Mesothelin-Targeted CARs: Driving T Cells to Solid Tumors

Aurore Morello¹, Michel Sadelain¹, and Prasad S. Adusumilli¹,²

ABSTRACT

Chimeric antigen receptors (CAR) are synthetic receptors that target T cells to cell-surface antigens and augment T-cell function and persistence. Mesothelin is a cell-surface antigen implicated in tumor invasion, which is highly expressed in mesothelioma and lung, pancreas, breast, ovarian, and other cancers. Its low-level expression in mesothelia, however, commands thoughtful therapeutic interventions. Encouragingly, recent clinical trials evaluating active immunization or immunoconjugates in patients with pancreatic adenocarcinoma or mesothelioma have shown responses without toxicity. Altogether, these findings and preclinical CAR therapy models using either systemic or regional T-cell delivery argue favorably for mesothelin CAR therapy in multiple solid tumors.

Significance: Recent success obtained with adoptive transfer of CAR T cells targeting CD19 in patients with refractory hematologic malignancies has generated much enthusiasm for T-cell engineering and raises the prospect of implementing similar strategies for solid tumors. Mesothelin is expressed in a wide range and a high percentage of solid tumors, which we review here in detail. Mesothelin CAR therapy has the potential to treat multiple solid malignancies. Cancer Discov; 6(2); 1–15. © 2015 AACR.

INTRODUCTION

Adoptive cell therapy using engineered T cells is emerging as a promising strategy to rapidly establish tumor immunity and eradicate small or large tumor burdens. T cells may be engineered to target a tumor antigen through a T-cell receptor (TCR) or a chimeric antigen receptor (CAR) (1, 2). In contrast to TCRs, which are restricted to human leukocyte antigen, CARs provide direct binding to cell-surface proteins, carbohydrates, or glycolipids. CARs intrinsically mediate T-cell activation as well as co-stimulation in the case of second-generation CARs (3). The use of CARs specific for CD19, a B-cell activation receptor, has recently been shown to induce durable remissions in patients with relapsed, chemorefractory B-cell malignancies, including acute lymphoblastic leukemia, chronic lymphocytic leukemia, and non-Hodgkin lymphoma, in multiple clinical trials (4–10). Second-generation CARs achieve these outcomes through both targeted tumor killing and functional T-cell enhancement (3, 11). Given the potential high efficiency of CAR therapy, it is critical to identify appropriate antigens to tackle solid tumors, in order to achieve tumor eradication with minimal or tolerable on-target/off-tumor toxicity to healthy tissues.

SEARCHING FOR CAR TARGETS IN SOLID TUMORS

Whereas CD19 provides a nearly ideal target for B-cell malignancies, no single antigen with equivalent characteristics has yet been identified for solid tumors. CD19 is highly and relatively homogenously expressed in most B-cell malignancies, possibly including their putative tumor-initiating cells. CD19 is functionally involved in B-cell activation, and may contribute to tumor survival and thus increase the likelihood of its expression in most tumor cells. Finally, in normal cells, CD19 expression is confined to the hematopoietic B-cell lineage, nonvital cells that can be dispensed (the B-cell aplasia induced by CD19 CAR T cells is not lethal and is clinically manageable). An ideal solid tumor CAR target would thus be overexpressed in all cancer cells, absent or with very low expression in nonvital normal tissue, and found in many patients. Furthermore, if expression of the target antigen is associated with tumor invasion or metastasis formation, CAR therapy may be directed to the more aggressive cancer cells and be less vulnerable to tumor relapse. Solid tumor CAR targets under investigation are altered gene products arising from genetic mutations or altered splicing (EGFRvIII), altered glycosylation patterns (MUC1), cancer-testis antigen-derived peptides (MAGE), overexpressed differentiation antigens [CEA, PSMA, GD2, MUC16, HER2/ERBB2, and mesothelin (MSLN)], or tumor-associated stroma (FAP and VEGFR; see...
cell-surface protein. After cleavage of the amino terminus by inositol (GPI) domain. It is initially synthesized as a 69 kDa anchored to the plasma membrane by a glycophosphatidyl exception of mesothelial cells (18). MSLN is a glycoprotein for solid tumors, Chang and colleagues discovered the MSLN (Fig. 2; Supplementary Table S2).

Although overexpressed antigens are numerous and relatively frequent, they raise concerns about “on-target/off-tumor” side effects due to the high sensitivity of T cells for low-level antigen expression, which can be greater than that of monoclonal antibodies (12). For instance, the use of ERBB2 CAR T cells, administered at a high cell dose, has resulted in a fatal adverse event, attributed in part to low-level ERBB2 expression in healthy lung epithelial and cardiovascular cells (13). Thus, an optimal solid-tumor antigen target is one whose expression either is restricted to tumor cells or occurs only at very low levels in expendable normal tissues. EGFRvIII and chondroitin sulfate proteoglycan-4 (CSPG4) have been suggested to be examples for such favorable scenarios (14, 15).

