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Review

Foreign or Domestic CARs: Receptor Ligands as Antigen-Binding Domains

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Abstract: Chimeric antigen receptors (CARs) are increasingly being used in clinical trials to treat a variety of malignant conditions and recent results with CD19-specific CARs showing complete tumor regressions has sparked the interest of researchers and the public alike. Traditional CARs have been generated using single-chain variable fragments (scFv), often derived from murine monoclonal antibodies, for antigen specificity. As the clinical experience with CAR T cells grows, so does the potential for unwanted immune responses against the foreign transgene. Strategies that may reduce the immunogenicity of CAR T cells are humanization of the scFv and the use of naturally occurring receptor ligands as antigen-binding domains. Herein, we review the experience with alternatively designed CARs that contain receptor ligands rather than scFv. While most of the experiences have been in the pre-clinical setting, clinical data is also emerging.

Keywords: chimeric antigen receptor; adoptive immunotherapy; immunology; cancer

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1. Introduction

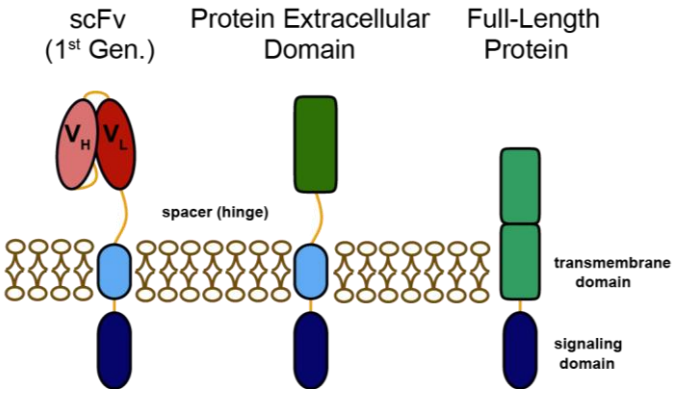
Immunotherapy for cancer is enjoying a resurgence in popularity as decades of research into basic immunological mechanisms have translated into powerful new therapies for patients with metastatic and refractory malignancies. Of late, adoptive cell therapies using tumor-infiltrating lymphocytes, viral-specific cytotoxic T cells, and genetically modified T or NK cells have received considerable attention as dramatic and sustained tumor regressions have been observed in some patients receiving these therapies [1–3].

Chimeric antigen receptors are one way of genetically endowing T cells with tumor specificity. The basic chimeric antigen receptor (CAR) design includes an antigen-binding domain, often but not always derived from a single-chain variable fragment, followed by a short spacer region, a transmembrane domain, and signaling domains usually derived from the CD3 ζ-chain (Figure 1) [4,5]. CARs provide several advantages over T cell receptors (TCRs) in that: (1) they recognize antigen in an MHC-independent fashion and therefore a single CAR may be used to treat cancer patients regardless of HLA type, (2) they are resistant to some of the tumor escape mechanisms as malignant cells that have down-regulated expression of class I MHC molecules or have decreased proteasome antigen processing are readily killed, (3) they provide a broader range of potential targets as virtually any protein, carbohydrate, or glycolipid antigen expressed on the tumor cell can be targeted, and (4) CARs can be readily modified with additional costimulatory domains (CD28, 4-1BB, OX40) to enhance T cell persistence and anti-tumor function *in vivo.*

Costimulation in CAR T cells has been the focus of intense research over the last several years [6], and will undoubtedly be covered in other reviews in this series. However, here we choose to focus on the antigen-binding domain of CARs and specifically on non-traditional CARs that have been designed using receptor ligands rather than single-chain variable fragments (scFv). Since many CAR constructs contain scFv of murine origin, there remains a risk of developing an immune response against the genetically modified cells in the form of human anti-mouse antibodies (HAMA) and even one report of anaphylaxis following transfer of CAR T cells [7]. Both of these unintended consequences can severely limit the effectiveness of the genetically modified cells and can cause potentially life-threatening complications. In this regard strategies to "humanize" the scFv may be beneficial; however humanization does not prevent the development of anti-idiotypic antibodies. Antigen-binding domains that use endogenously expressed receptor ligands, rather than scFv, could be advantageous in avoiding unwanted immune responses to the transgene. In particular, CARs using full-length native proteins (Figure 1) expressed on the surface of T cells are unlikely to elicit an antibody-mediated immune response—although novel epitopes created at the fusion site of the protein and the signaling domain could potentially be immunogenic if presented to T cells. Indeed, pre-clinical and clinical data suggest receptor ligands can be effective antigen-binding domains for redirecting CAR T cells to a variety of tumor-associated antigens.

