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Case Report of a Serious Adverse Event Following the Administration of T Cells Transduced With a Chimeric Antigen Receptor Recognizing *ERBB2*

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In an attempt to treat cancer patients with *ERBB2* overexpressing tumors, we developed a chimeric antigen receptor (CAR) based on the widely used humanized monoclonal antibody (mAb) Trastuzumab (Herceptin). An optimized CAR vector containing CD28, 4-1BB, and CD3ζ signaling moieties was assembled in a γ-retroviral vector and used to transduce autologous peripheral blood lymphocytes (PBLs) from a patient with colon cancer metastatic to the lungs and liver, refractory to multiple standard treatments. The gene transfer efficiency into autologous T cells was 79% CAR^+ in $CD3^+$ cells and these cells demonstrated high-specific reactivity in *in vitro* coculture assays. Following completion of nonmyeloablative conditioning, the patient received 10^{10} cells intravenously. Within 15 minutes after cell infusion the patient experienced respiratory distress, and displayed a dramatic pulmonary infiltrate on chest X-ray. She was intubated and despite intensive medical intervention the patient died 5 days after treatment. Serum samples after cell infusion showed marked increases in interferon-γ (IFN-γ), granulocyte macrophage-colony stimulating factor (GM-CSF), tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6), and IL-10, consistent with a cytokine storm. We speculate that the large number of administered cells localized to the lung immediately following infusion and were triggered to release cytokine by the recognition of low levels of ERBB2 on lung epithelial cells.

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INTRODUCTION

ERBB2 (HER-2/neu) is a member of the epidermal growth factor receptor family. Epidermal growth factor receptor–ligand interaction induces the heterodimerization of receptors, which in turn results in the activation of intracellular tyrosine kinase domain signaling cascades that mediate cell growth, differentiation, and survival.[1–3] Overexpression of *ERBB2* can induce dimerization of ERBB2 and initiates signal transduction activities without ligand binding. *ERBB2* overexpression/amplification occurs in ~15–25% of human breast cancer patients, and is associated with more aggressive disease.[4] A proportion of other human cancers are also associated with *ERBB2* gene amplification and protein overexpression; including cancers of the colon, ovary, stomach, kidney, melanoma, and others.[5–7] Investigation of agents that target the *ERBB2* protein led to the development of Trastuzumab (Herceptin), a humanized monoclonal antibody (mAb) that binds to the extracellular domain of the receptor.[8] Trastuzumab has been shown to be of clinical benefit for metastatic breast cancer patients with *ERBB2* overexpression/amplification, either alone or in combination with chemotherapy regimens.[9,10] *ERBB2* has also been the target of several cancer vaccine trials,[11–13] as well as, adoptive cell therapy using anti-ERBB2 cytotoxic T lymphocyte lines.[14]

Adoptive cell therapy has emerged as the most effective treatment for patients with metastatic melanoma. Adoptive cell therapy using tumor-reactive autologous tumor infiltrating lymphocytes (TIL) in combination with nonmyeloablative but lymphodepleting conditioning resulted in 50% objective clinical regression in melanoma patients.[15] Intensifying the lymphodepletion by adding total-body irradiation to the chemotherapy conditioning regimen improved the objective response rate to 72%.[16] This potent therapy, however, has been limited by the requisite surgery to procure tumor-reactive TIL, by *ex vivo* identification and expansion of these cells, and by the failure to reproducibly isolate similar cells from common epithelial tumors.

The transfer of genes into primary human lymphocytes permits the introduction of tumor antigen receptor molecules that can endow the engineered cell with antitumor specificity.[17–19] We reported the first clinical trials using autologous peripheral blood lymphocytes (PBLs) modified to express a tumor antigen-reactive T-cell receptor in the treatment of patients with metastatic melanoma that resulted in objective tumor regressions.[20,21] These strategies, however, have a lower response rate than TIL, and only a minority of patients are eligible for current protocols, as they must express human leukocyte antigen-A*0201 in order to be recognized by the T-cell receptor-engineered cells.

