Clinical Cancer Research

Targeting Alpha-Fetoprotein (AFP)–MHC Complex with CAR T-Cell Therapy for Liver Cancer

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Abstract

Purpose: The majority of tumor-specific antigens are intracellular and/or secreted and therefore inaccessible by conventional chimeric antigen receptor (CAR) T-cell therapy. Given that all intracellular/secreted proteins are processed into peptides and presented by class I MHC on the surface of tumor cells, we used alpha-fetoprotein (AFP), a specific liver cancer marker, as an example to determine whether peptide–MHC complexes can be targets for CAR T-cell therapy against solid tumors.

Experimental Design: We generated a fully human chimeric antigen receptor, ET1402L1-CAR (AFP-CAR), with exquisite selectivity and specificity for the $AFP_{158–166}$ peptide complexed with human leukocyte antigen (HLA)-A*02:01.

Results: We report that T cells expressing AFP-CAR selectively degranulated, released cytokines, and lysed liver cancer cells that were HLA-A*02:01$^+$/AFP$^+$ while sparing cells from multiple tissue types that were negative for either expressed proteins. *In vivo*, intratumoral injection of AFP-CAR T cells significantly regressed both Hep G2 and AFP_{158}-expressing SK-HEP-1 tumors in SCID-Beige mice ($n = 8$ for each). Moreover, intravenous administration of AFP-CAR T cells in Hep G2 tumor-bearing NSG mice lead to rapid and profound tumor growth inhibition ($n = 6$). Finally, in an established intraperitoneal liver cancer xenograft model, AFP-CAR T cells showed robust antitumor activity ($n = 6$).

Conclusions: This study demonstrates that CAR T-cell immunotherapy targeting intracellular/secreted solid tumor antigens can elicit a potent antitumor response. Our approach expands the spectrum of antigens available for redirected T-cell therapy against solid malignancies and offers a promising new avenue for liver cancer immunotherapy. *Clin Cancer Res; 23(2); 478–88. ©2016 AACR.*

Introduction

The use of chimeric antigen receptor (CAR) or T-cell receptor (TCR) engineered T cells for cellular immunotherapy has shown significant promise in the fight against cancer, particularly for hematologic malignancies (1–4). The success of this method relies on autologous or allogeneic T cells genetically modified to express synthetic CARs/TCRs that redirect them to attack and kill tumor cells expressing a particular antigen. Despite recent successes in the field, the use of redirected T-cell therapy for the treatment of solid tumors remains challenging. Insufficient localization/persistence in solid tumors (5) and an immunosuppressive microenvironment (6, 7) have been implicated in the low efficacy observed to date. Furthermore, toxicity associated with off-target or on-target/off-tumor effects has proved to be a serious risk factor that has highlighted the lack of tumor-specific antigens for solid tumors (8, 9). Thus, identifying the precise target antigen and designing CARs/TCRs to be highly selective are critical for the clinical application of such therapies.

Hepatocellular carcinoma (HCC) is the predominant type of liver cancer, affecting over 700,000 people each year worldwide (10). Incidents of HCC are gradually rising, while survival rates remain stagnantly low (11, 12). Current treatments of HCC are limited to resection (for small localized tumors), radiation, ablation, chemoembolization, liver transplantation, and targeted therapy (namely sorafenib), which show limited efficacy and marginal survival benefits in the majority of treated patients (12, 13). For many patients the only treatment offered is palliative.

AFP is a secreted glycoprotein that is commonly overexpressed in tumors of endodermal origin including pediatric hepatoblastoma and HCC (14–17). AFP is also expressed in the fetal yolk sac, liver, and gastrointestinal tract, but rarely in adult tissues, making it an attractive candidate for T-cell immunotherapy. Elevated expression of AFP in tumors and serum is found in 60%–80% of HCC patients (14) and correlates with poor prognosis (18). Although the function of AFP remains unclear, it has been reported to promote cell proliferation, suppress apoptosis, and act as an immunosuppressive agent (19–23), suggesting a potential role in disease progression.

Despite the attractiveness of AFP as a cancer-specific antigen, AFP is expressed intracellularly and secreted, and therefore has been considered undruggable with conventional antibody-based

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Note: Supplementary data for this article are available at Clinical Cancer Research Online (http://clincancerres.aacrjournals.org/).

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Translational Relevance

Liver cancer is the fifth most prevalent and third most lethal cancer worldwide, with incidence rates on the rise and limited treatment options. Alpha-fetoprotein (AFP) is overexpressed, specifically in liver cancer, making it an ideal target for chimeric antigen receptor (CAR) T-cell immunotherapy. However, AFP is intracellularly expressed and secreted, and thus "untargetable" by conventional CARs. Given that peptides derived from intracellular/secreted proteins are processed and presented by class I MHC on the surface of tumor cells, we designed a highly specific antibody against AFP/MHC complexes and engineered it into a CAR. AFP-CAR T cells significantly regressed established liver cancer xenograft models *in vivo*. Our work demonstrates that CAR T-cell therapy can be successfully used to target a secreted/intracellular antigen for the treatment of liver cancer. Importantly, this approach will be extensible to targeting other cancer-specific targets that are currently deemed "undruggable."

therapies. However, all proteins in nucleated human cells are processed into peptides and presented by class I MHCs on the surface of cells. We have previously demonstrated that targeting such peptide–MHC complexes is feasible by engineering a "TCR-like" antibody against a peptide of Wilms Tumor 1 (WT1), an intracellularly expressed cancer-specific antigen, complexed with HLA-A*02:01 (24). AFP/MHC–specific TCRs have been identified in mice and humans, indicating that AFP–MHC complexes can be immunogenic. Furthermore, vaccines employing AFP plasmids or dendritic cells pulsed with AFP peptides elicit immune reactions in mice (25, 26), and several HLA-A*02:01–restricted AFP peptides have been found to be immunogenic in humans (27, 28). However, attempts to immunize HCC patients against AFP by activating endogenous TCRs have only shown modest therapeutic responses to date (29–31), potentially due to the inability of vaccine-related strategies to overcome tumor-related immunosuppression.

On the basis of the expression pattern of AFP in HCC and its demonstrated immunogenicity, we developed ET1402L1, a fully human antibody that selectively binds to $AFP_{158–166}$ (AFP_{158}) peptide presented by HLA-A*02:01, the most prevalent class I MHC in the human population (26, 32). We then engineered this "TCR-like" antibody into a second-generation CAR to combine antigen recognition with direct and potent immune effector activity. We show that T cells transduced with ET1402L1-CAR (herein referred to as AFP-CAR) selectively degranulated, released cytokines, and killed HLA-A*02:01$^+$/AFP$^+$ cells. Moreover, AFP-CAR T cells effectively induced tumor regression in multiple established xenograft models of human HLA-A*02:01$^+$/AFP$^+$ liver cancer, demonstrating the effectiveness of this immunotherapeutic approach in a solid tumor setting.

Materials and Methods

Cell lines

Cell lines were obtained from ATCC or Caliper Life Sciences. To generate SK-HEP-1-MG cells, parental SK-HEP-1 cells were stably transduced with a pLenti vector carrying a fragment of the AFP gene spanning the peptide region. Healthy human donor peripheral blood leukocytes were obtained from Blood Centers of the Pacific and T cells were isolated using an EasySep Human T Cell Isolation Kit (StemCell Technologies).

Peptide synthesis

Peptides were synthesized by Elim Biopharmaceuticals. Biotinylated AFP_{158}/HLA-A*02:01 and control peptide-HLA-A*02:01 complexes were generated by refolding recombinant HLA-A*02:01 in the presence of peptide and β2-microglobulin (β2M). Streptavidin–PE–conjugated AFP_{158}/HLA-A*02:01 tetramer (PE-Tetramer) was made by Eureka Therapeutics.

Phage panning and generation of full-length IgG1 antibodies

Selection of human antibodies specific to AFP_{158}/HLA-A*02:01 by cell panning and generation of IgG1 antibody toward AFP_{158}/HLA-A*02:01 complexes were performed as described previously (24).

Identification of epitopes by alanine scanning mutagenesis

AFP_{158} peptides with alanine substitutions were synthesized by Elim Biopharmaceuticals. Binding of phage clones A-D to T2 cells pulsed with mutated peptides at 50 μg/mL was detected by flow cytometry using mouse anti-M13 antibody (GE Healthcare) and R-PE–conjugated anti-mouse IgG(H+L) antibody (Vector Laboratories).

