

Immune responses to transgene and retroviral vector in patients treated with ex vivo-engineered T cells

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Adoptive transfer of immune effector cells that are gene modified by retroviral transduction to express tumor-specific receptors constitutes an attractive approach to treat cancer. In patients with metastatic renal cell carcinoma, we performed a study with autologous T cells genetically retargeted with a chimeric antibody receptor (CAR) directed toward carbonic anhydrase IX (CAIX), an antigen highly expressed in renal cell carcinoma. In the majority of patients, we observed distinct humoral and/or cellular anti-CAIX-CAR

T-cell immune responses in combination with a limited peripheral persistence of transferred CAIX-CAR T cells in the majority of patients. Humoral immune responses were anti-idiotypic in nature and neutralized CAIX-CAR-mediated T-cell function. Cellular anti-CAIX-CAR immune responses were directed to the complementarity-determining and framework regions of the CAR variable domains. In addition, 2 patients developed immunity directed against presumed retroviral vector epitopes. Here, we document the

novel feature that therapeutic cells, which were ex vivo engineered by means of transduction with a minimal γ -retroviral vector, do express immunogenic vector-encoded epitopes, which might compromise persistence of these cells. These observations may constitute a critical concern for clinical ex vivo γ -retroviral gene transduction in general and CAR-retargeted T-cell therapy in particular, and underscore the need to attenuate the immunogenicity of both transgene and vector. (*Blood*. 2011;117(1):72-82)

Introduction

The adoptive transfer of autologous T cells that are gene-transduced to express antigen-specific receptors represents an attractive alternative experimental therapy to provide tumor-specific immunity to a broader group of cancer patients.¹⁻⁵ In this approach, T cells are either gene-modified by T-cell receptors (TCR), or by chimeric antibody-based receptors (CARs) composed of the antigen-specific variable regions of antibodies linked to TCR signaling chains.⁶ The first clinical successes of this approach have been reported with TCR-gene-modified T cells directed against metastatic melanoma, however,^{1,3} the objective clinical responses reached in these studies were markedly lower (ie, 12%-30%) than those in studies using nongene-modified tumor-infiltrating T cells (51%-72%).^{1,3,7,8} Furthermore, clinical application of T cells that are gene-modified with CARs to treat renal cell carcinoma (RCC), ovarian cancer, neuroblastoma, or lymphoma has, despite some successes, generally have not yet shown antitumor responses in a substantial number of patients.^{2,4,5,9}

Inadequate T-cell persistence is currently considered a major challenge of adoptive T-cell therapy of cancer. Peripheral persistence of CAR or TCR-engineered T cells is limited, ranging from weeks^{2,9} to months.^{1,4,5} But even despite relatively high levels of circulating gene-modified T cells in some of patients, not all such patients showed vigorous clinical responses.

The limited therapeutic effectiveness of CAR- or TCR-transduced T cells may result, among others,¹⁰ from the potential immunogenicity of the chimeric receptor transgenes. In immunocompetent hosts, the adoptive transfer of gene-modified immune effector cells may induce immune responses against the trans-

gene,¹¹⁻¹⁴ resulting in limited persistence and hence limited antitumor effects of transferred gene-modified cells.^{1,3} In addition, upon retroviral transduction, residual vector sequences that are integrated in the host cell genome theoretically might result in expression of immunogenic epitopes, which might further compromise their peripheral persistence.

We have conducted a clinical trial on the treatment of metastatic RCC patients with autologous T cells that were gene-modified to express a CAR. The CAR was based on the murine monoclonal antibody G250 that recognizes an epitope on carbonic anhydrase IX (CAIX), which is frequently overexpressed on RCC.¹⁵⁻¹⁷ Eleven patients were treated in 3 cohorts, each with different schedules, but with administration of similar numbers of CAIX-CAR-engineered T cells (CAR T cells). Clinical results of patients 1-3 (cohort 1) have been reported previously.⁹

Here we report the limited functional peripheral persistence of the transferred CAR T cells in the 11 treated patients. In addition, we have set out to evaluate and characterize humoral and cellular immune responses directed against CAR T cells as possible underlying cause for the observed phenomenon. Indeed, in conjunction with the limited peripheral persistence of CAR-engineered T cells, we observed distinct anti-CAR humoral and cellular immune responses and were able to characterize the transgene-specific immunogenic epitopes. Unexpectedly, we also identified, in addition to the antitransgene responses, cellular immune reactivity directed against retroviral vector-derived immunogenic epitopes expressed by the ex vivo gene-modified T cells.

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Table 1. Treatment schedule, circulating CAR T cells, and development of anti-CAR immunity

Cohort/ patient	Infusion, d*	CAR T cells infused, ×10 ⁹	No. of IL-2 doses	Observation period, d	Circulating CAR T cells† detection period, d	Circulating CAR DNA detection period, d	Human anti-CAIX- CAR antibodies		Anti-CAR cellular immunity detection first/last day
							Detection first/last day	Peak day/ level, ng/mL	
1									
1	1-4‡	2.1	9	1-57	3-23	tfd	37/57	57 /706	ne
2	1-5, 17-19	0.85	36	1-100	3-17:18-32	tfd-17:18-53	100/100	100 /190	60/60
3	1-4‡	0.38	9	1-240	3-12	3-32	79/240	79/292	38/240
2									
4	1-5, 29-33	1.0	40	1-184	3-29:30-39	3-29:30-50	50/184	95/686	36/184
5	1-5, 29-33	1.0	40	1-180	2-29:30-35	2-29:30-35	—/—¶	—/—	36/180
6	1-5, 29-33§	1.0	35	1-276	2-29:30-44	2-29:30-51	44/276	86/1241	36/122
7	1-5, 29-33	1.0	40	1-180	2-29:30-51	2-29:30-51	86/86	86/20	86/180
8	1-3‡	0.3	7	1-23	2-15	2-15	—/—¶	—/—	ne
3									
9	1-5, 29-33	1.0	40	1-256	2-29:30-51	2-29:30-51	—/—¶	—/—	36/50#
10	1-5, 29-33	1.0	40	1-198	2-29:30-53	2-29:30-53	—/—¶	—/—	51/86
11	1-5, 36-40	1.0	40	1-74	2-36:37-74	2-36:37-74	—/—¶	—/—	58#/74#

tfd indicates too few data points to allow data assessment; and ne, not evaluable. Patient 1: treatment with corticosteroids at days 8-36; patient 8: last blood collection at day 23.

*Treatment schedule: CAR T-cell infusion days: cohort 1, days 1-5 and days 17-19, cohort 2 and 3, days 1-5 and days 29-33 (patient 11 days 36-40); IL-2 doses: 5×10^5 IU/m²/12 hours at days 1-10 (patient 1-11) and days 17-26 (patient 1-3) or days 29-38 (patient 4-10; patient 11 days 36-45); Second treatment cycle patient 11 was postponed for 7 days due to a line infection.

†Detection (days) during and after treatment cycle 1: treatment cycle 2; assay sensitivity (lower limit of detection) anti-CAR Id flow cytometry: 0.06% of CD3 T cells (patients 1-3) and 0.01% of CD3 T cells (patients 4-11).

‡Treatment stopped due to SEA ≥ grade 3 liver toxicities.

§Patient developed grade 3 liver toxicities after completion of CAR T-cell infusions.

¶No anti-CAIX-CAR antibody detected during observation period.

||Last day of observation.

#Very weak or poorly reproducible responses.