An alternative to T-cell receptor gene therapy is the use of a chimeric antigen receptor (CAR) that is capable of relaying excitatory signals to T cells in a non-Major histocompatibility

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complex-restricted manner. These hybrid proteins, composed of an extracellular antigen recognition domain fused to an intracellular T-cell activation domain,[22,23] may therefore be used in patients regardless of their human leukocyte antigen genotype. The absence of human leukocyte antigen-restricted antigen recognition is achieved by harnessing the antigen-binding properties of mAb; this recognition is also independent of antigen processing, thus bypassing a potential mechanism by which tumor cells can evade the immune system *in vivo*. Several clinical trials using CAR-transduced T cells have been reported.[24–27] ERBB2-based CARs reported thus far are composed of single-chain Fv fragment from murine mAb, which have been shown to induce anti-CAR immune responses in humans.[25,26] The anti-ERBB2 CAR used in this case report was a next generation CAR containing both the humanized Herceptin single-chain Fv fragment and optimized costimulatory signaling domains designed for increased cytokine secretion, lytic activity, and shown to display robust *in vivo* antitumor activity in a human breast cancer xenograft model.[28]

RESULTS

In vitro characteristics of the ErbB2-CAR transduced T cells for patient treatment

Leukophoresis was performed to obtain patient peripheral blood mononuclear cells (PBMCs), which were stimulated with an anti-CD3 mAb and interleukin-2 (IL-2) to initiate T-cell expansion followed by transduction with the 4D5-CD8-28BBZ ERBB2-CAR vector as described in Materials and Methods section. At 4 days before infusion, cells were analyzed for the expression of the ERBB2 CAR using an ERBB2-Fc fusion protein as previously described.[28] As shown in **Figure 1**, 79% of $CD3^+$ T cells expressed the CAR with gene transfer into both $CD4^+$ (17%) and $CD8^+$ (63%) T-cell subsets. To determine functional activity, transduced T cells were cocultured with $ERBB2^+$ melanoma, breast cancer, and ovarian cancer cell lines, or $ERBB2^-$ breast cancer and T lymphoblastoid cell lines. ERBB2-specific reactivity was demonstrated by production of effector cytokine interferon-γ (IFN-γ) only in cell lines expressing ERBB2 (**Table 1**). Background cytokine production of ERBB2-CAR transduced cells, when cocultured with ERBB2-targets, was similar to untransduced control cells. To complete the certificate of analysis for patient treatment, transduced cells were also tested to be negative for the presence of replication competent retrovirus by PCR and passed sterility testing (data not shown). Retrospective testing using an amplification-based S^+/L^- assay was also negative for replication competent retrovirus.

Clinical course

The patient was a 39-year-old female who 3 years earlier had undergone a sigmoid resection for colon cancer that on pathologic analysis exhibited lymphatic invasion and vascular involvement, with spread to 6 of 21 lymph nodes and the presence of synchronous liver metastases. She was treated with a

