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Efficient Selection of Genetically Modified Human T Cells Using Methotrexate-Resistant Human Dihydrofolate Reductase

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Abstract

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 based on the pharmaceutical drug methotrexate (MTX), a potent allosteric inhibitor of human dihydrofolate reductase (DHFR). Here, we describe the development of SIN lentiviral vectors that direct the coordinated expression of a CD19-specific CAR, the human EGFRt tracking/suicide construct, and a methotrexate-resistant human DHFR mutein (huDHFR^{FS}; L22F, F31S). Our results demonstrate that huDHFR^{FS} co-expression renders lentivirally transduced primary human CD45RO$^+$CD62L$^+$ central memory T cells resistant to lymphotoxic concentrations of MTX up to 0.1 μM. Our modular cDNA design insures that selected MTX-resistant T cells co-express functionally relevant levels of the CD19-specific CAR and EGFRt. This selection system based on huDHFR^{FS} and MTX has considerable potential utility in the manufacturing of clinical-grade T cell products.

Keywords

Lentivirus; T cell selection; Methotrexate; DHFR

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Conflict of Interest

M.C.J. 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.

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 based on flow cytometric cell sorting or immunomagnetic techniques. While 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 based on expressing enzymes that confer resistance to cytotoxic selection drugs. While 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.[1–5]

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-benzylguanie (0^6-BG).[11–13] While *in vivo* selection of gene-modified hematopoietic stem cells (HSC) is achieved with this approach, it is not readily transferable to *in vitro* selection, nor is a genotoxic alkylator drug such as temolozomide 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. Additional desirable features of the system is a small transgene footprint for 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 DHFR constructs that confer resistance to lymphotoxic concentrations of MTX.[14–17]

In the present study, we evaluate the utility of a huDHFR^{FS}/MTX selection system for generating therapeutic T cells expressing 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, huDHFR^{FS} expressing T cells are enriched after a single round of MTX selection. Moreover, when huDHFR^{FS} is arrayed in a single transcript in combination with a CD19-specific CAR (CD19CAR) and EGFRt, 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.[18] We identified a threshold MTX concentration of 0.05 μM that rendered cultured Jurkat T cells non-viable (Figure 1a), a level consistent with previous observations of MTX cytotoxicity against hematopoietically derived cells in culture.[14,15,19] Therefore, in all subsequent experiments, ≥0.05 μM MTX was used to examine $DHFR^{FS}$-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 ($DHFR^{FS}$) that retains 2.3% of its catalytic activity while exhibiting a 478-fold resistance to MTX inhibition.[14,20] To determine whether $huDHFR^{FS}$ 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 $huDHFR^{FS}$-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 cytocidal 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, $huDHFR^{FS}$ expressing Jurkat cells retained viability with diminished proliferative activity in concentration 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 $huDHFR^{FS}$ transgene expressed in Jurkat T cells confers resistance to otherwise cytocidal concentrations of MTX *in vitro*.

We next sought to incorporate the $huDHFR^{FS}$-MTX selection system into our third generation SIN lentiviral vector system, and design trifunctional transgene constructs that place our CD19CAR and huEGFRt in frame with $huDHFR^{FS}$, each separated by a T2A ribosomal skip linker (Figure 2a).[21–23] As proof of concept, IL-2 dependent murine CTLL-2 T cells were first transduced with this CD19CAR:huEGFRt:$huDHFR^{FS}$ lentiviral vector and evaluated for their resistance to MTX. Ten days after lentivirus transduction, 7–8% of cells expressed CD19-CAR and huEGFRt 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 to 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 where enriched from the baseline of 7–8% huEGFRt$^+$ cells to 98% huEGFRt$^+$ 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 to 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 (i.e., 29-fold and 10-fold expansion over 8 days, respectively). These data clearly demonstrate that, as seen with huDHFR^{FS} transfected Jurkat cells, CTLL-2 cells transduced to express huDHFR^{FS} 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 (T_{CM}) following CD19CAR:huEGFRt:huDHFR^{FS} lentiviral vector transduction. Purified CD62L$^+$CD45RO$^+$ healthy donor peripheral blood T_{CM} 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 T_{CM} using anti-CD3/CD28 Dynal beads, 50 U/ml rhuIL-2 and 0.5 ng/ml rhuIL-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 lymphotoxic effects of MTX at concentrations as low as 0.05 μM (Figure 3a). Transduced T cells maintained a viability of 77.2 ± 1.8% after 10 days MTX selection at ≥0.05 μM 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% huEGFRt$^+$ upon culture in ≥0.05μM MTX from pre-selection levels of 17.9% and 28% percent, respectively (Figure 3b). Comparison of the frequency of CD19CAR$^+$/huEGFRt$^+$ 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$^+$ (see Figure 4a), comparable levels of expansion and MTX-mediated selection were observed with transduced CD8$^+$ T_{CM}-derived cells (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 (Figure S2). There also appears to be some flexibility in the timing of MTX addition to cultures, since 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% (Figure S3). Together these data support the utility of the huDHFR^{FS}/MTX strategy for selecting gene-modified T cells *in vitro*.

