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Mesothelin-Targeted CARs: Driving T cells to Solid Tumors

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Abstract

Chimeric antigen receptors (CARs) 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, 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 immune-conjugates 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.

Keywords

cancer immunotherapy; chimeric antigen receptor (CAR); lung cancer; adoptive T-cell therapy; mesothelioma; ovarian cancer; cancer of the pancreas; triple-negative breast cancer; T-cell engineering; tumor invasion

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 costimulation 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, chemo-refractory B-cell malignancies, including acute lymphoblastic leukemia, chronic lymphocytic leukemia and non-Hodgkin lymphoma in multiple clinical trials.(4–6) Second-

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generation CARs achieve these outcomes through both targeted tumor killing and functional T-cell enhancement.(3) 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, non-vital cells that can be dispensed (thus, the B-cell aplasia induced by CD19 CAR T cells is not lethal and clinically manageable). An ideal solid tumor CAR target would thus be overexpressed in all cancer cells, absent or very lowly expressed in non-vital 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),(7) altered glycosylation patterns (MUC-1),(8) cancer-testis antigen-derived peptides (MAGE),(9) differentiation antigens (CEA),(10) overexpressed antigens in tumors (PSMA, GD2, CA125, Her-2, and mesothelin [MSLN]), or tumor-associated stroma (FAP, VEGFR) (see Supplementary Table 1).(11) Examples of solid tumor antigens currently investigated in CAR T-cell clinical trials are shown in Figure 1.

While 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 is 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 is either restricted to tumor cells or only occurs at very low levels in expendable normal tissues. EGFRvIII and chondroitin sulfate proteoglycan-4 (CSPG-4) have been suggested to be examples for such favorable scenarios.(14, 15)

Mesothelin (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 (Figure 2 and Supplementary Table 2).

DISCOVERY AND EARLY CHARACTERIZATION OF MSLN

In searching for targets for monoclonal antibody therapy for solid tumors, Ira Pastan and Mark Willingham 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 glycophosphatidyl 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 MPF (megakaryotic potentiating factor), 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 TNF-α–converting enzyme ADAM 17.(19)

MSLN FUNCTION

The biological 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 NF-κB, MAPK, and PI3-kinase intracellular pathways promoting cell proliferation and resistance to apoptosis.(25) Several preclinical studies, including ours, support the finding that MSLN over-expression promotes cell migration and invasion by inducing activation and expression of the matrix metalloproteases MMP-7(26) and MMP-9.(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, are 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 *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, tonsil, 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 3 & 4) in the U.S. alone. The frequency and distribution patterns of MSLN expression differ for each tumor subtype, as summarized in Figure 2 and Supplementary Table 2. Using the 5B2 MSLN-specific

antibody, we developed a MSLN expression score integrating MSLN intensity and distribution.(21) In our series, MSLN expression was found in 90% of epitheloid malignant pleural mesothelioma (n=139),(21) 69% of lung adenocarcinoma (n=1209),(22) 60% of breast (n=314),(24) and 46% of esophageal cancers (n=125).(23) Furthermore, we observed that MSLN expression is more prevalent in aggressive histological subtypes of lung (KRAS + tumors),(22) breast (triple-negative)(24) and esophageal cancers (high grade dysplasia and adenocarcinoma).(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.(21) In lung adenocarcinoma, we and others have found that 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)

MSLN VACCINES AND IMMUNO-CONJUGATES

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) (Figure 3).