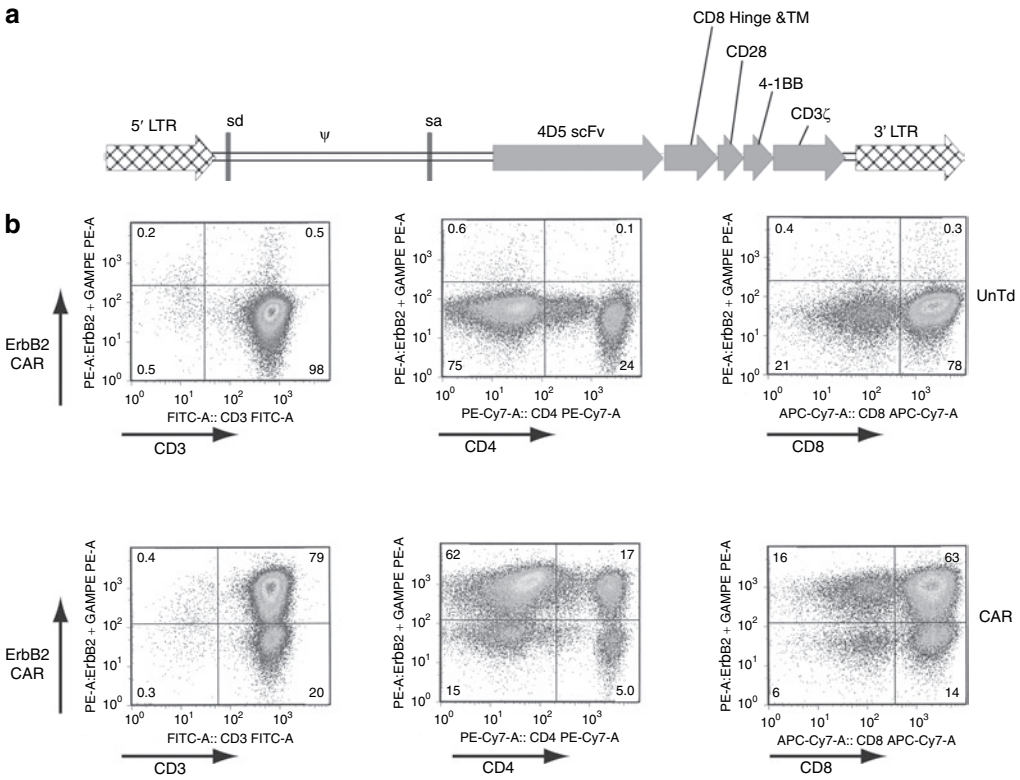

Figure 1 Expression of the ERBB2 CAR. Diagram of the ERBB2-CAR vector (MSGV1-4D5-CD8-28BBZ) used in this trial is as shown on the top of the figure. As described in Materials and Methods section, patient PBMC were stimulated to induced T-cell division and then transduced with the CAR vector. Four days before cell infusion samples were removed for analysis of *ERBB2-CAR* gene expression by FACS (along with the CD3, CD4, or CD8 T-cell markers). CAR, chimeric antigen receptor; LTR, long terminal repeats; PBMC, peripheral blood mononuclear cell; scFv, single-chain Fv

Table 1 ErbB2-CAR certificate of analysis

	ErbB2$^+$ target								ErbB2$^-$ target	
Effector	**Media**	**Mel888**	**Mel938**	**Mel526**	**Mel624**	**MDA361**	**SK-BR3**	**SK-OV3**	**CEM**	**MDA468**
None	24	49	68	44	66	57	143	82	37	99
MART-1 TCR	26	37	35	**3,700**	**4,900**	37	68	47	29	55
Patient CAR	46	**12,745**	**13,210**	**11,430**	**>21,065**	**>26,815**	**>33,385**	**>58,480**	114	134
Mock Td PBL	46	69	81	93	127	82	144	146	117	103

Abbreviations: CAR, chimeric antigen receptor; IFN-γ, interferon-γ; PBL, peripheral blood lymphocyte.
Cell reactivity following coculture of 1e5 effectors with 1e5 targets. Data are IFN-γ (pg/ml) following overnight incubation. Mock Td PBL, untransduced PBL maintained under identical culture conditions; MART-1 TCR, MART-1 TCR-transduced PBL; patient CAR, ErbB2 CAR-transduced patient PBL. ErbB2 antigen expression was as indicated. Only melanoma lines 526 and 624 expressed both HLA-A2 and MART-1, required for TCR recognition.