We next sought to quantitate the level of MTX resistance of these expanded selected T_{CM} after an additional round of expansion using OKT3 and irradiated feeder cells.[23,25] The non-

transduced (i.e., un-selected) cells and the transduced cells initially selected in 0.1 μM 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 μM, 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 (i.e., >90% transgene positive in ≥0.025 μM MTX) (Figure 3e). These data document the utility of the MTX/huDHFR^{FS} system for purifying primary human T_{CM} derived T cells following lentiviral transduction and expansion to clinically relevant cell doses for adoptive therapy trials.

The effect of huDFHR^{FS} expression and MTX selection on skewing the phenotype and function of primary human T_{CM} is unknown. Accordingly, we examined the phenotype of our MTX-selected CD19CAR:huEGFRt:huDHFR^{FS} T_{CM} lines and mock transduced non-selected control counterparts based on surface expression of CD4, CD8, CD28, CD62L, TCRαβ, CD127 and CD45 (Figure 4a). Expression of each of these T cell markers was equivalent to that observed on non-transduced, and on transduced but non-MTX-selected controls. Functionally, we tested selected and control lines for CD19CAR redirected cytolytic activity in 4-hr chromium release assays against CD19$^+$ SupB15 and LCL targets. MTX selection of transduced cells significantly increased the CD19CAR redirected cytolytic potency over that of their transduced non-selected counterparts (Figure 4b). Furthermore, this cytolytic activity of the selected T cells was not affected by the addition to culture of 0.1 μM MTX during the assay. CD19CAR regulated activation of these T cells for cytokine secretion (IL2, IFN-γ, TNF-α, 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 μM MTX during the assay (Figure 4c). These data indicate that the functional profile of CD19CAR redirected T_{CM}-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^{FS}-mediated strategy to other cellular enrichment systems including those that utilize alternate DHFR mutants.[14,20]

Based on the *in vitro* analysis presented here, we speculate that the expression of DHFR^{FS} 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 concombinant MTX chemotherapy with CD19-specific T cells may affect additive or synergistic anti-tumor effects. Additional potential attributes of this system unique to the cross-over 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 transferred T cells over the repopulating repertoire of endogenous T cells. Of potential benefit, human drug-resistant genes such as huDHFR^{FS} are also generally thought to be of limited immunogenicity as compared to other non-human transgene drug selection strategies (i.e., 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 $DHFR^{FS}$ mediated *in vitro* enrichment of therapeutic (i.e., CAR^+) primary human T cells with MTX. $DHFR^{FS}$-expressing Jurkat, CTLL-2 and primary human T cells consistently were resistant to the cytocidal effects of MTX at concentrations ranging from 0.025 to 0.25 μM *in vitro*. Such MTX concentrations are achievable *in vivo* and are clinically relevant, in that levels of 0.1μM to 1 μM MTX have been detected in patients after administration of low doses of MTX (10–500 mg/m^2).[26] Furthermore, Zaharko *et al.* have shown that low dose continuous MTX infusion in mice (1 μg/hr) produced a sustained plasma concentration of 0.01 μM, and was sufficient to block cellular thymidylate synthesis.[26,27] Thus, we are currently performing studies in animal models to assess the $huDHFR^{FS}$/MTX-mediated selection of CAR^+ T cells *in vivo*.

