Efficient selection of genetically modified human T cells using methotrexate-resistant human dihydrofolate reductase

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Genetic modification of human T cells to express transgene-encoded polypeptides, such as tumor targeting chimeric antigen receptors, is an emerging therapeutic modality showing promise in clinical trials. The development of simple and efficient techniques for purifying transgene+ T cells is needed to facilitate the derivation of cell products with uniform potency and purity. Unlike selection platforms that utilize physical methods (immunomagnetic or sorting) that are technically cumbersome and limited by the expense and availability of clinical-grade components, we focused on designing a selection system on the basis of the pharmaceutical drug methotrexate (MTX), a potent allosteric inhibitor of human dihydrofolate reductase (DHFR). Here, we describe the development of self inactivating (SIN) lentiviral vectors that direct the coordinated expression of a CD19-specific chimeric antigen receptor (CAR), the human EGFRt tracking/suicide construct, and a methotrexate-resistant human DHFR mutein (huDHFRFS, L22F, F31S). Our results demonstrate that huDHFRFS expression renders lentivirally transduced primary human CD45RO+ memory T cells resistant to lymphotoxic concentrations of MTX up to 0.1 μM. Our modular complementary DNA (cDNA) design insures that selected MTX-resistant T cells co-express functionally relevant levels of the CD19-specific CAR and EGFRt. This selection system on the basis of huDHFRFS and MTX has considerable potential utility in the manufacturing of clinical-grade T cell products.

INTRODUCTION

The selection of genetically modified cells in human gene therapy is a methodological challenge when potency and safety are linked to the purity of cell products manufactured ex vivo. A major focus of ex vivo cell engineering involves hematopoietically derived cells, in particular T cells modified to express chimeric antigen receptors for redirected tumor recognition. Selection following transfection/transduction typically involves physical purification on the basis of flow cytometric cell sorting or immunomagnetic techniques. Although these methodologies have several advantages, such as the use of human-encoded markers of transduction, these methods require expensive infrastructure such as GMP-compliant clinical cell sorting facilities, and clinical-grade reagents such as conjugated monoclonal antibodies. Alternately, cell selection can be achieved by chemical means on the basis of expressing enzymes that confer resistance to cytotoxic selection drugs. Although a number of drug-resistance enzymes have been employed for selection of gene-modified cells, such as bacterial phosphotransferases that confer resistance to hygromycin, neomycin and zeocin, these selection enzymes and drugs have proven disadvantages including the immunogenicity of the xenogeneic enzymes and the lack of GMP-grade selection drugs.1–3

Human selection enzyme systems would carry the advantage of limited immunogenicity and, if coupled with pharmaceutical selection drugs, excellent applicability in the setting of cGMP-compliant manufacturing. Several enzyme systems have been described that employ human enzymes capable of conferring resistance to cytotoxic chemotherapeutic drugs for human hematopoietic stem cell selection in vivo.2,6–10 The methyl guanine methyltransferase (MGMT) mutein can render cells resistant to the alkylator drug temozolomide when administered in combination with O6-benzylguanine (O6-BG).11–13 Although in vivo selection of gene-modified hematopoietic stem cells is achieved with this approach, it is not readily transferable to in vitro selection, nor is a genotoxic alkylator drug such as temozolomide a favorable agent for this purpose. In an effort to circumvent these challenges, we sought to develop a drug-selection platform that uses a human-resistance enzyme and a non-genotoxic lymphotoxic pharmaceutical anti-metabolite drug. Other desirable features of the system include a small transgene footprint for the incorporation into gene-transfer vectors, a rapid mechanism of action for culling non-transduced cells from culture, and a high-expression threshold of the resistance gene such that linked therapeutic transgenes are also expressed at high levels following selection. Accordingly, we focused on the adaptation of mutant human dihydrofolate reductase (DHFR) constructs that confer resistance to lymphotoxic concentrations of MTX.14–17