MSLN is emerging as an attractive target for cancer immunotherapy, considering its low expression on normal mesothelial cells and high expression in a broad spectrum of solid tumors. The MSLN-targeted immunotherapies reported to date support a favorable safety profile (16, 17). MSLN is a potential CAR target in a number of common solid tumors (Fig. 2; Supplementary Table S2).

DISCOVERY AND EARLY CHARACTERIZATION OF MSLN

In searching for targets for monoclonal antibody therapy for solid tumors, Chang and colleagues discovered the MSLN protein, which they found to be specifically expressed on ovarian cancer cells but not on normal human tissues, with the exception of mesothelial cells (18). MSLN is a glycoprotein anchored to the plasma membrane by a glycosphatidyl inositol (GPI) domain. It is initially synthesized as a 69 kDa cell-surface protein. After cleavage of the amino terminus by the furin protease, a 40-kDa C-terminal fragment remains attached to the membrane and a soluble 32-kDa N-terminal fragment, named megakaryocyte-potentiating factor (MPF), is released (17). A soluble form of MSLN has also been detected in the sera of patients with solid tumors, which is referred to as soluble MSLN-related protein (SMRP). SMRP is generated either by alternative splicing or by proteolytic cleavage of the MSLN mature form, induced by the TNFα-converting enzyme ADAM17 (19).

MSLN FUNCTION

The biologic function of MSLN seems to be nonessential in normal tissues, given that MSLN knockout mice exhibit normal development, reproduction, and blood cell count (20). In contrast, preclinical and clinical studies increasingly show that aberrant MSLN expression plays an active role in both malignant transformation of tumors and tumor aggressiveness by promoting cancer cell proliferation, contributing to local invasion and metastasis, and conferring resistance to apoptosis induced by cytotoxic agents (21–24). MSLN can act bidirectionally, either by directly activating intracellular pathways via its GPI domain or by interacting with its receptor, CA125/MUC16. Overexpression of MSLN alone is sufficient to constitutively activate the NFκB, MAPK, and PI3K intracellular pathways promoting cell proliferation and resistance to apoptosis (25). Several preclinical studies, including ours, support the finding that MSLN overexpression promotes cell migration and invasion by inducing activation and expression of the matrix metalloproteases MMP7 (26) and MMP9 (21). In addition, the high-affinity interaction between MSLN and CA125 leads to heterotypic cell adhesion, which facilitates metastasis of ovarian cancer cell lines (27). These observations correlate with clinical observations showing that MSLN expression, as well as
elevated serum SMRP levels, is associated with progressing tumor burden, increasing stage, and poor overall survival (22–24, 28, 29). Cancer cells that possess an invasive phenotype express high amounts of membranous MSLN, rather than the cytoplasmic form (30, 31). Our group (22) and others (29) have reported that in patients with lung adenocarcinoma, MSLN is expressed at metastatic sites and correlates with tumor aggressiveness and \(\text{KRAS}\) mutation. These discoveries strengthen the rationale for targeting MSLN-expressing cancer cells with CARs.

**MSLN EXPRESSION IN SOLID TUMORS**

Physiologically, MSLN is expressed on mesothelial cells of the peritoneal and pleural cavities and pericardium; it is expressed minimally on the epithelial cell surface of the trachea, ovaries, rete testis, tonsils, and fallopian tubes (32). Overexpression of MSLN was initially observed in mesothelioma and ovarian cancer, and subsequently in lung, esophageal, pancreatic, gastric, biliary, endometrial, thymic, colon, and breast cancers (17, 21–24). MSLN overexpression thus has an estimated incidence of 340,000 patients and prevalence of 2 million patients a year (Supplementary Tables S3 and S4) in the United States alone. The frequency and distribution pattern of MSLN expression differ for each tumor subtype, as summarized in Fig. 2 and Supplementary Table S2. Using the 5B2 MSLN-specific antibody, we developed an MSLN expression score integrating MSLN intensity and distribution (21). In our series, MSLN expression was found in 90% of epithelioid malignant pleural mesothelioma (\(n = 139; \text{ref. 21}\)), 69% of lung adenocarcinoma (\(n = 1,209; \text{ref. 22}\)), 60% of breast cancers (\(n = 314; \text{ref. 24}\)), and 46% of esophageal cancers (\(n = 125; \text{ref. 23}\)). Furthermore, we observed that MSLN expression is more prevalent in aggressive histologic subtypes of lung (\(\text{KRAS}\^*\) tumors; ref. 22), breast (triple-negative; ref. 24), and esophageal cancers (high-grade dysplasia and adenocarcinoma; ref. 23). These findings have been corroborated in other studies (29, 33). Within the cancer cell, MSLN expression may be luminal/membranous or cytoplasmic. In mesothelioma tumors, MSLN expression is homogeneously distributed on the cell surface.
In lung adenocarcinoma, we and others have found that the MSLN expression pattern is heterogeneous, with expression in the cytoplasm and on the cell surface (22, 29). In gastric cancer, cytoplasmic expression was found to be more prevalent than membranous expression (30). In addition to the studies characterizing MSLN expression by IHC analysis, functional genomic mRNA profiling studies in a large cancer database (n = 19,746) have reported MSLN expression in other solid tumors, such as thyroid, renal, and synovial sarcoma tumors, which were not previously reported (34).