Generation of AFP-CAR T cells

Anti-AFP_{158}/HLA-A*02:01 scFvs were grafted onto a second-generation CAR with CD28 and CD3ζ signaling domains engineered *in cis* to provide intracellular T-cell stimulation signals (33). The CAR sequence was cloned into a pCDH lentiviral vector (Systems Biosciences) for delivery into T cells. Human T cells were cultured in RPMI1640 supplemented with 10% FBS and activated with CD3/CD28 Dynabeads (Thermo Fisher Scientific). One day after activation, human T cells were transduced with concentrated lentivirus in RetroNectin (Takara) coated plates. Transduced T cells were then expanded in the presence of either 100 U/mL IL2 (Sigma) or 10 ng/mL IL7 and 5 ng/mL IL15 (Peprotech) for 8–12 days. Transduction efficiency was assessed by flow cytometry using Streptavidin–PE–conjugated AFP_{158}/HLA-A*02:01 tetramer.

Mouse xenograft models of liver cancer

Animal experiments were conducted at Murigenics. Female SCID-Beige or NSG mice aged 6–8 weeks were used. For subcutaneous tumor models, cells were mixed with 50% Matrigel prior to implantation and tumors were measured by calipers. For Hep G2 tumor models, 2.5×10^6 cells were implanted per mouse; for SK-HEP-1-MG models, 5×10^6 cells were implanted. When tumors reached approximately 100 mm^3, mice were randomized to treatment groups. For intraperitoneal studies, 2.5×10^6 Hep G2-luc2 cells were injected in PBS and tumors were measured by total luminescent flux using an IVIS Imaging System (Caliper Life Sciences). Serum AFP was measured by ELISA (Affymetrix). Human anti-CD3 IHC was performed by Ensigna Biosystems.

Statistical analysis

Statistical analyses were performed using Prism GraphPad software. For studies comparing two groups, we used a Student *t* test. For studies with multiple groups, we used a one-way

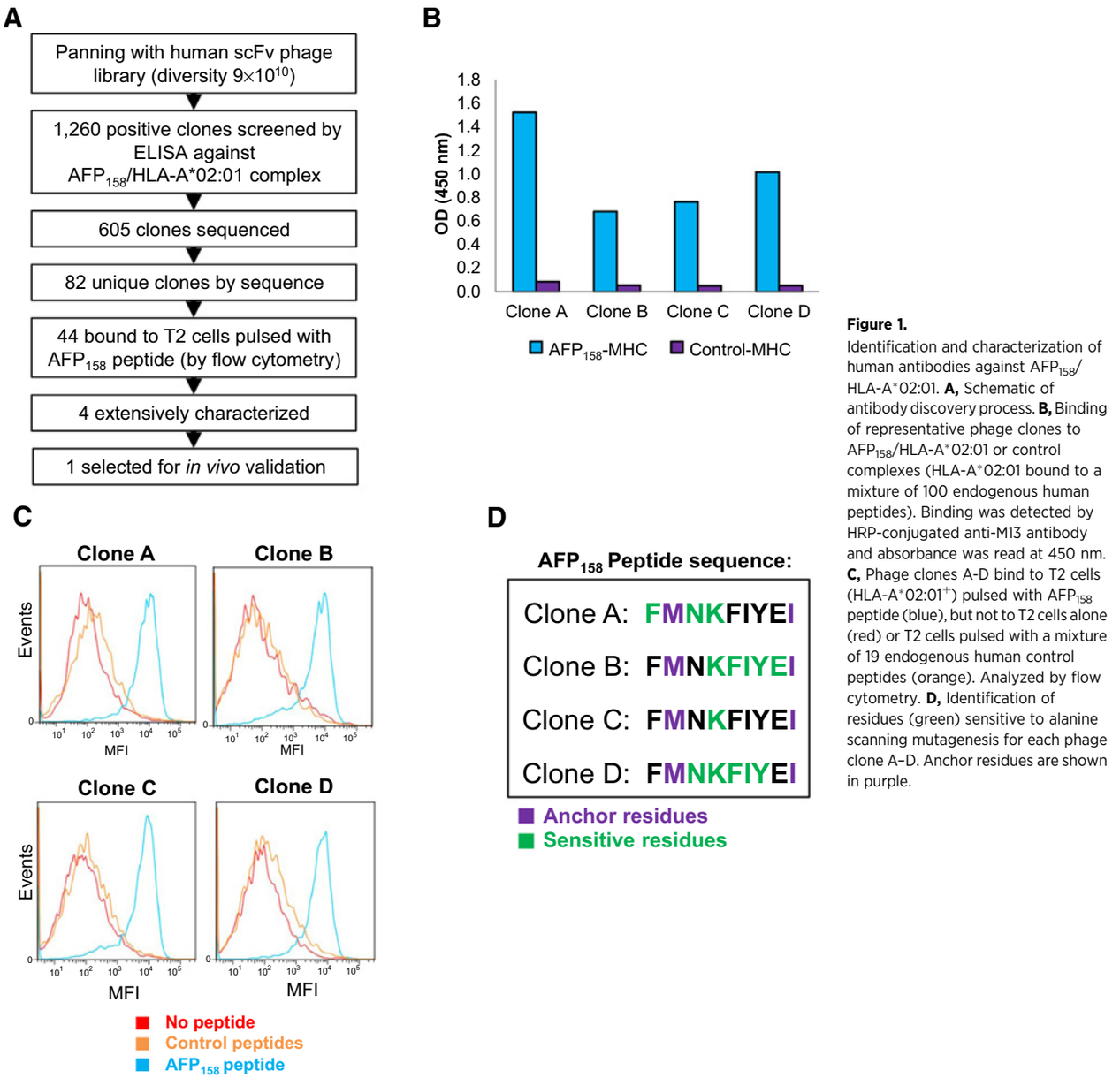

Figure 1.
Identification and characterization of human antibodies against AFP_{158}/HLA-A*02:01. **A,** Schematic of antibody discovery process. **B,** Binding of representative phage clones to AFP_{158}/HLA-A*02:01 or control complexes (HLA-A*02:01 bound to a mixture of 100 endogenous human peptides). Binding was detected by HRP-conjugated anti-M13 antibody and absorbance was read at 450 nm. **C,** Phage clones A-D bind to T2 cells (HLA-A*02:01$^+$) pulsed with AFP_{158} peptide (blue), but not to T2 cells alone (red) or T2 cells pulsed with a mixture of 19 endogenous human control peptides (orange). Analyzed by flow cytometry. **D,** Identification of residues (green) sensitive to alanine scanning mutagenesis for each phage clone A–D. Anchor residues are shown in purple.

ANOVA followed by Dunnett test which accounts for multiple comparisons. All analyses were two-tailed.

Results

Identification of clones against AFP_{158}/HLA-A*02:01 complex

A schematic of our screening and selection process is shown in Fig. 1A. A phage library (E-ALPHA™ Phage Display, Eureka Therapeutics) comprised of both naïve and semisynthetic human single-chain variable fragment (scFv) B-cell antibodies was screened for clones that selectively bound T2 cells (HLA-A*02:01$^+$) pulsed with the AFP_{158} peptide but not to cells pulsed with a mixture of 100 endogenous HLA-A2 peptides derived from various disease-related and house-keeping proteins. We validated the selective binding of 1,260 individual phage clones from three rounds of panning to AFP_{158}/HLA-A*02:01 complexes versus control complexes (HLA-A*02:01 bound to a mixture of 100 endogenous human peptides) by ELISA (representative clones are shown in Fig. 1B). We identified 605 positive clones by ELISA which corresponded to 82 unique clones by sequence, 44 of which demonstrated selectivity for AFP_{158}-loaded T2 cells over native T2 cells or T2 cells loaded with control peptides by flow cytometry (representative clones are shown in Fig. 1C).