Herein we will review CARs that have been engineered with receptor ligands as antigen-binding domains for adoptive immunotherapy of cancer and finish with a discussion of the potential advantages and disadvantages of this alternative design.

Figure 1. First generation scFv *vs.* receptor ligand-based chimeric antigen receptors (CARs). Traditional single-chain variable fragments (scFv) CARs derive specificity from an extracellular domain comprised of a variable heavy and variable light chain from a monoclonal antibody (MAb). Receptor ligand-based CARs may have just the extracellular domain of a protein or the full-length native protein as antigen binding domains. Transmembrane and signaling domains vary in scFv-based CARs, but identical configurations can be used with protein extracellular domains. In the full-length protein the transmembrane and cytoplasmic portion of the receptor ligand may be included in the CAR construct.



2. Heregulin-ζ CARs for Redirecting T Cells to HER3 and HER4

Heregulin, also known as neuregulin-1 (NRG1), is the ligand for HER3 and HER4 (ErbB3 and ErbB4) and is involved in a variety of cellular processes including proliferation, differentiation, invasion and survival. Alternative splicing creates many heregulin isoforms that are differentially expressed across tissues and allow for this wide variety of functions [8]. Since HER3 and HER4 are overexpressed on many cancers from breast to brain, there has been substantial interest in immunotherapeutic approaches to target these receptors.

One of the earliest examples that a receptor-ligand could be used as an antigen-binding domain in CAR T cells was an attempt to target HER3 and HER4 expressing tumor cells using heregulin β1 (hrgβ). A CAR was constructed by taking a 70 amino acid fragment of hrgβ fused to a flexible hinge (spacer) region from CD8α and the transmembrane and signaling domains of the CD3-ζ chain. Retroviral transduction was used to express hrgβ-CARs on the surface of primary rat and mouse T lymphocytes and resulted in efficient cytolysis of the HER3/HER4-expressing mouse tumor cell line HC11R111 [9].

Muniappan and colleagues also engineered heregulin-ζ CARs using the heregulin α1 or β1 isoforms as antigen-binding domains fused to a spacer region derived from the CH2CH3 domains of IgG, the CD34 transmembrane domain, and the signaling domain of the CD3-ζ chain [10]. After being expressed on Jurkat cells, both hrgα and hrgβ CARs induced the secretion of IL-2 in the presence of HER3- and HER4-positive breast cancer cell lines, but not in the presence of HER3- and HER4-negative cells. Similarly, CD8-positive T cells from a healthy donor expressing hrgα or hrgβ CARs recognized and killed HER3/HER4-positive MDA-MB453 cells. Specificity was confirmed in blocking experiments using soluble hrgα peptide.

Both of these studies showed that naturally occurring protein ligands could be used as antigen-binding domains in CAR constructs and could redirect T cell specificity and effector functions to tumor cells expressing the complementary receptor.

3. Targeting the Tumor Vasculature: Vascular Endothelial Growth Factor CARs

Several decades ago it was suggested that the growth and maintenance of solid tumors requires angiogenesis and a constant blood supply [11]. Thus, substantial investment has been made in anti-cancer therapies targeting the tumor vasculature. Angiogenesis is a complex process involving several growth factors (and their multiple isoforms) binding to one or more complementary receptors, which are primarily expressed on endothelial cells. Vascular endothelial growth factor (VEGF) is overexpressed by many different types of tumors and stimulates angiogenesis by binding to VEGF receptor-2 (VEGFR-2) on endothelial cells in the tumor microenvironment, allowing malignant growth and metastasis [12]. The VEGF antibody, bevacizumab, blocks VEGF from binding to its receptors and gained FDA approval in 2004 as the first angiogenesis inhibitor showing antitumor activity in patients with metastatic colorectal cancer [13]. Subsequently, small molecules that inhibit the tyrosine kinase activity of VEGF receptors have gained FDA approval and antibodies targeting VEGF receptors are also under investigation [14].

