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ARTICLE

Moving Receptor Redirected Adoptive Cell Therapy Toward Fine Tuning of Antitumor Responses

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Adoptive cell transfer (ACT) is emerging as a powerful modality of cancer treatment. While ACT has proved able to induce massive clinical responses, genetic modification of T lymphocytes further improved clinical responses obtained. One of the major current limitations of ACT is the inability to discern healthy from malignant cells, leading to on target/off tumor responses that can limit its application. We here discuss some of the approaches currently under development and potential solutions to circumvent these limitations and extend this potentially curative therapy to different tumors by targeting a variety of antigens.

Keywords: adoptive cell therapy, cancer, chimeric antigen receptor, gene therapy, immunotherapy, off-target, tumor antigen

Abbreviations: ACT: adoptive cell transfer; BBIRs: biotin-binding immune receptors; CAIX: carbonic anhydrase IX; CAR: chimeric antigen receptor; CEA: carcinoembryonic antigen; CRS: cytokine release syndrome; CTLA4: cytotoxic T-lymphocyte antigen 4; FAP: fibroblast activated protein; GVHD: graft versus host disease; HLA: human leukocyte antigen; HMW–MAA: high molecular weight – melanoma-associated antigen; HSV–TK: herpes simplex virus – thymidine kinase; iCasp9: induced caspase 9; IL: interleukin; MAGE: Melanoma-associated antigen; MART-1: melanoma associated antigen recognized by T cells 1; MHC: major histocompatibility complex; NCI: National Cancer Institute; NK: natural killer; PD-1: programmed death 1; PD-L1: programmed death ligand 1; rdACT: receptor redirected adoptive cell transfer; scFv: single chain fragment variable; SHP-1: Src homology region 2 domain-containing phosphatase-1; TAA: tumor-associated antigens; Tan-CAR: tandem CAR; TCR: T-cell receptor; TGF-β: transforming growth factor β; TIL: tumor infiltrating lymphocytes; TNFα: tumor necrosis factor α; VEGFR: vascular endothelial growth factor receptor

INTRODUCTION

Adoptive cell immunotherapy is emerging as a powerful methodology for cancer treatment. It has initially relied on the *in vitro* expansion and reinfusion of tumor

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infiltrating lymphocytes (TILs) isolated from tumor biopsies, particularly from patients with melanoma. This approach is based on the concept that TILs recognize tumor antigens through the interaction of their T-cell receptors (TCRs) with tumor antigen-derived peptides loaded in HLA class I (or II in certain tumors) molecules. Response rates of approximately 50% were achieved when patients with melanoma received the cells in combination with a non-myeloablative chemotherapy, and up to 72% when total body irradiation was added [1, 2]. Despite these striking results, the extension of this approach to other types of tumors is limited by lack of resectable lesions, inability to generate TIL cultures that recognize the tumor, and functional impairment of the expanded cells [3, 4].

The identification of the proper tumor antigens, the derived peptides loaded in HLA class I molecules and the TCR receptors recognizing these antigens has allowed different groups to bypass the hurdle of expanding very low-frequency TILs by simply transferring the tumor antigen-specific alpha and beta TCR chain sequences into polyclonal T cells harvested from patient aphaeresis. This strategy was successful in several preclinical studies and was used in patients with melanoma and synovial cell sarcoma, achieving objective clinical responses in a fraction of them [5, 6]. However, this approach relies in the TCR-HLA + peptide interaction and is thus limited to peptides + HLA allele combinations that are well established, limiting the clinical application to the most well-known (and more frequent) HLA alleles.

In the early 1990s, Zelig Eshhar and colleagues hypothesized that a fusion molecule carrying an antigen recognition domain derived from an antibody and the signaling body from a TCR could function as a mean of redirecting T-cell specificities in a HLA independent fashion [7]. These molecules were named T bodies or chimeric antigen receptors (CARs) and offer potential advantages such as high affinity, possibility to directly target any membrane antigen (protein, carbohydrate, or lipids), and customizable intracellular signaling domains. Preclinical studies showed that the addition of costimulatory domains derived from CD28 and/or 4-1BB increase the *in vivo* persistence and efficacy of the infused cells [8–10]. These results prompted researchers to initiate phase I clinical trials targeting several antigens in diverse tumor types, and striking results were obtained using anti-CD19 CAR for the treatment of chronic lymphocytic leukemia and acute lymphoblastic leukemia [11–15]. These results pushed into the field many new groups, biotech companies, and the pharma industry in an attempt to expand CAR-based therapy applicability both in terms of number of patients treated and tumor types approached. To date, more than 30 clinical studies are underway, including phase II clinical trials (http://www.clinicaltrials.gov/ct2/results?term=chimeric+antigen+receptor&pg=1). The long-term cancer remissions observed in protocols based on immune functions place immunotherapy as one approach capable of inducing clinical cure for some tumors [16], with the deeper understanding of this process showing potential to extend this benefit to most of the patients.