We identified the epitope recognized by our top four antibodies (clones A–D) using alanine scanning mutagenesis. Human AFP_{158} peptides with alanine substitutions at positions 1, 3, 4, 5, 6, 7, and 8 were synthesized and pulsed onto T2 cells. The anchor residues at positions 2 and 9 were not altered as mutations at these positions are known to ablate peptide–MHC binding (34). Antibody phage clones A–D were then tested for binding to

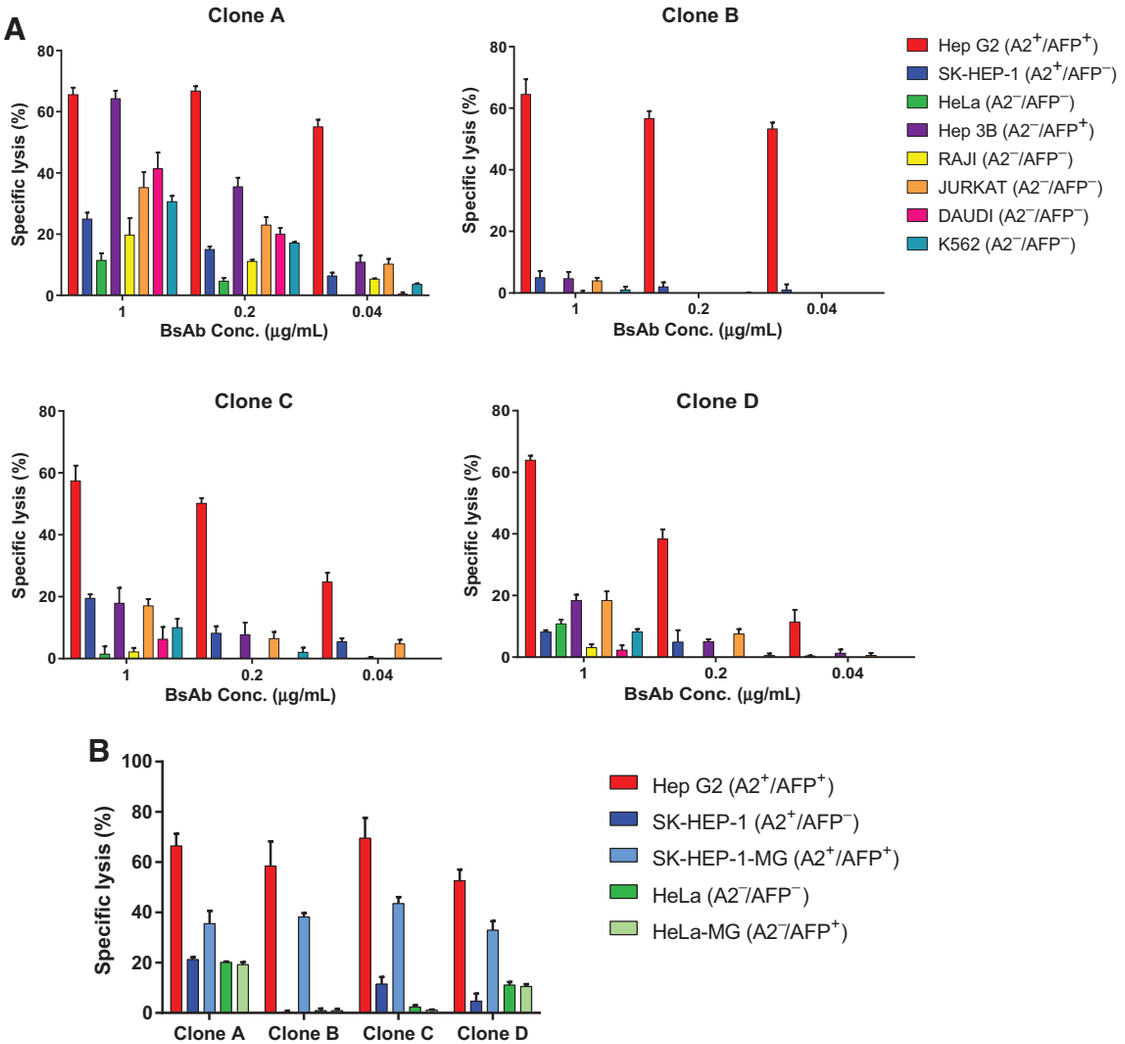

Figure 2.

Evaluation of top candidate clones for selective killing in bispecific antibody format. **A,** Clones A–D in bispecific antibody (BsAb) format were incubated at the indicated concentrations with T cells and a panel of target cells for 16 hours at an E:T ratio of 5:1. Cytotoxicity was measured by LDH release assay ($n = 3$ technical replicates for each condition). Error bars, SD. **B,** Clones A–D in BsAb format were incubated at 0.2 μg/mL with T cells and target cells engineered to express a mini-gene (MG) spanning the AFP_{158} peptide region for 16 hours at an E:T ratio of 5:1. Cytotoxicity was measured by LDH release assay ($n = 3$ technical replicates for each condition). Error bars, SD.

these peptide-loaded T2 cells and analyzed by flow cytometry (Supplementary Fig. S1). Although all antibodies recognized the small conformational epitope formed by the AFP_{158} peptide and its surrounding MHCα chain residues, the number and position of key peptide residues interacting with different antibodies varied among the clones (Fig. 1D and Supplementary Fig. S1). Clones B and D had the largest epitope, with 5 sensitive positions within the 9-mer peptide, indicating high selectivity for the AFP_{158} peptide sequence.

To measure binding affinities to the target antigen (AFP_{158} complexed with HLA-A*02:01), we engineered clones A–D into full-length antibodies with mouse IgG1 heavy- and light-chain constant regions as described previously (35). Binding affinities of the mouse chimeric antibodies against AFP_{158}/HLA-A*02:01 were measured by surface plasma resonance (SPR) using the BIACore X-100 system. K_d values were calculated to be in the subnanomolar range (Supplementary Table S1). A*02:01 is the most common subtype of HLA-A02 in the human population; however, other subtypes exist at relatively high frequencies in various ethnic groups. We therefore evaluated the binding affinities of our antibodies towards AFP_{158} complexed with additional HLA-A02 subtypes including 02:02, 02:03, 02:05, 02:06, 02:07, and 02:11 using the ForteBio Octet system. Results indicate that clones A–D also bound other HLA-A02 subtypes to varying degrees, which might broaden the eligible patient population (data not shown).

Validating the selectivity of clones against AFP_{158}/HLA-A*02:01

We next tested the selectivity of clones A–D to antigen-positive cells in bispecific antibody (BsAb) format in a T-cell–mediated

killing assay. Hep G2 cells, a human liver cancer cell line (hepatoblastoma; ref. 36), expressing both HLA-A*02:01 and AFP ($A2^+/AFP^+$) served as a positive control, along with 6 negative control tumor cell lines: the liver-derived cell line SK-HEP-1 which is HLA-A*02:01 positive but does not express AFP ($A2^+/AFP^-$), Hep 3B, an AFP^+ HCC cell line that does not express HLA-A*02:01 ($A2^-/AFP^+$), and $A2^-/AFP^-$ cell lines: HeLa (cervical), Jurkat (T-cell), Raji and Daudi (B-cell), and K562 (myeloid). At low antibody concentrations (0.04 μg/mL), all four BsAbs showed selectivity towards Hep G2 cells, although at higher concentrations clone B demonstrated superior specificity for lysing Hep G2 cells (Fig. 2A). Ectopic expression of the AFP_{158} peptide region (AFP mini-gene, MG) in AFP-negative, HLA-A*02:01–positive SK-HEP-1 cells (SK-HEP-1-MG) conferred sensitivity to killing mediated by the BsAb clones, while it had no effect on HLA-A*02:01-negative HeLa cells transduced with the same AFP mini-gene (HeLa-MG) (Fig. 2B). Similar to the results in Fig. 2A, clone B showed the highest degree of specific killing activity.

On average, each nucleated cell in the human body expresses about half a million different peptide–MHC class I complexes (37). To develop anti-peptide–MHC complex antibodies into anticancer drugs with high specificity and therapeutic index, it is essential for the antibodies to specifically recognize the target peptide–MHC complex, but not the MHC molecule itself or MHC molecules bound to similar or unrelated peptides. Therefore, we tested the binding of our top performing clone (clone B, named ET1402L1), in mouse chimeric IgG1 format against 100 known HLA-A*02:01 peptides derived from diabetes-, autoimmune disease-, cancer- and virus-related proteins. T2 cells were loaded with individual peptides and binding of ET1402L1 or an isotype control was measured by flow cytometry. While the isotype control did not bind any of the peptide-pulsed T2 cells, ET1402L1 bound in an exquisitely selective manner to T2 cells pulsed with AFP_{158} (Supplementary Fig. S2).