Methods

Chimeric antibody receptor

We have constructed a single-chain antibody-type (scFv) chimeric receptor (CAR) based on the murine monoclonal antibody (mAb) G250,¹⁶ entitled scFv(G250)-CD4_{TM}-γ as described. This mAb recognizes an epitope on CAIX, which is frequently overexpressed on clear cell RCC. After retroviral introduction of the transgene into primary human T cells, using a SFG-derived retroviral vector,^{15,18,19} the scFv(G250)-CD4_{TM}-γ chimeric receptor, further indicated as CAIX-CAR, is expressed on surface of these cells, which enables them to recognize CAIX and to exert antigen-specific effector functions such as cytokine production after exposure to CAIX and killing of CAIX⁺ RCC cell lines.^{19,20}

Patients

Patients had disseminated clear cell RCC, measurable progressive disease not amenable for surgery, and a primary tumor expressing CAIX. The clinical protocol (DDHK97-29/P00.0040C, "A phase I study with therapeutic intent of the treatment of metastatic renal cell cancer with autologous gene-modified T lymphocytes") was approved by the governmental regulatory authorities and the Erasmus Medical Center institutional medical ethical review board. The inclusion and exclusion criteria for patients participating in this study have been reported previously.⁹ Following written informed consent obtained in accordance with the Declaration of Helsinki, patients were treated with autologous T lymphocytes that were transduced with the anti-CAIX CAR using retroviral transduction.¹⁹⁻²¹ Here we describe results obtained in 3 patient cohorts (Table 1 and supplemental Figure 1, available on the *Blood* Web site; see the Supplemental Materials link at the top of the online article).

In cohort 1, we treated 3 patients with CAIX-CAR T cells in an in-patient dose escalation scheme of intravenous doses of 2×10^7 T cells at day 1; 2×10^8 T cells at day 2; 2×10^9 T cells at days 3-5 (treatment cycle 1) and 2×10^9 cells at days 17-19 (treatment cycle 2), in combination with subcutaneous injections of 5×10^5 IU/m² human recombinant inter-

leukin-2 (IL-2; Chiron), twice daily administered at days 1-10 and days 17-26. After 4 T-cell infusions, liver enzyme disturbances reaching CTC grades 3-4 developed in patients 1 and 3, which necessitated cessation of treatment in patients 1 and 3, corticosteroid treatment in patient 1, and reduction of the maximal T-cell dose to 2×10^8 T cells in patients 2 and 3.^{9,21}

In cohort 2, we treated patients with CAR T cells in a conventional phase I strategy with a maximum of 10 T-cell infusions at days 1-5 and days 29-33 in combination with IL-2, subcutaneous, 5×10^5 IU/m² twice daily administered at days 1-10 and days 29-38. We treated 5 patients at the lowest dose of 1×10^8 gene-modified T cells, of whom 2 developed CTC grade 3 liver toxicity after 10 and 3 infusions, respectively.

In cohort 3, we treated patients as in cohort 2, but applied a strategy to block CAIX CAR recognition of cognate antigen on normal liver tissue. To that end we included an extra intravenous infusion of 5 mg anti-CAIX mAb cG250 (kindly provided by L. Old, LIRC) 3 days before start of the CAR T-cell infusions. This low dose of 5 mg cG250 mAb blocks CAIX in the liver and leaving accessible CAIX at RCC tumor sites.^{22,23} Three patients who were treated at the lowest dose of 1×10^8 gene-modified T cells are included in this report.

Laboratory operations

All presented data were generated in a laboratory certified according CCKL (the CCKL Code of Practice has been incorporated into the worldwide standard for medical laboratories: ISO 15189; see www.cckl.nl) and using established laboratory protocols and standing operation protocols of qualified and validated assays.

Blood samples

We obtained blood samples at regular intervals before, during, and after treatment for flow cytometric analysis, isolation of peripheral blood mononuclear cells (PBMCs) and genomic DNA and serum, as described.^{24,25} We aliquoted and cryopreserved PBMCs in liquid nitrogen and stored genomic DNA and serum samples at -70°C .

Flow cytometric analysis

Fluorochrome-conjugated mAbs were all purchased from BD Biosciences, except for CD8 mAb conjugated to phycoerythrin (PE), which was from Dako. We studied the membrane expression of CAR and irrelevant antigen human CD24 on transduced T cells using the G250 anti-idiotypic (Id) mAb NuH82 as described^{24,26} and anti-huCD24/PE mAb, respectively. We assessed the percentages and absolute numbers of CAR T cells in the blood of patients 1-3 before and after T-cell infusions as described²⁴; limit of quantification (LOQ): 0.1% CAIX-CAR⁺ cells within the CD3⁺ T cells. An improved anti-CAR Id flow cytometric analysis (LOQ: 0.01% CAIX-CAR⁺ cells within the CD3⁺ T cells) was applied for patient 4-11 (see supplemental Figure 2). For flow cytometric detection of cellular immune responses following mAbs were used: CD3 conjugated to peridinin chlorophyll protein (CD3/PerCP), CD8 conjugated to allophycocyanin (CD8/APC), CD107a/PE, and CD137/APC, all from BD Biosciences.

Assessment of transgene copy number by quantitative real-time PCR

We isolated genomic DNA from blood using the QIAamp DNA mini kit (QIAGEN). We performed the quantitative real-time polymerase chain reaction (PCR) as described,²⁴ and presented data as number of CAIX-CAR DNA copies; with a LOQ of 1000 CAIX-CAR DNA copies/mL.²⁴

HACA response

We used a sandwich enzyme-linked immunosorbent assay (ELISA), developed to analyze human antibody responses to chimeric G250 (HACA)²⁷ to monitor antibody responses to CAIX-CAR in patients after treatment with CAIX-CAR T cells. As “catch” and “detection” antibodies, we used murine G250, chimeric G250 (cG250), and an irrelevant chimeric mAb c323A (with identical constants chains as cG250), allowing discrimination between anti-murine and anti-G250 variable part immune reactivity. The human anti-CAIX-CAR antibody (HACA) assay²⁷ was calibrated using the anti-Id G250 mAb NuH82.²⁶ The LOQ of this ELISA was 27 ng HuH82 mAb equivalent/mL. We expressed results in nanogram NuH82 mAb equivalent/mL serum.

Inhibition of CAIX-CAR-mediated cytotoxicity

We assessed the CAIX-CAR-mediated cytotoxic activity of a CAIX-CAR T-cell clone²⁵ in a standard 4-hour ⁵¹Cr-release assay using the CAIX⁺ RCC cell line SKRC-17 MW1-clone 4 as target cell and calculated the percentage of specific cytotoxicity (%CTX) as described.²⁰ To assess the level of anti-Id G250 antibodies in patient serum, we added serum to the test medium (final volume 50% patient serum) and calculated the percentage inhibition of CAIX-specific cytotoxicity by patient serum according: %CTX in serum [(day 0 - day x)/day 0] × 100.

Cellular anti-CAIX-CAR immune response

We tested patient PBMCs for anti-CAIX-CAR cellular immune reactivity in autologous-mixed lymphocyte cultures, using PBMCs as responder cells and autologous CAR transduced T cells as stimulator cells. Stimulator cells were either (1) irradiated, read out: ³H-Thymidine incorporation by PBMCs; (2) labeled with ⁵¹Cr, read out: ⁵¹Cr release by stimulator cells (%CTX of 4 tested effector cell:target cell [ET] ratios is expressed as weighted mean of specific cytotoxicity calculated for the ET ratio of 20:1 [WMSL_[ET 20:1]])²⁵; or (3) labeled with PHK67 fluorescein isothiocyanate (labeling kit; Sigma-Aldrich), read out: flow cytometric analysis of membrane expression of CD137 or CD107a by responder PBMCs detailed in supplemental Figure 3. Responses were considered weak in case the flow tests (see supplemental Figure 3) yielded a net %CD107⁺ or %CD137⁺ responder cells < 1%, or the cytotoxicity result WMSL_[ET 20:1] was < 20%. For weak responses, assays were repeated, and considered “poorly reproducible” in case in repetitive testing not all tests yielded positive results; typically, 2 of 4 tests were positive.