Materials and Methods

Plasmid and lentiviral vector

The $DHFR^{FS}$_pcDNA3.1(−) plasmid was generated by creating two point mutations in the wild type human DHFR enzyme through site directed mutagenesis ($DHFR^{FS}$; L22F/F31S) and ligated into pcDNA3.1(−). The CD19CAR-T2A-EGFRt-T2A-$DHFR^{FS}$_epHIV7 lentiviral vector was generated by cloning the cDNA encoding a) CD19-specific chimeric antigen receptor;[21] b) truncated EGFR sequence (EGFRt);[23] c) ribosomal skip T2A sequence;[28,29] and d) $DHFR^{FS}$.[14] The cDNA encoding the CD19CAR:huEGFRt:$huDHFR^{FS}$ was then incorporated into the lentiviral vector packaging plasmid epHIV-7 under control of human EF-1α 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 Type Culture Collection (ATCC) and maintained according to ATCC guidelines. EBV-transformed lymphoblastoid cell lines (LCL), LCL that expressed OKT3 (LCL-OKT3) cells were cultured as previously described.[25,27]

Human peripheral blood mononuclear cells (PBMCs) were isolated from healthy donors with the approval of Institutional Review Board (#09025) of the City of Hope National Medical Center (COHNMC) as described.[23] T_{CM}-derived T cell isolation, stimulation, expansion (Rapid expansion method; REM) and lentiviral-mediated transduction were done as described.[23]

In vitro selection of gene-modified T cells

The MTX (Parenta Pharmaceuticals, PA) 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 0.8×10^6 transduced primary human T cells in 24- well tissue culture plates or 0.4×10^6 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 was enumerated by Guava Personal Cell Analysis (Guava Technologies, Hayward, CA) method. The working concentrations of MTX were diluted in PBS. Transduced T cells were immunomagnetically selected for EGFRt expression with cetuximab as previously described.[23]

Flow cytometry

Cell surface phenotype was analyzed with fluorescent-conjugated antibodies specific for CD4, CD8, CD28, TCRαβ, CD62L, CD45, and CD127 and isotype controls (BD Biosciences). Biotinylated anti-human Fc-specific mAb was purchased from Jackson ImmunoResearch Laboratories, Inc. PE-conjugated anti-Biotin was purchased from Miltenyi Biotec. The generation of biotinylated-cetuximab has been previously described.[23] Stained cells were analyzed by FACScalibur, 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-hour chromium-release assay (CRA) as previously described.[30]

