the patient is determinant for therapeutic success. In fact, some studies have shown that enhanced CAR-T cell persistence and survival actively promotes a better therapeutic response [52,53].

Moreover, the infused T cells may lose their effector functions as a result of T-cell exhaustion. This phenomenon can be driven by different factors, such as persistent antigen stimulation, which leads to T-cell hyperactivation, the presence of certain CSDs and negative regulation by inhibitory receptors and immunosuppressive factors [54]. As previously mentioned, this can also be reminiscent of a strong CAR-dependent tonic signal.

The long-term efficiency of CAR-T cell-based treatment can be compromised by the tumour cells once they manage to downregulate or even lose all the expression of the target antigen. In 2014, Maude and colleagues reported that a group of patients who received CAR-Ts targeting CD19 initially showed a great response but subsequently relapsed due to the presence of malignant B cells lacking CD19 expression [13]. Similarly, a study reported that the treatment of HER2-positive cells with the anti-HER2 mAb trastuzumab leads to the downregulation of the target molecule and, as a resistance mechanism, the concurrent overexpression of additional oncogenic receptors, such as EGFR and MET [55]. The mechanisms by which such dramatic loss of tumour antigens takes place remain to be understood, but we envision that cancer stem cells and tumour heterogeneity may play a role.

Regarding the safety of CAR-T cell technology, concerns include the potentially dangerous side effects, such as on-target off-tumour toxicity, and cytokine release storms [7,8]. Regarding the former, the most decisive factor for the success of the CAR-T cell therapy is the target antigen recognized by the chimeric receptor. Ideally, this target should be exclusively expressed by the tumour cells to avoid immune responses against non-malignant cells and tissues. However, most of the times this cannot be achieved, as the target antigen can also be residually expressed by normal cells. The cytokine release storms, an uncontrolled secretion of cytokines that results from a rapid and intense CAR-T cell-derived response following antigen binding, may result in severe inflammation and morbidity [7,19]. Recent studies have shown that the use of anti-IL-6 mAb tocilizumab can control the secretion of cytokines, such as IL-6, without affecting CAR-T cell efficacy [45]. Nonetheless, additional solutions are warranted to circumvent the safety- and toxicity-related obstacles challenging the clinical implementation of CAR-T cell technology.

Emerging improvements of CAR-T cell therapy

Despite all the progress made in the field of protein and CAR design, different types of toxicity are still a major concern incontrovertibly linked to CAR-T cell therapy, as discussed in the previous section. In this sense, several approaches have been proposed to improve the safety associated with CAR-based anticancer therapies [56], such as the inclusion of suicide genes within the receptors and the development of advanced systems, including the dual and the switchable CAR systems [56].

One of such strategies relies on the incorporation of inducible cell death-associated genes in CAR-T cells, as is the case of caspase 9. These caspase-based systems are engineered to express a binding protein domain that dimerizes when activated by a chemical inducer of dimerization, such as the AP1903 synthetic drug and, consequently, to induce T-cell apoptosis. In this way, when the mounted antitumour response becomes excessive, it becomes possible to eliminate the reactive T cells, at the expense of reducing CAR-T cell efficiency [57–59].

To minimize the off-tumour toxicity associated with several CAR-T cell therapies, dual CAR systems have been developed. The concept of this approach relies on the design of a CAR construct with two different extracellular domains with specificity against distinctive antigens that need to be simultaneously expressed by the tumour cells. These extracellular domains are associated with different signalling regions, an activation domain and a CSD [60–63]. Ideally, CAR-T cells displaying such constructs will only produce a strong antitumour response when bound to cells that express both antigens. Although this system has already been implemented, residual signalling through the binding of only one antigen was still observed, generating

enough CAR-T cell activity and, consequently, off-target damage [60,62].

Another way to prevent unwanted CAR-mediated signalling is the use of the so-called switchable CARs. This system consists of using an exogenous signal, such as a small molecule, to control the activation and antigen specificity of the CAR-T cells. Wu and colleagues have incorporated a switch that activates the T cell when triggered by both said molecule and the target antigen expressed by the tumour cells [64]. Using this positive regulation system, it is possible to control the potency of the CAR-T cells by tightly controlling the signalling molecule dosages.