We used autologous nontransduced (NTD) expanded T cells and T cells transduced with the irrelevant antigen human CD24 (huCD24) as stimulator

cell controls. CD24 is not expressed by mature T cells. To further define anti-CAR T-cell immune reactivity, we generated alternative CAR T cells that expressed various CAR elements (see also Figure 3A) and huCD24 T cells using the same SFG-derived retroviral vector pBullet.^{18,28} CAR T cells and huCD24 T cells were enriched by mini-MACS (Miltenyi Biotec) using NuH82-biotin plus streptavidin/APC, or CD24/PE (BD Biosciences) and anti-APC or anti-PE paramagnetic mAbs (Miltenyi Biotec), respectively, to > 90% positivity. In addition, we also generated CAR T cells and huCD24 T cells by nucleofection of T cells using plasmids pColt-CAIX-CAR and pColt-huCD24, respectively, using the human T-cell nucleofector kit (Amaxa) and the Amaxa Nucleofector 1 apparatus.

As we could not detect cellular immune responses in fresh patient PBMCs with any of the described methods, we set up a feeder system to amplify frequencies of putative reactive T cells, in which patient PBMCs were cocultured with irradiated autologous CAR T cells in an in vitro stimulation culture system for 2 to 9 weeks, with weekly restimulation as modified from Berger et al.¹² Subsequently, we assayed these amplified PBMC cultures for cellular anti-CAR immune responses as described above. Detection of anti-CAR T-cell cellular immune reactivity required 2-5 amplification cycles of coculture of posttreatment PBMCs with autologous CAR T cells (Figure 1). Remarkably, no anti-CAIX-CAR T-cell cellular immune reactivity was generated during the 5 in vitro stimulations in the pretreatment samples, while this activity reproducibly could be expanded in posttreatment samples, with apparently very low levels of responding effector cells, which points to high antigen-specificity and sensitivity of the method, and the need for an in vivo priming event to expand this population.

Epitope mapping of anti-CAIX-CAR cellular immune responses

We used CAIX-CAR protein (347 amino acids [aa]) spanning peptides, consisting of 84 11 aa overlapping 15-mer peptides (JPT) to assess the epitope specificity of the anti-CAR T-cell responses. First, we incubated patient PBMCs after repeated stimulation with autologous CAR T cells with the complete 15-mers protein spanning peptide pool (containing 2 nmol of each of the 84 peptides) for 20 hours and assessed responder PBMCs for membrane expression of CD137. Next, we tested positive samples with 19 matrix pools containing 8-10 peptides each and subsequently with the identified candidate individual 15-mer peptides.

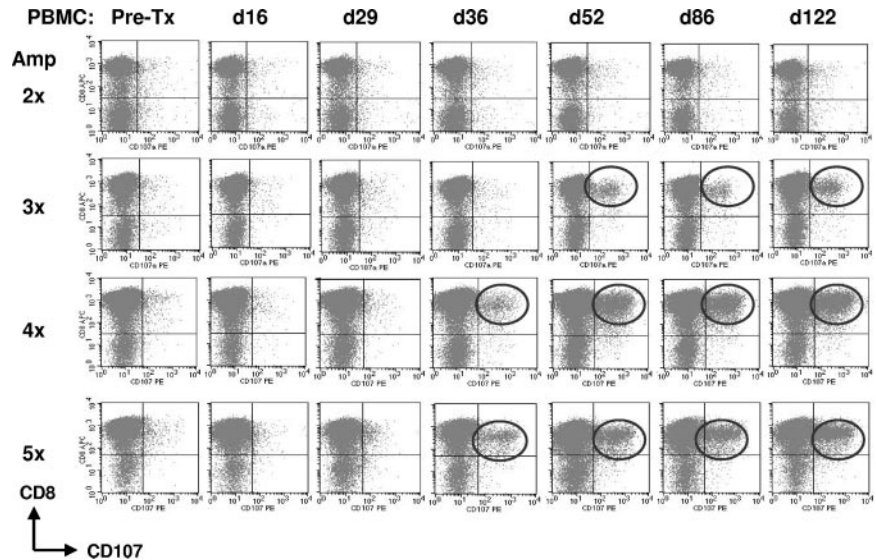
CAIX-CAR epitope prediction

We performed a CAIX-CAR CTL epitope prediction using 3 programs (ie, SYFPEITHI database for major histocompatibility complex [MHC] ligands and peptide motifs, access via: <http://www.syfpeithi.de>²⁹; the NetCTL 1.2 Server, at <http://www.cbs.dtu.dk/services/NetCTL/>,³⁰ and the human leukocyte antigen [HLA] Peptide Binding Predictions, at http://www.bimas.cit.nih.gov/molbio/hla_bind/).³¹ The NetCTL 1.2 Server (combined) prediction score is based on (1) predicted MHC binding affinity, (2) C-terminal cleavage affinity, and (3) the TAP transport efficiency. We analyzed the complete CAIX-CAR sequence (347 aa) for potential 9-mer immunoreactive peptides for all available relevant HLA-types. We considered MHC ligands with a SYFPEITHI score > 15 (max score = 36), and a NetCTL score > 0.75 (sensitivity 0.80, specificity 0.97). We performed HLA peptide binding predictions only for detected immunoreactive peptides and set the threshold for binding at a predicted dissociation half-life of 3.

Sequence analyses of SFG scFvG250-CD4/γ proviral DNA

Open reading frames (ORFs) in viral sequences of SFG scFvG250-CD4/γ proviral DNA sequences were analyzed by the ORF Finder program at <http://www.ncbi.nlm.nih.gov/projects/gorf/> and using a cutoff of > 100 nucleotides. Viral sequences per reading frame were analyzed for homologies using BLASTN 2.2.23⁺ program at (NCBI/ BLAST/ blastn suite).³² Putative amino acid sequences per reading frame were assayed for homologies using BLASTN 2.2.23⁺ program at (NCBI/ BLAST/ blastp suite).^{33,34}

Figure 1. Detection of cellular immune reactivity toward retrovirally transduced T cells requires 2-5 amplification cycles of coculture of posttreatment PBMCs with autologous CAR T cells. (A) Patient 6 PBMCs obtained before (Pre-Tx), during (days 16, 29, 36) and after treatment (days 52, 86, 122) with CAR T cells were cocultured as described (see “Methods”) with irradiated autologous CAR T cells in an autologous mixed lymphocyte culture. At 2-5 cycles of weekly stimulation, PBMCs were assayed for up-regulated CD107 expression by flow cytometry upon 2-hour stimulation with PHK67 fluorescein isothiocyanate-labeled autologous CAR T cells. Autologous NTD cultured T cells were used as negative control stimulator cells. The plots show CD107 versus CD8 expression within the CD3 T-cell population of PBMC cultures obtained from patient 6 after 2-5 cycles of coculture. Ellipsoids indicate CD107 positivity.



Results

Adoptively transferred CAIX-CAR T cells show limited peripheral persistence

Eleven patients were treated in 3 cohorts, receiving infusions with about similar numbers (median 1×10^9 , range $0.3\text{-}2.1 \times 10^9$) of CAR T cells. Patients treated in cohort 3 received an infusion with cG250 3 days before start of the CAR T-cell infusions to block CAIX sites in the liver (see supplemental Figure 1 and Table 1).

