Efficacy of CAR T-cell Therapy in Large Tumors Relies upon Stromal Targeting by IFNγ

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Abstract

Adoptive T-cell therapy using chimeric antigen receptor–modified T cells (CAR-T therapy) has shown dramatic efficacy in patients with circulating lymphoma. However, eradication of solid tumors with CAR-T therapy has not been reported yet to be efficacious. In solid tumors, stroma destruction, due to MHC-restricted cross-presentation of tumor antigens to T cells, may be essential. However, CAR-Ts recognize antigens in an MHC-independent manner on cancer cells but not stroma cells. In this report, we show how CAR-Ts can be engineered to eradicate large established tumors with provision of a suitable CD28 costimulatory signal. In an HER2-dependent tumor model, tumor rejection by HER2–specific CAR-Ts was associated with sustained influx and proliferation of the adoptively transferred T cells. Interestingly, tumor rejection did not involve natural killer cells but was associated instead with a marked increase in the level of M1 macrophages and a requirement for IFNγ receptor expression on tumor stroma cells. Our results argue that CAR-T therapy is capable of eradicating solid tumors through a combination of antigen-independent stroma destruction and antigen-specific tumor cell targeting. Cancer Res; 74(23); 6796–805. © 2014 AACR.

Introduction

T cells can be redirected with new antigen specificity and used for adoptive T-cell therapy (ATT) by introducing either a T-cell receptor (TCR) or chimeric antigen receptor (CAR). The CAR consists of an antigen (Ag)-binding single-chain variable fragment (scFv) antibody domain and a signaling domain, most often the CD3ζ endodomain (1). While T cells with CARs (CAR-T) containing CD3ζ had a moderate antitumor effect and poor persistence in vivo (2), addition of costimulatory signals as provided by dual signaling domains (e.g., CD28-CD3ζ) has improved the therapeutic effects of CAR-Ts in experimental models (3) and in clinical trials targeting CD19 on B-cell malignancies (4, 5).

Tumor transplantation models can be of clinical relevance if large established tumors grown for at least 2 weeks are treated (6). Such solid tumors are difficult to reject but can be successfully eradicated if the target antigen is recognized through the TCR (7–11). On the other hand, CAR-mediated recognition leads to regression but not complete eradication (3). This could be due to the suboptimal affinity of the CAR to the target antigen or the different mode of antigen recognition of CARs versus TCRs. In contrast to TCRs, which recognize peptide antigen presented by MHC class I (MHC I), CARs recognize the cognate cell surface antigen by an antibody domain independently of the MHC I. This can be an advantage as tumors escape TCR-mediated ATT by MHC I downregulation (12), but it can also be a disadvantage because tumor stroma cells cross-presenting surrogate tumor antigen on MHC I needs to be recognized by T cells to prevent tumor escape (7). However, it appears that direct recognition of the tumor stroma is less important for tumor rejection if cancer-driving antigens (CDA) are targeted by TCR-mediated ATT (10, 11). CDAs are arguably the best targets because cancer cell proliferation/survival often depends on its continuous expression, as is the case for the human cell line SKOV3 and HER2 (13), which is termed oncogene addiction (14). HER2 is normally an over-expressed self-antigen, but in this study, we used a mouse model where human HER2 was expressed only on the SKOV3 tumor cells and not on mouse cells, which makes it a relevant model for tumor-specific antigens targetable by CAR-Ts [e.g., mutant EGF receptor (EGFRvIII) or the chaperon Cosmc; refs. 15, 16].