In the present study, we evaluate the utility of a huDHFRFS/MTX selection system for generating therapeutic T cells expressing chimeric antigen receptors (CARs) and suicide genes following in vitro lentiviral vector transduction. Our results demonstrate that MTX is an effective lymphotoxic selection drug for activated, proliferating human T cells in vitro, rendering DHFR wild-type cells non-viable in 5–7 days. Following lentiviral vector transduction, huDHFRFS-expressing T cells are enriched after a single round of MTX selection. Moreover, when huDHFRFS is arrayed in a single
transcript in combination with a CD19-specific CAR (CD19CAR) and EGFr, each separated by the T2A, MTX selection results in high-level expression of each of the three transgene-encoded proteins, thus resulting in potent CAR-redirected functional outputs. By combining a human-encoded DHFR mutein with pharmaceutical grade MTX, this selection platform is well suited for in vitro selection of gene-modified T cells, and is a promising platform for the in vivo selection of gene-modified T cells.

RESULTS AND DISCUSSION

We first sought to define the minimum in vitro concentrations of MTX that render activated proliferating human T cells non-viable. Using Jurkat T cells, dose-viability response curves were generated at MTX concentrations up to 0.1 μM. As described previously, MTX acts through competitive binding with the dihydrofolate-binding site, which inhibits the ability of DHFR to convert dihydrofolate to tetrahydrofolate, resulting in inhibition of purine biosynthesis, and consequently, cell death of activated proliferating lymphocytes. We identified a threshold MTX concentration of 0.05 μM that rendered cultured Jurkat T cells non-viable (Figure 1a), a level consistent with the previous observations of MTX cytotoxicity against hematopoietically derived cells in culture. Therefore, in all subsequent experiments, ≥0.05 μM MTX was used to examine DHFR-mediated rescue of primary human T cell viability and proliferation.

Mutagenesis of the DHFR coding sequence at codon positions 22 and 31 has been reported to result in a mutant DHFR (DHFR<sup>FS</sup>) that retains 2.3% of its catalytic activity while exhibiting a 478-fold resistance to MTX inhibition. To determine whether huDHFR<sup>FS</sup> expressed from a human EF1-α promoter results in MTX resistance to concentrations of drug in excess of 0.05 μM, Jurkat cells were electroporated with a huDHFR<sup>FS</sup>-containing plasmid (Figure 1b) then selected in media supplemented with 0.05 μM MTX for 7 days. Selected cells were then assayed for their susceptibility to cytotoxic concentrations of MTX by re-plating in increasing concentrations (0–0.25 μM) of MTX. After 8 days of culture in these conditions, transfected/MTX-selected Jurkat cells were found to have expanded 101 ± 2.0-fold in 0.05 μM MTX (Figure 1c). In this culture system, huDHFR<sup>FS</sup> expressing Jurkat cells retained viability with diminished proliferative activity in concentrations of MTX up to 0.25 μM. This is in contrast to mock-transfected/non-selected cells, which were rendered non-viable in 0.05 μM MTX (Figure 1a). These data demonstrate that the huDHFR<sup>FS</sup> transgene expressed in Jurkat T cells confers resistance to otherwise cytotoxic concentrations of MTX in vitro.

We next sought to incorporate the huDHFR<sup>FS</sup>-MTX selection system into our third generation SIN lentiviral vector system, and design trifunctional transgene constructs that place our CD19CAR and huEGFr in frame, each separated by a T2A ribosomal skip linker (Figure 2a). As proof of concept, IL-2-dependent murine CTLL-2 T cells were first transduced with this CD19CAR/huEGFr/huDHFR<sup>FS</sup> lentiviral vector and evaluated for...