chemotherapy regimen consisting of 5-fluorourocil, leucovorin and oxaliplatin plus the antivascular endothelial growth factor mAb, bevacizumab. The tumor progressed and the patient was then treated with an alternate chemotherapy regimen in which irinotecan was substituted for oxaliplatin (FOLFIRI). The patient again progressed and after desensitization to oxaliplatin for an allergic reaction, she received a third chemotherapy regimen consisting of capecitabine, oxaliplatin, and bevacizumab. The tumors in the lung and liver continued to progress and the patient was referred to the Surgery Branch, National Cancer Institute (NCI; Bethesda, MD) and signed an informed consent for our protocol. The protocol was reviewed and approved by the National Institutes of Health Institutional Biosafety Committee, the NCI Institutional Review Board, the National Institutes of Health Office of Biotechnology Activities, and the Food and Drug Administration (all Bethesda, MD). Patient inclusion criteria included metastatic cancer that expressed ERBB2 (Her-2/neu) at ≥2$^+$ as assessed by immunohistochemistry.

To facilitate homeostatic expansion of the transduced cells, the patient received a lymphodepleting regimen (60 mg/kg cyclophosphamide daily for 2 days followed by flurodarabine 25 mg/m^2 for the next 5 days). On the day following the last chemotherapy dose the patient received an intravenous infusion of 10^{10} cells transduced with the ERBB2 CAR in 125 ml over 30 minutes. This was the largest number of cells permitted in the first dose-escalation cohort. Within 15 minutes after completing the infusion, the patient developed respiratory distress with decreased blood oxygen saturation that worsened over the next hour. Chest X-ray obtained 40 minutes after completion of the infusion showed pulmonary edema, which appeared worse on chest X-rays repeated at 2 and 4 hours after the infusion. Because of decreasing respiratory function the patient was transferred to the intensive care unit and was intubated about 1 hour after the cell infusion. The patient then developed severe hypotension requiring vasopressors. Dexamethasone, 8 mg every 6 hours, was administered starting at about 5 hours after the cell infusion (it was continued for 2 days, after which the dose was tapered). The patient experienced two cardiac arrests in the next 12 hours after cell infusion both requiring cardiopulmonary resuscitation. She was maximally supported with vasopressors and ventilatory support. She remained severely ill with maximum intensive care unit support for the next 5 days at which time progressive hypotension and bradycardia as well as gastrointestinal bleeding resulted in cardiac arrest from

Postmortem analysis

At autopsy, multiple organs exhibited signs of systemic ischemia and hemorrhagic microangiopathic injury. The lungs also showed diffuse alveolar damage consistent with the clinical findings of acute respiratory distress syndrome. Autopsy also revealed a generalized rhabdomyolysis. Copious blood in the small intestine indicated that the patient succumbed to hemorrhage in the setting of multiple organ failure secondary to systematic microangiopathic injury. The autopsy findings appeared to be a combination of the initial pulmonary injury followed by the sequela of several days of hypotension and organ ischemia.

Beginning at about 4 hours after cell infusion serum samples were obtained and stored for analysis. Compared to pretreatment samples, the patient's serum displayed a rapid and marked increase in the levels of five cytokines; IFN-γ, granulocyte macrophage-colony stimulating factor (GM-CSF), tumor necrosis factor-α (TNF-α), IL-6, and IL-10 (**Figure 2**). At 4 hours after infusion, four of the five cytokines displayed peak serum levels: IFN-γ,11, 456 pg/ml; TNF-α, 380 pg/ml; GM-CSF, 10,191 pg/ml; and IL-6, 34,467 pg/ml. Preinfusion levels for IFN-γ, GM-CSF, and IL-6 were undetectable, whereas TNF-α values varied from 0 to 51 pg/ml. The levels of these cytokines decreased over the next 3 days but remained above baseline values. The levels of seven additional cytokines (IL-1β, IL-2, IL-4, IL-7, IL-10, IL-12, and TRAIL) were determined by cytokine array (SearchLight assay) with only IL-10 showing increased levels after infusion, which unlike the other cytokines, was sustained through the study period (8 pg/ml preinfusion, 219 pg/ml at about 100 hours after infusion, **Figure 2**). An increased amount of IL-2 was observed at 4 hours only and was likely associated with the administration of the cell product, which was given in saline with 300 IU/ml IL-2 (the patient received no other IL-2). The possible involvement of an anaphylactic response to the infused cells was deemed unlikely because we measured only a modest twofold increase in serum tryptase levels at the 4 hours time point (from 7–9 pg/ml preinfusion to 15 pg/ml at 4 hours).