Previous studies using CARs for treatment of xenografted solid human tumors used polyclonal human T cells as recipient cells for the CAR (3, 17), a model containing several confounding factors (Supplementary Fig. S1). Human CAR-Ts were allogeneic with regard to the tumor and xenogeneic with regard to the host. In this setting, it is difficult to exclude
allo-MHC T-cell responses (through the TCR) contributing to therapy effects. Conversely, lack of overt graft-versus-host reactions to the xenogeneic mouse tissues indicates that human T cells perform poorly in mice. An unknown number of species-specific factors necessary for survival and proliferation may impair the function of human T cells in mice. For example, IFNγ function is species-specific (18), so that human T-cell–derived IFNγ cannot act on mouse tumor stroma cells, which had been shown in syngeneic models to be critical in preventing tumor recurrence (8, 11). To avoid confounding factors with polyclonal human T cells in mice and better dissect the mechanism of tumor eradication by CAR-Ts, we used mouse monoclonal CD8+ T cells with tumor-unrelated specificity as CAR recipients (OVA-specific OT-1 cells derived from Rag1 knockout mice). This ensured that the CAR-Ts could act only through their CAR but not TCR and that IFNγ could act on the tumor stroma but not the human cancer cells and also excluded a potential contribution of CD4+ T cells on the therapeutic outcome (Supplementary Fig. S1). Here, we first established that eradication of large established tumors can be achieved by HER2-specific CAR-Ts if provided with costimulatory CD28 signaling (28-30). This rejection was associated with sustained accumulation, proliferation, and differentiation of CAR-Ts to effector memory (TEM) cell type at the tumor site. We finally demonstrated that tumor rejection by CAR-Ts involved destruction of tumor stroma through IFNγR, independently of natural killer (NK) cell contribution.

Materials and Methods

Mice

All mouse studies were conducted in accordance with institutional, state, and federal (Landesamt für Arbeitsschutz, Gesundheits- und technische Sicherheit, Berlin) guidelines. Albino Rag1−/− or Rag2−/− (Rag−/−) mice, OT-1/Rag1−/−, and ChRLuc/OT-1/Rag1−/− mice were recently described (9). IFNγR−/− mice were obtained from Jackson Laboratory (003288) and bred at the MDC animal facility to Rag1−/− mice to obtain IFNγR−/−/Rag1−/− mice.

Retroviral vectors and cells

HER2-specific ζ-CARs constructs with scFv of different affinities and the 9-28ζ-CAR of the intermediate affinity (20) were introduced into the MSCV expression plasmid as earlier described (21). pMSCV vector encoding for GFP (pMIG) was used as mock control. Retroviral supernatants were generated by cotransfecting HEK-T cells with different MSCV-CAR constructs and gag, pol, and env encoding pCL-eco vector (Imgenex) as described previously (21). Virus supernatants were collected 48 and 72 hours posttransfection and used for transducing T cells. Human ovarian carcinoma cell line SKOV3 expressing CBG luciferase was described earlier (21). It was authenticated by flow cytometry as described below.

Expansion of T cells and retroviral transduction

Spleens were isolated from OT-1/Rag−/− or ChRLuc/OT-1/ Rag−/− mice and prepared as a single-cell suspension with 0.8% NH4Cl-mediated lysis of red blood cells. A total of 1 × 10⁶ to 2 × 10⁶ cells were cultured in 24-well plates in 1 mL of complemented RPMI media (10% FCS, PAN Biotech; 50 μg/mL gentamicin, Gibco; and 50 μM L-mercaptoethanol, Gibco), supplemented with 1 μg/mL anti-(α-)CD3, 0.1 μg/mL anti-CD28 antibody (Ab; BD Biosciences), and 10 IU/mL IL2 (Proleukin, Prometheus Laboratories) for 24 hours at 37°C in 5% CO2 humidified incubator. Virus supernatants of different CAR constructs and mock control produced by HEK-T cells were collected, filtered (0.45-μm pore size), and either used directly for transduction or stored at −80°C. After 24-hour activation, media were removed from the splenocytes and replaced with 1 mL/well virus supernatant containing 10 μg/mL polybrene (Sigma-Aldrich). The cells were spinoculated for 2 hours at 800 × g and 32°C. Virus supernatant was removed and replaced with 1-mL RPMI containing 10 IU IL2. Cells were transduced twice, with an interval of 24 hours. The level of surface CAR expression was measured 24 or 48 hours after the last transduction.