The need for cancer-specific antigens

The ideal target for a CAR-T cell would be a molecule specifically expressed at the cellular surface of neoplastic cells, and essential for cancer development and progression. Although there is a vast list of candidates, few antigens meet the requirements for a successful CAR-T cell therapy, especially in the solid tumour setting. The identification of such molecular targets with sufficient cancer-specific expression, although proven rather challenging, would represent an extraordinary advance in the field of CARs. These required characteristics of the molecular target open a window of opportunity for the development of CAR-T cells targeting cancer-associated glycan antigens (Box 2) [65].

Aberrant glycosylation, an ubiquitous and fundamental molecular hallmark of both liquid and solid tumours, leads to the emergence of a dense array of cancer-specific glycan antigens that are mainly restricted to the plasma membrane of cancer cells [16,66,67]. Altered glycan expression has been described in cancer for decades and has been applied in the clinical setting for prognostic and disease monitoring purposes [16,68]. Genetic and epigenetic alterations occurring in cancer lead to changes in the expression of glycosyltransferases [69]. These enzymes control the biosynthesis of glycans, leading to an aberrant expression of specific carbohydrate structures in cancer cells that are absent or show neglectable expression in healthy tissues (Fig. 3) [66,67,69].

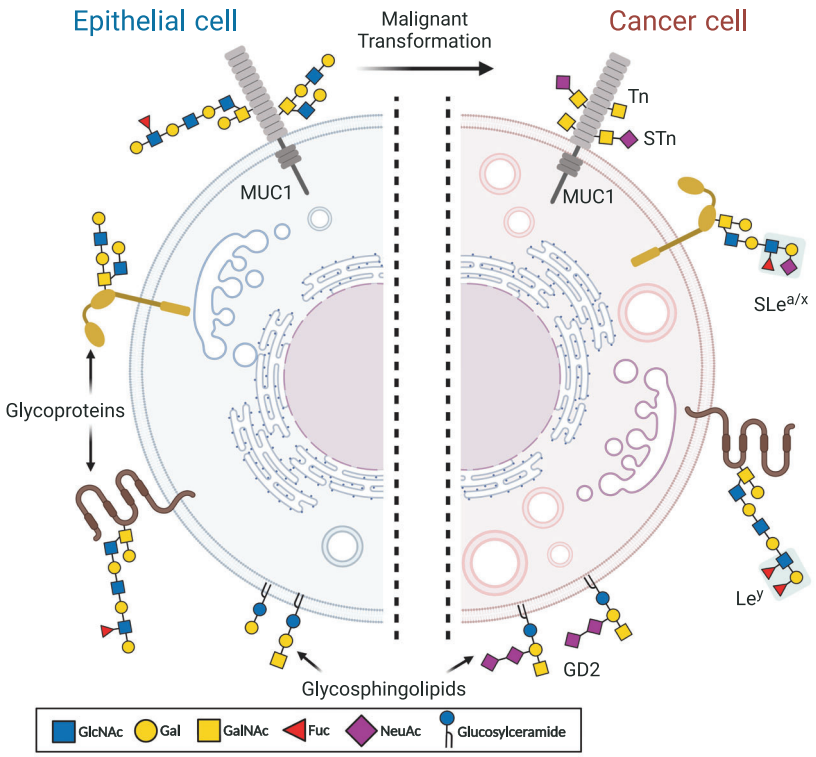

Fig. 3. Tumour-specific carbohydrate antigens are potential targets for CAR-T cells. Genetic and epigenetic alterations occur during the development and progression of cancer, leading to the expression of the so-called cancer-associated carbohydrate antigens, which vastly decorate the surface of cancer cells. These structures, namely, the truncated *O*-glycans Tn and sialyl Tn (STn), and both neutral and sialylated Lewis antigens such as Lewis y (Le^{y}) and sialyl Lewis x (SLe^{x}), have been used as targets for the development of CAR-T cells. Aberrant glycan motifs comprising specific proteins and lipids, such as MUC1 modified with STn and highly sialylated GD2, respectively, also represent promising cancer therapeutic targets.