AFP-CAR T cells specifically kill $A2^+/AFP^+$ cells *in vitro*

We next engineered ET1402L1 into a second-generation CAR containing the CD28/CD3ζ costimulatory domains as described previously (33). The CAR construct was then cloned into a lentiviral vector for transduction of primary human T cells. T cells expressing ET1402L1-CAR (AFP-CAR) and untransduced donor-matched T cells (mock) were first tested against HLA-A*02:01–positive liver cancer cell lines. After 16 hours of incubation with AFP-CAR T cells at an effector:target (E:T) cell ratio of 5:1, specific lysis of $A2^+/AFP^+$ cells was approximately 60% for Hep G2 cells and approximately 75% for SK-HEP-1-MG cells, with minor lysis (<5%) of $A2^+/AFP^-$ SK-HEP-1 cells (Fig. 3A, left). Mock T cells showed mild activity (<10% specific lysis) against all three cell lines in a non-antigen–dependent manner. AFP-CAR and mock T cells were also tested against a panel of HLA-A02–positive and -negative human cell lines from various tissues of origin including skin, cervix, ovaries, breast, pancreas, hematologic, prostate, colon, and kidney. We observed little or no lysis of antigen-negative cells (lacking either HLA-A2 or AFP) by AFP-CAR or mock T cells (Fig. 3A, right). Furthermore, to address the possibility (although unlikely) that circulating AFP can be taken up by normal cells, crosspresented on class I MHC and therefore, targetable by AFP-CAR T cells, we incubated AFP-CAR T cells with $A2^+/AFP^-$ COLO-205 (colon), A204 (muscle), or HUVEC (endothelial) cells cultured with a high concentration of recombinant human AFP (0.4 μg/mL). However, we observed no specific killing of these cell lines (Supplementary Fig. S3A and S3B). Overall, these data demonstrate that AFP-CAR T cells exhibit superb specificity for the target antigen complex.

AFP-CAR T cells selectively degranulate and release cytokines

To further characterize the biological activity of AFP-CAR T cells, we used flow cytometry to detect CD107a surface expression as a measurement of degranulation activity. Consistent with the cell killing assay results, degranulation only occurred when AFP-CAR T cells were cocultured with the antigen-positive Hep G2 or SK-HEP-1-MG cells (Fig. 3B). Cytokine measurements by multiplex ELISA showed that TNFα, IFNγ, GM-CSF, IL2, IL4, IL6, IL8, and IL10 were elevated in the supernatants from AFP-CAR T cells incubated with Hep G2 or SK-HEP-1-MG, but not from cocultures with the antigen-negative SK-HEP-1 cells (Fig. 3C and Supplementary Fig. S4A). Flow cytometry analysis of AFP-CAR T cells stained for intracellular IL2, IL6, TNFα, and IFNγ after 4 hours of incubation with antigen-positive Hep G2 or SK-HEP-1-MG cells showed that IL2, TNFα, and IFNγ, but not IL6, are produced by the CAR T cells themselves (Fig. 3D and Supplementary Fig. S4B). The source of IL6 measured in the killing assays is presumably the target cells. No cytokines were produced when AFP-CAR T cells were cocultured with the antigen-negative SK-HEP-1 cells (Fig. 3C and D and Supplementary Fig. S4A and S4B). To test whether AFP-CAR T cells can penetrate the extracellular matrix in solid tumors to kill cancer cells, we embedded Hep G2, SK-HEP-1, and SK-HEP-1-MG cells in Matrigel where they grew into clusters encapsulated by a thick layer of laminin-rich matrix. AFP-CAR and mock T cells were laid on top of the Matrigel and incubated for 2 days. While both mock and AFP-CAR T cells effectively penetrated the matrix and reached the cell clusters after 48 hours of incubation, only AFP-CAR T cells specifically killed both Hep G2 and SK-HEP-1-MG cells with high efficiency (Supplementary Fig. S5).

Adoptive transfer of AFP-CAR T cells significantly inhibits the growth of tumors in liver cancer xenograft models

We tested the *in vivo* antitumor activity of AFP-CAR T cells in several established human liver cancer xenograft models. For all *in vivo* experiments, injected T cells were approximately 100% $CD3^+$, 60% $CD4^+$, and 40% $CD8^+$ (Supplementary Fig. S6A and S6B). To confirm specific lysis of cells expressing the target antigen, we tested each batch in an *in vitro* killing assay before administration to mice (Supplementary Fig. S6C). Analysis of T-cell differentiation markers in AFP-CAR T cells using anti-CCR7 and CD45RA showed that $CD4^+$ CAR^+ T cells were approximately 26% naïve (T_N), 21% central memory (T_{CM}), 41% effector memory (T_{EM}), and 13% effector (T_E) T cells while $CD8^+$ CAR^+ T cells were enriched for T_N (~68%) and TE (~24%) with approximately 3% of T_{EM} and 5% of T_{CM} (Supplementary Fig. S7). These ratios were similar in mock T cells.

First, HLA-$A^*02:01^+/AFP^+$ Hep G2 cells were implanted subcutaneously (s.c.) over the right flank of SCID-Beige mice and then randomized into five groups: (i) no treatment, (ii) intravenous (i.v.) injection and (iii) intratumoral (i.t.) injection of Mock T cells, (iv) intravenous injection, and (v) intraperitoneal injection of AFP-CAR T cells. Tumors in untreated or mock-treated mice grew at a steady rate until reaching a size that required euthanasia. Intravenous administration of AFP-CAR T cells in Hep G2 tumor-bearing mice resulted in a delayed inhibition of tumor growth starting 28 days after the first dose (Fig. 4A). Approximately 17% reduction in tumor size relative to controls was observed by the

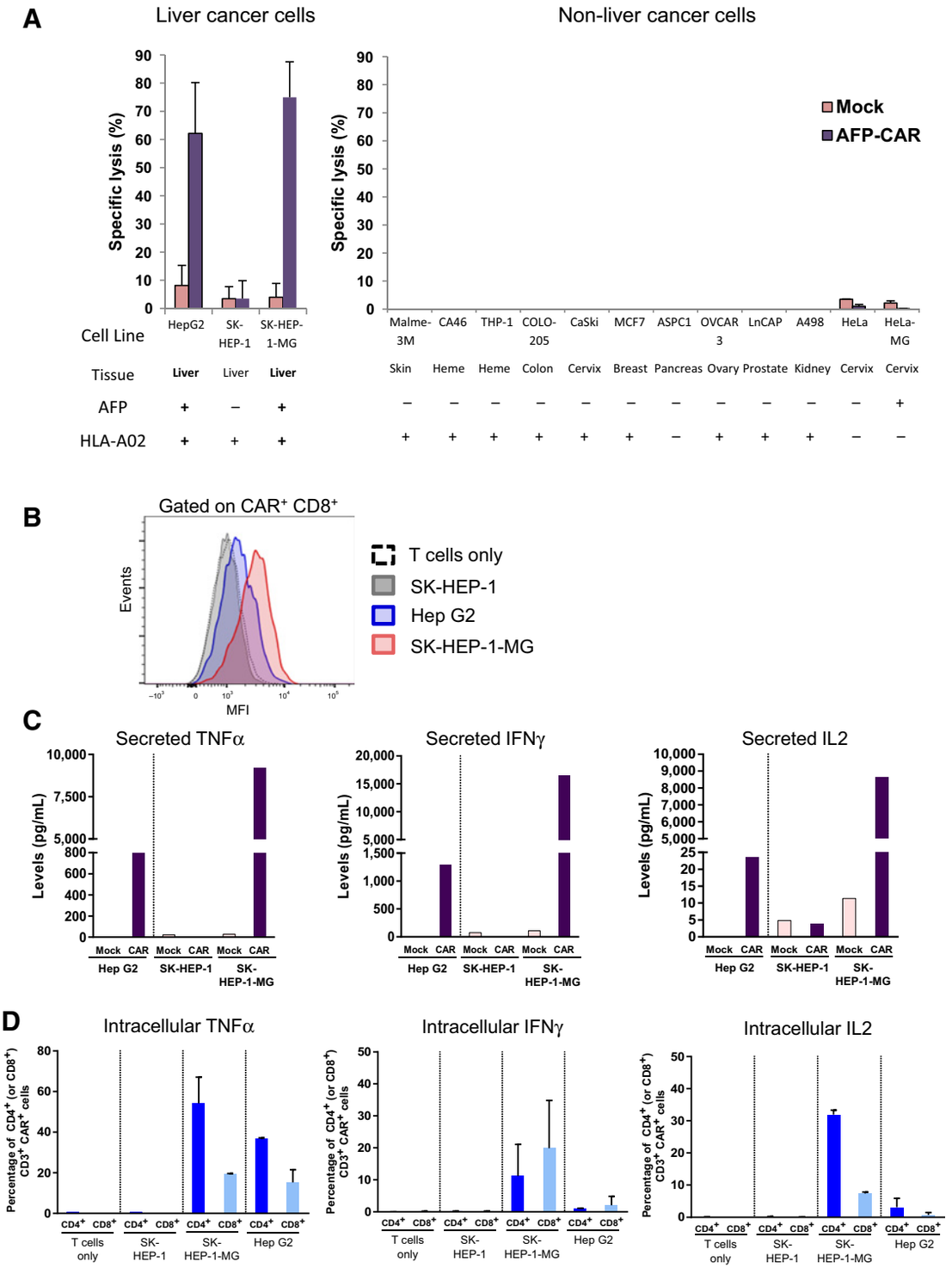

Figure 3.