Flow cytometry

Surface expression of the CARs was measured by staining with the F(ab′)2 fragment from goat anti-human Ig polyclonal Ab conjugated to phycoerythrin (PE), allophycocyanin (APC), or DyLight 649 (Southern Biotech or Jackson). In addition, cells were stained with a-CD4-APC (or FITC; clone 53-6.7, BD Biosciences) and a-CD3-FITC Ab (clone G4.18, BD Biosciences). CAR-Ts were also analyzed for activation markers using a-CD44-FITC (clone IM7, BD Biosciences) and a-CD62L-PE (clone clone MEL-14, BD Biosciences) and for proliferation markers by a-Ki-67 (Alexa Flour 488 clone B56, BD Biosciences) and propidium iodide staining Solution (BD Biosciences). NK cells were analyzed using a-NK1.1-APC (clone PK136, Biologend) and a-CD49b-PE (pan-NK-cells, clone DX5, Biologend) Ab. Macrophages were identified using a-F480-BV421 (clone BM8, Biologend), a-CD11b-PE (clone M1/70, Biologend) and additionally stained with a-IA-IE-PeCy7 (clone M5/114.15.2, Biologend). Tumor samples were additionally analyzed by a-CD45-APC (clone 104, Biologend) and a-Her2neu-PE (clone Neu 247, BD Biosciences) Ab. SKOV3 cells were stained with a-HER2neu-PE and with a-HLA-ABC (clone G46-2.6, BD Biosciences) as described earlier to confirm the species origin and HER2 expression (21). Data acquisition was performed on a FACSAria Calibur (BD Biosciences), MACSQuant (Miltenyi Biotec), or FACS Canto (BD Biosciences), and the analysis was done by FlowJo (Tree Star) software.

Tumor digestion and cytokine release assay

A third or a half of the isolated tumor was sliced into small pieces and incubated for 1 hour at 37°C in 10 mL digestion solution (complete RPMI medium with collagenase II (1 mg/mL, Gibco), Dispase II (1 mg/mL, Roche), and DNAse I (10 μg/mL, Roche). Tumor cells were passed through a cell strainer (40 μm), washed with PBS, and treated with ACK lysis buffer. Counted cells were prepared for flow cytometric analysis by incubation with a-FC receptor Ab (TruStain fcX, Biolegend) for 15 minutes at 4°C. CD8 T cells were purified from tumor cells by using a-CD8a (Ly-2) Microbeads (Miltenyi Biotec) according to the manufacturer’s protocol.
To measure cytokine release, CAR-Ts were mixed with mock-transduced T cells to equalize the CAR+ cells between the different constructs in total of 2 × 10⁶ cells per construct, from which 1 × 10⁴ were CAR+. The CAR-Ts were then cocultured with titrated numbers of target SKOV3 tumor cells in 96-well flat-bottom plates, and 24 to 48 hours later, IFNγ and IL2 levels were measured in the supernatants by ELISA (BD Biosciences) according to the manufacturer’s protocol.

Tumor challenge and ATT

Age- and sex-matched mice were injected with 5 × 10⁶ SKOV3-CBG tumor cells subcutaneously. On the day of treatment, mice received i.v. injection of (unless otherwise indicated) 2 × 10⁶ CAR-Ts or mock T cells, resuspended in 100 μL PBS. Tumor size was measured by an electronic caliper, and the average tumor diameter was calculated from the measurements of length, width, and the depth of the tumor (9). Mice were sacrificed when the tumors reached 15 mm in any one dimension. To confirm the complete tumor rejection, at the end of the treatment experiments (at least 60 days after no palpable tumor was detected), tumor-free mice were imaged for CBG luciferase signal emitted by any potentially remaining SKOV3 cells as described (21). NK cell depletion was achieved by weekly intraperitoneal injection of 160 μg/mouse of a-NK1.1 Ab (clone PK136, BioXCell) or isotype control (IgG2a, BioXCell) throughout the experiment. NK cell depletion was confirmed several times over the time course of the experiment. Tumor-free mice from this experiment were observed for at least 1 week following tumor rejection and the rejection was confirmed by bioluminescence imaging (BLI).

Bioluminescence imaging

In vivo imaging was performed using a Xenogen IVIS 200 (Caliper Lifescience). A maximum of 5 anesthetized mice were imaged at once. Each mouse received an i.v. injection of freshly prepared coelenterazine (Biosynth) that was dissolved in DMSO (Sigma) and diluted in PBS (100 μg/100 μL per mouse) as earlier described (9). Images were acquired for 1 minute using small binning, unless saturated signal was obtained, in which case the acquisition was repeated using 10-second imaging time. All data were analyzed using Living Image analysis software (Caliper Lifescience). The region of interest (ROI) for the measured signal was drawn at the tumor site identically for all mice and was set anew for each experiment.