Peripheral persistence of CAR T cells was assessed by flow cytometric analysis, using the anti-Id G250 mAb NuH82, and by quantitative PCR (qPCR). As previously published for the first 3 patients,⁹ circulating CAR T cells were detectable in all 8 patients treated in the second and third cohort after the first series of infusions (days 1-5) and persisted until day 29 (the start of the second treatment cycle, days 29-33). However, after completion of the second series of infusions, CAR T cells were only detectable for another 2-18 days in cohort 2 and for 18-34 days in cohort 3. An improved anti-CAR Id flow cytometric analysis (supplemental

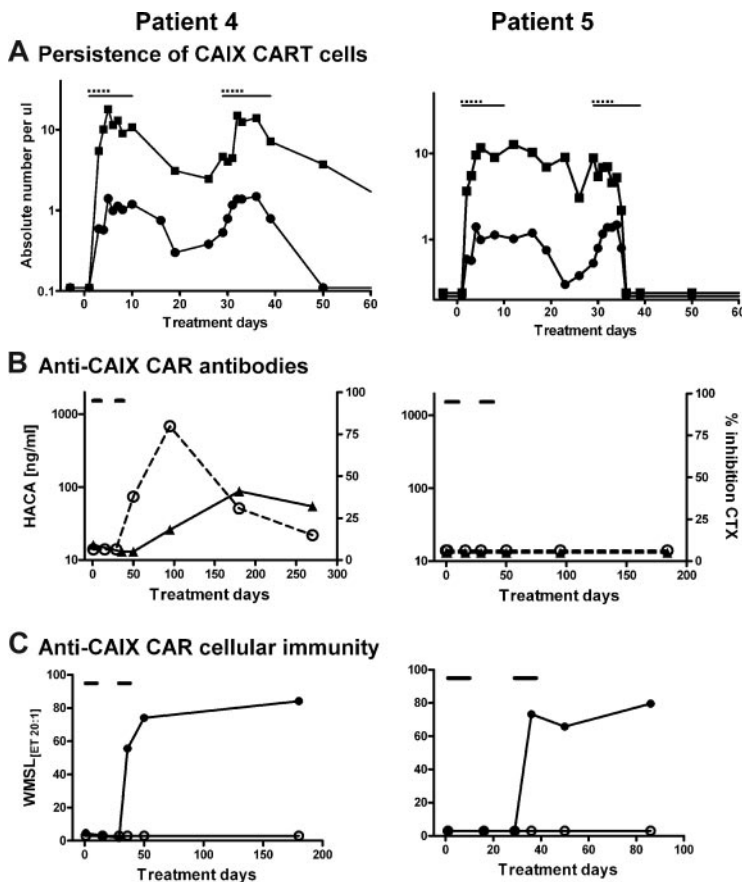


Figure 2. Adoptively transferred CAIX-CAR T cells show limited peripheral persistence and induce both humoral and cellular immunity. (A) Kinetics of absolute numbers of circulating CAIX-CAR T cells (●) and CAIX-CAR DNA copies (■) in blood samples of patients 4 and 5 obtained before, during, and after immunogene therapy with CAIX-CAR⁺ T cells. Treatment periods are indicated at the top of each panel. Individual CAIX-CAR infusions are represented by dots; IL-2 treatment by lines; (B) Kinetics of HACA (○; expressed as ng/μL at left y-axis) blood levels and capacity of patient plasma to inhibit CAIX-CAR mediated cytotoxicity (▲; expressed as percent inhibition compared with pretreatment serum at right y-axis). Data are shown for 2 representative patients (patient 4 and 5), showing, respectively, 1 development of HACA and serum inhibitory capacity (patient 4 representative for patients 1-4, 6, and 7), and no development of HACA and inhibitory activity (patient 5 representative for patients 5, 8-11). Negative tests were repeated 2-3× for confirmation. Inhibition of CAIX-CAR-mediated cytotoxicity was expressed relative to cytotoxicity in autologous pretreatment serum. Treatment intervals are indicated by horizontal lines in the top of each panel. (C) Kinetics of cellular anti-CAIX-CAR immune reactivity in patients 4 and 5. PBMC obtained before, during, and after treatment were cocultured for 5 weeks on autologous CAIX-CAR T cells as described (see Figure 1) and subsequently assayed for anti-CAIX-CAR T-cell reactivity in a cytotoxicity assay using ⁵¹Cr-labeled autologous CAIX-CAR T cells as “positive control” target cells (●) and autologous NTD cultured T cells (○) as “negative control” target cells. Percent cytotoxicity of target cells is expressed as weighted mean of specific cytotoxicity at effector cell to target cell ratio of 20:1 (WMSL_[ET 20:1]). Representative experiments are shown.