Specificity Still Limits Clinical Safety and a Broader Application of Redirected T Cells

Although the astonishing results reported so far pushed gene therapy to the spotlight in cancer immunotherapy, receptor redirected adoptive cell transfer (rdACT) is now facing the consequences of its high capacity of selectively killing cells expressing the target antigen. Besides the technological hurdles involved in the application of rdACT, which is beyond the scope of this review, another problem that limits its broad use is the on target/off tumor response, characterized by the recognition of the selected target antigen in healthy cells. The use of TILs in patients with melanoma was often associated with vitiligo and/or uveitis amongst other side effects owing to the recognition of melanocyte differentiation antigens in the normal skin and in the eye [17,

18]. On target/off tumor response were also observed in patients treated with T cells expressing a TCR recognizing the melanoma antigen MART-1 [5]. Although these responses impose a drawback for the patients, they are not life threatening and are easily controlled in the clinical setting.

However, targeting antigens expressed in vital organs (or even those targets with not fully characterized expression patterns) is a difficult and risky task in immunotherapy, and the results already published demonstrate that this can be an important limitation. In one report, three patients with metastatic colorectal cancer were treated with T cells expressing TCRs specific for CEA, a protein that is overexpressed in these tumors but is also expressed in the normal epithelial cells of the gastrointestinal tract. Despite one patient achieving objective responses, all three patients developed severe inflammatory colitis and the study was closed [19]. Recently, two reports using TCR-modified T cells recognizing the MAGE-A3 antigen led to four deaths (2 in each study) owing to different mechanisms. In the study by Rosenberg's group, nine patients with different tumors (predominantly melanoma) were treated and five patients achieved clinical regression. However, three patients showed severe neurological toxicity, with two entering in coma and dying. Postmortem analysis showed widespread white matter damage and infiltration of T lymphocytes, and subsequent experiments demonstrated that anti-MAGE-A3 TCR possibly recognized a related peptide from MAGE-A12, a protein weakly expressed in the brain [20]. Another work from June's group used T cells expressing anti-MAGE-A3 TCR for the treatment of melanoma or myeloma. Despite no evidence of adverse effects in preclinical data, two patients died after experiencing acute cardiotoxicity. Although no expression of MAGE-A3 was detected at the heart, histopathological analysis showed high T-cell infiltration in this tissue. Experiments using cardiomyocytes cultures derived from iPS cells showed that a related peptide derived from titin, a protein expressed in striated muscle cells, was recognized by the TCR-modified cells [21].

The use of CARs is also subjected to on target/off tumor responses. Preclinical models using CARs showed that recognition of the target antigen in healthy cells could impair treatment [22] and induce long-lasting elimination of B cells [23]. The first report of these responses using receptor-redirected T cells in humans was in a study by Lamers and colleagues, which used a CAR anti-CAIX, an antigen overexpressed in renal cell carcinoma, to treat three patients [24]. However, the patients developed liver toxicity due to a previously unknown expression of CAIX in bile duct cells and the treatment was stopped. In a more serious event, a patient with colon cancer treated with anti-ErbB2 CAR+ cells developed pulmonary edema and cytokine release syndrome (CRS) shortly after the infusion, dying after 5 days. The recognition of low levels of ErbB2 antigen in the lungs was probably associated with this outcome [25]. Finally, there was a death in a trial using anti-CD19 CAR for the treatment of chronic lymphocytic leukemia, but in that case it could not be clearly associated with the infused cells and/or the recognition of the target antigen, despite the increased serum cytokine levels [26].