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**Figure 1.** Expression of double-mutant DHFR transgene (DHFR<sup>FS</sup>) in Jurkat cells confers MTX resistance. (a) Cells were plated in triplicate with equal cell number in 24-well plate. Total viable cell number, percentage of viable cells and fold expansion of non-transduced Jurkat cells (mean ± s.d.) at indicated concentrations of MTX are depicted. (b) Plasmid construct containing the huDHFR<sup>FS</sup> transgene that was used to genetically alter Jurkat T cells. Location of CMV promoter (CMVp), bovine growth hormone polyadenylation (BGH PolyA), f1 origin of replication (f1 ori), ColE1 origin of replication (ColE1 ori), SV40 polyadenylation signal (SV40), neomycin (NeoR) and ampicillin resistance (AmpR) sequences inherent in the pcDNA3(−) plasmid are also depicted. (c) Pre-selected huDHFR<sup>FS</sup>-transfected Jurkat T cells (7 days in 0.05 μM MTX) were re-plated in triplicate with equal cell number in 24-well plates at the indicated concentrations of MTX. The data represent the mean ± s.d. There was a significant difference in their total viable cell number (P < 0.0001) and percentage of viable cells (P < 0.0001), as compared with that in the non-transduced group at >0 MTX concentration over 8 days. The data are a representative of four separate experiments.
Figure 2. Transduction with a lentiviral construct containing huDHFR<sup>FS</sup>, CD19CAR and huEGFR<sup>t</sup> transgenes confers MTX resistance in CTLL-2 cells. (a) Construct within lentiviral vector containing the CD19CAR, huEGFR<sup>t</sup> and huDHFR<sup>FS</sup> transgenes linked by T2A ribosomal skip sites. Location of EF1α promoter (EF1p) and GM-CSF receptor signal sequences (GM-CSFR<sub>ss</sub>) relative to transgenes are also depicted. (b) CTLL-2 cells were assessed for CD19CAR (Fc) and EGFR<sup>t</sup> expression (gray histograms) by flow cytometry 8 days after transduction with the vector described in (a). Percentages of positive cells above staining with secondary reagent alone (open histograms) are indicated. (c) Non-transduced (non-Txd, gray line) vs transduced (Txd, black line) CTLL-2 cells were plated in triplicate with equal cell number in 24-well plates in the indicated concentrations of MTX and evaluated for their viable cell number and percentages of viable cells (mean ± s.d.) over 8 days. Data for a representative experiment are shown. (d) Flow cytometric evaluation of huEGFR<sup>t</sup> transgene expression (gray histograms) on transduced CTLL-2 cells after 8 days of culture in the indicated concentrations of MTX. A representative example (n = 3) of the percentage of huEGFR<sup>t</sup>-positive cells and mean fluorescence activity above staining with secondary reagent alone (open histograms) are indicated. (e) Pre-selected transduced (Txd + Slxd, black line) CTLL-2 cells (8 days in 0.1 μM MTX) vs non-transduced CTLL-2 cells (non-Txd, gray line) were re-plated at the indicated concentrations of MTX. There was a significant difference in their total viable cell number (P < 0.0001) and percentage of viable cells (P < 0.0001), as compared with that in the non-transduced group at > 0 MTX concentrations over 8 days. Data for a representative experiment are shown.

their resistance to MTX. Ten days after lentivirus transduction, 7–8% of cells expressed CD19CAR and huEGFR<sup>t</sup>, as assessed by flow cytometry (Figure 2b). In the absence of MTX, the non-transduced and transduced CTLL-2 cells expanded at 124 ± 15 and 127 ± 9.5-fold over 8 days, respectively (Figure 2c). After incubation with MTX (0.025–0.1 μM) for 8 days, a 5–11-fold expansion of viable transduced cells was observed (Figure 2c), whereas exposure of non-transduced CTLL-2 cells to ≥ 0.025 μM MTX rendered cultures non-viable. Transduced CTLL-2 cells were enriched from the baseline of 7–8% huEGFR<sup>t</sup> cells to 98% huEGFR<sup>t</sup> following the 8-day culture in ≥ 0.05 μM MTX (Figure 2d).