Oncogenesis-induced aberrant glycosylation can, therefore, generate a wide range of candidate epitopes, exposed at the cell surface, eligible for the development of highly specific CAR-based therapeutic strategies. Several methodologies have been used to identify the aberrant glycosylation occurring in cancer, including the use of lectin/antibody-based detection of aberrant glycans, as well as mass spectrometry-based glycomics and glycoproteomics [70,71]. These cancer-specific carbohydrate structures include the short-truncated *O*-glycans Tn and sialyl Tn (STn) that arise from incomplete *O*-glycan biosynthesis, and both neutral and sialylated Lewis antigens such as Lewis y (Le^y) and sialyl Lewis x (SLe^x) [15,16]. In addition, aberrant glycan motifs comprising specific peptidic sequences, such as MUC1 glycopeptides modified with STn [16], are also promising cancer targets.

The development of antiglycan antibodies with high specificity is a challenging process. Recently, a study has identified scFv variants with higher affinity for the Tn epitope and broadly reactive towards a variety of Tn-presenting glycoproteins, allowing the genetic modification of T cells with respective novel glycan-directed CARs. These engineered T cells demonstrated strong cytotoxic responses against both mouse and human cancer cell lines defective in *O*-linked glycosylation [72].

In addition to the Tn epitope, the corresponding sialylated glycan structure – the STn antigen – also represents an ideal candidate for CAR-T cell therapy. This truncated *O*-glycan epitope depicts high tumour specificity and is associated with a variety of glycoproteins, such as the tumour-associated glycoprotein TAG72, commonly overexpressed by different cancer types. Previous studies have reported that the anti-TAG72 CAR-T cells have the ability to selectively elicit a potent cytotoxic response and to inhibit tumour expansion in xenograft models [73]. However, clinical studies showed a heterogeneous clearance rate of the anti-TAG72 CAR-T cells and a challenging T-cell trafficking to the TAG72-expressing metastatic sites [74].

Cell surface hypersialylation is a common feature of tumour cells that actively supports the establishment of an immunosuppressive microenvironment [15,75,76]. The membrane-expressing sialylated antigens support cancer cell immune evasion through the establishment of a multitude of inhibitory synapses with the sialic acid-binding immunoglobulin-type lectins (siglecs), which constitute transmembrane protein receptors expressed at the cell surface of immune effector cells. The incorporation of sialic acid-binding domains naturally found on siglecs, such as siglec-7 and siglec-9, in the structure of the CAR construct allows the resulting genetically modified T cells to detect and eliminate the cancer cells displaying cell surface sialoglycan antigens, both *in vitro* against different cell lines and *in vivo,* in a tumour xenograft model of melanoma [77].

CAR-Ts designed against gangliosides, a group of sialylated glycosphingolipids, such as the case of the GD2 di-sialoganglioside, have also been developed for the treatment of neuroblastoma. In particular, a CAR has been engineered in Epstein–Barr virus (EBV)-specific cytotoxic T lymphocytes for improved co-stimulation [52]. Such CAR-T cells have improved survival rates compared to control cells lacking viral specificity, and their injection led to significant tumour regression and/or necrosis in half of the tested individuals. Furthermore, such CAR-T cells exhibited prolonged low-level circulatory persistence, which was robustly correlated with improved patient clinical outcome [78]. Several phase I/II clinical trials are underway to evaluate the therapeutic potential of GD2-targeting late-generation CAR-T cells in the treatment of various GD2-expressing solid tumours (NCT03356782; NCT03635632; NCT04539366).