Given MSLN’s distribution, protumorigenic functions, and immunogenicity (see below), various immunotherapeutic strategies have been devised, some of which have shown encouraging results in early-phase clinical trials (Table 1). These strategies include the use of (1) tumor vaccines, (2) antibody-based therapies, and (3) adoptive T-cell therapy (CAR T cells; Fig. 3).

Figure 3. MSLN-targeted immunotherapy strategies. Several therapeutic strategies have been designed for targeting MSLN on tumor cells: (1) tumor vaccine strategy; (2–4) antibody-based therapies; and (5) adoptive CAR T-cell therapy. These therapies are being evaluated in phase I and/or phase II clinical trials.
Table 1. Phase I/II clinical trials for MSLN-targeted immunotherapies

<table>
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<tr>
<th>Agent</th>
<th>Phase</th>
<th>Intervention</th>
<th>Cancer/s targeted</th>
<th>Status/results</th>
<th>Clinicaltrials.gov Identifier</th>
<th>References</th>
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<td>CRS-207</td>
<td>II</td>
<td>GVAX and cyclophosphamide with or without CRS-207</td>
<td>Metastatic pancreatic cancer</td>
<td>31% SD, 51% PD</td>
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<td>II B</td>
<td>CRS-207 alone or plus GVAX therapy and cyclophosphamide</td>
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<td>I B</td>
<td>CRS-207 plus pemetrexed and cisplatin with and without cyclophosphamide</td>
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<td>SS1P</td>
<td>I</td>
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<td>I</td>
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<td>Amatuximab (MORAb-009)</td>
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<td>and other cancers</td>
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<td>CAR T cells plus fludarabine, cyclophosphamide, and aldesleukin</td>
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(Continued)
**Table 1. Phase I/II clinical trials for MSLN-targeted immunotherapies (Continued)**

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<th>References</th>
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<td>I</td>
<td>CART T cells plus cyclophosphamide</td>
<td>Mesothelioma, metastatic lung, and breast cancers</td>
<td>Recruiting</td>
<td>NCT02414269</td>
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</table>

Abbreviations: NSCLC, non–small cell lung cancer; PD, progressive disease; PR, partial response; SD, stable disease.

*Listeria monocytogenes* vector that overexpresses human MSLN, either alone (35) or in combination with cyclophosphamide and GVAX (irradiated allogeneic cell line–secerting GM-CSF; refs. 36, 37). Although no clinical responses were reported, MSLN-specific CD8 T-cell responses were induced following cyclophosphamide, GVAX, and CRS-207 administration, along with a modest increase in survival (36). Significantly, no toxicities were observed in the patients. In addition to CD4 and CD8 T-cell responses (38), MSLN-specific antibody immune responses (39) were observed in patients with ovarian and pancreatic cancers, confirming the immunogenicity of MSLN and further supporting the safety of its immunotherapeutic targeting.

Phase I studies with SS1P, an anti-MSLN immunotoxin engineered by fusing a murine anti-MSLN variable antibody fragment to the PE38 portion of *Pseudomonas exotoxin*, enrolled patients with advanced mesothelioma, ovarian cancer, or pancreatic cancer (40). As a single agent, SS1P exhibits moderate antitumor efficacy. Impressive antitumor responses were seen in patients with mesothelioma who received SS1P, together with pentostatin and cyclophosphamide, to deplete T and B cells (41). Leveraging the knowledge that chemotherapies act in concert by disrupting the tumor structure, thereby allowing better penetration of the SS1P molecule, SS1P, in combination with cisplatin and pemetrexed, has resulted in partial responses in 77% of 13 patients with mesothelioma (42). A limitation of the strategies that use SS1P immunotoxin is the development of neutralizing antibodies specific for the toxin portion of the construct and possibly the chimeric SS1 antibody as well. Fully human anti-MSLN monoclonal antibodies have been evaluated in the preclinical setting (43, 44), with the goal of identifying agents with a lower immunogenicity profile—an important concern in the development of CARs as well.