Phase I and II clinical studies have been conducted at Johns Hopkins University with CRS-207, an attenuated form of *Listeria monocytogenes* vector that overexpresses human MSLN, either alone(35) or in combination with cyclophosphamide and GVAX (irradiated allogeneic cell line–secreting GM-CSF).(36, 37) Although no objective 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 cancer, 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 PE38 to a portion of *Pseudomonas* exotoxin, enrolled patients with advanced mesothelioma, ovarian cancer, and 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 been investigated and 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 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).(45) Amatuximab binds to MSLN, thereby inhibiting adhesion between cell lines expressing CA125, and it elicits antibody-dependent cell-mediated cytotoxicity (ADCC). 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).(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 (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 signaling domains derived from CD3ζ and costimulatory receptors) (Figure 4A). Several MSLN-specific scFv's have been reported, including the murine SS1 scFv (47–50) and two fully human scFv's,(51, 52) spanning all three generations of CAR design based on their signaling domains (Figure 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 sole activation signal provided by the CD3ζ cytoplasmic domain. Costimulatory 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 (IFN-γ and IL-2), and reducing AICD (activation-induced cell death) through the recruitment of the PI3-kinase, TRAF, and/or other pathways.(3, 47, 52) Third-generation CARs comprise three signaling domains, typically encompassing those of CD3ζ and two costimulatory domains, for example CD28 and 4-1BB or CD28 and OX40.(47, 56) Compared to second-generation CARs, third-generation CARs have shown inconsistent antitumor activity *in vivo*.(47, 57) A recently described MSLN CAR construct was generated to provide the DAP12 killer immunoglobulin-like receptor with signaling activation that includes the ITAM domain.(58) Preclinical experiments

demonstrated that T cells engineered with this CAR displayed increased potency *in vivo* associated with retention of CAR expression, compared to second-generation MSLN CARs comprising either CD28 or 4-1BB signaling domains.(58) Other costimulatory domains have been tested in combination with other antigens, including FcRγ, OX40, DAP10, and CD27.(3, 56, 59) Choosing an appropriate costimulation domain is essential to sustain CAR T-cell activity and calibrate T-cell persistence. However, the ideal costimulation 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 immunological features of the tumor microenvironment.(65–68)

A particular concern regarding mesothelin CARs is interference from soluble MSLN, which in principle could occupy and block the scFv portion. Reassuringly, mesothelin CAR T-cell activation (cytokine secretion and cytotoxic activity) is dependent on MSLN expression on the cell-surface.(47, 52) Significantly, 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 CEA(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 intrapleurally.(52) Intrapleurally 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 re-challenge 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, and 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 US alone (mostly from lung and breast cancer),(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).(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 observed (pleuritis, pericarditis, or peritonitis). Encouragingly, moderate clinical responses were observed in this phase I study, and MSLN CAR T cells were detected in the tumor. The antitumoral activity of MSLN CAR T cells *in vivo* was established by the transient elevation of inflammatory cytokines in the sera, including IL-12, IL-6, G-CSF, MIP-1β, MCP-1, 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 classical 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 et al., as one study subject developed severe anaphylaxis and cardiac arrest after the third infusion of MSLN CAR T cells.(72) 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 herpes simplex thymidine kinase (*HSV-tk*) gene,(76) inducible Caspase-9(77) or EGFRΔ 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 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 (1) promoting CAR T-cell infiltration, (2) augmenting the functional persistence of CAR T cells, (3) enhancing CAR T cells to overcome inhibitory signals encountered in the tumor microenvironment, and (4) 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, since mesothelioma cells highly express its chemokine ligand, CCL2, and the T cells express a minimal amount of CCR2b.(54) CRR2b overexpression significantly improves 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, have 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 since solid tumors overexpress CCR2 and CCR4 chemokine ligands.

Upon T-cell activation following tumor infiltration, multiple intracellular factors, such as diacyl-glycerol kinase (dgk), impair T-cell effector functions and promote T-cell anergy. Riese et al. demonstrated that genetic deletion of dgkζ 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 (IL-12, IL-7, and IL-15 secretion) in other models which are detailed 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, overexpress inhibitory molecules, such as TGF-β, IDO, and PD-L1, which limiting 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) costimulation alone is not 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 TGF-β 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 HER2+ antigen (clinicalTrials.gov, NCT00889954), could readily be adapted for use with MSLN CAR T-cell therapy, as TGF-β is an immunosuppressive factor in lung cancer, ovarian cancer, and mesothelioma. Overexpression of PD-L1 by tumor cells has been shown to induce MSLN

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 by the cointroduction of a PD-1 dominant negative receptor. Our results demonstrate that coexpression of a PD-1 dominant negative receptor together with a 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 costimulatory signal through the transduction of two CARs specific for different antigens (Figure 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 costimulation domain (Figure 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 cancer-specific 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 been 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. Mesothelin 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 mesothelin-targeted immunotherapies have been very well tolerated. These clinical outcomes, combined with the preclinical data obtained with mesothelin CARs, argue favorably for a series of clinical trials targeting breast, lung, mesothelioma, ovarian and pancreatic cancer, which will soon be performed at multiple

centers (NCT01355965, NCT01897415, NCT011583686, NCT02159716, NCT02414269, and NCT02465983).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Significance

Recent success obtained with adoptive transfer of CAR T cells targeting CD19 in patients with refractory hematological malignancies have generated much enthusiasm for T-cell engineering and raise 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.