Strategies to Manage or Reduce Undesired Toxicities During Antitumor Responses

Unfortunately, there is no pre-clinical model that can faithfully predict on target/off tumor side effects in humans in part because animal models cannot reproduce human tissue antigen expression and distribution. Maybe more challenging, the levels and the pattern of expression of a target molecule in different patients cannot be precisely predicted. This is true for physiologically healthy individuals and the scenario is probably even more complex for nonphysiological conditions such as patients with cancer, acute or chronic inflammation, and other chronic conditions [27]. Even the exposure of cancer patients to some pharmacological agents can change gene expression

profiles in different cells, leading sometimes to improved immunological responses to tumors [28]. Consequently, the broad application of adoptive T-cell transfer is dependent on the development of robust strategies to manage and avoid the on target/off tumor response, some of which will be reviewed in the next topics.

Control of cytokine release syndrome

The infusion of high quantities of antitumor T cells may induce the rapid secretion of cytokines, mainly IL-6 and TNFα, owing to the recognition of target cells, culminating in an acute inflammatory response called CRS [13, 15, 29, 30]. The cytokines induce the activation of other cells, like macrophages and NK cells, augmenting the response and leading to endothelial damage, organ failure, and, in advanced cases, death. As stated in the previous section, the two deaths reported in CAR trials were associated with CRS, making this phenomenon an important limitation of rdACT. Importantly, one of these patients harbored mutations in *IL-6* and *IL-10* genes associated with increased production, showing that these alterations might be helpful in the prediction of the syndrome [25]. Similar events were observed in a trial treating pediatric B-cell leukemia. In this study, antibodies anti-IL6 (tocilizumab) and anti-TNFα (etanercept) were used to limit the CRS, and no impact was observed in the expansion and function of lymphocytes [14, 29]. The use of these drugs in combination with other approaches, like dose escalation and detection of polymorphisms in cytokine genes, might decrease the occurrence of CRS. A recent report identified elevations in C-reactive protein levels along with fever as an early indicator of severe CRS [15]. The same group reported a correlation between higher levels of residual leukemia burden and the occurrence of severe CRS [13, 15]. Additional genetic or functional modifications can potentially render T lymphocytes capable of responding to target cells without generating massive production of inflammatory cytokines. This scenario would allow the larger application of this modality of immunotherapy, although the manipulations required to achieve such responses are not clear at this moment.

Inclusion of suicide genes

The suicide gene approach has been extensively studied and is a potential solution for the side effects observed in clinical trials. It allows the controlled elimination of the infused cells by administrating a drug (or inducer), thereby limiting the damage induced by T cells. Several systems were developed and are reviewed elsewhere [31], but these systems have potential disadvantages like immunogenicity and slow elimination of cells (HSV-TK) or elimination of B cells (CD20/Rituximab) [32, 33]. Recently, a system based on the caspase 9 (iCasp9), a self-protein, was created [34]. The proteolytic domain of caspase 9 was fusioned to a FK domain modified to bind a small molecule dimerizing agent. Upon the administration of the drug, the casp9 dimerizes and induces the apoptosis pathway, eliminating the majority of cells in a short period. Indeed, this suicide system was successfully used in a preclinical model of on target/off tumor response [35] and in patients with GVHD [36], showing that it has the potential to control chronic side effects of rdACT. *In vitro* data support the equivalent efficiency of the CD20 and iCasp9 systems as suicide gene approaches [37].

Evaluating TCR/CAR affinity

The first trial with TCR-modified T cells used a moderate affinity TCR anti-MART1 for the treatment of patients with melanoma. Despite the low response rate (2/15 patients), no adverse events were detected [38]. In an attempt to augment the function of the infused cells, a second study was performed by the same group using an enhanced affinity TCR isolated from a TIL clone. The response rate increased to 30% when using human-derived TCRs, but the patients experienced destruction of normal

melanocytes [5]. The low number of patients in these studies impairs the establishment of a direct correlation between TCR affinity and on target/off tumor reactions, but evidence from literature supports this relation, at least in the range of physiological affinities (1–100 μM) [39, 40]. However, T cells expressing TCRs with supraphysiological affinities (K_d <1 μM) were shown to lose antigen specificity [41] and functionality, with increased expression of the inhibitory receptor PD-1 and SHP-1 [42]. Indeed, as shown in above sections, similar on target/off tumor responses were obtained with enhanced affinity TCRs anti-MAGE-A3 and with the trastuzumab-derived anti-ErbB2 CAR ($K_d = 0.1$nM). Therefore, using a TCR or CAR with an appropriate affinity could favor the response against the tumor while having minimal activity on the normal cells. Nonetheless, recent data using anti-gp100 TCRs showed that the antitumoral and autoreactivity response are coupled and its maximal effect is achieved at $K_d = 10$ μM, suggesting that higher-affinity TCRs might not improve the efficacy of rdACT [43]. The use of defined parameters, like the recently developed K_{off} assay, might help to select TCRs with optimal affinities, increasing the functionality of T cells while maintaining the specificity [36].