Another therapeutic strategy based on the MSLN antibody uses amatuximab (also called MORAb-009; ref. 45). Amatuximab binds to MSLN, thereby inhibiting adhesion between cell lines expressing CA125, and it elicits antibody-dependent cell-mediated cytotoxicity. Phase II clinical trials have been conducted with amatuximab treatment alone or in combination with pemetrexed and cisplatin. Combination treatment is well tolerated; objective tumor response and stable disease were achieved in 40% and 51% of patients with nonresectable pleural mesothelioma, respectively (n = 89; ref. 45).

Therapeutic agents have been linked to anti-MSLN antibody, with the idea that the drugs will be released into the cytoplasm following internalization of the antibody: (1) duocarmycin, a DNA alkylating agent, which led to the development of MDX-1382 MED2460 (Medarex); and (2) DM4, a tubulin polymerase inhibitor, which led to the development of BAY 94-9343 (46). Interestingly, *in vitro*, BAY 94-9343 is able to induce a bystander-killing effect on neighboring MSLN-negative cancer cells without affecting nonproliferating cells, an observation that is of particular interest in the context of heterogeneous antigen-expressing tumors (46). A phase I clinical trial investigating the safety of BAY 94-9343 is currently under way. Taken together, these reports demonstrate the safety and feasibility of MSLN as a target for CAR T-cell immunotherapy.

**MSLN CAR DESIGN AND PRECLINICAL EVALUATION**

CARs consist of an ectodomain [commonly derived from a single-chain variable fragment (scFv)], a hinge, a transmembrane domain, and an endodomain (typically comprising signaling domains derived from CD3ζ and costimulatory receptors; Fig. 4A). Several MSLN-specific scFvs have been reported, including the murine SS1 scFv (47-50) and two fully human scFvs (51, 52), spanning all three generations of CAR design based on their signaling domains (Fig. 4B). First-generation CARs contain the CD3ζ cytoplasmic domain, which is sufficient to initiate T-cell activation and enable T-cell-mediated cytotoxicity (3, 52, 53). Second-generation CARs further enhance T-cell function and persistence through the incorporation of signaling domains that rescue and amplify the activation signal provided by the CD3ζ cytoplasmic domain. Co-stimulatory elements may be derived from receptors such as 4-1BB (33, 48, 54, 55), CD28 (47, 51, 52), or ICOS (50). Dual signaling prevents T-cell anergy and increases persistence and function by augmenting T-cell proliferation and cytokine production (IFNγ and
IL2) and reducing activation-induced cell death through the recruitment of the PI3K, TRAF, and/or other pathways (3, 47, 52). Third-generation CARs comprise three signaling domains, typically encompassing those of CD3ζ and two co-stimulatory domains, for example CD28 and 4-1BB or CD28 and OX40 (47, 56). Compared with second-generation CARs, third-generation CARs have shown inconsistent anti-tumor activity in vivo (47, 57). A recently described MSLN CAR construct was generated to provide the DAP12 killer immunoglobulin-like receptor activation (58). Preclinical experiments demonstrated that T cells engineered with this CAR displayed increased potency in vivo, compared with second-generation MSLN CARs comprising either CD28 or 4-1BB signaling domains (58). Other co-stimulatory domains have been tested in combination with other antigens, including FcRγ, OX40, DAP10, and CD27 (3, 56, 59). Choosing an appropriate co-stimulation domain is essential to sustain CAR T-cell activity and calibrate T-cell persistence. However, the ideal co-stimulation domain may depend on context, as CAR function depends on multiple extraneous factors, such as antigen density (60, 61), CAR stoichiometry (61), CAR affinity (62–64), and the immunologic features of the tumor microenvironment (65–68).

A particular concern regarding MSLN CARs is interference from soluble MSLN, which in principle could occupy and block the scFv portion. Reassuringly, MSLN CAR T-cell activation (cytokine secretion and cytotoxic activity) is dependent on MSLN expression on the cell surface (47, 52). Significantly, the presence of serum SMRP does not alter MSLN CAR T-cell efficiency in vitro or in vivo, even at high levels (51, 52). Similar findings have been reported with carcinoembryonic antigen (CEA; ref. 69) and HER2-targeted CARs (70). The lack of CAR blockade by serum protein may be explained by the avidity of CAR T cells for membranous target antigen, which may be increased by interactions between adhesion molecules and other accessory molecules present on the surface of the T cell and tumor cells (71).