AFP-CAR T cells are selectively activated by and kill $A2^+/AFP^+$ cells. **A,** AFP-CAR or mock T cells were incubated with HLA-A02–positive and -negative human cells that were either positive or negative for AFP for 16 hours at an E:T ratio of 5:1. Cytotoxicity was measured by LDH release assay ($n = 4$ independent experiments for liver cancer cell lines; $n = 3$ technical replicates for each non-liver cell line tested). Error bars, SD. **B,** Representative flow cytometry plot showing CD107a expression on AFP-CAR^+ $CD8^+$ T cells incubated with Hep G2, SK-HEP-1, and SK-HEP-1-MG cells for 4 hours at an E:T ratio of 5:1. **C,** AFP-CAR or mock T cells were incubated with Hep G2, SK-HEP-1, and SK-HEP-1-MG for 16 hours at an E:T ratio of 5:1. Secreted cytokine levels in the supernatant were measured by multiplex ELISA. Data shown are representative of three independent experiments. **D,** AFP-CAR T cells were incubated alone or with Hep G2, SK-HEP-1, and SK-HEP-1-MG cells and a protein transport inhibitor for 4 hours at an E:T ratio of 5:1. Bar graphs show percentage of $CD4^+$ or $CD8^+$ ($CD4^-$) $CD3^+$ $Tetramer^+$ cells that express intracellular TNFα, IFNγ, and IL2 ($n = 2$ independent experiments).

time control mice had to be euthanized (Fig. 4A). This tumor growth inhibition was reproduced in a consistent manner in a second independent study with similarly treated mice (data not shown). In contrast to the delayed and modest effects of intravenous administration, intratumoral injections of AFP-CAR T cells caused rapid, profound, and lasting tumor regression. Remarkably, 75% of mice treated with AFP-CAR T cells experienced complete regression with no palpable tumor remaining. In mice with palpable tumors, approximately 97% reduction in tumor size relative to controls was observed by the time control mice had to be euthanized (Fig. 4A).

We further assessed the efficacy of AFP-CAR T cells against large/advanced Hep G2 tumors through intratumoral delivery. In this study, intratumoral injection of mock or AFP-CAR T cells was given when the average tumor volume reached 600 mm^3.Even in this setting, AFP-CAR T cells significantly inhibited the growth of tumors, causing a 53% reduction in tumor volume by 1 week after dosing (Fig. 4B).

We next evaluated the *in vivo* activity of AFP-CAR T cells in a SK-HEP-1-MG subcutaneous xenograft model in SCID-Beige mice. For these experiments, mock or AFP-CAR T cells were given via intratumoral or intravenous delivery. Similar to the results obtained with Hep G2 tumors, intratumoral injection of AFP-CAR T cells resulted in robust tumor growth inhibition (Fig. 4C). Approximately 72% reduction in tumor size relative to mock-treated mice was observed by the time control mice had to be euthanized (Fig. 4C). Interestingly, in this model, intravenous administration of AFP-CAR T cells resulted in a more immediate and pronounced tumor growth inhibition than seen in the Hep G2 model (Fig. 4D). Approximately 28% reduction in tumor size was observed by the time control mice had to be euthanized (Fig. 4D). These data clearly show that while intratumorally delivered AFP-CAR T cells significant inhibit tumor growth *in vivo*, the efficacy of AFP-CAR T cells administered intravenously is relatively weak in SCID-Beige mice.

Previous studies have demonstrated better efficacy with intravenous administration in NSG mice (38–40), which have defective macrophages and dendritic cells and no natural killer cells, thus increasing the likelihood for *in vivo* CAR T-cell survival. To test this, NSG mice were implanted subcutaneously with Hep G2 cells and treated intravenously with either mock or AFP-CAR T cells. Here, intravenous injection of AFP-CAR T cells rapidly and significantly inhibited tumor growth (Fig. 4E). Approximately 64% reduction in tumor size relative to mock-treated mice was observed by the time control mice had to be euthanized (Fig. 4E). Importantly, we were able to detect $CD3^+$ T cells in Hep G2 tumors in these mice 4 weeks after T-cell dosing. Moreover, T cells were enriched in AFP-CAR–treated mice relative to controls (Supplementary Fig. S8A). This is in contrast to the low number of detectable $CD3^+$ T cells observed in intravenously treated Hep G2 tumors from SCID-Beige mice (Supplementary Fig. S8B). Overall, these data show a marked improvement in CAR T-cell survival and antitumor activity following intravenous administration in NSG mice compared with SCID-Beige.

Finally, we tested whether serum AFP levels in tumor-bearing mice might serve as a pharmacodynamic marker for response to treatment. Blood was collected from Hep G2 tumor-bearing mice 4 weeks after a single intratumor injection of either mock, 10^6, or 10^7 AFP-CAR T cells, and AFP levels were analyzed by ELISA. While serum AFP levels were very high (~2 mg/mL) in mock-treated mice, they were substantially lower (~1 mg/mL) in mice treated with the low dose of AFP-CAR T cells and even further reduced (~100 μg/mL) in mice treated with the high dose of AFP-CAR T cells, which experienced near complete tumor regression. Thus, serum AFP levels correlate well with tumor size in these models ($R = 0.9391$) and can potentially be used as a marker for tumor response (Fig. 4F).

AFP-CAR T-cell therapy shows efficacy in a disseminated peritoneal liver cancer model

Although rare, peritoneal dissemination of liver cancer occurs in a subset of patients who are left with very limited treatment options. Therefore, we tested the antitumor activity of AFP-CAR T cells in an established intraperitoneal liver cancer xenograft model. Luciferase-tagged Hep G2 cells (Hep G2-luc2) were implanted intraperitoneally (i.p.) and tumor burden was assessed weekly by measuring tumor-derived bioluminescence. One week after tumor implantation, animals were randomized on the basis of total bioluminescent flux into four groups: (i) untreated, (ii) intraperitoneal injection of 10^7 mock T cells, (iii) intraperitoneal injections of 10^6 AFP-CAR T cells, and (iv) intraperitoneal injection of 10^7 AFP-CAR T cells per mouse.

Tumor burden measured via luciferase photon emission in mock-treated animals showed no difference from that observed in the untreated control group (Fig. 5A and B). In contrast, mice treated with AFP-CAR T cells at either 10^6 or 10^7 cells per mouse showed robust tumor regression as early as one week after the first injection, which persisted for the duration of the study (11 weeks). No dose-dependent antitumor activity was observed; indicating that 10^6 total T cells per mouse exceeds the maximum efficacious dose in this model. Collectively, these data demonstrate that AFP-CAR T cells can successfully inhibit the growth of peritoneal disseminated tumors as well.

Discussion

The recent progress with redirected T-cell therapies against hematologic malignancies has revitalized the field of cancer immunotherapy. Despite the excitement surrounding this technology, many aspects of CAR/TCR–based therapies remain to be optimized to harness the full curative potential of tumor-specific immune responses. This is particularly true for solid tumors, where T-cell therapies have yet to demonstrate a significant therapeutic impact.