Results

Increasing the affinity of a ζ-CAR does not improve the T-cell function

HER2-specific CARs were cloned into the pMSCV retroviral vector, resulting in five CARs with CD3ζ signaling domain (ζ-CAR) with the affinities of their scFv’s between 10⁻⁷ and 10⁻⁹ mol/L (Fig. 1A), and one CAR with CD3ζ and CD28 signaling domains (28-ζ-CAR) with the affinity of 10⁻⁹ mol/L (Fig. 1A). Following retroviral transduction of mouse splenocytes, the percentage of CAR+ CD8+ T cells was generally lower for the ζ-CARs (8.5%; SD ± 5%) when compared with the 9-28-ζ-CAR (18.5%; SD ± 13%; Fig. 1B and Supplementary Fig. S2). Human T cells targeted with these same ζ-CARs responded at a very similar level above the affinity threshold of 1.6 × 10⁻⁸ mol/L (20). Similarly, 8-ζ-CAR-Ts responded by secreting more IFNγ compared with 7-ζ-CAR-Ts, and levels of IFNγ did not increase with CARs of higher affinity, including the 9-28-ζ-CAR (Fig. 1C). CAR-Ts can secrete IL2 when stimulated through their endogenous CD28 receptor (22) or the chimeric CD28-ζ receptor (19). Accordingly, we found that CAR-Ts engineered with 9-28-ζ but not ζ-CARs secreted IL2 (Fig. 1C).

We next investigated the ability of ζ-CAR-Ts to reject SKOV3 tumors in vivo and whether an increase in scFv affinity influences therapeutic outcome. Rag²−/− mice were subcutaneously injected with 5 × 10⁶ SKOV3 cells. About 3 weeks later, when the tumors were 6.6 mm (SD, ±1.1 mm) in average diameter, the mice were treated intravenously with 2 × 10⁶ CAR-Ts transduced with mock (GFP) retrovirus or the different affinity ζ-CARs. Tumors in mice that received 7-ζ-CAR-Ts progressed unimpaired comparable with mock-treated mice (Fig. 2 and Table 1). Similarly, ζ-CAR-Ts of the other affinities (including the highest affinity 11-ζ-CAR) did not reject SKOV3 tumors and, if at all, only slightly delayed tumor progression (Fig. 2 and Table 1).

Costimulation by the 28-ζ-CAR leads to rejection of large established tumors

To determine whether addition of a costimulatory CD28 signaling to the CAR molecule would lead to rejection of SKOV3 tumors, tumor-bearing mice were treated with CAR-Ts expressing either 9-ζ or 9-28-ζ-CAR, which contain the same scFv domain (κ: 1 × 10⁻⁹ mol/L) but different signaling domains. As before, tumors grown for about 3 weeks (average tumor diameter, 7.1 mm; SD, ±0.4 mm) progressed in mice receiving 9-ζ-CAR-Ts or mock treatment but were long-term rejected by 9-28-ζ-CAR-Ts (mice remained tumor-free 60 days postrejection; Fig. 3A and Table 1). Although both groups received 2 × 10⁶ transduced CD8+ T cells, due to the different transduction efficiency, 9-28-ζ-CAR-T–treated mice received more CAR-Ts (5.6 × 10⁶) than 9-ζ-CAR-T–treated mice (1.4 × 10⁶). To account for that and exclude that the different therapeutic outcome was due to transferring different numbers of CAR-Ts, tumor-bearing mice were treated with titrated numbers of 9-28-ζ-CAR-Ts. Either 2 × 10⁴ or 5 × 10⁵ CD8+ T cells were transferred, which equals to 5.6 × 10⁶ or 1.4 × 10⁷ CD8+ T cells, respectively. In both cases, tumors were again long-term rejected (Fig. 3B and Table 1). To further confirm these results, mice with large established tumors (average tumor diameter, 10.5 mm; SD, ±1.8 mm) received the same number of CAR+ T cells; either mock treated or injected with 2 × 10⁶ CAR-Ts with similar percentage of 9-28-ζ- or 9-ζ-CAR+ cells (8% and 10%, respectively). Once again, 9-28-ζ-CAR-Ts rejected the tumors (n = 2), whereas tumors in mock-treated and 9-ζ-CAR-T–treated mice progressed (Fig. 3C and Table 1).
CARs require costimulation to accumulate at the tumor site and persist in vivo