Brain	EGFRvIII, HER2, IL-13RA
Head and Neck	ERBB family
Lung	CEA, HER2, MSLN
Pleura	FAP, MSLN
Breast	CEA, cMet, HER2, MSLN
Gastric	CEA, HER2
Liver	GPC3
Colon	CEA
Pancreas	CEA, MSLN
Renal	VEGFR2
Ovarian	FR, HER2, MSLN, MUC16
Prostate	PSMA
Skin	GD2, VEGFR2
Bone	GD2, HER2
Soft Tissue	GD2, HER2
Neural	GD2, L1-CAM

Figure 1. Antigens targeted in solid tumor CAR T-cell therapy clinical trials

Antigens listed in the figure were compiled by search of the active clinical trials in the clincialtrials.gov. CEA: Carcinoembryonic Antigen; EGFR: Epidermal Growth Factor Receptor; FAP: Fibroblast Associated Protein; FR: Folate Receptor; GD2: Diasialoganglioside; GPC3: Glypican 3; HER2: Human Epidermal Growth Factor Receptor; L1-CAM: L1 Cell Adhesion Molecule; MSLN: Mesothelin; MUC-16: Mucin 16; VEGFR: Vascular Endothelial Growth Factor Receptor.

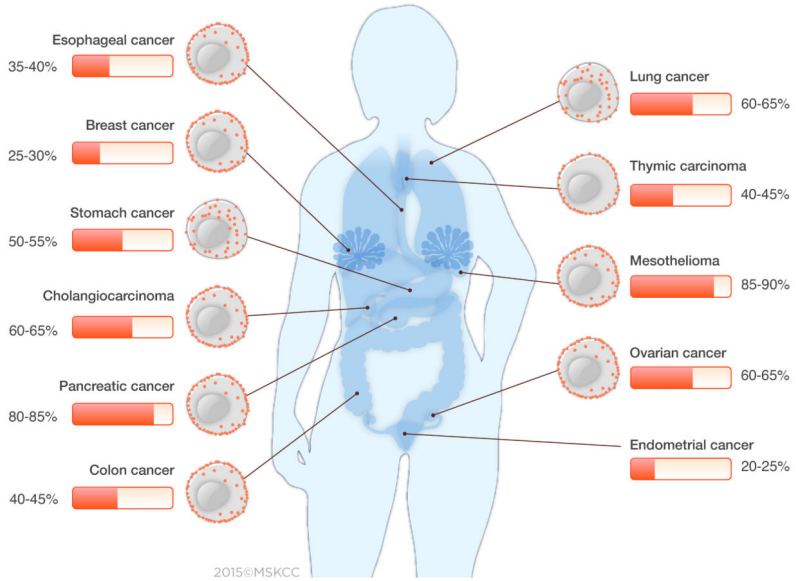

Figure 2. Frequency and distribution pattern of the mesothelin protein in solid malignancies

Mesothelin is expressed in a wide range of solid tumors. For most cancers, MSLN expression is homogeneously distributed on the cell-surface, with low cytoplasmic expression. For stomach and lung cancers, MSLN is frequently expressed in the cytoplasm, as well as on the cell-surface. The distribution of MSLN in cytoplasm and/or cell-surface was represented in the figure, where data was available. The frequency and distribution of MSLN were calculated from the published literature (Supplementary Table 2).

