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Plasma IFN-γ and IL-6 levels correlate with peripheral T-cell numbers but not toxicity in RCC patients treated with CAR T-cells

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ABSTRACT

Autologous T-cells genetically modified to express a chimeric antigen receptor (CAR) against carboxy-anhydrase-IX (CAIX) were administered to twelve patients with CAIX-positive metastatic renal cell carcinoma. Here, we questioned whether plasma cytokine levels following treatment or in vitro cytokine production from the T-cell infusion products could serve as predictors for peripheral T-cell persistence or in vivo T-cell activity. We demonstrated that CAR surface as well as gene expression are down-regulated following T-cell infusion, and that peripheral numbers of CAR T-cells are best captured by flow cytometry and not by qPCR. Numbers of CAR T-cells in blood correlated with plasma levels of IFN-γ and IL-6, but not with any of the other cytokines tested. Plasma IFN-γ or IL-6 levels did not correlate with liver enzyme values. Thus, out of 27 cytokines tested, IFN-γ and IL-6 levels in plasma are potential surrogate markers for CAR T-cell persistence in solid tumors.

1. Introduction

With the use of gene transfer technologies, T-cells can be genetically modified to stably express antibody molecules on their cell surface. The first generation of chimeric antigen receptors (CARs) consisted of constructs in which an extracellular antibody-based recognition domain was combined with the intracellular signaling domain of the CD3-zeta (CD3ζ) or Fc(ε)RIγ chains into one single protein [1]. In the so-called second or third generation CARs, one or two additional intracellular co-signaling domains that are generally derived from the CD28 or 4-1BB co-stimulatory molecules, are added [2,3]. The introduction of such an extra intracellular co-stimulatory domain increases the clinical effectivity of CAR T-cells, and coincided with enhanced in vivo persistence, and in vivo expansion of CAR T-cells [2,4–6]. In recent years, CAR T-cell therapy has shown impressive clinical responses in hematological B-cell malignancies [7]. Also correlations between T-cell persistence and clinical effectivity have been described [2,4–6]. In solid tumors, however, the number of clinical CAR T-cell studies has lagged behind with only a few clinical responses reported [8,9].

Obviously, in this emerging field there is a need for markers that provide information about early CAR T-cell persistence and in vivo activity [7]. With the increasing number of studies valuable data becomes available to perform such analysis.

One of the first CAR T-cell trials, performed at Erasmus MC Rotterdam, used a CAR:Fc(ε)RIγ directed to carboxy-anhydrase-IX (CAIX), an antigen that is over-expressed on renal cell carcinoma (RCC). T cells were transduced with the CAIX CAR by the SFG γ-retroviral vector. [10] A total of twelve RCC patients were treated in this phase-I dose escalating trial with CAR T-cells and low dose IL-2 without prior chemotherapy in three separate cohorts (see Section 2). Four out of eight patients in cohorts 1 and 2 experienced severe, but transient liver toxicities, which were most likely due to CAIX antigen expression on the surface of epithelial cells lining the bile ducts in the liver, and its recognition by the administered CAIX CAR T-cells [11,12]. Another four patients in cohort 3 were pre-treated with CAIX monoclonal antibodies (mAb) to preferentially block CAIX in the liver but not in RCC lesions, a scheme that successfully prevented severe liver toxicity in these patients [11,12]. Though we could demonstrate in vivo activity of CAIX CAR T-cells, as measured by the observed on-target liver toxicities, objective clinical responses were not seen [11,13]. In the study described here, we measured the concentration of an extended set of cytokines in blood samples taken at multiple time points during treatment, as well as in culture supernatants from T-cell infusion products, and assessed whether or not cytokine values correlated with numbers of circulating T-cells that express CAR and/or liver toxicity. To the best of our knowledge, such information was not yet available for CAR T-cell treatments of a solid tumor.

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2. Materials and methods

2.1. Patient treatment schedule and evaluation

Patients were diagnosed with clear cell RCC with progressive disease, not suitable for curative surgery, for whom no standard treatment existed, and with the primary tumor expressing CAIX [12]. Specific patient characteristics are described elsewhere [11]. Patients were treated after written informed consent, and treated according to three patient cohorts due to serious adverse events observed in the first patients treated [11]. In short, in cohort 1, it was aimed to assess toxicity and to establish the maximum tolerated dose (MTD) of CAIX CAR T-cells by an in-patient dose escalation scheme. Treatment consisted of intravenous administration of 2×10^7 T-cells at day 1; 2×10^8 T-cells at day 2; and 2×10^9 T-cells at days 3–5 (treatment cycle 1) and days 17–19 (treatment cycle 2). Simultaneously patients received twice daily subcutaneous injections of IL-2, 5×10^5 IU/m^2 on days 1–10 and days 17–26. Because of liver toxicity, the schedule was changed in cohort 2. It was aimed to assess several dose levels starting at 1×10^8 CAR T-cells per infusion and extending to 2, 4, 8, 16, 20, 25, and 30×10^8 cells in subsequent dose levels, and applying a maximum of 10 T-cell infusions at days 1–5 and days 29–33 combined with IL-2, subcutaneously, 5×10^5 IU/m^2 twice daily at days 1–10 and days 29–38.

In cohort 3, patients were treated as in cohort 2, but received an extra intravenous infusion of 5 mg of the anti-CAIX mAb G250, 3 days before start of each series of CAR T-cell infusions, in order to block CAIX antigen in the liver and leaving accessible CAIX antigen at RCC tumor sites [14–16]. Patients from this latter cohort were not included in the analyses of in vivo parameters because of additional pretreatment with anti-CAIX mAb, which has led to differences in T cell persistence between the first two cohorts versus the third cohorts [11]. For the analyses of infusion products, however, all patients and treatment cycles were included, as preparations of CAR T-cells were independent of patient cohorts and treatment cycles.