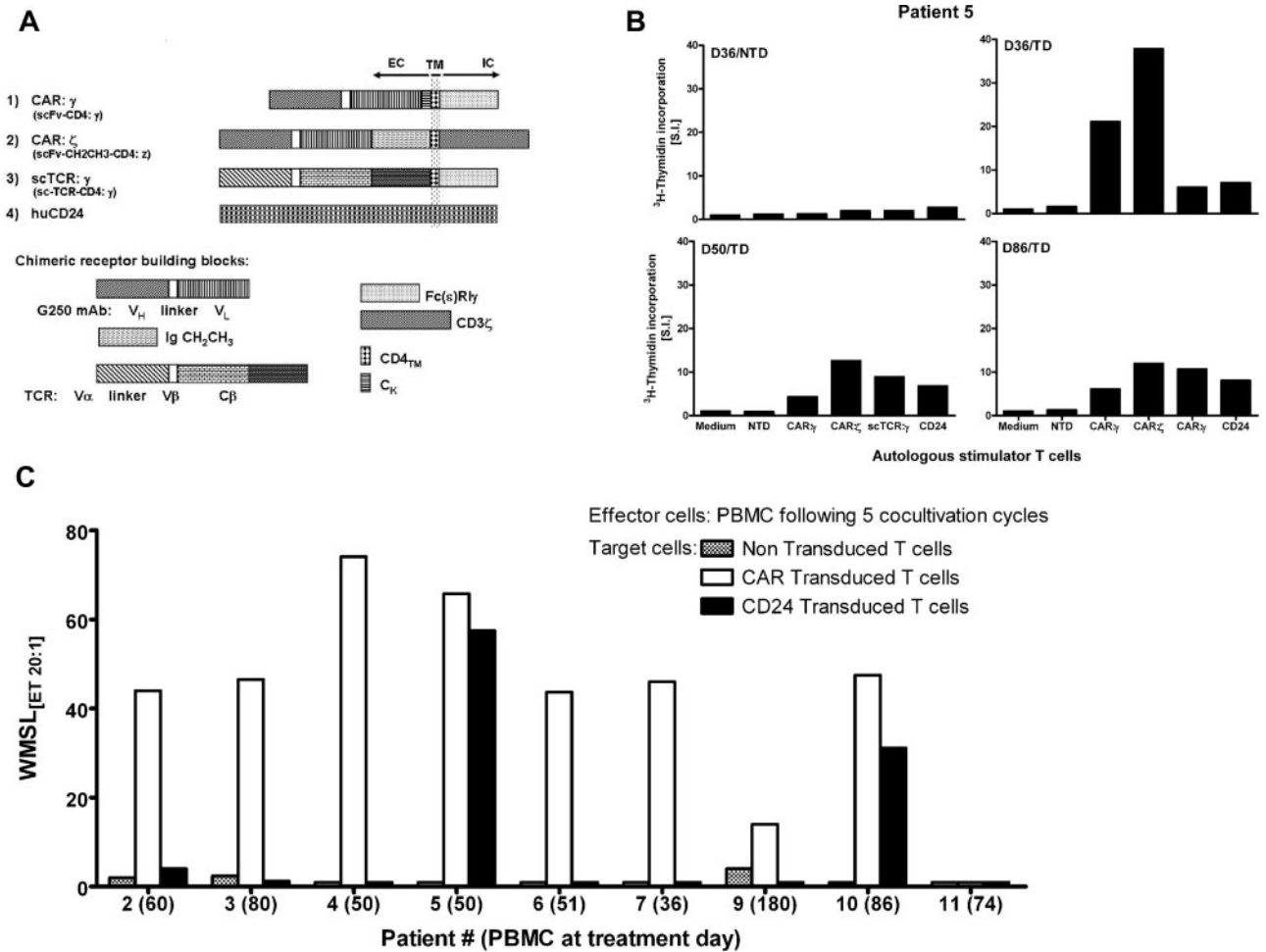


Figure 3. Specificity of anti-CAIX-CAR cellular immunity. (A) Schematic representation of receptor transgenes used to stimulate PBMCs. CAR and TCR constructs comprised one or several of the following domains: the hinge domains of IgG1, a short C κ fragment, CD4 transmembrane region, Fc(ϵ)R γ intracellular domain, complete CD3 ζ molecule, as described.^{18,28} Constructs: (1) CAR: γ^9 ; (2) CAR: ζ^{18} (including IgG1 hinge domain CH₂CH₃) comprising intracellular CD3 ζ instead of Fc(ϵ)R γ ; (3) scTCR: γ^{28} comprising identical transmembrane and intracellular domains as original CAR (Construct 1); (4) irrelevant antigen human CD24 (huCD24). (B) PBMCs from patient 5 obtained at days 36, 50, and 86 of treatment were cocultured with irradiated autologous NTD cultured T cells (top left panel) or with CAIX-CAR T cells. PBMC cultures after 5 cycles of cocultivation were assayed for ³H-thymidine incorporation assay upon stimulation with irradiated autologous stimulator T cells, either NTD or transduced with transgenes described in panel A. ³H-Thymidine incorporation in responder PBMCs is expressed as stimulation indexes (SI) to relate values to the medium controls. (C) PBMC cultures after 5 cycles of cocultivation (patient 2-11) were assayed for CAR transgene- and nontransgene-related cellular immune reactivity in a cytotoxicity assay using ⁵¹Cr-labeled autologous CAR T cells as target cells (□), NTD T cells (▨), or T cells transduced with human CD24 (■) as negative control target cells. Percent specific cytolysis of target cells is expressed as WMSL_{ET 20:1} (see also legend to Figure 2C). Representative experiments are shown.

Figure 2) was applied for patients 4-11. In these patients, flow cytometric analysis and qPCR showed equal persistence of CAR T cells in 6 patients, whereas in 2 patients (4 and 6) circulating CAR T cells were detected for a longer period by qPCR than by flow cytometric analysis. Notably, in patient 5, a sudden disappearance of CAR T cells from the circulation was observed by both flow cytometric analysis and qPCR at day 36 (ie, only 3 days after completion of the second treatment cycle; Table 1 and Figure 2A).

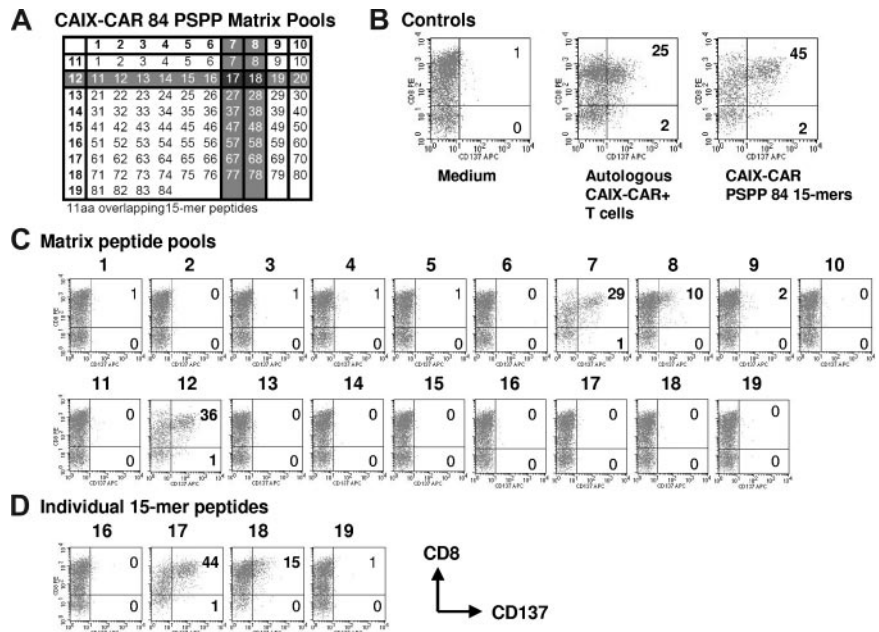
Adoptively transferred CAR T cells induce anti-Id CAR antibodies

We assayed the HACA serum levels by ELISA in all patients. HACA became detectable after the second treatment cycle in 6 patients from days 37 to 100 after treatment start. Remarkably, no HACA were detected in patient 5, and in patients treated in cohort 3 (patient 9-11) receiving pretreatment with G250 antibody. Antibody titers were highest between days 57-100 with a gradual

decline to still detectable levels at the end of immunologic follow-up (ie, days 57-276; Table 1 and Figure 2B). ELISA revealed that HACA reactivity was directed against the variable part of the CAIX-CAR (ie, the cG250 idotype; data not shown). This feature is further illustrated by the observation that the first detection of HACA in patient 4 (day 50) and 6 (day 44) coincided with an inability to detect circulating CAR T cells by anti-Id CAR flow cytometric analysis, whereas qPCR still showed their presence, suggesting a block of the recognized CAR epitope by antibodies generated in patients. To assess whether HACA could neutralize CAIX-CAR-mediated T-cell responses, we tested the functional blocking capacity of HACA containing serum in a CAIX-CAR-mediated cytolysis assay. We showed inhibition of CAIX-CAR-mediated cytolysis by HACA containing patient sera, with the level of inhibition being associated with the HACA concentration (Figure 2B). These data confirm the anti-Id CAR nature of the HACA, which limit the functional persistence of CAR T cells.

Figure 4. CAR cellular immune reactivity is directed against V_H -CDR and V_K -FR epitopes in the CAIX-CAR protein. CAR protein spanning peptides, consisting of 84 11 aa overlapping 15-mer peptides, were generated to assess epitope specificity of the anti-CAIX-CAR cellular immunity. Responder cells were day 50 PBMCs of patient 4, and cocultivated for 5 weekly cycles with CAR T cells.

(A) Design of the matrix peptide pools with numbers indicated in left column and top row. Individual 15-mer peptides in these pools correspond to the numbers in the respective columns and rows. (B) PBMCs were incubated in medium (negative control, left plot) or stimulated with CAIX-CAR⁺ T cells (middle plot) or the complete protein spanning peptide pool (84 15-mer peptides; right plot) as 2 positive stimulation controls and (C) 19 matrix pools, each containing 8-10 15-mer peptides and subsequently with the individual candidate peptides (D). (A) The gray shading in the matrix illustrates how candidate peptides were identified. Stimulated PBMC were assayed for CD137 expression by flow cytometry. Plots in panels B-D show CD137 versus CD8 expression within the CD3⁺ T-cell population. Numbers in the top right quadrant of each dotplot indicate the percentage of cells that express CD137 in response to stimulation. Controls were stimulated by medium alone or by autologous CAR T cells (B).



CAR T cells induce anti-CAR T-cell cellular immunity

We tested patient PBMCs for cell-mediated immunity (CMI) toward autologous CAR T cells using several read-out systems (see Methods). No CMI was detected when testing fresh PBMCs taken from patients before, during, and after CAR T-cell therapy. As we anticipated the frequency of putative anti-CAR T cells in PBMC to be low, we set up a method to amplify their frequency to enable detection. Detection of anti-CAR T-cell CMI depended on 2-5 cycles of weekly cocultivations of PBMCs with irradiated autologous CAR T cells to amplify the frequencies of reactive T cells before their functional testing (Figure 1). Negative controls included fresh PBMCs (not exposed to cycles of cocultivation with autologous CAR T cells), PBMCs cocultivated with NTD autologous T cells, and amplified PBMCs tested versus NTD T cells, which all showed negative results (data not shown).