Selection and screening of target antigens

Considering the adverse events reported and the capacity of T cells to recognize even minor amounts of antigen, the choice of the target molecule is an important step in the design of clinical trials using genetic engineered cells. Excluding the tumors of viral etiology, there is no known antigen expressed exclusively in neoplastic cells. In an effort of organizing and prioritizing the strategies in vaccine development, the NCI has developed a list of cancer antigens based on predefined parameters (therapeutic function, immunogenicity, role of the antigen in oncogenicity, specificity, expression level and percent of antigen-positive cells, stem cell expression, number of patients with antigen-positive cancers, number of antigenic epitopes, and cellular location of antigen expression), each with a different weight in the final score [44]. These parameters could also be useful in the context of rdACT, providing a theoretical basis for antigen selection. A recent report suggests that using alloreactive T cells, HLA-loaded peptides (including TAAs) can be identified in high frequencies, allowing the empirical identification of new HLA-associated epitopes and eventually cloning TCR sequences specific for these antigens [45].

Along with antigen ranking, it is of great importance to identify new tumor-associated antigens and to precisely define the pattern of expression among healthy tissues. In this regard, new technologies such as high throughput sequencing and proteome analysis are playing an important role. Using whole-exome sequencing, a recent work showed that melanoma cells have mutated peptides loaded in HLA class I, and these are recognized by TILs isolated from the tumor. Importantly, wild-type peptides were not recognized, making these antigens unique to tumor, but further studies are required to show the proportion of patients expressing such peptides [46]. A similar profiling study using Nanostring technology identified potentially overexpressed antigens in melanoma, increasing the possible targets in this tumors [47].

Proteome analysis of surface proteins (called “surfomics” or “surfaceome”) can be another useful method for screening of tumor-specific membrane proteins, and can also be used for the generation of a normal tissue expression database that might help prevent on target/off tumor responses. Initial studies relied on the biochemical fractionation of the membrane and posterior isolation of proteins [48, 49], but this method has disadvantages like contamination from other cellular compartments. In other approach, intact cells are “shaved” by proteases and the fragments are purified and analyzed by mass spectrometry, bypassing the membrane fractionation step [50, 51]. *In vivo* analysis using phage libraries are being tested [52] and such approaches can be

used to identify patterns of on target (off tumor) scFv binding predicting potential tissue damage. Finally, the Human Protein Atlas project (http://www.proteinatlas.org/) is a repository of protein expression profiles based on immunohistochemistry of cell lines, normal and cancer tissues, as well as transcript information. Currently, it covers about 16 000 genes in 185 tissues and cell lines, being constantly updated, and represents an invaluable source of information [53].

Ultimately, the combination of the different methods described can contribute to a wiser choice of the target antigen for rdACT [54]. The advent of faster and cheaper molecular technologies will probably enable the emergence of personalized rdACT protocols, aiming at unique mutations and/or specific antigen expression patterns of each patient.