To further delineate the range of MTX concentrations that could be tolerated by selected CTLL-2 cells, transduced cells selected in 0.1 μM MTX for 8 days were re-plated in a range of MTX concentrations (up to 0.75 μM). We observed 97–105-fold expansion of viable transduced cells at MTX concentrations up to 0.25 μM, which is equivalent to the fold expansion of non-transduced control CTLL-2 in the absence of MTX (Figure 2e). In addition, these cells continued to expand and maintain at least 67% viability in MTX concentrations of 0.5 and 0.75 μM (that is, 29-fold- and 10-fold expansion over 8 days, respectively). These data clearly demonstrate that, as seen with huDHFR<sup>FS</sup>-transfected Jurkat cells, CTLL-2 cells transduced to express huDHFR<sup>FS</sup> in combination with two other functional transgenes acquire resistance to high concentrations (up to 0.75 μM) of MTX.

We next sought to define the utility of this selection system in the context of selecting primary human central memory T cells (TCM), following CD19CAR:huEGFR<sub>thuDHFR<sup>FS</sup></sub> lentiviral transduction. Purified CD62L<sup>+</sup>CD45RO<sup>+</sup> healthy donor peripheral blood TCM were used in these experiments due to the capacity of expanded T cell products derived from these precursors to engraft, persist and re-populate memory T cell niches following
adoptive transfer.\(^{23,24}\) Following activation of purified TCM using anti-CD2/CD28 Dynal beads, 50 U ml\(^{-1}\) rhU-2 and 0.5 mg ml\(^{-1}\) rhU-15, transduced and control non-transduced T cells expanded equally (71.7 ± 8 and 79.6 ± 5.1) in the absence of MTX, whereas non-transduced T cells exhibited sensitivity to the lymphotrophic effects of MTX at concentrations as low as 0.05 \(\mu\)M (Figure 3a). Transduced T cells maintained a viability of 77.2 ± 1.8% after 10 days MTX selection at \(\geq 0.05 \mu\)M and the yield of selected cells was 1.7–6.2% of the number of viable cells at the initiation of selection (Figure 3a). Flow cytometric evaluation of transduced T cells after 10 days in culture with varying concentrations of MTX revealed significant MTX-mediated enrichment of transgene-expressing cells up to 70–76% CD19CAR\(^{+}\) and 87–89% huEGFR\(^{+}\) upon culture in \(\geq 0.05 \mu\)M MTX from pre-selection levels of 17.9% and 28% percent, respectively (Figure 3b). Comparison of the frequency of CD19CAR\(^{-}/huEGFR\(^{+}\)) expression at day 6 vs day 10 of MTX selection revealed the progressive enrichment of MTX-resistant transgene expressing T cells over time (Figure 3c). Of note, while these cells were predominately CD4\(^{-}\) (Figure 4a), comparable levels of expansion and MTX-mediated selection were observed with transduced CD8\(^{+}\) TCM-derived cells (Supplementary Figure S1). Furthermore, when a direct comparison between MTX-mediated selection and EGFRt-based immunomagnetic selection was performed with these same cells, the total yields of transgene\(^{-}\) cells were found to be comparable, if not better when using MTX (Supplementary Figure S2). There also appears to be some flexibility in the timing of MTX addition to cultures, as addition of MTX on either day 0, day 4 or day 8 during an 18-day expansion of transduced cells resulted in fold expansion and transgene expression that only differed by 10–20% (Supplementary Figure S3). Together these data support the utility of the huDHFR\(^{5}/\)MTX strategy for selecting gene-modified T cells in vitro.