To visualize the in vivo dynamics of the CAR-Ts associated with tumor rejection compared with CAR-Ts associated with failed therapy, we introduced CARs into T cells derived from Renilla luciferase transgenic mice (ChRLuc/OT-1/Rag-/-; ref. 9) and imaged tumor-bearing mice by in vivo BLI at various time points following T-cell transfer. The BLI background signal was set at $1 \times 10^{3}$ counts per second (cps)/sr, which is the lowest signal emitted at the tumor site by mock-treated mice, probably reflecting the homeostatic expansion of transferred T cells (Fig. 4A). Similar to TCR-mediated tumor rejection (9), the presence and persistence of the CAR-T signal was associated with tumor regression as only 9-28-ζ-CAR-Ts accumulated at the tumor site (Fig. 4A and B). Typically, the signal appeared and reached its peak during the second week after ATT (Fig. 4B), where it persisted for 2 to 3 weeks and then gradually declined (Fig. 4A and B). The signal from the in vivo imaging of 9-28-ζ-CAR-transduced T cells indicate that this population contracted in a fast kinetic following three cycles of expansion. This is similar to what we have earlier observed during the rejection of large established tumors by adoptively transferred TCR transgenic T cells (9). It is likely that, following the expansion, the T cells contracted because of the consumption of the limited amount of the homeostatic cytokines required for T-cell persistence. In line with this, a similar
This indicates that during successful ATT, a new cycle of T-cell expansion is repeatedly initiated as long as the cognate antigen is available. Because the SKOV3 cells used in this study were transduced with a click beetle luciferase (CBG)-expressing retrovirus (21), we confirmed complete tumor rejection by showing that no remaining tumor cells could be detected by BLI (Fig. 4C). The values for the ζ-CAR–T–imaged signals did not vary much between the different treatment groups, and only a slight increase over background was observed during the BLI time period of 50 days (Fig. 4A). By calculating the maximal T-cell signal from mock as compared with 9-28-ζ-CAR–T–treated mice (9), we estimated that homeostatic expansion contributed to about 5% of Ag-driven proliferation. Decreasing the lower scale to 2 × 10^4 p/s/cm^2/sr to visualize the homeostatic expansion of the transferred T cells showed that the signal of ζ-CAR–Ts was similar to control-treated mice, suggesting these cells were largely ignorant, which was in contrast to the large signal increase of 9-28-ζ-CAR–T–treated mice (Supplementary Fig. S3).

Costimulation by the 28-ζ-CAR leads to T-cell differentiation and proliferation at the tumor site
Using BLI, we observed that 9-28-ζ-CAR–Ts expanded in the tumor-bearing mouse and accumulated preferentially at the tumor site (Supplementary Fig. S4). Although BLI allowed us to visualize this accumulation, we could not address the question of whether these cells were also able to proliferate inside the tumor or to evaluate their differentiation status. Because retroviral transduction required antigen-independent T-cell activation, 9-ζ-CAR–, 9-28-ζ-CAR–, and mock-transduced CAR–Ts contained similar numbers of similarly activated T cells. This was confirmed by the high (hi) expression of CD44 and CD62L (CD44hi, CD62Lhi; Fig. 5A), which is typical for memory phenotype. Nevertheless, additional in vivo activation by costimulation was required as only 9-28-ζ-CAR–Ts rejected the tumors whereas 9-ζ-CAR–Ts did not. Because TCR-mediated tumor rejection favors TEM activity of the transferred T cells showed that the signal of ζ-CAR–Ts was similar to control-treated mice, suggesting these cells were largely ignorant, which was in contrast to the large signal increase of 9-28-ζ-CAR–T–treated mice (Supplementary Fig. S3).

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CAR-mediated rejection depends on IFNγR expression on the tumor stroma

By analyzing the tumors for the cancer cells and stroma cells content, we found that large established SKOV3 tumors contained about 15% of HER2+ cancer cells whereas the other 85% were stroma cells (Supplementary Fig. S5A). The majority of the stroma cells in the SKOV3 tumors was of nonhematopoietic origin (>90% CD45.2-negative, Supplementary Fig. S5A). To investigate whether CAR-mediated tumor rejection requires targeting of the tumor stroma by IFNγ, we treated Rag-/- and IFNγR-/-/Rag-/- mice bearing about 3-week-old SKOV3 tumors (6.6 mm; SD ±1.5 mm) with mock or 9-28-ζ-CAR-Ts. In Rag-/- mice, tumors were again rejected following treatment with 9-28-ζ-CAR-Ts (Fig. 6A and Table 1). However, following treatment with 9-28-ζ-CAR-Ts, tumors progressed in IFNγR-/-/Rag-/- mice at a comparable kinetic to that seen in mock-treated mice (Fig. 6A and Table 1).