One of the major factors hindering the success of T-cell therapies against solid tumors is the lack of targetable tumor-specific antigens. First, unlike the blood cell lineages, which express unique cell surface markers, antigens expressed in solid tumors are often found in a variety of normal tissues. While patients can survive ablation of the hematologic cell compartment due to on-target/off-tumor effects, this is not the case with solid tumor antigens. As seen with T-cell therapies targeting MAGE-A3, MART-1, CEA, or ERBB2, even trace amounts or transient expression of a target antigen in cells of vital tissues can have adverse consequences (41). Second, the majority of proteins (including mutated and viral oncogenes) identified as highly specific markers for solid tumors are intracellularly localized, and therefore inaccessible by conventional antibodies or CARs. To circumvent this, one alternative strategy is to select intracellular antigenic targets that are processed and presented as peptide products by class I MHCs on the surface of tumor cells. Indeed, in prior work, we

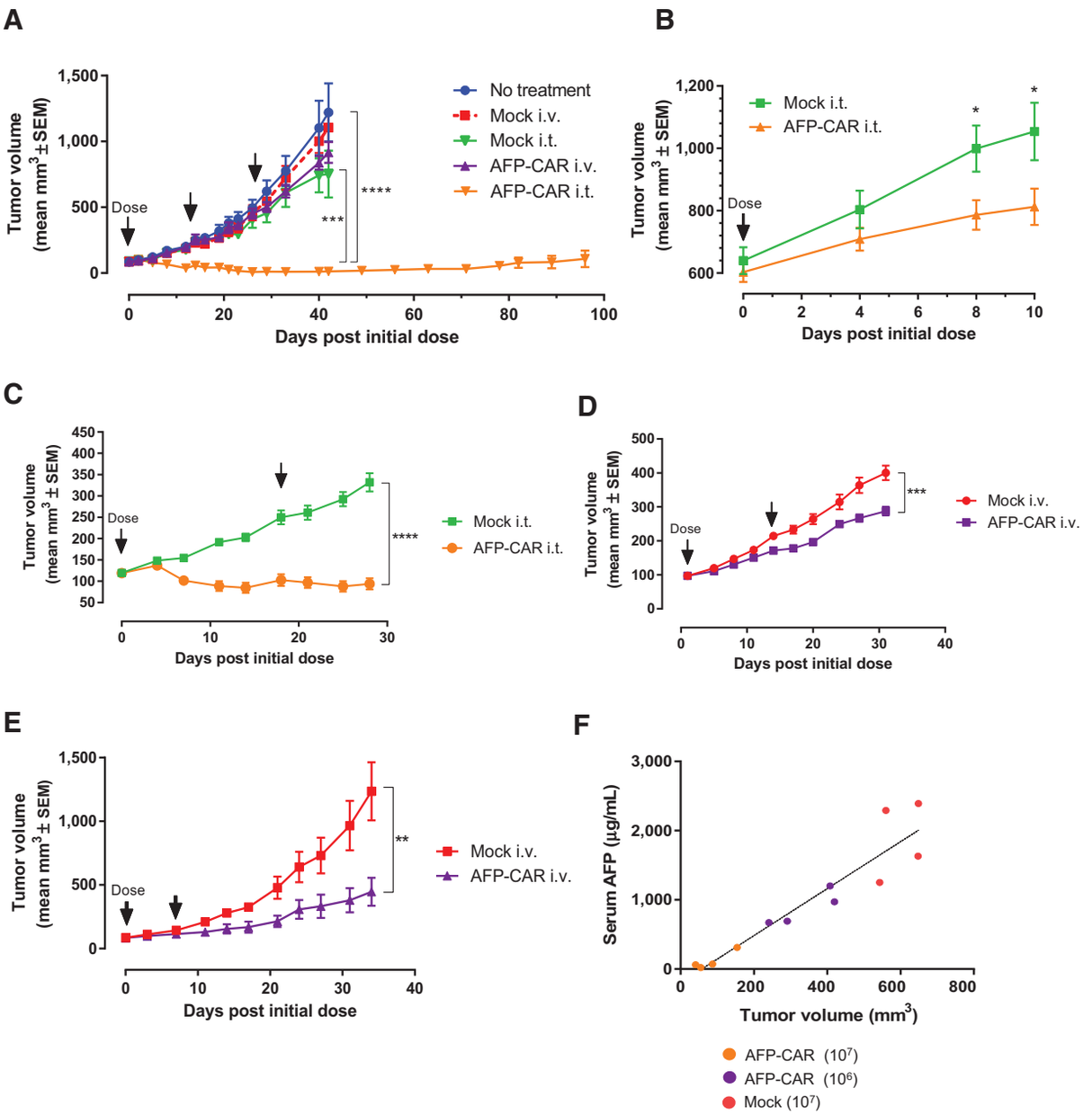

Figure 4.

Adoptive transfer of AFP-CAR T cells significantly inhibits the growth of multiple liver cancer xenograft models. **A,** Average volume of Hep G2 subcutaneous tumors treated as indicated by intravenous or intratumoral administration of 3 doses (every 2 weeks) of 10^7 mock or AFP-CAR T cells (50%–75% AFP-CAR$^+$). Dosing was initiated when tumors reached approximately 100 mm^3; $n = 8$ SCID-Beige mice/group. **B,** Average volume of Hep G2 subcutaneous tumors treated by intratumoral administration of 10^7 mock or AFP-CAR T cells (50%–75% AFP-CAR$^+$). Dosing was initiated when tumors reached approximately 600 mm^3; $n = 8$ SCID-Beige mice/group. **C,** Average volume of SK-HEP-1-MG subcutaneous tumors treated by intratumoral administration of mock or 5×10^6 AFP-CAR$^+$ T cells. Dosing was initiated when tumors reached approximately 100 mm^3. A second dose of T cells was administered at the time tumor regrowth was observed ($n = 6$ SCID-Beige mice/group). **D,** Average volume of SK-HEP-1-MG subcutaneous tumors treated by intravenous administration of mock or AFP-CAR T cells. Dosing was initiated when tumors reached approximately 100 mm^3. Mice were administered 2 doses 2 weeks apart, each consisting of 10^7 total T cells (70% CAR$^+$) per mouse ($n = 8$ SCID-Beige mice/group). **E,** Average volume of Hep G2 subcutaneous tumors from NSG mice treated by intravenous administration of mock or a total of 9×10^6 AFP-CAR$^+$ T cells. Dosing was initiated when tumors reached approximately 100 mm^3 (two doses: first dose at 2×10^6 CAR$^+$ T cells and second dose at 7×10^6 CAR$^+$ T cells); $n = 4$–6 NSG mice/group. **F,** Serum AFP levels correlate with tumor volume in Hep G2 tumor-bearing mice treated with either mock, 10^6, or 10^7 AFP-CAR T cells (70% CAR$^+$; $n = 4$ SCID-Beige mice/group. Correlation coefficient, $R = 0.9391$. Arrows indicate time of dosing. Statistical significance was determined using Dunnett test. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$.

developed a human "TCR-like" mAb against WT1, an intracellular oncoprotein that is overexpressed in a myriad of leukemias and solid cancers. This "TCR-like" mAb (ESK1) is specific for a 9-mer peptide of WT1 in complex with HLA-A*02:01 (24). Importantly, we demonstrated that ESK1, either as a naked antibody or incorporated into a bispecific antibody, shows potent *in vivo* antitumor

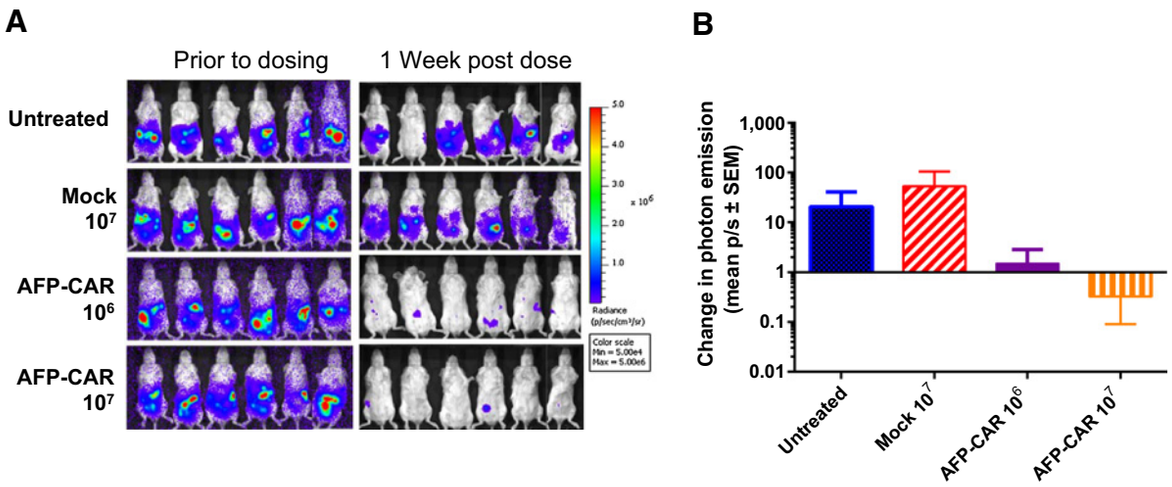

Figure 5.