Seven of 9 evaluable patients presented with strong cellular responses against autologous CAR T cells, but not toward autologous NTD T-cells. Anti-CAR CMI was detected after the second treatment cycle from day 36-86 after start of treatment and persisted until the end of observation (days 60-240; Table 1 and Figure 2C). Two patients (9 and 11) treated in cohort 3 showed only weak or poorly reproducible responses. Onset of anti-CAR CMI preceded detectable HACA serum levels, and in 3 patients,⁴⁻⁶ CMI was detectable as early as from 3 days after completion of the second series of CAR T-cell infusions onwards. These combined HACA and CMI data show that immunogenicity contributes to the limited peripheral persistence of CAR T cells.

To further characterize specificity of the anti-CAR CMI, we studied the reactivity of the amplified PBMCs obtained from patient 5 against a series of autologous stimulator T cells retrovirally transduced with different CAR formats that differ in their use of the various CAR elements (Figure 3A) as well as with a retrovirus harboring an irrelevant antigen (human CD24, normally not expressed by mature T cells). Remarkably, all transgene expressing stimulator T cells, including those expressing huCD24, were recognized by PBMCs collected at day 36-86 and amplified in presence of autologous CAR T cells, but not by day 36 PBMCs amplified in presence of NTD stimulator T cells (Figure 3B).

Extension of this specificity survey confirmed in 7 of 9 evaluable patients clearly detectable cellular responses against autologous CAR T cells, but not to autologous NTD T cells. In addition, 2 of 9 patients (5 and 10) also displayed additional reactivity against the transduction control (ie, autologous stimulator T cells transduced with a retrovirus harboring the irrelevant antigen huCD24 huCD24; Figure 3C), suggesting the development of cellular immune reactivity against retrovirus backbone derived epitopes.

Epitope mapping of anti-CAR cellular immunity

To further characterize epitope specificity of the CMI toward the CAR transgene, we generated a panel of 84 different 11-aa overlapping 15-mer peptides spanning the 347-aa CAIX-CAR protein (Figure 4A). First, we confirmed reactivity of the patient-amplified PBMCs with the autologous CAR T cells and the 15-mers protein spanning peptide pool (84 peptides; Figure 4B). Subsequently, we tested reactive PBMC samples with 19 matrix pools containing 8-10 peptides each and subsequently with the identified candidate individual 15-mer peptides, as illustrated for patient 4 (see Figure 4A-D). In this way, we identified CAR immunoreactive peptides in 5 of 6 tested patients. In patient 10, no CAIX CAR immunoreactive peptides were detected despite the presence of a strong anti-CAR T-cell reactivity. Remarkably, in the other 5 patients, only a single CAR epitope per patient was identified. Three epitopes were located in the V_H -complementarity-determining region (CDR) and 2 in the V_K -framework region (FR) of the CAR protein (Figure 5 and Tables 2-3). Of interest, immunogenic peptides within the CAR fusions sites, joining the variable region of G250 to the transmembrane and T-cell signaling domains, were not detected in these patients.

Furthermore, we observed that repeatedly stimulating patient PBMCs with autologous CAR T cells reduced the diversity of TCR-V β usage to only 2-3 dominances, which was further reduced to only one (ie, V β 2 in patient 4) after isolation of CD107 or CD137-expressing PBMCs after CAIX-CAR-specific stimulation (supplemental Figure 4). These results support the observation that

CAIX CAR amino acid sequence (aa = 347)

	<u>Signal peptide</u> VH	<u>Mature</u> VH	
1	MGFGLRLIFLVLVLKGVLC	CDVKLVESGGGLVKLGGSLKLS	
41	CAASGFTTFSNYMSWV	<u>RQTPEKRLELVAAINS</u> <u>DGGITYYL</u>	Immuno reactive epitopes Patient 4: aa 65 – 83 VH-CDR2 Patient 5: aa 117 – 131 VH-CDR3 Patient 6: aa 257 – 275 Vκ-FR4 Patient 7: aa 257 – 275 Vκ-FR4 Patient 9: aa 57 -75 VH-CDR2 Patient 10: no epitope Epitopes: underlined and bold
81	<u>DVT</u> KGRFTISRDNAKNTLYLQMSLLKSEDTALFYCAR <u>RHRS</u>		
		<u>Linker</u>	
121	<u>GYFSMDYWGQ</u> TSVTVSSGSGSTSGSGKSSEGGKTRDIYM		
	<u>Mature</u> VK		
161	TQSQRFMSTTVGDRVSI	TKCKASQNVVSAVAWYQQKPGQSP	
201	KLIIYSASNRYTGVPDRFTGSGSGTDFTLTIS	<u>NMQSEDLA</u>	
241	<u>DFFCQY</u> SNYPWTFGGGT <u>KLEIKRADAAPT</u> <u>VSQS</u> FGLLDR		
	<u>CD4Tm</u>	<u>Gamma</u>	
281	SQPMALIVLGGVAGLLLF	IGLGIFFRLKIQVRKAAITSYE	
321	KSDGVYTGSLSTRNOET	YETLKHKEKPPQ	

Figure 5. Amino acid sequence of the CAIX-CAR protein with identified immunoreactive epitopes. Amino acid sequence of the CAIX-CAR protein: aa 1-19, signal peptide G250 mAb VH; aa 20-137, mature VH; aa 138-159, linker; aa 160-278, mature VK; aa 279-301, CD4 transmembrane region (abbreviated CD4Tm); aa 302-347, intracellular Fc(ε)RI γ-chain (abbreviated γ). Identified immunoreactive epitopes are presented in bold (see also Table 3).

only a single immunoreactive CAIX-CAR-specific epitope was identified in this patient.

Supportive toward our experimental characterization of the immunogenic CAR T-cell epitopes is the close overlap between these epitopes and the computationally predicted epitopes for specific HLA alleles (Table 3) using programs, such as SYFPEITHI, NetCTL 1.2, and BIMAS, although not all experimentally characterized epitopes are found among the 10 strongest predicted epitopes (supplemental Figure 5). Of note, epitope prediction could not be performed for all involved alleles.

Immune reactivity toward retroviral vector epitopes

To investigate whether the additional anti-huCD24 T-cell CMI observed in patients 5 and 10 was indeed related to the retroviral transduction/applied SFG retroviral vector,^{19,20} we generated CAIX-CAR and huCD24 transgene expressing stimulator T cells both by retroviral transduction and nucleofection. For the latter technique, we have used nonretroviral vectors encoding the same genes as those used for retroviral transduction to avoid genomic integration of vector elements or transgenes. In patient 5, the CAR positivity on stimulator T cells was lower after nucleofection (23%) versus transduction (97%), whereas the percentages of huCD24-positive T cells were similar after nucleofection (78%) and transduction (74%). Amplified day 50 PBMCs were stimulated with transduced or nucleofected stimulator T cells, and at 2 hours and 24 hours were assayed for CD107 and CD137, respectively. The proportion of responder PBMCs showing expression of CD107 or CD137 upon CAR T-cell stimulation, relative

to the proportion of CAR positivity of stimulator cells, was about equal for stimulator cells generated by nucleofection (8.0%/23% = 0.35) versus transduction (25%/97% = 0.26; Figure 6A). In contrast, huCD24 transgene expressing stimulator T cells generated by retroviral transduction induced CD107 or CD137 expression on amplified PBMC, whereas stimulator T cells generated by nucleofection did not (Figure 6A). In addition, amplified PBMCs derived from patient 10 only recognized transduced, but not nucleofected stimulator T cells that express either CAR or huCD24 (Figure 6B), supporting our observation on the inability to identify a CAIX-CAR immunoreactive epitope for this patient (see above). From these results we conclude that retroviral transduction of T cells introduced vector-derived immunogenic epitopes in patient T cells. Thus, the anti-CAR T-cell CMI in patient 5 was directed to both CAR and retroviral vector epitopes, whereas in patient 10 it was uniquely restricted to retroviral vector epitopes. So far, no unique shared features (ie, HLA genotype; Table 3), could be identified for patient 5 and 10.