While these clinical developments have been exciting for the angiogenesis field, their anti-cancer efficacy has been fairly modest, with bevacizumab therapy generally increasing overall survival in patients by a few months [15]. One potential reason for the modest anti-tumor effects is that these approved therapies are "cytostatic" in that they prevent new blood vessel formation, but do not necessarily destroy the existing tumor vasculature. Thus, T cells redirected to target the tumor vasculature could potentially improve the anti-cancer effects of anti-angiogenic therapy.

Niederman and colleagues redirected mouse T cells to murine VEGFR-2 (Flk-1) by engineering a CAR with the murine VEGF isoform 165 (VEGF-165) fused to the CD8α hinge region, and the transmembrane and signaling domain of the murine CD3-ζ chain [16]. Primary T cells expressing VEGF-CARs specifically lysed B16.F10 cells genetically modified to express Flk-1, but not unmodified B16.F10 cells. Similarly, murine islet endothelial (MILE) cells that naturally express Flk-1 were lysed when co-cultured with T cells expressing VEGF-CARs. However, lysis by VEGF-CAR T cells was completely abrogated when MILE cells were pre-incubated with anti-Flk-1 antibodies. In animal models VEGF-CAR T cells significantly inhibited the growth of subcutaneously implanted CT26 adenocarcinoma cells as well as B16.F10 melanoma cells. Likewise, VEGF-CAR T cells significantly suppressed the growth of LS174T human colon adenocarcinomas growing in SCID mice.

These experiments showed that targeting angiogenesis significantly inhibits tumor growth in xenograft and immune competent mouse models. Importantly, the investigators observed no acute toxicity in mice treated with VEGF-CAR T cells with respect to weight loss, appearance and general behavior. However, it is worth noting that to achieve the observed anti-tumor effects the mice had to be treated with multiple injections of VEGF-CAR T cells and exogenous IL-2, suggesting limited *in vivo* persistence of the CAR T cells.

4. IL13 Zetakine T Cells for the Treatment of Glioblastoma

Malignant gliomas are the most common primary brain tumor in the United States with an annual incidence of 17,000 cases per year. Glioblastoma, the most aggressive and lethal of all gliomas, accounts for 82% of these new cases [17]. Glioblastoma is a particularly devastating disease as it causes significant detriment to quality life and cognitive abilities of those affected. Current treatments are largely inadequate as the 1-year survival stands at 35% with few patients surviving to 5 years [17]. Thus, new therapies are desperately needed for patients with glioblastoma.

T cells represent a particularly attractive therapeutic modality for patients with glioblastoma as they have potent cytotoxic capabilities and have been shown in mouse models to traffic across the cerebral hemispheres in response to tumor [18]. This ability to migrate to the tumor cells is particularly important given glioblastoma's invasiveness. As with any T cell immunotherapy, target antigen selection is critically important and perhaps even more so when attempting to treat brain tumors, as collateral damage could have catastrophic consequences.

IL-13 receptor alpha-2 (IL-13Rα2) is expressed on >80% of high-grade gliomas, but has little to no expression in normal tissues of the central nervous system, making it an ideal antigen for T cell immunotherapy of glioblastoma [19]. In the physiologic setting, IL-13 binds to IL-13Rα2 with approximately 10-fold higher affinity than to IL-13Rα1, which is more widely expressed in tissues. However, IL-13Rα1 will form a complex with IL-4Rα that is of very high affinity for the IL-13 cytokine [20]. To generate a CAR with enhanced specificity for IL-13Rα2, Kahlon and colleagues took advantage of the E13Y IL-13 mutein, which has a 50-fold higher affinity for IL-13Rα2 and a 5-fold lower affinity for the IL-13Rα1/IL-4Rα complex than wild-type IL-13 [21,22].