Measurement of cytokine production

T cells (5×10^4) were co-cultured overnight with 5×10^4 SupB15, LCL-OKT3, LCL, or K562 in 96-well tissue culture plates. Supernatants were measured by cytometric bead array assay using a bioplex human cytokine panel (Bio-Rad Laboratories) 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. Total viable cell numbers, percentage of viable cells between non-transduced and transduced cells were analyzed using repeated one-way ANOVA by R program (http://www.r-project.org/). An effect is considered to be statistically significant when the P-value is <0.05.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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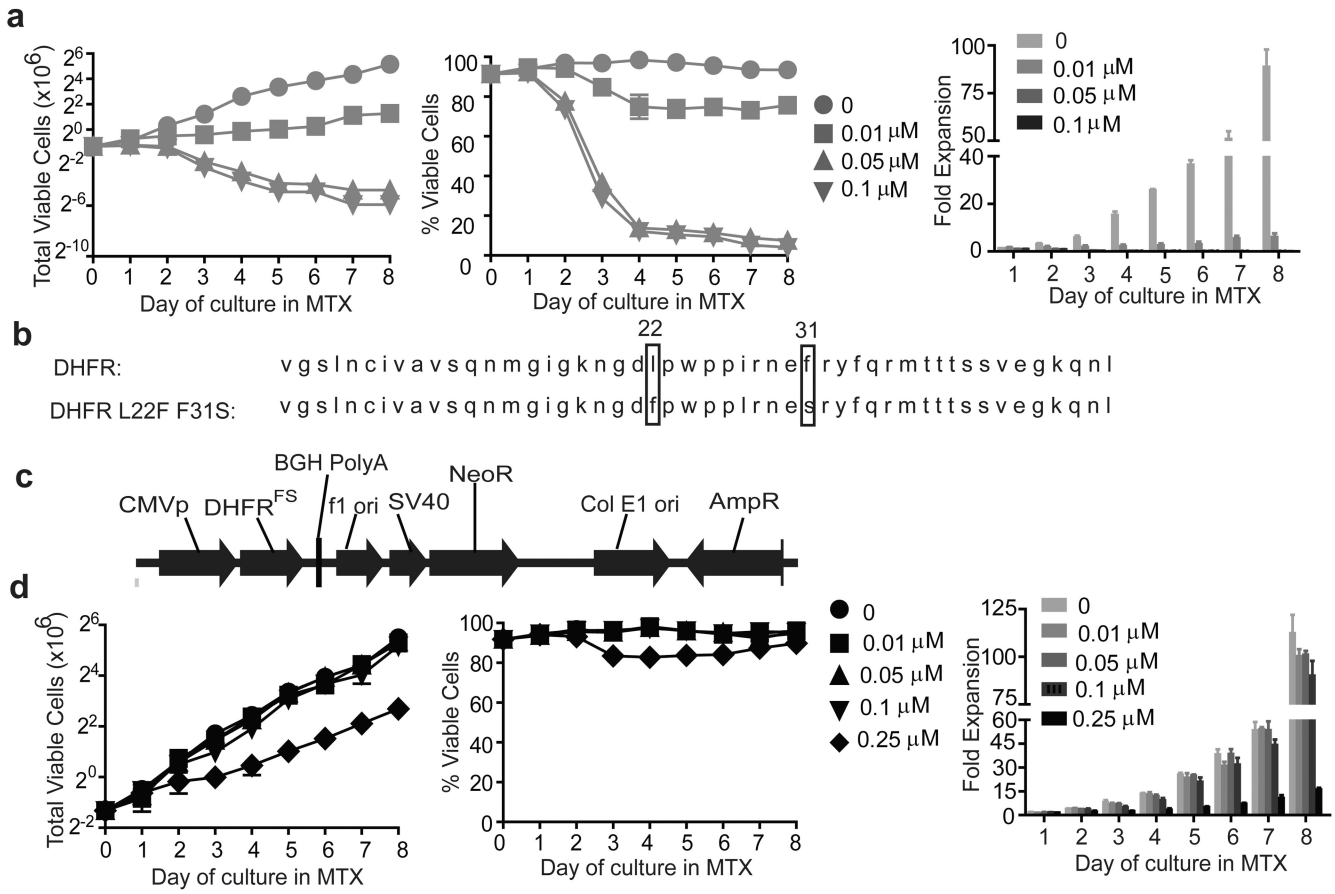

Figure 1.

Expression of double mutant DHFR transgene ($DHFR^{FS}$) 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 $huDHFR^{FS}$ 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 $huDHFR^{FS}$-transfected Jurkat T cells (7 days in 0.05 μM MTX) were re-plated in triplicate with equal cell number in 24-well plate 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.