In the current settings, IFNγ secreted by the transferred T cells could have contributed to tumor eradication either by direct stroma destruction (10, 25) or indirectly by activating NK cells and M1 macrophages (21, 26). To answer this question, we compared the NK cells and macrophages infiltration in the tumors of mock, 9-ζ-CAR-T-, and 9-28-ζ-CAR-T-treated tumor-bearing mice. Tumors from the mice that received 9-28-ζ-CAR-Ts had a high percentage of infiltrating NK cells (30%) compared with tumors from 9-ζ-CAR-T (10%) and mock (4%) treated mice (Fig. 6B). Tumors isolated from 9-ζ-CAR-T-treated mice contained about two times more NK cells than tumors from mock-treated mice (Fig. 6B), which is not surprising as we showed that 9-ζ-CAR-Ts secrete IFNγ upon antigen recognition. The numbers of tumor-infiltrating macrophages (F4/80/CD11b double positive) did not vary much between 9-28-ζ-CAR-T-, 9-ζ-CAR-T-, and mock-treated mice (Fig. 6C). Nevertheless, macrophages in tumors from 9-28-ζ-CAR-T-treated mice had a marked increase in MHC class II expression (80%), which is typical for M1 macrophage population, whereas the percentages were much lower for 9-ζ-CAR-T (43%) and mock (28%)-treated mice (Fig. 6C). These findings
indicate that the IFNγ secreted by 9-28-ζ-CAR-Ts activated NK cells and M1 macrophages. To investigate the role of NK cell activation in tumor rejection, we performed an in vivo NK cell depletion experiment. Tumor-bearing mice received a-NK 1.1 antibody or isotype (iso) control weekly over the period of 85 days (starting 2 days before ATT), which depleted the NK cells in the blood (Supplementary Fig. S5B). Mock-treated mice did not receive any additional treatment and tumors progressed (Fig. 6D). Mice that were treated with a-NK 1.1 antibody and 11 1 1 81 6 3 0 4 9 26 40
×106 p/s/cm2/sr
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×105 p/s/cm2/sr
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2
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Figure 4. CAR-Ts expressing ζ-CAR fail to expand and to accumulate specifically at the tumor site whereas 28-ζ-CAR-Ts do. A, T-cell signal at the tumor site of the adoptively transferred CAR-Ts (derived from ChRLuc/OT-1/Rag−/− mice) was followed over time. The shaded box represents the background signal, which was set to 1 × 10^7 p/s/cm²/sr. Each line represents the signal emitted at the tumor site from a single mouse (n = 2 for mock, n = 3 for the all five ζ-CARs, n = 4 for the 9-28-ζ-CAR). B, T-cell signal for one or two representative mice is shown on different indicated days after ATT for mice receiving mock, 9-ζ-, or 9-28-ζ-CAR-Ts. C, shown are mice with tumor rejection imaged to detect Fluc signal emitted by the tumor cells. Data are representative of two independently performed experiments.

Figure 5. 9-28-ζ-CAR-Ts differentiate into effector memory T cells and proliferate at the tumor site. A, splenocytes from ChRLuc/OT-1/Rag−/− mice were either left untreated (naïve) or were transduced with mock, 9-ζ-CAR, or 9-28-ζ-CAR retroviruses. Two days after last transduction, cells were stained with a-CD3, a-CD44, and a-CD62L antibodies and analyzed by flow cytometry. Shown are CD3-gated cells. B, single tumor cells (day 16 after ATT) were labeled with a-CD8, a-CD44, and a-CD62L antibodies. Shown are CD8/CD44-gated cells of one representative tumor sample per group of two. C, shown are CD8+ cells that were MACS sorted with 85% purity from two pooled tumor samples for each treatment (mock, 9-ζ-CAR, or 9-28-ζ-CAR) and labeled with PI and a-Ki-67 antibody (day 31 after ATT).
isotype control received 9-28-ζ-CAR-Ts, and during the observation time of 82 days after ATT, majority of the tumors were rejected for both groups (2 of 3 for isotype control and 4 of 5 for NK-depleted; Fig. 6D). This indicates that NK cells were not necessary for the CAR-mediated tumor rejection.