An IL13 "zetakine" was engineered by fusing the IL-13 (E13Y) mutein to a hinge region from human $_{\gamma 4}$Fc, the CD4 transmembrane domain and the intracellular cytoplasmic tail of CD3-ζ [22]. When expressed on $CD8^+$ CTL clones, the IL13 zetakine mediated specific lysis of Daudi lymphoma cells genetically modified to express IL-13Rα2, but not wild-type Daudi cells. U251 glioma cells that naturally express IL-13Rα2 were also killed, but cells that expressed IL-13Rα1, and not α2, were not killed, indicating a high degree of specificity for IL-13Rα2. IL13 zetakine CTLs released IFN-γ, TNF-α, GM-CSF, and IL-2 in response to IL-13Rα2-positive tumor cells and this release could be blocked by the addition of both recombinant human IL-13 and IL-13 antibody. To assess the *in vivo* anti-tumor activity of IL13 zetakine CTLs, firefly luciferase-expressing U87 cells were stereotactically implanted into the forebrain of NOD-SCID mice and mice were treated intratumorally with IL13 zetakine or CD19-CAR CTLs. By 3 days after treatment all mice given IL13 zetakine T cells showed a complete elimination of the U87 bioluminescence signal, whereas mice treated with CD19-CAR CTLs showed increasing bioluminescence signal, indicative of tumor progression. Impressively, IL13 zetakine-treated mice showed no tumor progression for up to 75 days.

This study led to the initiation of a clinical trial to assess the safety of IL13 zetakine T cells in patients with glioblastoma and to our knowledge is the first human study using a receptor ligand-based CAR to treat human cancer (clinicaltrials.gov: NCT00730613). While the final results from this trial have not been published, the study was presented at the American Society of Gene & Cell Therapy's 14th annual meeting in Seattle, WA, USA.

Brown and colleagues presented three patients treated with IL13 zetakine CTLs; no patient experienced significant side effects from the cells and anti-glioma responses were observed [23]. However, heterogeneous expression of IL-13Rα2 on glioma cells appears to be driving antigen escape and the recurrence of IL-13Rα2-negative glioma tumors may be a major contributor to disease progression in these patients after therapy.

Recently Kong and colleagues described a "2nd generation" IL13 zetakine, which included a CD28 costimulatory signaling domain and contained two amino acid mutations in IL-13 (E13K and R109K) [24]. These mutations increase affinity of IL-13 for IL-13Rα2 while decreasing affinity for IL-13Rα1. As with the original IL13 zetakine, they observed strong T cell proliferation, cytokine secretion and cytolysis in response to target cells expressing IL-13Rα2, but not IL-13Rα1. Unfortunately, these authors did not perform a head-to-head comparison of their receptor with the originally described IL13 zetakine, so it is impossible to determine if the additional R109K mutation provides an advantage over the single E13K mutation. One would presume that the addition of the CD28 costimulatory domain would make this a superior CAR, but it could also increase the reactivity of the CAR to cells expressing IL-13Rα1. Indeed, Kong and colleagues see significant IL-2 secretion in response to IL-13Rα1^{pos}/α2^{neg} THP-1 cells, whereas Kahlon and colleagues observed no IFN-γ secretion in response to these same cells [22,24]. Clearly, further studies are needed to determine the ideal IL13 zetakine for the treatment of glioblastoma.

5. Targeting NKG2D Ligands with NKG2D CARs

Natural killer (NK) cells are a critical component of the innate immune system guarding against tumor formation and providing a fast-acting defense against viral infections. Activation of NK cells is controlled through the absence of MHC molecules on target cells and a combination of activating and inhibitory receptors. One of the best-characterized activating receptors is NKG2D, a type-2 transmembrane homodimer containing charged residues in the transmembrane segments and associating with DNAX-activating protein of 10 kDa (DAP10). Several ligands binding NKGD2D have been discovered; in humans these are MICA, MICB, ULBP1, ULBP2, ULBP3, RAET1L and RAET1E [25]. These ligands provide optimal targets for immunotherapy because they are often overexpressed on tumor cells, but not by normal cells, and have been shown to mediate anti-tumor immunity in mice and humans [26,27].