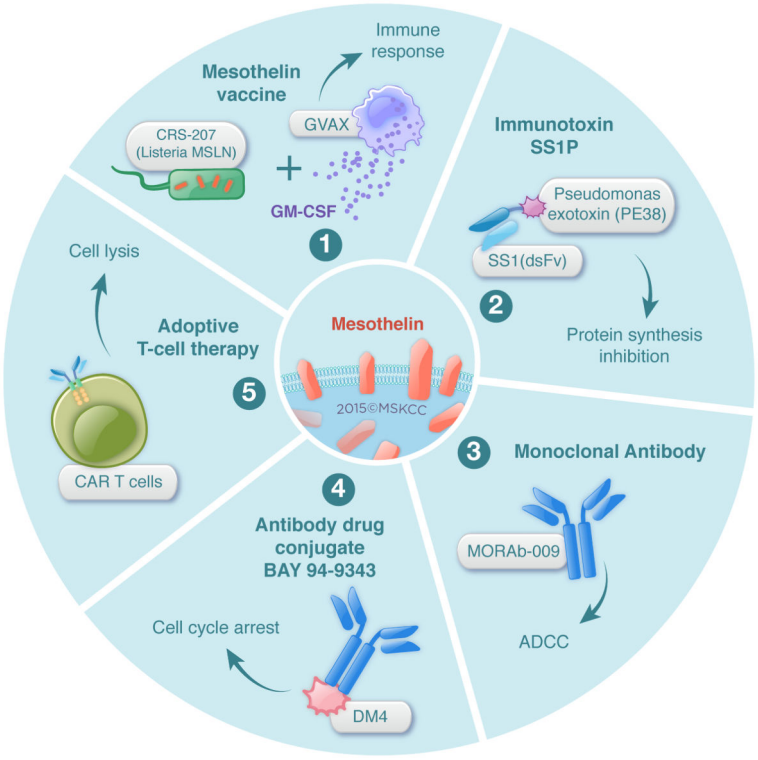

Figure 3. Mesothelin-targeted immunotherapy strategies

Several therapeutic strategies have been designed for targeting mesothelin on tumor cells (1) tumor vaccine strategy (2) antibody-based therapies (3) adoptive CAR T-cell therapy. These therapies are being evaluated in phase I and/or phase II clinical trials.

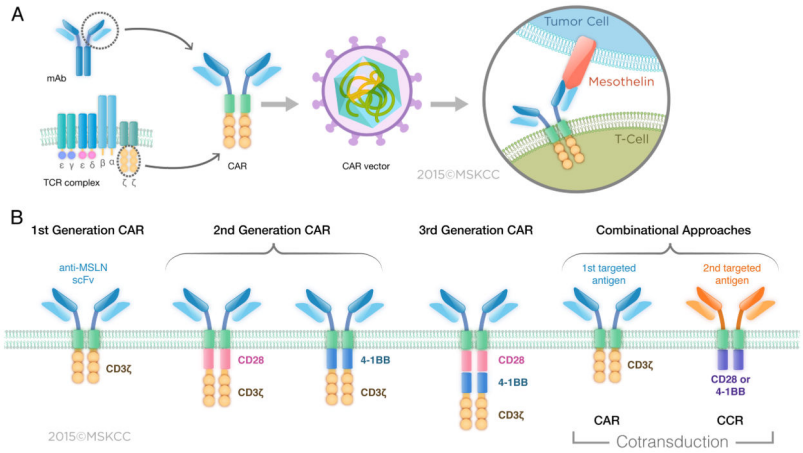

Figure 4. CAR T-cell design. (A) Structure of the CAR

The CAR typically contains a single chain fragment variable (scFv) binding domain specific for mesothelin fused to a transmembrane domain and intracellular signaling domain (CD3ζ and CD28 or 4-1BB). The CAR expressed into patient's own T cells after transduction provides both specificity and effector function activation **(B) Different generations of the MSLN CAR.** Three generations of CAR T cells differing by their signaling domains have been designed to increase the activation strength of T cells.