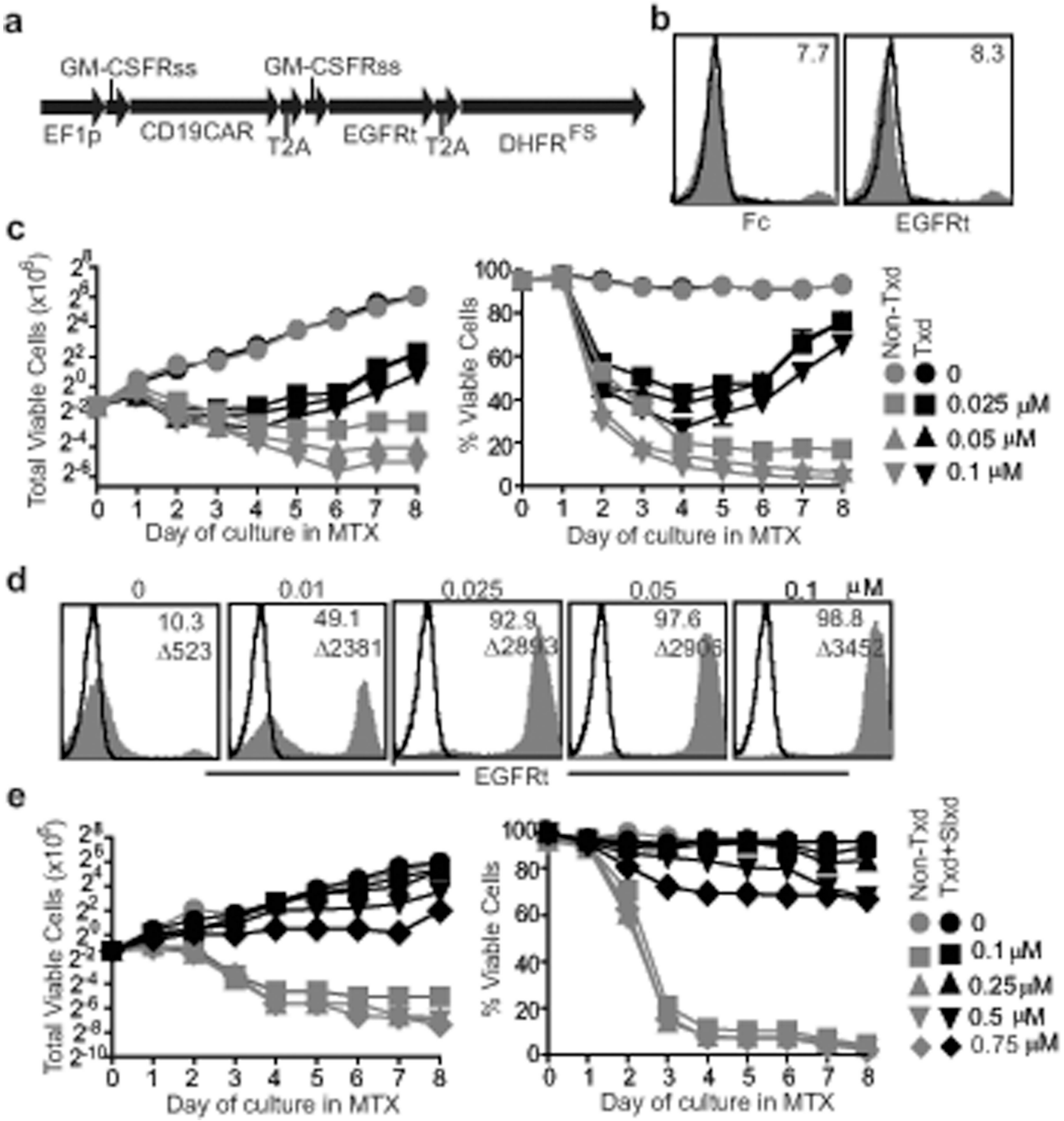

Figure 2.

Transduction with a lentiviral construct containing huDHFR^{FS}, CD19CAR and huEGFRt transgenes confers MTX resistance in CTLL-2 cells. (**a**) Construct within lentiviral vector containing the CD19CAR, huEGFRt and huDHFR^{FS} transgenes linked by T2A cleavage sites. Location of EF1-α promoter (EF1p) and GM-CSF receptor signal sequences (GM-CSFRss) relative to transgenes are also depicted. (**b**) CTLL-2 cells were assessed for CD19CAR (Fc) and EGFRt expression (grey 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, grey line) vs. transduced (Txd, black line) CTLL-2 cells were plated in triplicate with equal cell number in 24-well plate in the indicated concentrations of MTX and evaluated for their viable cell number and percentage viable cells (mean ± S.D) over 8 days. (**d**) Flow cytometric evaluation of huEGFRt transgene expression (grey 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 huEGFRt-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, grey line) were re-plated at the indicated concentrations of MTX. 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. Data for a representative experiment are shown.