The efficiency of MSLN CAR T-cell therapy has been investigated in subcutaneous or orthotopic mouse models of mesothelioma, ovarian cancer, and lung cancer (47, 51, 52). We established a clinically relevant orthotopic mouse model of malignant pleural mesothelioma in which the tumor is aggressive loco-regionally with extensive lymphangiogenesis and mediastinal lymph node metastases mimicking human pleural mesothelioma (21, 72, 73). In this model, we administered MSLN CAR T cells systemically or

Figure 4. CAR T-cell design. A, structure of the CAR. The CAR contains an scFv binding domain specific for MSLN fused to a transmembrane domain and intracellular signaling domain (CD3ζ). The CAR expressed in the patient's own T cells after transduction provides both specificity and effector function activation. B, different generations of the MSLN CAR. Three generations of CART cells differing by their signaling domains (CD28 and/or 4-1BB) have been designed to increase the activation strength of T cells. Combinational antigen recognition with balanced signaling has been described. CCR, chemokine receptor.
intrapleurally (52). Intrapleural delivery resulted in greater T-cell proliferation, T-cell redistribution to extra-thoracic metastatic sites, tumor eradication, and survival, than a 30-fold higher T-cell dose administered systemically (52). Systemically administered CAR T cells are sequestered in the lungs prior to tumor infiltration. Regional administration of MSLN CAR T cells facilitated earlier antigen encounter and T-cell activation, cytokine secretion, and effector function of CD4+ CAR T cells, which was associated with increased CD8+ CAR T-cell proliferation and function. Furthermore, intrapleurally administered MSLN CAR T cells persisted long-term and eliminated a tumor rechallenge 200 days after the initial tumor eradication. On the basis of these findings, we are now initiating a phase I study to investigate MSLN CAR T cells administered regionally to subjects with mesothelioma, lung cancer, or breast cancer with pleural metastases (NCT02414269, Table 1).

### MSLN Cars in Clinical Trials

With more than 150,000 individuals diagnosed with primary and metastatic pleural disease each year in the United States alone (mostly from lung and breast cancers; ref. 52), a treatment that is effective against these diseases has the potential to make a significant impact. Multiple phase I clinical trials are currently being initiated to determine the safety and the maximum tolerated dose of MSLN CAR T cell therapy. The risk of on-target/off-tumor toxicity has led to different strategies to address this safety concern.

One such strategy is based on the transfection of mRNA that encodes the MSLN CAR, which results in transient CAR expression for only a few days. In preclinical models, this approach has shown promise; multiple infusions of mRNA CAR T cells have produced a robust antitumor effect in vivo (49). However, transient expression of the CAR may limit the long-term efficacy of the therapy. A clinical trial conducted at the University of Pennsylvania administered autologous T cells electroporated with the mRNA encoding for a second-generation MSLN CAR (SS1–4-1BB CAR; refs. 74, 75). In this study, 4 patients with advanced mesothelioma or pancreatic tumors were treated with MSLN CAR T cells infused intravenously or intratumorally. Multiple MSLN CAR T-cell infusions were well tolerated, with no off-target toxicities (pleuritis, pericarditis, or peritonitis) observed. Encouragingly, moderate clinical responses were observed in this phase I study, and MSLN CAR T cells were detected in the tumor. The antitumor activity of MSLN CAR T cells in vivo was established by the transient elevation of inflammatory cytokines in the sera, including IL12, IL6, G-CSF, MIP1β, MCP1, IL1RA, and RANTES (74, 75). No severe cytokine release syndrome (CRS) was reported with MSLN CARs (74, 75). Interestingly, the clinical evidence also highlights the capacity of CAR T-cell therapy to elicit a systemic antitumor cellular and humoral immune response by favoring epitope spreading (74). The detection of a polyclonal IgG antibody response is consistent with the classic process of epitope spreading, where tumor lysis and inflammation induced by MSLN CAR T cells lead to the release of multiple antigens that are cross-presented on dendritic cells and activate the host immune response. This observation underscores the indirect mechanism present in MSLN CAR T-cell therapy to potentiate a broad antitumor immune response. A serious adverse event was subsequently reported by Maus and colleagues, as one study subject developed severe anaphylaxis and cardiac arrest after the third infusion of MSLN CAR T cells. This reaction was related to the high production of IgE antibodies directed against the MSLN CAR (75), which included the murine SS1 scFv. This effect may be attributable to the multiple injections of the CAR T cells, which may have resulted in an effective prime/boost regimen to stimulate the host humoral immune response. The use of a fully human MSLN CAR (51, 52) will hopefully abrogate or at least reduce the risk of developing such an anti-CAR antibody response.

Another approach to increase T-cell safety is to utilize a suicide gene to eliminate T cells in the event of an emerging adverse event. CAR T cells can be eliminated by drug-induced activation of a suicide gene, such as the herpes simplex thymidine kinase (HSV-TK) gene (76), inducible caspase-9 (iCaspase-9; ref. 77), or EGFRα gene (78). Unlike HSV-TK, the latter two are human proteins with a minimal immunogenic potential. These “safety-switch systems” have been successful in clinical investigation (77) and can rapidly deplete CAR T cells if required. The clinical-grade construct that incorporates an iCaspase-9 safety switch—which we will use in an upcoming clinical trial of intrapleural MSLN-targeted CAR T-cell therapy (NCT02414269, Table 1)—has been shown to be safe in preclinical experiments wherein a single dose of the AP1903 small molecule eliminated intrapleurally administered MSLN CAR T cells at the peak of their proliferation within 4 hours.