The Le^y tetrasaccharide antigen, although depicting residual expression in healthy tissues, has also been considered a suitable target candidate for CAR-T cells due to its aberrant overexpression on the surface of cancer cells of the majority of epithelial-derived tumours [79,80]. In fact, a previous study has reported that T cells bearing a CAR against Le^y have the ability to proliferate and secrete cytokines *in vitro* and also can lead to tumour growth inhibition when delivered to xenograft mice models of ovarian cancer [81]. A phase I clinical study exploring autologous anti-Le^y CAR-T cell therapy for the treatment of a small group of patients with AML reported that two patients achieved partial remission. In the subjects with the most favourable clinical outcome, CAR-T cells trafficking to the bone marrow were observed, with concomitant circulatory CAR-T cells persistence [82]. Currently, CAR-Ts targeting this di-fucosylated glycan structure are under evaluation for the treatment of Le^y-positive solid and liquid tumours (e.g. NCT03198052, NCT03851146).

MUC1 is a transmembrane glycoprotein overexpressed in most carcinomas. In the cancer context, this mucin is aberrantly glycosylated, enriched in Tn- and STn-containing glycopeptides. Previous studies have developed antibodies that are directed to glycopeptide epitopes, providing highly specific antibodies against cancer-associated glycoforms [83]. Through the manipulation of the previously developed 5E5 antibody directed to a glycopeptide epitope [83], Posey and colleagues engineered a T cell with a CAR targeting the

cancer-associated Tn and STn antigens bound to MUC1. This novel CAR successfully demonstrated to specifically bind to its cognate target *in vitro* and capable of inhibiting tumour growth in xenograft models of several cancers [84]. Due to its potential therapeutic efficacy, the performance of CAR-T cells against this abnormal MUC1 glycoform is currently being evaluated in a series of clinical trials for the treatment of different malignancies (e.g. NCT03525782, NCT03633773).

Glycoengineering strategies are being applied for the optimization of CAR-T cell homing and tissue colonization [85]. During inflammation, selectins are expressed on endothelial cells and, through the interaction with the tetrasaccharide SLe^x-containing glycoproteins expressed on circulating leucocytes, they promote lymphocyte rolling, arrest and endothelial transmigration to an infection or inflammatory site [86]. Attempts to mimic the process of vessel extravasation have been applied to CAR-T cell therapy. In fact, *in vitro* manipulation of CAR-T cells leading to the expression of SLe^x, the E-selectin ligand, led to an increased infiltrative capacity of CAR-Ts into bone marrow following intravascular administration into mice [87].

Concluding remarks

Over the past few years, the field of CAR-Ts has demonstrated substantial therapeutic potential, mainly for the treatment of haematologic malignancies. However, there are still numerous challenges undermining the effectiveness, safety and widespread clinical implementation of CAR-T technology for the treatment of other tumour types, such as solid neoplasms. These limitations could be attributed in part to the lack of cancer-specific antigens. Indeed, most of the surface antigen discovery platforms have focused on cancer-related proteins. It is widely recognized that the glycosylation profile of cancer cells is strongly affected during tumour development and progression. Therefore, the tumour cell surface glycome and glycoproteome arise as ideal, yet still poorly explored source of tumour-specific target antigens. Although challenging to produce, antibodies specific for these altered carbohydrate groups should be developed and further conceived as a potential novel class of innovative CAR molecules.

Box 1. Chimeric antigen receptors

Chimeric antigen receptors (CARs) are genetically engineered proteins composed of an extracellular antigen-binding domain, usually in the form of a single-chain variable fragment (scFv), an extracellular hinge region, a transmembrane (TMD) and an intracellular T-cell signalling module. Due to their design, CARs provide both the ability of antibodies to detect predefined antigens with a high degree of specificity and the effector functions of T cells.

Box 2. Glycans

Glycans constitute the most complex and abundant group of molecules in living organisms. Also denominated as oligosaccharides or carbohydrates, glycans can be found at the surface of all living cells, playing important roles in different biological processes, such as in cell–cell and cell–matrix interactions and recognition, pathogen infection and in the modulation of protein function and signalling. The enzymatic process responsible for the synthesis of these glycan structures is a very complex and organized process known by post-translational glycosylation.

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