Table 1

Phase I/II clinical trials for mesothelin-targeted immunotherapies

Agent	Phase	Intervention	Cancer/s targeted	Status/Results	Clinicaltrials.gov Identifier	References
CRS-207	II	GVAX and cyclosphosphamide with or without CRS207	Metastatic pancreatic cancer	31% SD, 51% PD	NCT01417000	(36)
	II B	CRS-207 alone or/plus GVAX therapy and cyclophosphamide	Metastatic pancreatic cancer	Recruiting	NCT02004262	
	I B	CRS-207 plus pemetrexed and cisplatin w/ and w/o cyclophosphamide	Mesothelioma	Recruiting	NCT01675765	
	I	CRS 207 alone	Mesothelioma, Pancreatic adenocarcinoma, NSCLC, Ovarian adenocarcinoma	Dose-dependent evidence of immune activation	NCT00585845	(35)
Immunotoxin SS1P	I	SS1P plus pemetrexed and cisplatin	Mesothelioma	77% PR, 8% SD, 15% PD	NCT01445392	(42)
	I	SS1P plus pentostatin and cyclophosphamide	Mesothelioma	30% PR, 30% SD, 40% PD	NCT01362790	(41)
	I	SS1P treatment plus carboplatin, paclitaxel and bevacizumab	NSCLC	Closed	NCT01051934	
	I	SS1P alone, continuous infusion	Ovarian, Pancreatic, Mesothelioma	4% PR, 50% SD, 46% PD	NCT00006981	(89)
	I	SS1P alone, bolus infusion	Ovarian, Pancreatic, Mesothelioma	12% PR, 58% SD, 30% PD	NCT00066651	(40)
Amatuximab (Morab-009)	II	Amatuximab plus pemetrexed and cisplatin	Mesothelioma	40% PR, 51% SD	NCT00738582	(45)
	II	Amatuximab plus gemcitabine	Pancreatic cancer	No objective response	NCT00570713	
	I	Amatuximab alone	Colorectal, Pancreatic, Head and Neck, Mesothelioma	18% SD, 82% PD	NCT01018784	(90)
	I	Amatuximab alone	Pancreatic, Mesothelioma, Ovarian	55% SD, 45% PD	NCT00325494	(91)
BAY94-9343	I	BAY94-9343 alone	Invasive ovarian cancer, Primary peritoneal, Fallopian tube cancer, Mesothelioma, and other cancers	Recruiting	NCT01439152 NCT02485119	
CAR T cells	I/II	CAR T cells plus fludarabine, cyclophosphamide and aldeslekin	Metastatic cancers, Pancreatic Cancer, Mesothelioma, Ovarian	Recruiting	NCT01583686	
	I	CAR T cells plus cyclophosphamide	Metastatic pancreatic ductal adenocarcinoma, Epithelial Ovarian cancer, Mesothelioma	Recruiting	NCT02159716	
	I	CAR T cells alone	Metastatic pancreatic ductal adenocarcinoma	Completed	NCT01897415	
	I	CAR T cells alone	Mesothelioma	Completed	NCT01355965	

Agent	Phase	Intervention	Cancer/s targeted	Status/Results	Clinicaltrials.gov Identifier	References
	I	CAR T cells plus cyclophosphamide plus CD19-CAR T cells	Pancreatic cancer	Recruiting	NCT02465983	
	I	CAR T cells plus cyclophosphamide	Mesothelioma, Metastases lung and breast cancers	Recruiting	NCT02414269	

NSCLC: Non Small Cell Lung Cancer; PR: Partial Response, CR: Complete Response, PD: Progressive Disease, SD: Stable Disease, CAR: Chimeric Antigen Receptor, MPM: Malignant Pleural Mesothelioma

Table 2

Genetic engineering strategies and combinational therapies potentiating CAR T-cell efficacy