AFP-CAR T-cell therapy shows efficacy in a disseminated peritoneal liver cancer model. **A,** Overlay of mouse images with tumor-derived bioluminescence from Hep G2-luc2 intraperitoneal tumors in SCID-Beige mice treated with either mock, 10^6, or 10^7 AFP-CAR T cells (~63% CAR^+; $n = 6$ mice/group). Two doses of T cells were administered to each group, 2 weeks apart. **B,** Fold change in tumor burden (measured by total photon emissions) for mice described in **A** over the length of the study. Total flux at 11 weeks after tumor implantation was divided by initial flux before the first dose.

efficacy against several human cancer xenografts (24, 42). However, whether such tumor-specific antigen products can be targets for CAR T-cell therapy against solid tumors has not yet been reported.

To address this, we employed our previously developed antibody discovery platform to identify and optimize a highly specific antibody against the AFP_{158} peptide in complex with HLA-A*02:01 for the treatment of liver cancer, one of the most pressing unmet medical needs. Notably, we show that redirected T-cell therapy employing a second-generation CAR constructed with this antibody displayed potent antitumor activity in multiple preclinical models of AFP/HLA-A*02:01–expressing human liver cancer. Thus, by expanding the repertoire of targetable antigens to include cancer-specific intracellular or secreted protein products, we have overcome a major obstacle to successful targeting of solid tumors by CAR T-cell therapy. These findings will be applicable to many other intracellular antigens as solid tumor-specific targets.

Furthermore, by engineering an scFv specific for a peptide–MHC complex into a second-generation CAR platform, we circumvented some of the disadvantages associated with conventional CARs (which cannot target intracellular antigens) and TCRs. For one, the mispairing of engineered TCRs with endogenous TCRs is avoided, which greatly reduces the chances for off-target effects. In addition, unlike conventional engineered TCRs, which compete with endogenous TCRs for surface expression and require external costimulatory signals to mount a full immune response, second-generation CARs can effectively modulate T-cell expansion and persistence through their dual-signaling receptors (i.e., CD28/CD3ζ). Thus, this scFv-CAR design allows us to target intracellular and secreted antigens with exquisite selectivity while providing the potency typically achieved with CARs.

One of the major potential side effects of conventional CAR T-cell therapy, however, is cytokine-release syndrome, commonly referred to as "cytokine storm." This is due, in part, to target selection, in that the majority (if not all) of current CARs target tumor antigens expressed at high levels on the surface of tumor cells, thus increasing the likelihood for overactivation of T cells and the release of toxic levels of cytokines. As AFP peptide–MHC complexes are present at low numbers on the surface of tumor cells (43), we anticipate that CAR T cells designed to target AFP peptide–MHC complexes may effectively kill tumor cells without the associated "cytokine storm". In support of this, we found that while AFP-CAR T cells killed Hep G2 cells (low level of HLA-restricted AFP_{158} complexes on their surface; Supplementary Fig. S9) just as potently as SK-HEP-1-MG cells (high level of HLA-restricted AFP_{158} complexes on their surface; Supplementary Fig. S9), AFP-CAR T cells produced substantially lower levels of cytokines when incubated with Hep G2 cells compared with SK-HEP-1-MG cells. Thus, these data underscore a few key points: (i) CAR T cells designed with sufficiently high binding affinity to their target peptide–MHC complex can effectively kill tumor cells with very low expression of the antigen on their surface; (ii) the ability of CAR T cells to be a strong cytokine producer upon antigen encounter may not always be a reliable predictor of CAR T-cell efficacy; and (iii) CAR T-cell therapy–induced "cytokine storm" may be mitigated or prevented by targeting tumor-specific peptide–MHC complexes.

Finally, for redirected T-cell therapy to be successful in solid malignances such as HCC, T cells must be able to reach the tumor and overcome its immunosuppressive microenvironment. CAR T cells that are administrated intravenously are required to survive in circulation long enough to penetrate the tumor, engage their target antigen, and become activated. Despite these hurdles, we show that intravenously administered AFP-CAR T cells are able to traffic to subcutaneous liver tumors in NSG mice and inhibit their growth. However, intratumoral injection of AFP-CAR T cells caused a much more profound, rapid, and lasting antitumor response. Thus, our data make a strong case that local delivery of T-cell therapy may be a more attractive clinical path as it not only shortens the time between CAR T injection and antigen encounter, but is potentially safer than systematic delivery. The

analogous routes of administration in humans would be direct injection into liver tumors or injection through the hepatic artery, both of which are commonly used in the clinic to administer chemotherapeutic agents to liver cancer patients (44). Intrahepatic artery is a particularly attractive delivery route as it infuses the entire liver vascular system with T cells which can reach multiple lesions. In support of this, Katz and colleagues used the intrahepatic artery for delivery of anti-CEA CAR T cells in a recent phase I trial and found that the engineered T cells were able to penetrate metastatic tumors in the liver (45). However, the low efficacy of anti-CEA CAR T cells observed in that trial was attributed to the presence of myeloid-derived suppressor cells (MDSCs; ref. 46). Thus, combining intrahepatic artery delivery with inhibitors of MDSCs and/or checkpoint inhibitor might be required to alleviate the immunosuppressive effects of the tumor microenvironment.

Given that the first criteria for a successful CAR T-cell–based therapy is specificity, our ability to generate fully-human antibodies specific for peptide–MHC complexes significantly increases the pool of targets for identifying safe and effective anticancer therapies. Our work demonstrates that targeting intracellular and secreted tumor antigen products with CAR T cells is feasible and efficacious, potentially safer, and has promising applications for future therapies against currently incurable cancers.

Disclosure of Potential Conflicts of Interest

H. Liu, Y. Xu, J. Xiang, L. Long, Z. Yang, J. Lu, N. Cheng, B. Liu, S. Yan, P. Wang, L. Jin, V.W. Chan, and C. Liu hold ownership interest (including patents) in Eureka Therapeutics. No potential conflicts of interest were disclosed by the other authors.