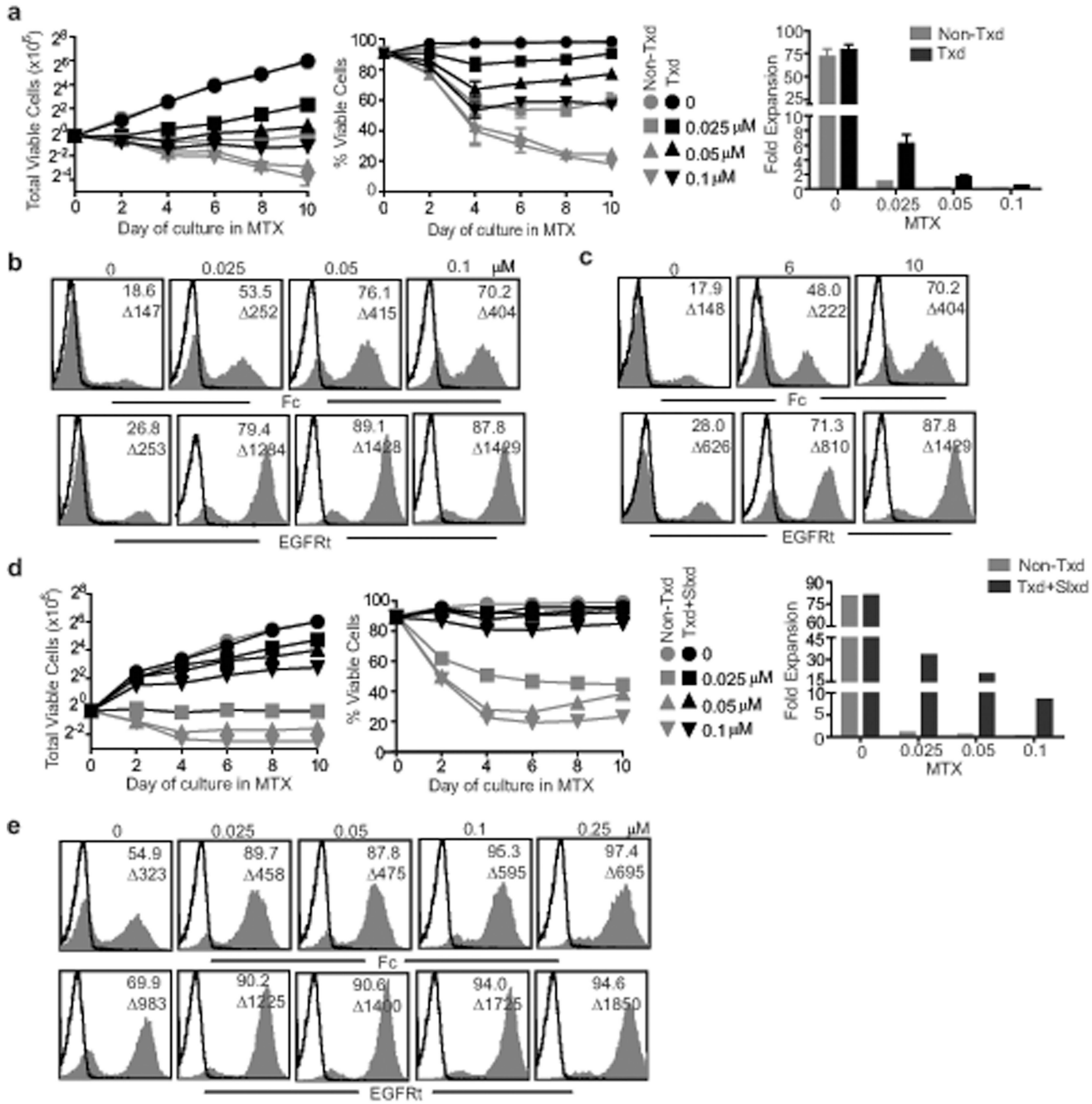

Figure 3.

Human T cells transduced to express the huDHFR^{FS} transgene are resistant to MTX. (**a**) Non-transduced primary human T cells (Non-Txd, grey line/bar) vs. primary human T cells transduced with the vector shown in fig. 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 (i.e., 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 (grey 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 fig. 2a and preselected for 6 days in 0.1 μM MTX (Txd+Slxd, black line/bar) vs. non-transduced T cells (Non-Txd, grey 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 combined the concentrations with > 0 MTX as compared to Non-Txd T cells over 10 days. (**e**) Flow cytometric evaluation of CD19CAR (Fc) and huEGFRt transgenes expression (grey histograms) on pre-selected transduced T cells after 10 days of culture in the indicated concentrations of MTX. Percentage of positive cells and mean fluorescence activity above staining with secondary reagent alone (open histograms) are indicated.