To determine the relative tissue distribution of vector-containing cells, DNA was isolated from samples obtained at autopsy and subjected to quantitative-PCR using vector-specific primers and probe. As a reference for this analysis, DNA was extracted from the infusion sample (79% ERBB2-CAR$^+$) and arbitrarily assigned a value of 100 for comparison to tissue samples. There was a wide variation in the presence of vector-containing cells found in mul-

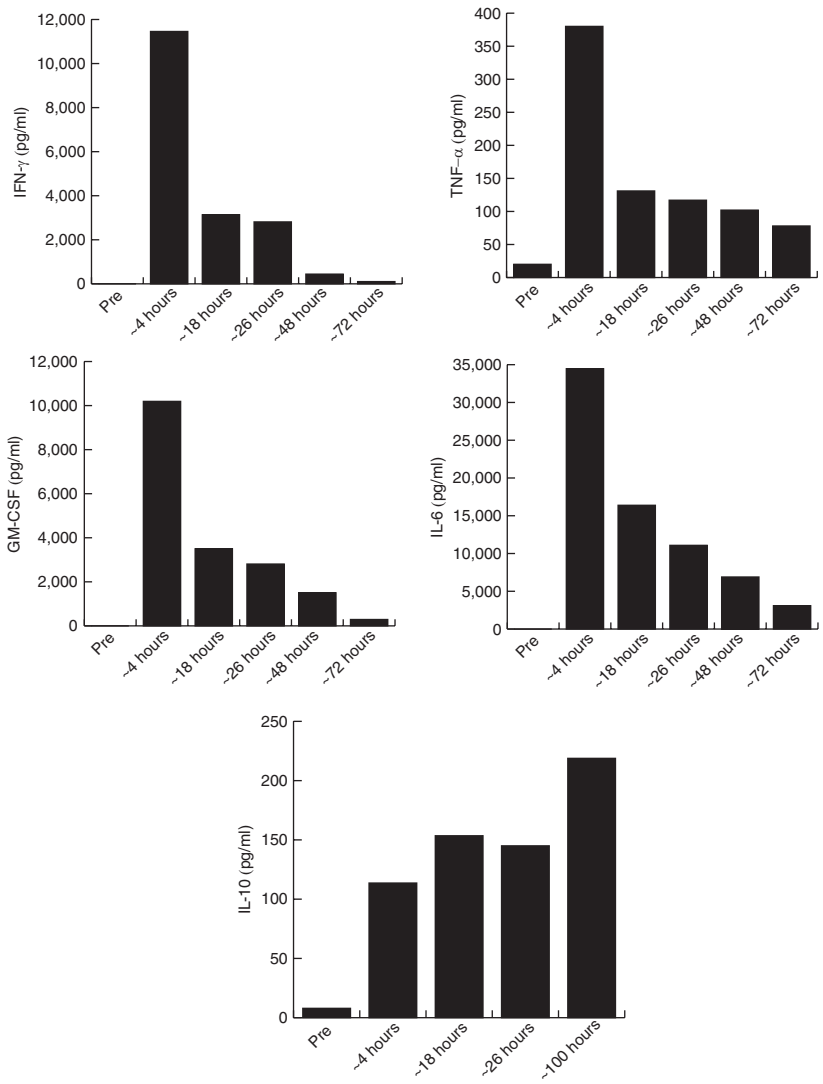

Figure 2 Serum cytokine levels. Serum samples obtained at the approximate times indicated after cell infusion were assayed for cytokine expression using commercial ELISA kits for cytokines IL-6, TNF-α, GM-CSF, and IFN-γ. The levels of cytokine IL-10 were independently determined by cytokine array (SearchLight) assay. All samples were diluted as necessary as to be in the linear range of the assay. ELISA, enzyme-linked immunosorbent assay; GM-CSF, granulocyte macrophage-colony stimulating factor; IFN-γ, interferon-γ; IL, interleukin; TNF-α, tumor necrosis factor-α.

abdominal/mediastinal lymph nodes (**Table 2**). There did not appear to be a preferential accumulation of vector-containing cells in metastatic deposits in the liver or lungs. DNA from the pretreatment PBMC was also subjected to analysis of single-nucleotide polymorphisms associated with the activity/function of five cytokine genes (*IL-6*, *IL-10*, *IFN*-γ, *TGF*-β*1*, and *TNF*-α). PCR using sequence-specific primers (**Figure 3**) indicated that the patients' genotype was: IL-6, heterozygous −741G/C; IL-10, homozygous −1082G, −819C, −592C; IFN-γ, homozygous 874A; transforming growth factor-β1, homozygous 10T, 25G; and TNF-α homozygous −308G. This genotype is consistent with a phenotype of increased synthesis of transforming growth factor-β, IL-10, and IL-6, but lower production of TNF-α and IFN-γ.