While the NKG2D receptor is expressed on certain T cell subsets, it acts in a co-stimulatory fashion. Zhang and colleagues asked whether NKG2D could serve as the antigen-binding domain of a CAR so that T cells could directly lyse tumors expressing NKG2D ligands [28]. Since NKG2D is a type II transmembrane protein (N-terminus located intracellularly), they engineered a murine NKG2D-CAR by fusing the CD3-ζ chain in front of full-length mouse NKG2D. With this design the orientation of the CD3-ζ cytoplasmic tail is reversed inside cells. As with wild-type NKG2D, the NKG2D-CAR required DAP10 for surface expression as transfection into Bosc23 cells resulted in no NKG2D surface expression unless DAP10 was co-transfected into cells. When the CAR was transduced into murine T cells, which endogenously express DAP10, a significant increase in NKG2D expression could be observed over vector transduced control T cells.

NKG2D-CAR was functionally active causing the release of large amounts of IFN-γ and other proinflammatory cytokines in response to YAC-1 cells that endogenously express murine NKG2D ligands, as well RMA cells genetically modified to express NKG2D ligands, but not the wild type RMA cell line. Likewise, T cells expressing NKG2D-CAR efficiently lysed ligand-positive targets, but not ligand-negative RMA cells. T cells transduced with wild-type NKG2D displayed no cytokine secretion or cytotoxic activity toward any of the target cells. Finally, *in vivo* activity was examined by mixing RMA cells (genetically modified to express NKG2D ligands) with T cells expressing NKG2D-CAR or vector control T cells and subcutaneously injecting the mixture into mice. Whereas tumors grew efficiently in mice given the vector control T cells, NKG2D-CAR T cells significantly inhibited the outgrowth of tumors. Perhaps more interestingly, NKG2D-CAR T cells appeared to induce broad anti-tumor immunity as mice not developing tumors after NKG2D-CAR therapy were completely protected upon rechallenge with wild-type RMA cells (expressing no NKG2D ligands) [28].

Subsequent investigations into NKG2D-CAR using therapeutic models (CAR T cells injected after tumor development) have shown anti-tumor activity against murine ovarian, lymphoma and myeloma tumors [29–31]. Likewise, a human version of NKG2D-CAR was engineered and shown to have activity against human leukemia and myeloma cells *in vitro* [32,33]. Histone deacetylase inhibitors may enhance this therapy as they can upregulate NKG2D ligand expression on tumor cells, thus increasing their susceptibility to lysis by NKG2D CAR-expressing T cells [34]. No clinical data is available for NKG2D-CAR therapy, but a phase I clinical trial is expected in the near future.

6. Immunotherapy of CD70-Positive Malignancies with a CD27-ζ CAR

CARs containing co-stimulatory signaling domains from molecules like CD28, 4-1BB, and OX40 are being actively investigated in pre-clinical and clinical trials and show superiority to CARs with only a ζ-chain [6,35]. One co-stimulatory pathway that has received less attention until recently is the CD27/CD70 pathway. CD27 is a member of the tumor necrosis factor superfamily of receptors and is expressed widely on peripheral blood T cells and on subsets of other hematopoietic cells, while its ligand CD70 has a far more restricted expression pattern on a subset of activated B, T and dendritic cells [36]. In the physiologic setting, CD27 ligation provides a co-stimulatory signal critical for T cell expansion, survival and the induction of long-term memory [37]. The CD70 ligand has a short cytoplasmic domain; however, the functional consequences of "reverse" signaling through CD70 are less clear, but may contribute to B cell activation and IgG production [38].