Enhancing the elimination of the target cell

An important aspect of targeting single antigens expressed in tumors is the selective pressure applied to the tumor cells. It is very clear now that tumors are very heterogeneous in terms of genetic mutations [55, 56] and that this heterogeneity can be reflected in the antigen collection displayed by single cells in the tumor mass (and, as such, in the tumor as a whole) [57–59]. Such concept has clear implications for target antigen selection and is likely to impact the outcome of therapies targeting one single antigen. Although the best results reported with CAR therapies (Figure 1A) targeted the lineage antigen CD19, at least one patient with pediatric acute lymphoblastic leukemia showed a CD19 negative escape variant after rdACT directed to CD19 [14]. This patient was previously treated with an anti-CD19 monoclonal antibody that could have potentially led to the development of the CD19 negative subclone, raising a note of caution for the previous treatments applied before rdACT. As an alternative for this case, cells against different targets such as CD10 or CD22 [60] can be generated and infused concomitantly or in a sequential approach, anticipating the raising of escape variants. For protocols using TCR-based gene transfer, escape variants lacking the expression of the transgenic TCR-targeted protein were also reported in the literature [61–63]. In this regard, different strategies (Figure 1) are emerging such as those described by recent reports of T cells carrying CARs recognizing multiple antigens [64] (Figure 1B) and by biotin-binding immune receptors (BBIRs) composed of an extracellular-modified avidin linked to an intracellular T-cell signaling domain [65]. By using BBIRs, multiple molecules can be specifically tagged with biotin and targeted in concomitant or sequential fashion, allowing the immunotherapy to induce multiple selective pressures on the tumor, thus limiting immune escape variants (Figure 1C). In a similar approach, T cells were modified to express a CAR containing the extracellular region of CD16, a receptor that binds to the Fc portion of antibodies, allowing the targeting of cells bound to antibodies [66] (Figure 1D). Such universal strategies might enhance the applicability of CAR-based immunotherapy by creating "off the shelf" reagents, decreasing costs and simplifying the manufacturing process.

These results advocate in favor of therapies targeting more than one antigen displayed by the tumor, and is still to be determined if this must be performed in a concomitant or sequential fashion. For instance, a recent report in mouse model bearing human glioblastoma xenografts shows that targeting Her2 and IL13Rα2 by T cells carrying two CARs rendered the immunotherapy approach much more potent by preventing antigen escape than targeting each single antigen [67]. The tumor cell killing by single antigen-targeted T cells can still induce antigen spreading responses [68] relying on the enhanced availability of antigens derived from lysed tumor cells, and these responses can favor indirect or bystander targeting of other antigens from the antigenically heterogeneous tumors [69, 70]. The possibility of enhancing such process by applying immune checkpoint blockade strategies such as CTLA4 [71] and/or