Analysis of the ERBB2-CAR transduced T cells before infusion demonstrated specific recognition of ERBB2-expressing tumor cells (**Table 1**). To confirm and extend this analysis, an retested. In addition to cell lines used for the certificate of analysis, the patient's PBMC were used to derive autologous dendritic cell and macrophage cultures and several cultures of allogeneic primary cells adapted for growth in culture by a commercial supplier were obtained. Coculture results presented in **Figure 4** confirm the data obtained in the certificate of analysis. There was no reactivity seen to the dendritic cell and macrophage autologous cell cultures. Cytokine release was observed in several cocultures using allogeneic primary cells adapted for growth in culture.

DISCUSSION

The *ERBB2* gene has been extensively studied as a target for both chemotherapy and immunotherapy. In breast cancer, overexpression of *ERBB2* is correlated with a poor clinical outcome and at the same time, is a positive predictive factor for those women who are most likely to respond to therapy with

Table 2 Vector tissue distribution

Normal tissue	Signal
Brain (left frontal lobe)	0.00
Pectoralis muscle (left)	0.00
Aorta	0.01
Atrium (right)	0.04
Neck LN (left)	0.05
Ventricle (left)	0.08
Small bowel	0.09
Liver (left lobe)	0.11
Liver (right lobe)	0.13
Abdominal para-aortic LN	0.14
Liver	0.15
Auxiliary LN	0.15
Kidney (left)	0.16
Adrenal gland (left)	0.17
Kidney (right)	0.25
Spleen	0.30
Lung left—A	0.44
Adrenal gland (right)	0.49
Lung left—B	0.56
Mediastinal	0.70
Lung right—A	0.78
Lung right—B	1.59
Para-aortic/mediastinal LN	1.99
Abdominal para-aortic LN	2.24
Abdominal LN	2.58
Mediastinal LN	2.64
Metastasis	**Signal**
Liver metastasis	0.03
Lung metastasis (left)	0.08
Liver metastasis	0.09
Liver metastasis (left lateral)	0.11
Lung metastasis (left)	0.29
Liver metastasis (left anterior)	0.34

Abbreviations: CAR, chimeric antigen receptor; FACS, fluorescence-activated cell sorting; LN, lymph node; PBL, peripheral blood lymphocyte; Q-PCR, quantitative-PCR.
DNA extracted from tissues and metastasizes at autopsy were subjected to Q-PCR using ErbB2 CAR-vector specific primers/probe. Values were normalized to the infusion PBL (arbitrarily assigned a value of 100). Gene transfer efficiency of the infusion PBL was 79% as measured by FACS.