### Future Cars and Their Paths

The solid-tumor microenvironment poses several obstacles for MSLN CAR T cells that may limit their antitumor efficacy (79). To optimize the efficiency of CAR T cells, numerous approaches are under evaluation to tame the host tumor microenvironment or generate “ armored” CAR T cells that can overcome immune barriers. Such strategies include (i) promoting CAR T-cell infiltration, (ii) augmenting the functional persistence of CAR T cells, (iii) enhancing CAR T cells to overcome inhibitory signals encountered in the tumor microenvironment, and (iv) improving safety by preventing on-target/off-tumor toxicity (Table 2A).

To enhance trafficking to solid tumors, MSLN CAR T cells have been engineered to overexpress the chemokine receptor CCR2B, because mesothelioma cells highly express its chemokine ligand, CCL2, and T cells express a minimal amount of CCR2B (54). CCR2B overexpression significantly improved specific homing of MSLN CAR T cells to the tumor microenvironment, as well as the efficiency of the therapy following systemic administration. Potentiation of trafficking by cotransduction of chemokine receptors, such as CCR2 or CCR4, has been demonstrated previously in other preclinical models for T-cell therapy engineered with CARs (80) as well as TCRs (81). This strategy is particularly relevant for MSLN CAR T-cell therapy because solid tumors overexpress CCR2 and CCR4 chemokine ligands.
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<th>Effects</th>
<th>Antigen targeted</th>
<th>Tumor targeted</th>
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<tr>
<td><strong>Improve CAR T-cell infiltration/migration</strong></td>
<td>CCR2 Promotes CAR T-cell trafficking to the tumor following systemic administration</td>
<td>MSLN, GD2</td>
<td>Mesothelioma, Neuroblastoma</td>
<td>(54, 80)</td>
</tr>
<tr>
<td></td>
<td>CCR4 Promotes CAR T-cell trafficking to the tumor following systemic administration</td>
<td>CD30</td>
<td>Lymphoma</td>
<td>(92)</td>
</tr>
<tr>
<td></td>
<td>Heparanase Degrades the extracellular matrix, thereby improving CAR T-cell tumor infiltration and efficacy</td>
<td>CSPG4, GD2</td>
<td>Melanoma, Neuroblastoma</td>
<td>(93)</td>
</tr>
<tr>
<td><strong>Improve CAR T-cell effector function</strong></td>
<td>Active AKT The constitutive AKT expression improves CAR T-cell survival, proliferation, cytokine secretion and renders them resistant to Treg suppression</td>
<td>GD2</td>
<td>Neuroblastoma</td>
<td>(82)</td>
</tr>
<tr>
<td></td>
<td>IL12 Increases effector cytokine secretion, renders CAR T cells resistant to Treg-mediated inhibition, and induces host innate immune response</td>
<td>CD19, CD30, MUC16, CEA, VEGFR</td>
<td>Leukemia, Lymphoma, Ovarian, Colon, Melanoma, sarcoma, and colon cancer stroma</td>
<td>(94–97)</td>
</tr>
<tr>
<td></td>
<td>IL15 Improves T-cell expansion and reduces PD-1 expression</td>
<td>CD19</td>
<td>Leukemia</td>
<td>(98, 99)</td>
</tr>
<tr>
<td></td>
<td>IL7 or IL7R Increases proliferation, survival, and effector function of CAR T cells even in the presence of Tregs</td>
<td>CD19, GD2</td>
<td>Leukemia, Neuroblastoma</td>
<td>(99, 100)</td>
</tr>
<tr>
<td></td>
<td>IL21 Increases CAR T-cell proliferation and cytotoxic efficacy</td>
<td>CD19</td>
<td>Leukemia</td>
<td>(99, 101)</td>
</tr>
<tr>
<td></td>
<td>CD80 or 4-1BB Trans-/autocostimulation between CAR T cells enhancing effector functions</td>
<td>PSMA</td>
<td>Prostate</td>
<td>(102)</td>
</tr>
<tr>
<td></td>
<td>CD40L Enhances tumor cell immunogenicity, stimulates moDC, and increases CAR T-cell cytotoxic efficacy</td>
<td>CD19</td>
<td>Leukemia</td>
<td>(103)</td>
</tr>
<tr>
<td></td>
<td>4αβ chimeric cytokine receptor generated by the fusion of IL4R ectodomain and IL2R and IL15R subunit enhances CAR T-cell long-term proliferation and cytotoxicity</td>
<td>MUC1, PSMA, ERBB</td>
<td>Breast, Prostate, Head and neck</td>
<td>(104)</td>
</tr>
<tr>
<td><strong>Counteract immunosuppression</strong></td>
<td>shRNA CTLA4 Decreased CTLA4 expression enhances CAR T-cell proliferation and antitumor activity</td>
<td>CD19</td>
<td>Leukemia</td>
<td>(105)</td>
</tr>
<tr>
<td><strong>Improve specificity and safety</strong></td>
<td>iCAR &quot;safety switch&quot; iCAR with PD-1 or CTLA-4 inhibitory intracellular domain linked to secondary antigen constrains CAR T-cell specificity to cancer cells expressing the primary antigen</td>
<td>PSMA</td>
<td>Prostate</td>
<td>(106)</td>
</tr>
</tbody>
</table>