We next sought to quantitate the level of MTX resistance of these expanded selected TCM after an additional round of expansion using OKT3 and irradiated feeder cells.\(^{25,26}\) The non-transduced (that is, unselected) cells and the transduced cells initially selected in 0.1 \(\mu\)M MTX exhibited similar expansion in the absence of MTX (Figure 3d). In contrast, upon re-plating in escalating concentrations of MTX on day 8 of their second expansion cycle, the transduced MTX-selected cells exhibited retention of viability (\(>80\%)\) and proliferative activity (\(>8.5\)-fold expansion after re-introduction of MTX) in MTX at concentrations up to 0.1 \(\mu\)M, while non-transduced T cells did not expand at any of the tested MTX concentrations (Figure 3d). In addition, this second cycle of MTX exposure resulted in further selection of transgene\(^{-}\) cells (that is, \(>90\%)\) transgene positive in \(\geq 0.025 \mu\)M MTX) (Figure 3e). These data document the utility of the MTX/huDHFR\(^{5}\) system for purifying primary human TCM-derived T cells following lentiviral transduction and expansion to clinically relevant cell doses for adoptive therapy trials.

The effect of huDHFR\(^{5}\) expression and MTX selection on cytokine secretion (IL-2, IFN-\(\gamma\), TNF-\(\alpha\), GM-CSF) in the supernatants of mixed lymphocyte-tumor cultures were augmented in cells selected in MTX, and not altered by the presence of 0.1 \(\mu\)M MTX during the assay (Figure 4c). These data indicate that the functional profile of CD19CAR redirected TCM\(^{-}\)-derived effector cells is enriched by virtue of selection in MTX using culture methods that are currently employed in cell manufacture for human adoptive therapy trials. Overall, given our success with this platform in T cells, it will now be important to continue to compare our DHFR\(^{5}\)-mediated strategy to other cellular enrichment systems including those that utilize alternate DHFR mutants.\(^{14,20}\)

On the basis of the in vitro analysis presented here, we speculate that the expression of DHFR\(^{5}\) by in vitro MTX selected T cells may be sufficient to render cells resistant to MTX administered to patients after adoptive transfer. MTX is an active drug against a variety of CD19\(^{+}\) hematologic malignancies and the ability to use concomitant MTX chemotherapy with CD19-specific T cells may affect additive or synergistic anti-tumor effects. Additional potential attributes of this system unique to the crossover use of MTX in vitro and in vivo include the ability of MTX administration to induce lymphopenia and reinforce transgene expression with the potential outcome of the selective homeostatic cytokine-driven engraftment of transfected T cells over the repopulating repertoire of endogenous T cells. Of potential benefit, human drug-resistant genes such as huDHFR\(^{5}\) are also generally thought to be of limited immunogenicity as compared with other non-human transgene drug-selection strategies (that is, hygromycin, neomycin)\(^{4,5}\) however, the immunogenic potential of this DHFR muteins needs to be further investigated. Furthermore, when MTX is converted to polyglutamate derivatives within the cytosol, it reduces drug efflux, as well as enhances binding to endogenous target enzymes such as DHFR,\(^{26}\) thus potentially enhancing its potency as a selection drug.

This is the first study to demonstrate the feasibility of DHFR\(^{5}\), mediated in vitro enrichment of therapeutic (that is, CAR\(^{+}\)) primary human T cells with MTX. DHFR\(^{5}\)-expressing Jurkat, CTLL-2 and primary human T cells consistently were resistant to the cytotoxic effects of MTX at concentrations ranging from 0.025–0.25 \(\mu\)M in vitro. Such MTX concentrations are achievable in vivo and are clinically relevant, in that levels of 0.1–1 \(\mu\)M MTX have been detected in patients after administration of low doses of MTX (10–500 mg m\(^{-2}\)).\(^{26,27}\) Furthermore, Zaharko et al.\(^{26,27}\) have shown that low-dose continuous MTX infusion in mice (1 \(\mu\)g h\(^{-1}\)) produced a sustained plasma concentration of 0.01 \(\mu\)M, and was sufficient to block cellular thymidylate synthesis.\(^{26,27}\) Thus, we are currently performing studies in animal models to assess the huDHFR\(^{5}/\)MTX-mediated selection of CAR\(^{+}\) T cells in vivo.