trials, trastuzumab in combination with chemotherapy lead to increased disease-free and overall survival in breast cancer patients.[9,29,30] The mechanism of action of trastuzumab appears to be multifactorial, but there are several studies that indicated the involvement of cell-mediated immunity (natural killer cell–based antibody-dependent cellular cytotoxicity) in patient responses.[31–33] Furthermore, patient immune responses (antibody-dependent cellular cytotoxicity) can be increased when IL-12) administration,[34,35] or in vaccine trials where anti-ERBB2 T cells responses have been reported.[11,13]

Safety considerations that preceded our clinical trial included the use of trastuzumab in thousands of cancer patients, the lack of toxicity seen in multiple studies immunizing against epitopes of ERBB2, and the lack of toxicity seen in a report of the adoptive transfer of autologous anti-ERBB2 cytotoxic T lymphocyte clones in the setting of breast cancer.[14] In this report, three different cytotoxic T lymphocyte clones were administered in five transfers given 2 weeks apart. A total of 2.65×10^9 total cells were administered along with low-dose IL-2. With the exception of low-grade fever and chills following the third and fourth infusions, no side effects were noted. The therapy was associated with a decrease in tumor cells within the patient's bone marrow, but larger metastatic sites (such as liver) were not impacted. Radioimaging was performed using ^{111}In-labeled cytotoxic T lymphocyte that demonstrated an immediate accumulation of cells in the lung that decreased over 72 hours, whereas uptake to the liver and spleen increased over the first 24 hours then remained stable for the 72-hour study period. We have demonstrated similar uptake of ^{111}In-labeled TIL[36] and have observed that lung uptake of TIL happens at the first pass (at the 90% retention level) after which the TIL then leak out of the lungs to the liver and other organs (ref. 36 and J.C. Yang, unpublished results).

The γ-retroviral vector construct used in this cancer gene therapy trial was designed for optimal *ERBB2-CAR* gene expression and anti-ERBB2 reactivity. It was demonstrated to be highly specific, was able to recognize a wide range of tumor histologies, and was able to significantly prevent the growth of human breast cancer cells orthotopically implanted into the mammary fat pad of severe combined immunodeficiency mice.[28] Part of the process of optimizing this anti-ERBB2-CAR vector was the inclusion of two T-cell costimulatory domains from CD28 and 4-1BB (CD137) that were linked to the CD3z signaling element. 4-1BB is essential for the optimal activity of $CD8^+$ T cells[37,38] and inclusion of 4-1BB signaling domains was shown to enhance the *in vivo* antitumor activity of CARs in tumor xenograft models.[28,39–41] To date, the reported clinical application of CAR-engineered T cells has been limited to constructs containing CD3z alone.[24–27,42] In one report targeting carbonic anhydrase IX, on-target toxicity was observed most likely due to recognition of antigen expression on biliary epithelium. The results with the carbonic anhydrase IX directed CAR suggest that on-target toxicity may be antigen dependant and does not require the presence of costimulatory signals (such as CD28 and 4-1BB) in the CAR construct.

In our initial report we observed, in some transductions, T-cell recognition of the $ERBB2^-$-tumor line MDA468 (this line is negative for ERBB2 expression by fluorescence-activated cell sorting, but ERBB2 mRNA can be detected by PCR). Transduction of the current patient's T cells with the identical ERBB2-CAR vector did not result in recognition of the MDA468 cell line (**Table 1**, **Figure 4**). The significance of the recognition of allogenic primary cells adapted for growth in culture (**Figure 4**) is not clear, as it is not known how the adaptation of these cells for growth in *ex vivo* culture influences the expression of ERBB2. For example, it was

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