(Continued)
### Table 2. Genetic engineering strategies and combinational therapies potentiating CAR T-cell efficacy (Continued)

#### (B) Preclinical investigation of combinational therapies potentiating CAR T-cell efficacy

<table>
<thead>
<tr>
<th>Agents</th>
<th>Effects</th>
<th>Antigen targeted</th>
<th>Tumor targeted</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Preconditioning</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Radiotherapy</td>
<td>Total body irradiation (TBI)–induced lymphodepletion in the host promotes CAR T-cell efficacy</td>
<td>EGFRvIII</td>
<td>Glioblastoma</td>
<td>(107)</td>
</tr>
<tr>
<td>Flutamide</td>
<td>Flutamide-induced androgen ablation acts in additive with CAR T cells in vitro</td>
<td>MUC1</td>
<td>Prostate</td>
<td>(108)</td>
</tr>
<tr>
<td>Valproate</td>
<td>Sodium valproate–induced upregulation of tumor cell-surface NKG2DL expression enhances the immune recognition of CAR T cells in vitro</td>
<td>NKG2DL</td>
<td>Ovarian</td>
<td>(109)</td>
</tr>
<tr>
<td><strong>Monoclonal antibodies</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PD-L1 or PD-1 immune checkpoint blockade</td>
<td>Blocking the PD-1 immunosuppressive signaling enhances CAR T-cell proliferation, cytotoxicity, and cytokine secretion</td>
<td>CEA</td>
<td>Liver metastases from colon cancer</td>
<td>(48, 65, 110)</td>
</tr>
<tr>
<td>Bispecific antibodies EGFR/cMET or EGFR/EPCAM</td>
<td>Bispecific antibodies link EGFR-transduced CAR T cells to antigen-expressing tumor cells enhancing CAR T-cell recruitment/retention and cytotoxicity</td>
<td>CEA</td>
<td>Colon</td>
<td>(111)</td>
</tr>
<tr>
<td>Anti GM-CSF or Gr-1</td>
<td>Reduction of myeloid-derived suppressor cell (MDSC) population</td>
<td>CEA</td>
<td>Liver metastases from colon cancer</td>
<td>(65)</td>
</tr>
<tr>
<td><strong>Small specific inhibitory drug</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ABT-737</td>
<td>Improves CAR T-cell killing by restoring apoptosis pathway in tumor cells</td>
<td>CD19</td>
<td>Leukemia</td>
<td>(112)</td>
</tr>
<tr>
<td>Rapamycin</td>
<td>Inhibition of mTOR kinase decreases the expression of antiapoptotic molecules and others (VEGF, PD-L1, IL10) leading to a superior antitumor effect of CAR T cells engineered with mTOR resistance</td>
<td>CD19</td>
<td>Leukemia</td>
<td>(113)</td>
</tr>
<tr>
<td>BRAFi/MEKi</td>
<td>Inhibition of MAPK pathway blocks tumor cell growth and enhances apoptotic killing by CAR T cells in vitro</td>
<td>GD2</td>
<td>Melanoma</td>
<td>(114)</td>
</tr>
<tr>
<td><strong>Oncolytic virus</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Adenovirus vector expressing Rantes and IL15</td>
<td>Adenovirus vector-mediated Rantes and IL15 expression in the tumor enhances CAR T-cell infiltration and persistence</td>
<td>GD2</td>
<td>Neuroblastoma</td>
<td>(115)</td>
</tr>
<tr>
<td><strong>Whole-cell vaccine</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Irradiated K562 cells expressing CD40L and OX40L</td>
<td>Vaccination boosts antitumor efficacy of CAR T cells</td>
<td>GD2</td>
<td>Lung Neuroblastoma</td>
<td>(116)</td>
</tr>
</tbody>
</table>

Abbreviations: iCAR, inhibitory CAR; moDC, monocyte-derived dendritic cells.
Upon T-cell activation following tumor infiltration, multiple intracellular factors, such as diacylglycerol kinase (DGK), impair T-cell effector functions and promote T-cell anergy. Riese and colleagues demonstrated that genetic deletion of DGKC, significantly increased the antitumor activity of MSLN CAR T cells, as shown by the enhancement of effector cytokine secretion, FASL, and TRAIL expression, and the cytotoxic functions in vitro (55). In addition to the strategies that investigated the CAR T-cell intracellular pathways such as DGKζ, or AKT (82), other genetic engineering strategies to enhance CAR T-cell effector function have been described (IL12, IL7, and IL15 secretion) in other models, with examples provided in Table 2A.