**MATERIALS AND METHODS**

Plasmid and lentiviral vector

The DHFR\(^{5}\)pcDNA3.1(\(\lambda\)) plasmid was generated by creating two point mutations in the wild-type human DHFR enzyme through site-directed mutagenesis (DHFR\(^{5}\); L22F/F31S) and ligated into pcDNA3.1(\(\lambda\)). The CD19CAR-T2A-EGFRt-T2A-DHFR\(^{5}\), ephIV7 lentiviral vector was cloned by connecting the cDNA encoding (a) CD19-specific chimeric antigen receptor;\(^{21}\) (b) truncated EGFR sequence (EGFRt);\(^{25}\) (c) ribosomal skip T2A sequence;\(^{28,29}\) and (d) DHFR.\(^{14}\) The cDNA encoding the CD19CARhuEGFRt/huDHFR\(^{5}\) was then incorporated into the lentiviral vector packaging plasmid ephIV7 under control of human EF-1\(\alpha\) promoter for production of the lentiviral vector. The correct assembly of the transgenes was verified with DNA sequence analysis. All DNA constructs and construction-associated PCR primer sequences are available upon request.

Cell lines and maintenance

Jurkat cells (Human T lymphoblast-like cell line), SupB15 leukemia, CTLL-2 cells (mouse T-cell line) and K562 cell lines were purchased from American
Figure 3. Human T cells transduced to express the huDHFR<sup>ts</sup> transgene are resistant to MTX. (a) Non-transduced primary human T cells (non-Txd, gray line/bar) vs primary human T cells transduced with the vector shown in Figure 2a (Txd, black line/bar) were REM stimulated, the indicated concentrations of MTX were added at day 8 of the REM cycle and cells were followed for viability and fold expansion for 10 days (that is, day 8–18 of the REM). There is a significant difference in the total viable cell number (P = 0.012) and percentage of viable cells (P < 0.0001) when compared the concentrations over >0 MTX with that non-Txd cells over 10 days. (b) Flow cytometric evaluation of CD19CAR (Fc) and huEGFRt transgene expression (gray histograms) on transduced T cells after 10 days of culture in the indicated concentrations of MTX. Representative percentage of positive cells and mean fluorescence activity above staining with secondary reagent alone (open histograms) are indicated for each histogram. (c) Enrichment kinetics of transduced human T cells by MTX selection. The transduced T cells (day 0) were cultured in 0.1 μM MTX and analyzed for positive selection by staining for CD19CAR (Fc) and huEGFRt expression on day 0 (pre-MTX), and days 6 and 10 following MTX addition. (d) Primary human T cells transduced with the vector shown in Figure 2a and pre-selected for 6 days in 0.1 μM MTX (Txd + Slxd, black line/bar) vs non-transduced T cells (non-Txd, gray line/bar) were REM stimulated. On day 8, T cells were re-plated at the indicated concentrations of MTX and Txd + slxd cells are significantly different in their total viable cell number (P = 0.025), percentage of viable cells and fold expansion (P = 0.002) when the concentrations >0 MTX were combined and compared to that of non-Txd T cells over 10 days. (e) Flow cytometric evaluation of CD19CAR (Fc) and huEGFRt transgene expression (gray histograms) on pre-selected transduced T cells after 10 days of culture in the indicated concentrations of MTX. Percentages of positive cells and mean fluorescence activity above staining with secondary reagent alone (open histograms) are indicated.
Type Culture Collection (ATCC) and maintained according to the ATCC guidelines. EBV-transformed LCL, that expressed OKT3 (LCL-OKT3) cells were cultured as previously described.\textsuperscript{25,27}

Human peripheral blood mononuclear cells were isolated from healthy donors with the approval of Institutional Review Board (#09025) of the City of Hope National Medical Center (COHNMC), as described.\textsuperscript{23} TCM-derived T
were calculated using FCS Express V3 (analyzed by FACScalibur, and the percentage of cells in a region of analysis with cetuximab as previously described.23