(A) Genetic engineering strategies potentiating CAR T cells

	Transgene Effects		Antigen targeted	Tumor targeted	References
Improve infiltration/migration	CCR2	Promotes CAR T-cell trafficking to the tumor following systemic administration	MSLN GD2	Mesothelioma Neuroblastoma	(54, 80)
	CCR4	Promotes CAR T-cell trafficking to the tumor following systemic administration	CD30	Lymphoma	(92)
	Heparanase	Degrades the extracellular matrix, thereby improving CAR T-cell tumor infiltration and efficacy	CSPG4 GD2	Melanoma Neuroblastoma	(93)
Improve CAR T-cell effector function	Active akt	The constitutive Akt expression improves CAR T-cell survival, proliferation, cytokine secretion and renders them resistant to Treg suppression	GD2	Neuroblastoma	(82)
	IL-12	Increases effector cytokine secretion, renders CAR T cells resistant to Treg-mediated inhibition, and induces host innate immune response	CD19 CD30 MUC-16 CEA VEGFR	Leukemia Lymphoma Ovarian Colon melanoma, sarcoma, and colon cancer stroma	(94–97)
	IL-15	Improves T-cell expansion and reduces PD-1 expression	CD19	Leukemia	(98, 99)
	IL-7 or IL-7R	Increases proliferation, survival and effector function of CAR T cells even in the presence of Tregs	CD19 GD2	Leukemia Neuroblastoma	(99, 100)
	IL-21	Increases CAR T-cell proliferation and cytotoxic efficacy	CD19	Leukemia	(99, 101)
	CD80 or 4-1BBL	Trans-/autocostimulation between CAR T cells enhancing effector functions	PSMA	Prostate	(102)
	CD40L	Enhances tumor cell immunogenicity, stimulates moDC and increases CAR T-cell cytotoxic efficacy	CD19	Leukemia	(103)
	4αβ chimeric cytokine receptor	4αβ generated by the fusion of IL-4R ectodomain and IL-2R and IL-15R subunit enhances CAR T-cell long term proliferation and cytotoxicity	MUC1 PSMA ERBBR	Breast Prostate Head and Neck	(104)
Counteract immunosuppression	shRNA CTLA4	Decreased CTLA4 expression enhances CAR T-cell proliferation and antitumor activity	CD19	Leukemia	(105)
Improve specificity and safety	iCAR ‘safety switch’	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	PSMA	Prostate	(106)

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

	Agents Effects		Antigen targeted	Tumor targeted	References
Preconditioning	Radiotherapy	Total body irradiation (TBI)-induced lymphodepletion in the host promotes CAR T-cell efficacy	EGFRvIII	Glioblastoma	(107)
	Flutamide	Fludamide-induced androgen ablation acts in additive with CAR T cells *in vitro*	MUC-1	Prostate	(108)
	Valproate	Sodium Valproate-induced upregulation of tumor cell-surface NKG2DL expression enhances the immune recognition of CAR T cells *in vitro*	NKG2DL	Ovarian	(109)
Monoclonal antibodies	PD-L1 or PD-1 immune checkpoint blockade	Blocking the PD-1 immunosuppressive signaling enhances CAR T-cell proliferation, cytotoxicity and cytokine secretion	CEA MSLN HER2	Liver metastases from colon cancer Mesothelioma Sarcoma	(48, 65, 110)
	Bispecific antibodies EGFR/cMet or EGFR/ Epcam	Bispecific antibodies link EGFR-transduced CAR T cells to antigen-expressing tumor cells enhancing CAR T-cell recruitment/retention and cytotoxicity	CEA cMet Epcam	Colon	(111)
	Anti GM-CSF or Gr-1	Reduction of Myeloid-derived suppressor cells population (MDSC)	CEA	Liver metastases from colon cancer	(65)
Small specific inhibitory drug	ABT-737	Improves CAR T-cell killing by restoring apoptosis pathway in tumor cells	CD19	Leukemia	(112)
	Rapamycin	Inhibition of mTor kinase decreases the expression of anti-apoptotic molecules and others (VEGF, PD-L1, IL-10) leading to a superior antitumor effect of CAR T cells engineered with mTor resistance	CD19	Leukemia	(113)
	BRAFi/MEKi	Inhibition of MAPK pathway blocks tumor cell growth and enhances apoptotic killing by CAR T cells *in vitro*	GD2	Melanoma	(114)
Oncolytic virus	Adenovirus vector expressing Rantes and IL-15	Adenovirus vector-mediated Rantes and IL-15 expression in the tumor enhances CAR T-cell infiltration and persistence	GD2	Neuroblastoma	(115)
Whole cell vaccine	Irradiated K562 cells expressing CD40L and OX40L	Vaccination boosts antitumor efficacy of CAR T cells	GD2	Lung Neuroblastoma	(116)

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