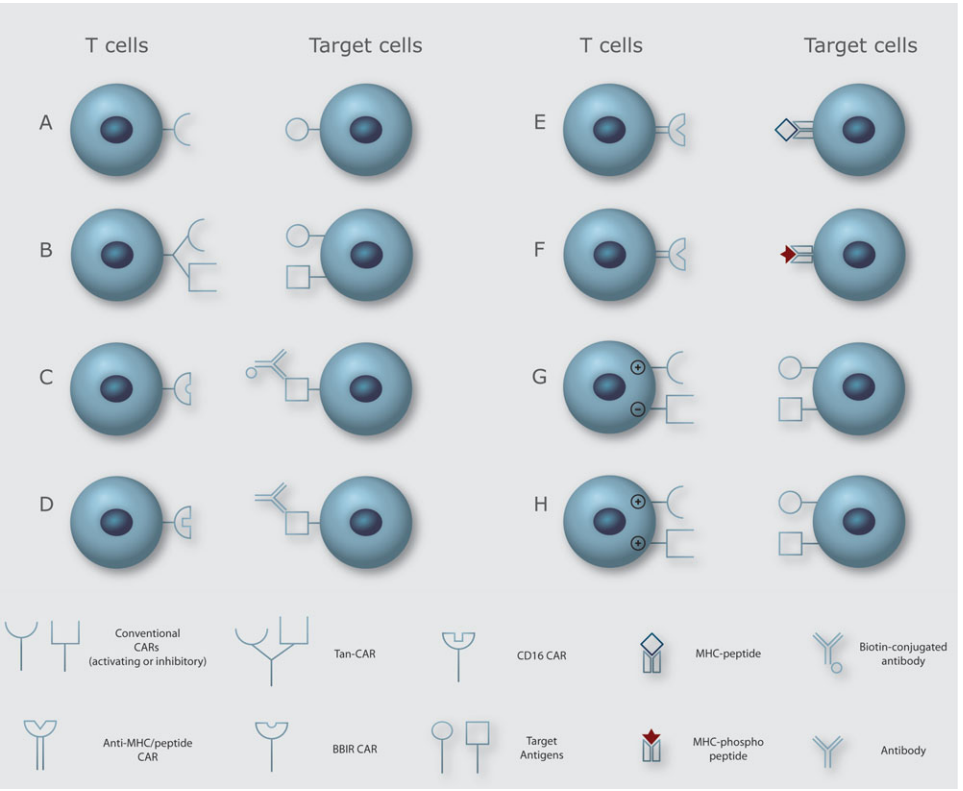

Figure 1. Different strategies for targeting tumor cells using CAR-modified lymphocytes. As described in the text, the cells can be modified with a conventional CAR (A) that recognizes the antigen in the surface of the target cell. CARs targeting multiple antigens, named Tan-CAR, were developed using two different scFv in tandem aiming to generate cells with multiple (and incremental) reactivity (B). CARs based on the recognition of biotinilated molecules by BBIR domains were developed allowing the sequential recognition of targets by switching the biotinilated molecule targeting tumor antigens (C). In a similar approach, CARs recognizing the Fc portion of antibody molecules through a CD16 extracellular domain were developed (D). Alternatively, T cells can express a CAR that recognizes a specific peptide–major histocompatibility complex (MHC) (E) or phosphopeptide–MHC (F) complex, allowing cytoplasmatic peptides to be targeted. Conditional responses were obtained using the inhibitory CAR (H) and split-CAR (G) approaches.

PD-1/PDL1 interaction-specific antibodies [72] must be considered in this scenario. Indeed, this approach was reported by a recent work combining anti-Her2 CARs with PD-1 blockade, showing enhanced tumor growth inhibition and increased function of CAR+ T cells [73].

Other than the classical CAR design and transgenic TCR developed, the emergence of monoclonal antibodies selected against HLA + peptide complexes [74, 75] can add a new layer of specificity to the ACT. TCRs are able to recognize peptides derived from both membrane and intracellular proteins, allowing T cells to screen for protein expression repertoire. CARs in contrast are restricted to surface antigens but deriving CARs from mAbs recognizing HLA + Peptide complexes could extend CAR specificities to intracellular antigens, broadening its target antigen collection (Figure 1E). The possibility of extending this approach to the recognition of HLA-phosphopeptide complexes can lead the targeting of rdACT to deeper levels of specificities restricting the response to cells displaying, for example, only the phosphorylated residue of a target protein (Figure 1F). In fact, mAbs specific for HLA-phosphopeptide have been recently

reported [76]. Theoretically, this could restrict immune response to cells displaying certain activated signaling pathways (a common pattern in cancer) by targeting combinations of phosphopeptides [77].

The ability to generate conditional responses to antigen combinations has the potential to deeply impact the field of rdACT. Some groups have been working in this context [78–80]. The strategy of using a combination of target antigens relies on the conditional activation of the effector T lymphocyte depending on the signals provided by different CARs. One attempt has been to use inhibitory CARs to counterbalance activating CARs, thus limiting the immune response to those cells lacking the inhibitory surface antigen (Figure 1G) [78, 81]. Until recently, this was a theoretical approach, but Sadelain's group showed that a CAR containing the PD-1 intracellular domain was capable of inhibiting the activation mediated by a transgenic TCR or an activating CAR [82]. The inhibitory CAR was functional *in vitro* and *in vivo*, and its activity was dependent on the target and CAR expression levels. Although the optimal combination of activating and inhibitory signaling is still to be determined, this approach could enhance the safety of CAR-based therapy. Such an approach could limit the off-target effects of strategies targeting antigens shared by tumors and healthy cells, avoiding off-target toxicities such as those reported for the CAIX [24, 83] and ErbB2 [25] trials. Inhibitory CARs can potentially counterbalance not only activating CARs but also transgenic TCRs designed to redirect T lymphocytes to tumor cells. This strategy could help reduce off-target effects such as those recently described in the clinical trial with TCRs for MAGE A3 [20].

A different approach can take advantage of the clearly demonstrated superiority of CARs carrying multiple domains for T-cell activation (especially the zeta chain as signal number one and costimulatory signaling domains such as those of CD28 or 4-1BB as signal number two) over those carrying only the zeta chain. In this approach, the requirement of complementary signaling for full T-cell activation leads to the opportunity of splitting these signals into two separate CARs, allowing now to restrict responses to situations when both CARs are engaged (Figure 1H) [78–80, 84]. If each CAR targets a different surface antigen, then conditional activation depends on the target antigen panel displayed by the cell, as has been earlier proposed [78]. A recent publication elegantly demonstrated that tumors implanted in mice can be selectively recognized on the basis of the target combination since infused T lymphocytes express two different CARs, one carrying the zeta chain and the other containing a signaling tail with endodomains of CD28 and 4-1BB [80]. In this system, the affinity of the interaction of the CAR carrying the zeta chain for its target had to be diminished in order to avoid tumor lysis by the engagement of the CAR triggering signal one, making the cell dependent on signal 2. This illustrates that other than choosing the correct antigen combination, the level of antigen expression, the affinity of the CAR and thus the threshold of activation of the T lymphocyte must be considered in order to fine tune the system. More importantly, this proof of principle work demonstrates that targeting antigen panels instead of a single antigen is a feasible approach *in vivo*. Other groups have also reported increased activations of T cells by partially and activating CAR engagement (signal 1 and 2 in different CARs) *in vitro* [79, 85] and *in vivo*, [84] or the stronger activation of T cells carrying CARs with two specificities once engaged by both target antigens [64], reinforcing the feasibility of this approach.