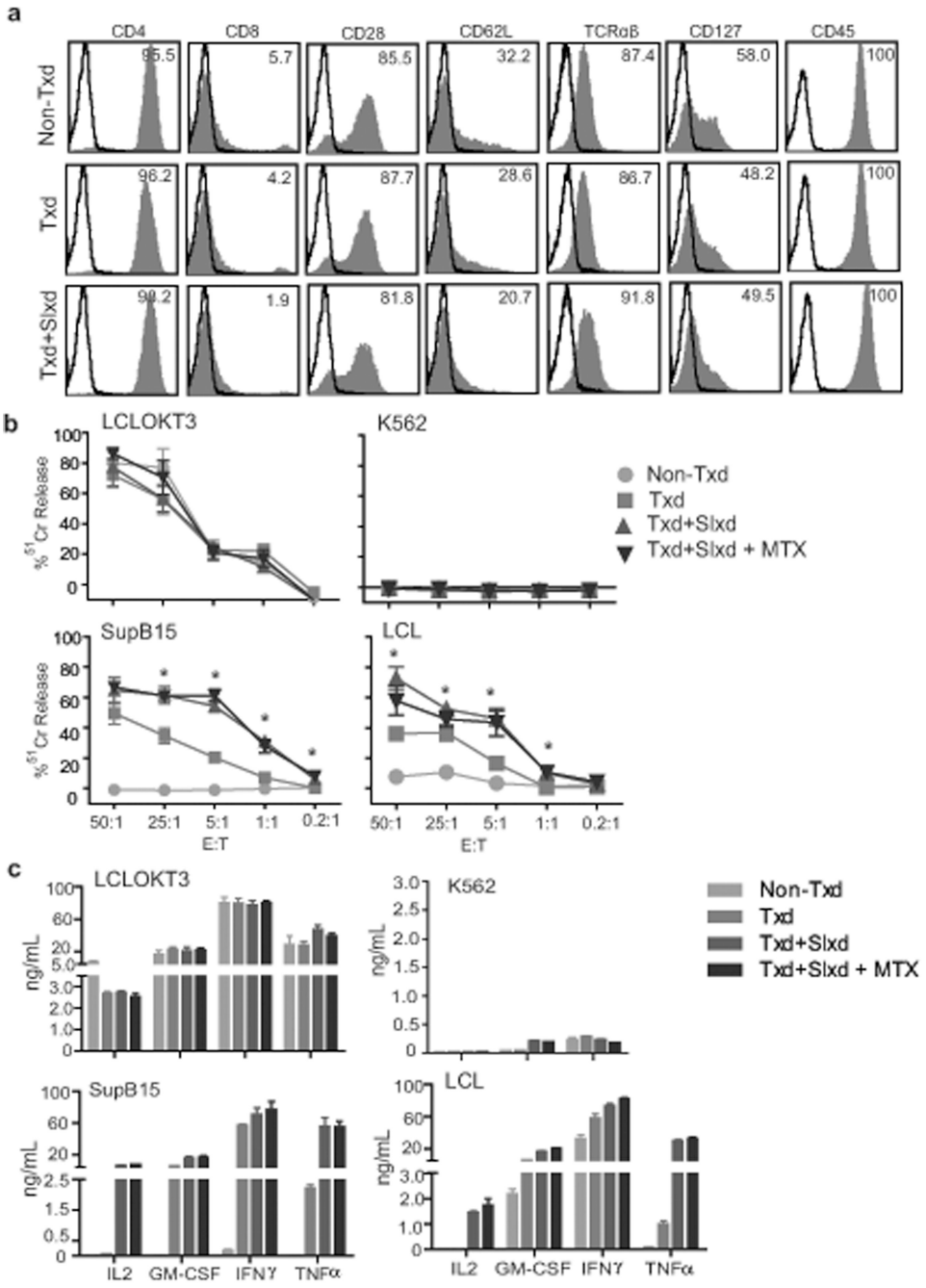

Figure 4.

Transduced and MTX selected T cells maintained their phenotype effector functions. (**a**) Non-transduced (Non-Txd) vs. transduced human T cells (Txd) +/− selection (Slxd, 6 days in 0.1 μM MTX) were REM stimulated, and 0.1 μM MTX was again added to just the Txd +Slxd cells on day 8 of the REM stimulation. Each cell line was then analyzed 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 hr chromium release assay, with the exception of the Txd+Slxd cells, which were washed to remove MTX 24hrs before the assay; and half of the washed Txd+Slxd cells then had 0.1 μM MTX added back just for the 4hr assay (Txd+Slxd +MTX). ^{51}Cr-labeled CD19^{+} SupB15 or LCL cells, CD19^{-} K562 cells, or OKT3-epressing 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 compare Txd vs. Txd+Slxd effectors using 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 percent cytokine release ± S.D. of triplicate wells are depicted.