Within the solid tumor, CAR T cells are confronted with a tumor-induced immunosuppressive microenvironment that can limit CAR T-cell potency. Tumor cells and associated-stroma cells, including Tregs and myeloid-derived suppressor cells, express inhibitory molecules, such as TGFβ, IDO, and PD-L1, which limit CAR T-cell efficacy (48, 55, 65, 67, 68). Although second-generation CARs are relatively efficient in the immunosuppressive microenvironment, as shown by us and other investigators (83), co-stimulation alone may not be sufficient (66). To potentiate CAR T cells in an immunosuppressive environment, multiple approaches have been investigated, including antibody-based therapy and genetic approaches, such as engineering T cells to express a dominant-negative TGFβ receptor that restores T-cell effector functions in an immunocompetent mouse melanoma model (84). This strategy, which is currently being investigated in a clinical trial utilizing CAR T cells targeting the HER2 antigen (clinicalTrials.gov, NCT00889954), could readily be adapted for use with MSLN CAR T-cell therapy, as TGFβ is an immunosuppressive factor in lung cancer, ovarian cancer, and mesothelioma. Overexpression of PD-L1 by tumor cells has been shown to induce CAR T-cell exhaustion (48, 65). The immunosuppressive effect of the PD-L1/PD-1 pathway can be reverted by the addition of PD-1/PD-L1-blocking antibody (48), a PD1/CD28 converter (85, 86), or a PD-1 dominant-negative receptor. Our results demonstrate that coexpression of a PD-1 dominant-negative receptor together with an MSLN CAR potentiates long-term eradication of mesothelioma tumors (87).

To improve the specificity and safety of CAR T cells, a trans-signaling strategy was developed where CD3ζ signaling is physically dissociated to the co-stimulatory signal through the transduction of two CARs specific for different antigens (Fig. 4B); these dual-CAR T cells eliminate only cancer cells that coexpress the two targeted antigens (53, 88). In one such strategy, T cells are engineered to express an MSLN-specific CAR containing a CD3ζ domain and a folate receptor–specific CAR containing a CD28 co-stimulation domain (Fig. 4B). Cotransduced T cells possess superior antitumor activity against cancer cells expressing both antigens, compared with first-generation CAR T cells, and equivalent activity compared with second-generation CAR T cells. Thus, this study demonstrates the ability to manage on-target toxicity on normal tissue, as well as the ability to combine MSLN CARs with another tumor antigen to improve the safety, specificity, and efficacy of MSLN CAR T-cell therapy.

POTENTIAL COMBINATION THERAPIES

In addition to the genetic engineering strategies described above, rational combinatorial approaches with therapeutic agents that are already in clinical practice are being investigated to enhance therapy response by improving T-cell engraftment, sensitizing tumor cells to apoptosis, and stimulating the host immune system. Examples of such combinations investigated in preclinical studies potentiating CAR T-cell efficacy are shown in Table 2B. Preconditioning to achieve host lymphodepletion by use of cyclophosphamide, fludarabine, or radiotherapy is commonly used to promote engraftment of adoptively transferred T cells. Other promising approaches include combining CAR T-cell therapy with small-molecule inhibitors, monoclonal antibodies, oncolytic viruses, or whole-cell vaccines. Although these studies are conducted in tumor models, such as melanoma or leukemia, some of these may be applicable to solid tumors, including MSLN-expressing solid tumors.

CONCLUSION

CAR therapy using second-generation CARs has rapidly translated to clinical impact in CD19+ malignancies, paving the way for unprecedented enthusiasm for adoptive cell therapy and engineered T cells. Having such a powerful technology at hand, one important future direction for CAR research is the identification of suitable targets for tackling solid tumors. MSLN offers exciting prospects based on its high expression in a variety of cancers and low-level expression in normal tissues. The latter commands a thoughtful targeting strategy, noting that MSLN-targeted immunotherapies have been very well tolerated. These clinical outcomes, combined with the preclinical data obtained with MSLN CARs, argue favorably for clinical trials targeting mesothelioma and breast, lung, ovarian, and pancreatic cancers, which will soon be performed at multiple centers (NCT01355965, NCT01897415, NCT011583686, NCT02159716, NCT02414269, and NCT02465983).

Disclosure of Potential Conflicts of Interest

M. Sadelain is a consultant/advisory board member for Juno Therapeutics. No potential conflicts of interest were disclosed by the other authors.

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