The co-stimulatory properties of CD27 notwithstanding, of particular interest to the field of tumor immunotherapy is the frequent aberrant or overexpression of CD70 by multiple tumor types. CD70 is expressed by diffuse large B-cell and follicular lymphoma and also by the malignant cells of Hodgkin lymphoma, Waldenström macroglobulinemia, and multiple myeloma, and by human T-lymphotropic virus type 1- and EBV-associated malignancies [39–43]. Solid tumors like renal cell carcinoma and glioblastoma have also been reported to express CD70 [44,45]. While some animal studies suggest that the expression of CD70 on tumor cells may promote anti-tumor immunity and delay the growth of tumors in mice, these studies have been performed with transplantable tumor models and may not accurately reflect the complex development of CD70-expressing tumors in humans [37]. We and

others have suggested that CD70 may be a targetable tumor antigen given its expression on a variety of different tumor types and limited expression in normal tissues.

To generate a CAR targeting CD70, we fused full-length human CD27 to the CD3-ζ chain [46]. CD27-ζ CAR could be stably expressed on primary human CD4 and CD8 T cells and induced the secretion of large amounts of IFN-γ and IL-2 in the presence of CD70-expressing tumor cells. Importantly, no cytokine secretion was observed with CD70-negative tumor cells or in the presence of B and T cells from healthy donors. In cytotoxicity assays, CD27-ζ-CAR T cells efficiently lysed CD70-expressing tumor cell lines and primary tumors, but not CD70-negative cells [47]. Since CD27 is itself a co-stimulatory molecule we suspected that CD27-ζ-CAR T cells would receive a beneficial co-stimulatory signal when ligated with CD70. To test this hypothesis, we generated a second CD27-ζ-CAR in which 23 amino acids (238–260) in the cytoplasmic domain of CD27 were deleted (ΔCD27-ζ-CAR). Whereas CD27-ζ-CAR was able to bind TRAF2, the key adaptor protein mediating CD27 signaling, ΔCD27-ζ-CAR could not. Functionally, both versions of the CAR were able to induce lysis of CD70-expressing targets, cytokine release and T cell proliferation. However, T cells receiving a CD27 signal from CD27-ζ-CAR were significantly more viable and expressed higher levels of the anti-apoptotic protein Bcl-xl. In a systemic lymphoma model, mice treated with CD27-ζ-CAR T cells had significantly fewer tumors and longer overall survival than mice treated with non-transduced T cells.

Subsequently, other groups have found CD27 signaling provides survival advantages to T cells when incorporated into a CAR construct. Song and colleagues generated a folate receptor-specific CAR with the CD27 cytoplasmic domain fused to CD3-ζ [48]. Addition of CD27 signaling increased cytokine secretion and cytotoxicity when compared with a CAR expressing only the CD3-ζ chain. Similar to our study, they also found CD27 signaling from the CAR caused an upregulation of Bcl-xl and resistance to apoptosis. This translated to increased persistence *in vivo* that was similar to CARs expressing a 4-1BB co-stimulatory domain and better than CARs expressing a CD28 domain. Another study used an unbiased screening approach to pull out novel combinations of co-stimulatory domains that would enhance CD69 expression and IL-2 production after antibody crosslinking of CARs expressed on Jurkat T cells [49]. Their screen discovered that a combination of DAP10 and CD27 signaling domains induced optimal activation of Jurkat T cells. When expressed in primary human T cells, CARs containing the DAP10/CD27 combination induced higher cytotoxic activity than CARs with a CD28-ζ domain and this translated to a small, but significant effect on inhibiting tumor growth in mice. Finally, it is worth nothing that studies using tumor-infiltrating lymphocytes (not genetically modified) for the treatment of metastatic melanoma have found that cells mediating the best anti-tumor response tend to be $CD8^+$ and have high expression of CD27 [50]. Whether this is solely because these cells tend to be less differentiated "young" cells or whether some benefit is derived from CD27 costimulation remains to be determined.

At present no clinical data exists for CARs that have incorporated a CD27 signaling domain, but it will be interesting to see if this domain could mediate improved anti-tumor activity in human patients. CD70 as a therapeutic target is being actively investigated by Seattle Genetics who have developed an antibody-drug conjugate to target CD70-expressing tumors and initiated a phase I clinical trial in 2012 (clinicaltrials.gov: NCT01677390).