Discussion

Our study showed for the first time that CAR-Ts were able to reject large solid tumors exclusively by CAR-mediated CD8⁺ T-cell effector function and independent of CD4⁺ T-cell or B-cell contribution. Costimulation provided by the CD28 signaling domain of the 9-28-ζ-CAR was essential for tumor rejection in our model and could not be compensated for by increasing the affinity of the scFv domain of the ζ-CARs. The inability of ζ-CAR-Ts to secrete IL2 may have contributed to their failure to proliferate, differentiate into TEM, and accumulate at the tumor site to mediate HER2-specific tumor rejection. Another reason for therapy failure might have been the tumor burden; perhaps, ζ-CAR-Ts would have been more effective against smaller tumors (27).

Both antigen-dependent and -independent mechanisms can contribute to stroma destruction as a requirement for cancer eradication (28). Previous studies of TCR-mediated ATT consistently reported that tumor stroma targeting was critical to prevent tumor recurrence and that T-cell–produced IFNγ needed to act on the tumor stroma (8, 11). However, likely due to the use of different experimental models, different
mechanisms were suggested to explain this. When using a surrogate antigen expressed at a high level, antigen cross-presentation by stroma cells was essential for bystander elimination of antigen loss variants (7, 8), whereas targeting a CDA (like SV40 large T antigen) did not require antigen cross-presentation for tumor eradication in a H-2 mismatched host (10, 11). Excluding that cross-dressing by the peptide-MHC (29) could have contributed to stroma recognition in the H-2 mismatched host, it appeared but was not formally proven that antigen recognition only on the cancer cells was sufficient for tumor rejection. This could have resulted from the induction of IFNγ production by the T cells, which then acted on stroma cells in an antigen-independent fashion either by direct stroma destruction or indirectly by activating the non–T-cell immune compartment (10, 11, 30, 31). We proved the initial assumption in our current study because CAR-Ts recognized HER2 exclusively on the cancer cells but not tumor stroma, yet IFNγ responsiveness by the stroma was essential for tumor rejection. There was a correlation between increase in the M1 macrophage numbers and tumor rejection in our model; however, it is not clear whether the macrophages directly contributed to tumor rejection or whether simply the increase was due to higher number of T cells secreting IFNγ in the tumors. Similarly, IFNγ led to NK cell activation, but despite the increased numbers of NK cell infiltrates in tumors of 28-¿-CAR-T–treated mice, the CAR-mediated tumor rejection was not dependent on NK cells. In accordance with our previous studies (10, 25), it is possible that the mechanism of IFNγ acting on tumor stroma has a direct effect involving destruction of tumor vasculature. However, we cannot completely exclude that direct targeting of some stroma cells may have been mediated by recognition of acquired tumor-derived microvesicles containing HER2, as such acquisition was observed for some surface receptors (32).

Because of species-specific binding, the IFNγ secreted by the mouse T cells could only act on the tumor stroma but not on the cancer cells, which might explain the prolonged rejection time required in our model. Simultaneous cancer and stroma cell targeting would have probably accelerated tumor rejection, as is the case in other models where both tumor compartments were targeted by IFNγ (7, 9, 10). Furthermore, the inability of 9-28-¿-CAR-T therapy to induce initial tumor regression in IFNγR−/−/Rag−/− mice could be attributed to this specific setting, in which both cancer and stroma cells were not responsive to IFNγ (11).

Although targeting stroma cells by IFNγ was essential for tumor rejection in our model, we cannot exclude that stroma cell targeting would have been dispensable in a setting where also cancer cells responded to IFNγ. However, in the cases when human CAR-Ts were used for therapy and cancer cells responded to IFNγ, established tumors could not be completely rejected (3, 17), further implying at the relevance of IFNγ stroma cell targeting for tumor rejection. Therefore, it will be important in future studies to elucidate the relative contribution of antigen-dependent (7, 8) and -independent (10, 11) destruction of tumor stroma and whether our data are related to targeting a CDA.

Despite the effectiveness of tumor rejection by 9-28-¿-CAR, we do not suggest using HER2-CARs in the clinic, because we did not address potential toxicity in regard to HER2 expression on normal cells. However, our data are clinically relevant because tumor-specific antigens accessible for CARs have been described (15, 16) and will be evaluated for clinical use (33). Taken together, our results show that antigen-independent tumor stroma destruction is required for CAR-mediated cancer eradication.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors’ Contributions
Conception and design: A. Textor, J.J. Listopad, H. Abken, T. Blankenstein, J. Charo
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