Analysis of the CAIX CAR proviral DNA (supplemental Figure 6A), that upon retroviral transduction is integrated in the host cell DNA, revealed diverse open reading frames (ORFs) in the residual GAG and POL vector sequences that potentially encode putative T-cell epitopes (supplemental Figure 6B). The predicted proteins per ORF showed multiple homologies with GAG and POL sequences of various viruses (supplemental Figure 6C). In addition, a significant homology was found with the human immunodeficiency virus type 1 NEF protein (supplemental Figure 6D). Thus, viral sequences in the proviral DNA contain several ORFs that can code for multiple vector derived proteins, which show homology with proteins with documented T-cell epitopes. This suggests that SFG proviral sequences might code for proteins containing immunoreactive T-cell epitopes.

Table 2. CDR-region sequences and amino acid positions in parental anti-CAIX G250 antibody chains and scFv(G250)-chimeric receptor

Antibody region	Amino acid sequence*	Amino acid position in	
		Parental antibody chains	scFv chimeric receptor
Heavy chain			
H-CDR1	NYYSMS	31-35	50-54
H-CDR2	AINSDGGITYYLDTVKVG	50-66	69-85
H-CDR3	HRSGYFSMDY	99-108	118-127
Light chain			
L-CDR1	KASQNVVSAVA	24-34	180-190
L-CDR2	SASNRYT	50-56	206-212
L-CDR3	QQYSNYPWT	89-97	245-253

*CDR amino acid sequences G250 mAb heavy and light chains from European patent application EP1733736A2; published 20-12-2006 Bulletin 2006/51.

Discussion

In a clinical trial we have treated 11 RCC patients with autologous T cells that were gene-modified with a first generation CAR⁶ specific for CAIX, which is frequently overexpressed on RCC.¹⁵⁻¹⁷ CAIX is to a minor extent also expressed on epithelial cells of scattered parts in the digestive tract, including the bile ducts.^{9,16} Patients were treated with multiple doses of CAR T cells, without prior lymphodepleting preconditioning,^{1,8} and patients 9-11 received an extra infusion of the parental cG250 mAb before the infusion of the CAR T cells to block the CAIX sites in the liver.^{22,23}

Table 3. Patient HLA class I genotypes and detected CAIX-CAR immunoreactive epitopes

Cohort/patient	HLA class I genotypes	Detected CAIX-CAR immune reactive epitopes	
		aa numbers	FR/CDR region
1/2	A*02,—; B*61, 62; Cw2, 3	ne	—
1/3	A*01, 24; B*08, 51; Cw4,—	ne	—
2/4	A*02 [0201/0203], —; B*15 [1501/1510], —	65-83	VH-CDR2
2/5	A*01, 24[2402]; B*51[5101], 57	117-131	VH-CDR3
2/6	A*03, 32; B*27,35	257-271	VL-FR4
2/7	A*02,11; B*35, 51	257-271	VL-FR4
3/9	A*02, 24; B*13, 15 [1521]	57-75	VH-CDR2
3/10	A*02,—; B*08, 35	—†	—
3/11	A*03, 29; B*07,—	ne	—

ne indicates not evaluated due to limited immune reactivity or technical restrictions.
 †No immunoreactive epitope detected.

After infusion, CAR T cells could be detected in the circulation, while maintaining transgene-specific immune functions.^{24,25} To date, no objective clinical responses have been observed (Lamers et al⁹ and C.H.J.L., unpublished data).

Here we reported that in these nonpreconditioned immune-competent RCC patients the limited peripheral persistence of CAR T cells coincided with distinct humoral and cellular anti-CAR T-cell immune responses, which interfered with the functionality of the CAR T cells. In addition, we showed that these anti-CAR T-cell immune responses were directed against both the CAIX CAR transgene and potentially retroviral vector-encoded epitopes expressed by the CAR-engineered T cells. This study extends on previous observations on immunogenicity of transgenes, such as tumor associated antigens, suicide genes, and selection markers,^{11-14,35} but is the first

to document the clinical immunogenicity of both CARs and potentially retroviral vector-derived epitopes.

Six of 7 evaluable patients treated in cohort 1 and 2 developed HACA, which was directed against the Id of scFv(G250) and functionally blocked CAR T cells both in ex vivo tests but also in vivo. The last phenomenon was documented in patient 4 and 6, in whom at day 44 in presence of serum HACA levels, circulating CAR T cells still could be detected by qPCR but no longer by anti-Id CAIX-CAR(G250) flow cytometric analysis. Our study underscores that treatment with CAIX-CAR transgene expressing T cells is a powerful means to induce specific humoral immune responses toward Id-CAR in immune competent patients. In contrast, only 0%-30% of patients treated with repeated high doses of the cG250 antibody developed anti-Id-G250 antibodies.^{27,36,37}

A Patient 5

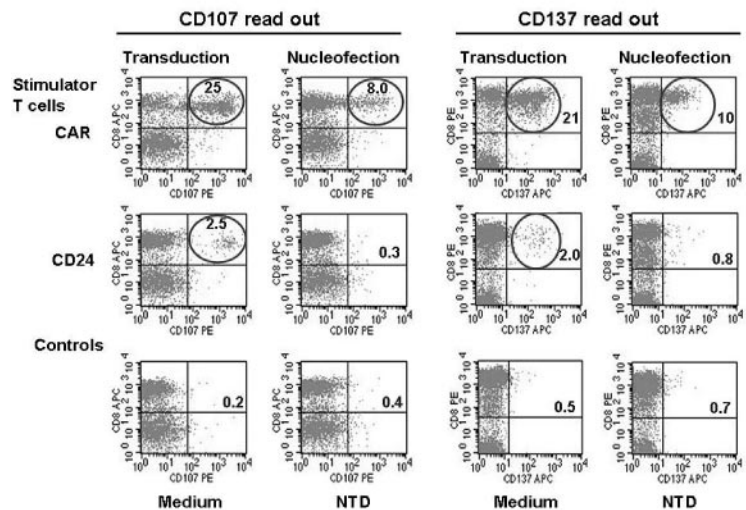
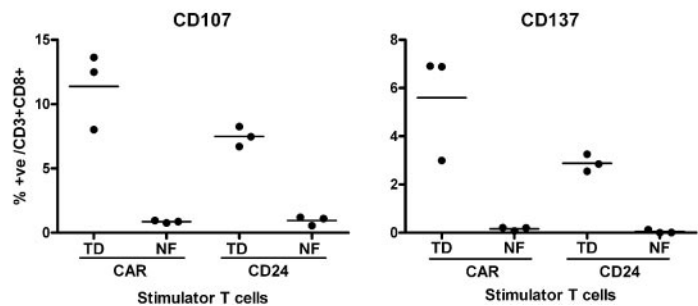