7. Peptides as CAR-Binding Domains

To this point we have discussed naturally occurring receptor ligands that can provide antigen specificity for CAR constructs; however novel tumor-specific peptides have also been described. For example, Pameijer and colleagues took advantage of a tumor-binding peptide specific for integrin alpha vβ 6 that was discovered by phage display [51]. Alpha vβ 6 integrin is overexpressed on squamous cell carcinomas of the head and neck, cervix, skin and esophagus, but has a limited and low level expression on normal tissues [52]. To generate an αvβ 6-specific CAR, a 12 amino acid tumor-binding peptide (RTDLDSLRTYTL) was fused to the IgG4 hinge region, the CD4 transmembrane domain and the CD3-ζ signaling domain. A leader sequence from GM-CSFR was also included at the N-terminus for surface expression of the CAR.

The tumor-binding peptide CAR could be stably expressed on the surface of primary T cells and led to the specific lysis of tumor cells expressing integrin αvβ 6, but not αvβ 6-negative tumor cells. Similarly, significant amounts of IFN-γ were released in the presence of αvβ 6-positive tumor cells and this release could be blocked by the addition of soluble tumor-binding peptide, but not an irrelevant peptide [51].

Lastly, Davies and colleagues have generated CARs containing the chimeric T1E polypeptide as an antigen binding domain, which consists of the N-terminal seven amino acids from human transforming growth factor (TGF)-α have and the C-terminal 48 amino acids of epidermal growth factor (EGF) [53]. T cells expressing T1E-CARs with a CD28-ζ endodomain recognized epidermal growth factor receptor (EGFR) members including HER1, HER2, HER2, and HER4, and heterodimers. T cells produced IL-2 and IFN-γ killed tumor cells in an antigen-dependent manner. *In vivo*, T1E-CAR T cells had potent anti-tumor activity in an intraperitoneal xenograft model using MDA-MB-435 cells.

These proof-of-concept studies highlight that it is feasible to generate antigen-specific CARs using peptides as binding domains. Since phage display libraries containing peptides are readily available, this approach should enable the generation of CARs against virtually any tumor-associated antigen of interest. While novel peptides can be immunogenic, their smaller size should reduce the risk of immune responses in comparison to scFv.

8. Conclusions

At present there is relatively little known about the clinical efficacy of receptor ligand-based CARs, especially in comparison to the more commonly used scFv-based CAR. Since even the smallest modifications to CAR constructs, such as spacer length, can greatly affect CAR function, careful experimentation in mouse models with these two types of CARs targeting the same antigen, in the same tumor types, with identical constructions would be of substantial interest [54]. A greater understanding of relative binding affinities, effect of costimulatory domains, and their subsequent *in vivo* efficacy will be needed to achieve maximal clinical efficacy from receptor ligand-based CARs.

Today most CARs used in clinical trials contain an scFv for antigen specificity. While these CARs have produced exciting clinical results, they have disadvantages. Many of the scFv being used are from antibodies raised in mice and are potentially immunogenic. This not only has the potential to induce life-threatening anaphylaxis [7], but can also limit the persistence of infused cells [55]. Receptor

ligand-based CARs derived from naturally occurring proteins may eliminate these types of immune responses. However, while potentially less immunogenic, many ligands bind not only to the intended target receptor, but also to other receptors with lower affinity, increasing the risk of 'off target' side effects. Thus, as for scFv, the specificity of each ligand has to be carefully assessed in the 'CAR context'. Most likely, there will not be a universal 'ideal CAR antigen-binding domain', and continued exploration of ligand-based CARs seems advisable given the encouraging results obtained so far.

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Author Contributions

DS, PZ, and SG wrote, reviewed and edited the manuscript.

Conflicts of interest

The Center for Cell and Gene Therapy has a research collaboration with Celgene and Bluebird Bio. DS, PZ, and SG have patent applications in the field of T-cell and gene-modified T-cell therapy for cancer.

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