Most of the T-cell responses based on multiple antigens have relied on CAR intracellular domains to promote T-cell activation or function modulation. As a consequence of the new synthetic biology circuits being described in recent publications [86–88], one can envision a future for conditional T-cell activation based on the engagement of artificial signaling circuits. This concept is starting to be applied to cellular therapies, with recent work demonstrating the control of T-cell proliferation based on a synthetic

RNA circuit responsive to the small molecule theophylline [89]. The development and use of orthogonal signaling components has the potential to provide a better control of activation, robust induction of the desired phenotype and minimal crosstalk with endogenous signaling pathways. The lymphocytes can be rendered responsive not only to the combination of membrane antigens on the target cell, but to the different conditions, molecules or cytokines present in the tumor microenvironment (e.g. lactic acid, hypoxia, TGF-β), integrating these responses in a controllable phenotype.

Targeting the most relevant cell population

Debulking big tumor burdens is important to achieve clinical and physiological relief but long-lasting and definitive cure is thought to be only achieved if tumor maintaining cells are targeted as well. In this context, identifying antigens restricted to tumor stem cells is one of the most promising approaches to maximize antitumor responses against whole tumor masses. Very few stem cell surface markers have been identified until now and the possibility of targeting intracellular targets by rdACT has potential to change this landscape [90]. Nonetheless the efforts for targeting tumor stem cells is still ongoing and recent data in the literature indicates that, at least for certain tumors, affordable stem cell surface markers are targetable by rdACT.

For melanoma patients, it has been shown that half of the tumors express the CD20 surface marker, whose expression was previously ascribed exclusively to B lymphocytes [91, 92]. More strikingly, these tumors can be targeted *in vivo* by the anti CD20 therapeutic mAb Rituximab, leading to antitumor response [93–95]. This somehow aberrant or "out of context" expression highlights the relevance of pursuing a better characterization of the pattern of expression of antigens in tumors and, especially, in tumor stem cells. The CD20 expression in these cells is also accompanied by the expression of the high molecular weight – melanoma-associated antigen (HMW-MAA). Both antigens were shown to be targetable by rdACT using CARs [93]. Exploiting the aberrant expression of surface (or even intracellular) markers has thus the potential to turn stem cell populations into targetable cells and multiple antigen combinations can further narrow the immune response sparing their healthy stem cell counterparts. For leukemia stem cells, the level of expression of some surface antigens such as CD45, CD90, and CD96 has been shown to discriminate leukemia from healthy stem cells [96, 97]. Although not exclusive form of leukemia cells, these markers could be potentially used as surface markers for conditional responses mediated by rdACT. For stem cell-targeted rdACT, *in vitro* and *in vivo* models that enable the selective evaluation of stem cell-based tumor cell renewal are valuable tools. Such models are available for normal [98] and leukemic [99] hematopoiesis *in vitro* and *in vivo* [100, 101], and for some models of xenografted tumors [93, 100, 102].