The working concentrations of MTX were diluted in PBS. As controls, non-transduced T cells were plated with equal cell number. The cells were cultured for 8–10 days at the increased concentrations of MTX. After this, some cells were harvested and the supernatants were stored at −70°C. The remaining cells were used on day 18 (of the REM stimulation) for T cell surface phenotype markers CD4, CD8, CD28, CD62L, TCRαβ, CD127 and CD45 (filled histogram) vs isotype control antibody (open histogram) by flow cytometry. Percentages of positive cells are indicated. (b) The same cells as in (a) were used as effectors in a 4-h chromium-release assay, with the exception of the TxD + Slxd cells, which were washed to remove MTX 24h before the assay; and half of the washed TxD + Slxd cells then had 0.1 µM MTX added back just for the 4-h assay (TxD + Slxd + MTX).23 Cr-labeled CD19+ SupB15 or LCL cells, CD19-K562 cells, or OKT3-expressing LCL cells were then used as targets at the indicated E: T ratios. Mean percent chromium release ± s.d. of triplicate wells are depicted. *P < 0.05 when comparing TxD vs TxD + Slxd effectors using an unpaired Student’s two-tailed t-test. (c) Using the same cells described in (b) IL-2, IFN-γ, TNF-α and GM-CSF levels in supernatants from overnight co-cultures were determined by bioplex. Mean percentages of cytokine release ± s.d. of triplicate wells are depicted.

In vitro selection of gene-modified T cells

The MTX (Parenta Pharmaceuticals, PA, USA) concentration required for in vitro selection of gene-modified T cells was determined by dose–response curve. Briefly, the selection was initiated on day 8 of REM stimulation cycle by plating 0.8 × 10⁶ transduced primary human T cells in 24-well tissue culture plates or 0.4 × 10⁶ transduced CTLL-2 or Jurkat T cells and cultured for 8–10 days at the increased concentrations of MTX. As controls, non-transduced T cells were plated with equal cell number. The total viable cell number and percentage of viable cells were enumerated by Guava Personal Cell Analysis (Guava Technologies, Hayward, CA, USA) method. The working concentrations of MTX were diluted in PBS. Transduced T cells were immunomagnetically selected for EGFR expression with cetuximab as previously described.23

Flow cytometry

Cell surface phenotype was analyzed with fluorochrome-conjugated streptavidin and antibodies specific for CD4, CD8, CD28, TCRαβ, CD62L, CD45, and CD127 and isotype controls (BD Bioscience). Biotinylated anti-human Fc-specific mAb was purchased from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA, USA) PE-conjugated anti-Biotin was purchased from Miltenyi Biotec (Auburn, CA, USA). The generation of biotinylated-cetuximab has been previously described.23 Stained cells were analyzed by FACS CALIBUR, and the percentage of cells in a region of analysis were calculated using FCS Express V3 (De Novo Software).

Chromium-release assays

The cytolytic activity of T cells was determined by 4-h chromium-release assay (CRA) as previously described.30

Measurement of cytokine production

T cells (5 × 10⁵) were co-cultured overnight with 5 × 10⁴ SupB15, LCL-OKT3, LCL or K562 in 96-well tissue culture plates. Supernatants were measured by cytokometric bead array assay using a bioplex human cytokine panel (Bio-Rad Laboratories, Hercules, CA, USA), according to the manufacturer’s instructions.

Statistical analysis

Data are expressed as mean ± s.d. Student’s two-tailed t-test was used to evaluate the significance of differences between experimental groups for CRA and bioplex by using Graphpad prism (San Diego, CA, USA). Total viable cell numbers, percentage of viable cells between non-transduced and transduced cells were analyzed using repeated one-way analysis of variance by R program (http://www.r-project.org/). An effect is considered to be statistically significant when the P-value is <0.05.

CONFLICT OF INTEREST

MCI is an inventor of licensed intellectual property and a co-founder/equity of ZetaRx Biosciences, Inc. All other authors have no conflicts of interest to disclose.

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Supplementary Information accompanies the paper on Gene Therapy website (http://www.nature.com/gt)