Figure 6. Two patients presented with cellular immune reactivity toward retrovirally transduced T cells independent of CAR transgene. (A) Patient 5 developed both anti-CAR and antivector immune reactivity. Patient 5 stimulator T cells were generated either by transduction or nucleofection with the transgenes CAIX-CAR or huCD24 (see “Methods”). Transduced T cells were enriched for transgene positivity by MACS-selection, whereas nucleofected T cells were used within 48 hours after nucleofection, without enrichment. Of note, the percentage CAR T cells was lower after nucleofection (23%) versus transduction (97%), whereas the percentages huCD24⁺ T cells were similar after nucleofection (78%) and transduction (74%). Responder cells were day 50 PBMCs cocultivated for 5 weekly cycles with CAR T cells. Propagated PBMCs were stimulated with transduced or nucleofected stimulator T cells, and at 2 hours and 24 hours assayed for CD107 and CD137 expression, respectively. The dot plots show CD107/CD137 versus CD8 expression within the CD3 T-cell population and values indicate the percentage positivity. Controls were stimulation by medium alone or NTD T cells. Of note, discrepancy with Figure 3C in the levels of immune recognition of CAIX CAR and huCD24 T cells might be attributed to biologic variation in the batches of responder cells used for both tests and assay sensitivity (cytotoxicity and flow cytometry). (B) Patient 10 developed antivector immune reactivity only. Patient 10 stimulator T cells were prepared and assayed as described for patient 5. Graphs show individual and mean results of 3 independent experiments obtained with PBMCs collected at day 86. The proportion of transgene expression on stimulator T cells was for CAR generated by transduction 94%-98% versus 36%-40% by nucleofection, and for huCD24 generated by transduction 88%-94% versus 56%-68% by nucleofection. TD, transduced stimulator T cells; NF, nucleofected stimulator T cells.

B Patient 10



Similar to our findings, anti-CAR humoral immunity has been described in nonpreconditioned immune-competent patients.^{2,9} In contrast, no such reactivity was detected in B-cell lymphoma patients treated with CD20-CAR T cells after cytoreductive chemotherapy.⁵ These observations suggest that preconditioning using immunosuppressive regimens attenuates humoral immunogenicity profile of CARs.

Remarkably, no HACA was induced in patients 5 and 9-11, the latter 3 patients receiving an infusion with the parental cG250 mAb before the CAR T cells. Preliminary data from these 3 patients suggest that pretreatment with cG250 attenuates liver toxicity and prolongs peripheral persistence (⁹ and C.H.J.L., unpublished data). It is tempting to speculate that the observed liver toxicity and resulting inflammatory reaction might have potentiated the anti-CAR T-cell immune response in patients treated in cohort 1 and 2.

In addition to humoral antitransgene reactivity, induction of antitransgene CMI has been described previously after adoptive transfer of T cells that were gene-modified to express suicide genes, selection markers, and the tumor-associate antigen MAGE 3.^{11-14,35} The sudden disappearance of circulating CAR T cells in patient 5 at day 36, without measurable levels of HACA, strongly suggested the existence of anti-CAR T-cell CMI. However, no such reactivity could be detected in fresh PBMCs taken from patients before, during, and after CAR T-cell therapy. By amplifying the frequency of the putative anti-CAR T cells in PBMCs, we were able to detect anti-CAR T-cell CMI in all 6 evaluable patients treated in cohort 1 and 2 from as early as day 36 onwards in 3 patients (ie, shortly after completion of the second series of 5 serial CAR T-cell infusions [day 29-33]). Notably, CMI was detected before HACA in 5 of 6 patients. In patients treated in cohort 3,⁹⁻¹¹ in addition to absence of HACA, also a very weak or poorly reproducible anti-CAR CMI was detected. Of note, the detected anti-CAR T-cell reactivity in patient 10 was not directed against the CAR. Recently, a direct method for detection of gene-modified T-cell immune reactivity was described, by combining V β -spectratyping and V β -specific flow cytometric analysis.³⁵

The specificity of the anti-CAR CMI was determined by epitope mapping using CAR-derived peptides, spanning the complete CAR-protein, with the caveat that *in vitro* expansion may have skewed against other *in vivo* relevant immune reactivities. Remarkably, in all 5 evaluable patients, only a single CAIX-CAR epitope per patient was recognized; all were located within the CAR V_H-CDR or V_K-FR variable domains and none in the “novel” epitopes created by fusion of the different CAIX-CAR domains. The skewing of the TCR-V β repertoire during the PBMC amplification cultures toward a single dominant V β in patient 4 supports the conclusion on the recognition of a single epitope in this patient. In contrast, in patients treated with HSV-TK T cells the reported anti-HSV-TK immunities hosted multiple specificities per patient.¹² Although the experimentally identified immunogenic CAIX-CAR epitopes show a close overlap with computationally predicted epitopes for specific HLA alleles, they are not found among the 10 strongest predicted epitopes in 4 of 5 patients. Of note, for some of the involved alleles epitope prediction is not yet available.

In concordance with the observation that clonal immunoglobulin (Ig) in B-cell malignancies bear immunogenic peptides in both CDR and FR-domains of the IgH chain and to a minor extent to the κ chain,³⁸ we have identified immunogenic epitopes in the murine V_H-CDR and V_K-FR variable domains of the CAIX-CAR. The observed immunogenicity of the V_H-CDR sequences suggests that

the strategy of humanizing murine CARs by CDR grafting³⁹ alone might not completely eliminate the potential immunogenicity of humanized CARs and will need additional manipulation, such as specificity-determining residue grafting and deimmunization methods,⁴⁰ or by using completely human mAbs⁴¹ to construct nonimmunogenic CARs.

Thus far, vector-specific immune responses have only been described in clinical studies applying viral vectors *in vivo*, using both replication-deficient and replication-competent adenoviruses^{42,43} and adeno-associated virus (AAV).⁴⁴ In a clinical trial in which an AAV vector expressing coagulating factor IX was introduced into the liver of hemophilia B subjects, an immune rejection of transduced hepatocytes was reported to be mediated by AAV capsid-specific CD8 T cells.⁴⁵ An adaptive immune response or earlier exposure to AAV virus have been proposed to have played a critical role in the recall of a memory T-cell response toward the AAV capsid, which affected the success of the clinical trial.^{44,46} In our clinical trial using an *ex vivo* approach, we identified 2 patients who developed anti-gene-modified T-cell CMI, irrespective of the transgene.

We conclude that therapeutic cells that were *ex vivo* gene-modified by means of retroviral transduction may also express immunogenic vector-encoded epitopes. Although no preexisting vector immunity was seen in these patients, it cannot be concluded whether this antivector epitope reactivity is a primary adaptive immune response to the CAR T cells or that it reflects a recall of a memory T-cell response against endogenous retroviral epitopes in these patients.⁴⁷ The residual SFG retroviral vector sequences that are integrated in the host cell genome bear multiple homologies with GAG, POL, and HIV-NEF sequences and code for diverse proteins for which multiple T-cell epitopes have been described.^{48,49} We hypothesize that this phenomenon will extend to other clinically *ex vivo* applied viral vectors. However, antivector immune reactivity in adoptive T-cell therapy may have been masked by the antitransgene immunity or suppressed by the applied immunosuppressive preconditioning regimens.

In conclusion, our study demonstrates the immunogenicity of CAR and of retroviral vector-engineered T cells, which limit the functional peripheral persistence of CAR T cells. In particular, the finding that cellular immunity may occur against retroviral vector-encoded epitopes is of major concern for adoptive T-cell therapies using retroviral transduction and needs further exploration. These results advocate minimization of residual viral sequences in vectors used to *ex vivo* generate gene-modified T cells for therapy. Immunogenicity of CARs can be minimized by using completely human CARs.⁴¹ Transfection methods other than retroviral, or extension of the therapeutic protocol with preconditioning using immunosuppressive regimens, may be crucial to exploit adoptive T-cell therapy to its full extent.

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Authorship

Contribution: C.H.J.L. designed and analyzed experiments and wrote the paper; P.v.E., R.W., S.v.S.-L., M.B., J.O.-W., and E.O. performed and analyzed experiments; R.W., S.S., R.D., and J.W.G. designed experiments; and S.S. and J.W.G. supervised the clinical study.

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