If tumor stem cells are intuitive targets for rdACT, the recent studies that show the presence in the tumor microenvironment of many targetable cellular components can further extend the applicability of rdACT. Cancer-associated fibroblasts play an important role in the maintenance of the tumor, producing growth factors and extracellular matrix components that modulate the proliferation and invasion of the tumor [103]. Fibroblast-activated protein (FAP) has been identified as a marker of tumor-associated fibroblast [104], with high expression in the tumor microenvironment of most types of cancer and absent in normal tissues. These properties led to the development of different strategies for elimination of this subset [105, 106]. However, recent work has demonstrated that targeting FAP+ cells using CARs leads to cachexia and elimination of bone marrow stromal cells, resulting in lethal bone toxicity in murine tumor models [107]. Importantly, human bone marrow stromal cells were also FAP+, limiting the application of this approach unless additional targets are used to narrow the response to those FAP+ cells exclusively associated to tumors. In pancreatic tumors, tumor

microenvironment and the associated stroma are potential targets for immune and pharmacological interventions, with fibroblasts, activated pancreatic stellate cells, and inflammatory cells playing important role in tumor progression [108]. Angiogenic pathways, targeted extensively by small molecule drugs, are also being evaluated in the field of adoptive immunotherapy. A recent report suggests that targeting VEGFR with T lymphocytes carrying CARs specific for this molecule can impair vascular tube

Table 1. Potential antigen combinations for conditional CAR therapy.

	"Split" approach		
Type of cancer	Signal 1	Signal 2	Rationale
Glioblastoma (GBM)	EGFRvIII	CD133	CD133: cancer stem cell (CSC) marker in glioblastoma; important for the maintenance of CSC phenotype. [110]; its expression in other tissues prevents the use of this antigen as an immunotherapeutic target. EGFRvIII: highly expressed in GBM; with low or absent expression in normal tissues; EGFRvIII+ CD133+ GBM stem cells have a high capacity of self-renewal and maintenance of the tumor [111]
Acute Myeloid Leukemia (AML)	CD123	CD96	CD96: marker of CSC in AML; expressed in various normal tissues like spleen, thymus and lung [96] CD123: marker of CSC in AML; expressed in immune cells and bone marrow
Melanoma	HMW-MAA	CD20	CD20: expressed in melanoma CSC; expressed in high levels in mature B cells [93] HMW-MAA: expressed in melanoma CSC; expressed in the basal layer of epidermis, endothelial cells, pericytes and smooth muscle [112]
Breast cancer	Her2	MUC1	MUC1: overexpressed in 90% of patients; expressed in normal cells of the gastrointestinal tract, liver, and pancreas [113] Her2: overexpressed in 25% of patients; expressed in normal cells of the bronchus, bladder and intestine
	"Inhibitory" approach		
Type of cancer	Activating	Inhibitory	Rationale
Chronic lymphocytic leucemia (CLL)	CD19	CD22	CD19: expressed in malignant CLL B cells and normal B cells CD22: expressed in normal B cells
Acute Myeloid Leukemia (AML)	CD44v6	CD14	CD44v6: variant of CD44 overexpressed in AML cells; expressed in monocytes [35]. CD14: expressed in monocytes
Melanoma	CD20	CD19	CD20: expressed in melanoma CSC and in normal B cells CD19: expressed in B lymphocytes

Different potential combinations of antigens targeted by receptor redirected adoptive cell transfer. Target combinations are listed along with the predicted pattern of expression of the different target molecules. The proposed approach (activating split or inhibitory vs. activating receptors) are also listed.
The expression pattern in the "Rationale" column was retrieved from the literature or from the Protein Atlas database.

formation *in vitro* and delay A549 cell line-mediated tumor growth and pulmonary metastasis in mice, especially if CAR+ T cells are modified to co-express IL-15 [109].

Potentially Relevant Antigen Combinations

To achieve such a level of refined antitumor responses, relevant antigen combinations must be identified and exploited in relevant preclinical and clinical settings. We know very few of such combinations yet and some of the potential panels of discrimination between tumor and healthy cells are listed in Table 1. Nonetheless, a deeper understanding of the pattern of expression of different membrane bound and intracellular antigens is likely to allow considering new antigens as immunotherapy targets, extending the list for conditional immunotherapy based responses using rdACT.

CONCLUSIONS

The field of immunotherapy has finally achieved its first striking clinical results moving from anecdotal tumor regressions to consistent patterns of clinical responses. The impressive clinical results obtained were accompanied by mild to severe side effects that can limit its wider applications on certain tumors and clinical settings. Despite the first outstanding clinical results with CARs targeting CD19 antigen, safe and effective additional targets are still to be validated, and the possibility of combining signals and antigen targets, as shown recently, opens the opportunity to fine tuning antitumor responses increasing its efficacy and safety. We are likely to witness a revolution on cancer therapy and the use of immune cells as effectors of tumor elimination.

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Declaration of Interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the article.

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