2.2. Preparation of CAIX CAR T-cell infusion products and their supernatants

Patient peripheral blood mononuclear cells (PBMCs) from leukapheresis were activated in a complete Mixed Medium (MixMed) [17] using 10 ng/ml CD3 mAb OKT3 (Janssen-Cilag Beerse, Belgium), without addition of exogenous IL-2. At days 2 and 3, T-cells were transduced with the CAIX CAR vector (batch #M4.50086; BioReliance, Sterling, UK) as described [10] in the presence of 100 IU/ml IL-2 (Chiron, Amsterdam, The Netherlands). From day 4 onward, T-cells were expanded in complete MixMed supplemented with 360 IU/ml IL-2. Lymphocytes were counted every 2–3 days and adjusted to 0.5×10^6 cells/ml by adding fresh culture medium and IL-2 until day 15. At culture days 2, 3, 4, 7, 9, 11 and 14 aliquots of culture supernatants were collected, cleared by centrifugation (10 min at 3000*g*) and stored at −70 °C for retrospective analysis.

2.3. Blood samples

We obtained blood samples at regular intervals before, during, and after treatment for flow cytometric analysis, isolation of PBMCs, genomic DNA, RNA and plasma was as described elsewhere [13,18]. We aliquoted and cryopreserved PBMCs in liquid nitrogen, and stored genomic DNA, RNA and serum samples at −70 °C.

2.4. Enumeration of transduced T-cells in infusion products and blood samples

Number of T-cells with membrane expression of CAIX CAR in cultures as well as blood was assessed by flow cytometry (FCM) using the anti-CAIX CAR idiotype mAb NUH82 [19] (kindly provided by Dr. E. Oosterwijk, Nijmegen, The Netherlands) (limit of quantification: 0.01% CAIX-CAR$^+$ cells within CD3$^+$ cells) [18,20]. Gene expression of CAIX CAR was assessed by qRT-PCR. To this end, RNA was isolated from T cell cultures or blood samples using the ChargeSwitch Total RNA Cell Kit (Invitrogen, Carlsbad, CA, USA) and QIAamp® RNA Blood Minikit (Qiagen, Valencia, CA, USA), respectively, according to the manufacturer's guidelines. Complementary DNA (cDNA) synthesis was done using Reverse Transcriptase Superscript III (Invitrogen) under standard conditions. The quantitative real time PCR to detect CAIX CAR cDNA copies was performed as described previously. [18] CAIX CAR RNA levels in blood were only assessed in cohorts 2 and 3. Additionally, genomic DNA was isolated from blood aliquots and T cell cultures using the QI Amp DNA mini kit (Qiagen, Hilden, Germany). The quantitative real time PCR to detect CAIX CAR DNA copies was again performed as described previously [18].

2.5. Assessment of cytokine levels

The concentrations of IL-1β, IL-2ra, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-12(p70), IL-13, IL-15, IL-17, Eotaxin, FGF-Basic, G-CSF, GM-CSF, IFN-γ, IP-10 (CXCL10), MCP-1, MIP-1α, MIP-1β, PDGF-bb, RANTES, TNF-α, and VEGF in plasma samples and culture supernatants were determined using a commercially available 27-multiplex bead assay (BioRab Laboratories, Inc., Veenendaal, The Netherlands/Minneapolis, MN, USA) according to the manufacturers' instructions. For array specificities and lower limit of quantification levels, see Supplementary Table S1.

2.6. Statistical analysis

Cytokine levels in culture supernatant were normalized to the levels at day 2 (i.e., 40 h after anti-CD3 mAb activation of PBMCs) and the cytokine levels in plasma were normalized to the baseline plasma levels at day 1. The Spearman correlation coefficient method was used to assess linear association between two continuous variables. P-values <0.05 were considered significant. Differences between two categories with respect to paired continuous parameters were determined using an exact Wilcoxon rank sum test. Statistical analyses were performed with SPSS software (version 21) for Windows (IBM Corporation, Illinois, U.S.A.). Graphpad Prism v5.0 was used to prepare graphs and calculation of the Area Under the Curve (AUC) for cytokines and CAR T-cell numbers was performed using non-normalized data in linear X- and Y-axis plots.

3. Results

3.1. Patients

In total, twelve RCC patients were treated with CAR T-cells. Specific patient characteristics are described elsewhere [11]. Four out of eight patients treated in the first two cohorts developed grades 3–4 liver toxicities and therefore three of them did not receive a second cycle of CAR T-cell infusions. Six out of eight patients developed anti-CAR cellular immune response, and 6 out of eight patients developed a humoral immune response. These responses became particularly prominent during and after treatment cycle two, compromising CAIX CAR T-cell numbers [20]. For this reason, we performed an analysis of circulating CAR T-cell numbers and cytokine levels during the first treatment cycle only (cohort 1: day 1 until 17, and cohort 2: day 1 until 29).

3.2. CAIX CAR T-cell numbers in patient blood peak between days 5–8 after onset of treatment

CAIX CAR T-cells and CAIX CAR DNA copies were quantified in patient blood by FCM and qPCR, respectively (Fig. 1A, B) and were clearly detectable in all patients during the first cycle of CAR T-cell treatment. Since some of the patients received a second cycle of CAR T-cells at