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References

1. Curran KJ, Brentjens RJ. Chimeric antigen receptor T cells for cancer immunotherapy. J Clin Oncol 2015;33:1703–6.
2. Maude SL, Frey N, Shaw PA, Aplenc R, Barrett DM, Bunin NJ, et al. Chimeric antigen receptor T cells for sustained remissions in leukemia. N Engl J Med 2014;371:1507–17.
3. Kochenderfer JN, Dudley ME, Kassim SH, Somerville RP, Carpenter RO, Stetler-Stevenson M, et al. Chemotherapy-refractory diffuse large B-cell lymphoma and indolent B-cell malignancies can be effectively treated with autologous T cells expressing an anti-CD19 chimeric antigen receptor. J Clin Oncol 2015;33:540–9.
4. Rapoport AP, Stadtmauer EA, Binder-Scholl GK, Goloubeva O, Vogl DT, Lacey SF, et al. NY-ESO-1-specific TCR-engineered T cells mediate sustained antigen-specific antitumor effects in myeloma. Nat Med 2015;21:914–21.
5. Kershaw MH, Westwood JA, Parker LL, Wang G, Eshhar Z, Mavroukakis SA, et al. A phase I study on adoptive immunotherapy using gene-modified T cells for ovarian cancer. Clin Cancer Res 2006;12:6106–15.
6. Gilham DE, Debets R, Pule M, Hawkins RE, Abken H. CAR-T cells and solid tumors: tuning T cells to challenge an inveterate foe. Trends Mol Med 2012;18:377–84.
7. Kakarla S, Gottschalk S. CAR T cells for solid tumors: armed and ready to go? Cancer J 2014;20:151–5.
8. Parkhurst MR, Yang JC, Langan RC, Dudley ME, Nathan DA, Feldman SA, et al. T cells targeting carcinoembryonic antigen can mediate regression of metastatic colorectal cancer but induce severe transient colitis. Mol Ther 2011;19:620–6.
9. Lamers CH, Sleijfer S, Vulto AG, Kruit WH, Kliffen M, Debets R, et al. Treatment of metastatic renal cell carcinoma with autologous T-lymphocytes genetically retargeted against carbonic anhydrase IX: first clinical experience. J Clin Oncol 2006;24:e20–2.
10. GLOBOCAN. Liver cancer: estimated incidence, mortality and prevalence worldwide in 2012; 2012. Available from:http://globocan.iarc.fr/Pages/fact_sheets_cancer.aspx.
11. Altekruse SF, McGlynn KA, Reichman ME. Hepatocellular carcinoma incidence, mortality, and survival trends in the United States from 1975 to 2005. J Clin Oncol 2009;27:1485–91.
12. American Cancer Society. Survival rates for liver cancer; 2015. Available from: http://www.cancer.org/cancer/livercancer/detailedguide/liver-cancer-survival-rates
13. European Association for the Study of the Liver, European Organisation for Research and Treatment of Cancer. EASL-EORTC clinical practice guidelines: management of hepatocellular carcinoma. J Hepatol 2012;56:908–43.
14. Johnson PJ. Role of alpha-fetoprotein in the diagnosis and management of hepatocellular carcinoma. J Gastroenterol Hepatol 1999;14 Suppl:S32–6.
15. Bei R, Mizejewski GJ. Alpha fetoprotein is more than a hepatocellular cancer biomarker: from spontaneous immune response in cancer patients to the development of an AFP-based cancer vaccine. Curr Mol Med 2011;11:564–81.
16. Herzog CE, Andrassy RJ, Eftekhari F. Childhood cancers: hepatoblastoma. Oncologist 2000;5:445–53.
17. Perkins GL, Slater ED, Sanders GK, Prichard JG. Serum tumor markers. Am Fam Physician 2003;68:1075–82.
18. Li P, Wang SS, Liu H, Li N, McNutt MA, Li G, et al. Elevated serum alpha fetoprotein levels promote pathological progression of hepatocellular carcinoma. World J Gastroenterol 2011;17:4563–71.
19. Li M, Li H, Li C, Wang S, Jiang W, Liu Z, et al. Alpha-fetoprotein: a new member of intracellular signal molecules in regulation of the PI3K/AKT signaling in human hepatoma cell lines. Int J Cancer 2011;128:524–32.
20. Li C, Wang S, Jiang W, Li H, Liu Z, Zhang C, et al. Impact of intracellular alpha fetoprotein on retinoic acid receptors-mediated expression of GADD153 in human hepatoma cell lines. Int J Cancer 2012;130:754–64.
21. Li M, Li H, Li C, Zhou S, Guo L, Liu H, et al. Alpha fetoprotein is a novel protein-binding partner for caspase-3 and blocks the apoptotic signaling pathway in human hepatoma cells. Int J Cancer 2009;124:2845–54.

22. Fang ZL, Fang N, Han XN, Huang G, Fu XJ, Xie GS, et al. Effects of AFP gene silencing on Survivin mRNA expression inhibition in HepG2 cells. Genet Mol Res 2015;14:3184–90.
23. Pardee AD, Shi J, Butterfield LH. Tumor-derived alpha-fetoprotein impairs the differentiation and T cell stimulatory activity of human dendritic cells. J Immunol 2014;193:5723–32.
24. Dao T, Yan S, Veomett N, Pankov D, Zhou L, Korontsvit T, et al. Targeting the intracellular WT1 oncogene product with a therapeutic human antibody. Sci Transl Med 2013;5:176ra33.
25. Vollmer CMJr., Eilber FC, Butterfield LH, Ribas A, Dissette VB, Koh A, et al. Alpha-fetoprotein-specific genetic immunotherapy for hepatocellular carcinoma. Cancer Res 1999;59:3064–7.
26. Butterfield LH, Meng WS, Koh A, Vollmer CM, Ribas A, Dissette VB, et al. T cell responses to HLA-A*0201-restricted peptides derived from human alpha fetoprotein. J Immunol 2001;166:5300–8.
27. Butterfield LH, Ribas A, Meng WS, Dissette VB, Amarnani S, Vu HT, et al. T-cell responses to HLA-A*0201 immunodominant peptides derived from alpha-fetoprotein in patients with hepatocellular cancer. Clin Cancer Res 2003;9:5902–8.
28. Liu Y, Daley S, Evdokimova VN, Zdobinski DD, Potter DM, Butterfield LH. Hierarchy of fetoprotein (AFP)-Specific T cell responses in subjects with AFP-positive hepatocellular cancer. J Immunol 2006;177:712–21.
29. Butterfield LH, Ribas A, Dissette VB, Lee Y, Yang JQ, De la Rocha P, et al. A phase I/II trial testing immunization of hepatocellular carcinoma patients with dendritic cells pulsed with four alpha-fetoprotein peptides. Clin Cancer Res 2006;12:2817–25.
30. Tada F, Abe M, Hirooka M, Ikeda Y, Hiasa Y, Lee Y, et al. Phase I/II study of immunotherapy using tumor antigen-pulsed dendritic cells in patients with hepatocellular carcinoma. Int J Oncol 2012;41:1601–9.
31. Butterfield LH, Economou JS, Gamblin TC, Geller DA. Alpha fetoprotein DNA prime and adenovirus boost immunization of two hepatocellular cancer patients. J Transl Med 2014;12:86.
32. Gonzalez-Galarza FF, Takeshita LY, Santos EJ, Kempson F, Maia MH, da Silva AL, et al. Allele frequency net 2015 update: new features for HLA epitopes, KIR and disease and HLA adverse drug reaction associations. Nucleic Acids Res 2015;43:D784–8.
33. Mackall CL, Merchant MS, Fry TJ. Immune-based therapies for childhood cancer. Nat Rev Clin Oncol 2014;11:693–703.
34. Ruppert J, Sidney J, Celis E, Kubo RT, Grey HM, Sette A. Prominent role of secondary anchor residues in peptide binding to HLA-A2.1 molecules. Cell 1993;74:929–37.
35. Tomimatsu K, Matsumoto SE, Yamashita M, Teruya K, Katakura Y, Kabayama S, et al. Production of human monoclonal antibodies against Fc(epsilon)RI(alpha) by a method combining in vitro immunization with phage display. Biosci Biotechnol Biochem 2009;73:1465–9.
36. Lopez-Terrada D, Cheung SW, Finegold MJ, Knowles BB. Hep G2 is a hepatoblastoma-derived cell line. Hum Pathol 2009;40:1512–5.
37. Mak TW, Saunders ME. The immune response: basic and clinical principles. Amsterdam; Boston: Elsevier/Academic; 2006.
38. Long AH, Haso WM, Shern JF, Wanhainen KM, Murgai M, Ingaramo M, et al. 4-1BB costimulation ameliorates T cell exhaustion induced by tonic signaling of chimeric antigen receptors. Nat Med 2015;21:581–90.
39. Song DG, Ye Q, Carpenito C, Poussin M, Wang LP, Ji C, et al. In vivo persistence, tumor localization, and antitumor activity of CAR-engineered T cells is enhanced by costimulatory signaling through CD137 (4-1BB). Cancer Res 2011;71:4617–27.
40. Casucci M, Nicolis di Robilant B, Falcone L, Camisa B, Norelli M, Genovese P, et al. CD44v6-targeted T cells mediate potent antitumor effects against acute myeloid leukemia and multiple myeloma. Blood 2013;122:3461–72.
41. Casucci M, Hawkins RE, Dotti G, Bondanza A. Overcoming the toxicity hurdles of genetically targeted T cells. Cancer Immunol Immunother 2015;64:123–30.
42. Dao T, Pankov D, Scott A, Korontsvit T, Zakhaleva V, Xu Y, et al. Therapeutic bispecific T-cell engager antibody targeting the intracellular oncoprotein WT1. Nat Biotechnol 2015;33:1079–86.
43. Ferrone S, Marincola FM. Loss of HLA class I antigens by melanoma cells: molecular mechanisms, functional significance and clinical relevance. Immunol Today 1995;16:487–94.
44. Song MJ.Hepatic artery infusion chemotherapy for advanced hepatocellular carcinoma. World J Gastroenterol 2015;21:3843–9.
45. Katz SC, Burga RA, McCormack E, Wang LJ, Mooring W, Point GR, et al. Phase I hepatic immunotherapy for metastases study of intra-arterial chimeric antigen receptor-modified T-cell therapy for CEA+ liver metastases. Clin Cancer Res 2015;21:3149–59.
46. Burga RA, Thorn M, Point GR, Guha P, Nguyen CT, Licata LA, et al. Liver myeloid-derived suppressor cells expand in response to liver metastases in mice and inhibit the anti-tumor efficacy of anti-CEA CAR-T. Cancer Immunol Immunother 2015;64:817–29.